

NEUROPROTECTION GENE THERAPY IN RETINAL DEGENERATION

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Haidong Shan

Merton College

UNIVERSITY OF OXFORD

Abstract

Retinal degenerative disease, which includes age-related macular degeneration and retinitis pigmentosa, is the main cause of blindness in developed countries. Degeneration of the retinal pigment epithelium (RPE) and photoreceptor cells through apoptosis is believed to be the main mechanism of cell death. X-linked inhibitor of apoptosis (XIAP) is an endogenous anti-apoptotic protein that mediates its effects through the inhibition of caspases - the proteins regulating the final stages of apoptosis. The neuroprotection of XIAP has been demonstrated in various neurodegenerative models. Retinal gene therapy based on adeno-associated virus (AAV) has recently been proven safe and effective in clinical trials of Leber's congenital amaurosis. However, studies are very limited so far about AAV-mediated XIAP effect on degeneration of the RPE and photoreceptor cells.

In this thesis, a comprehensive study of AAV-mediated XIAP was performed in the RPE and photoreceptor degenerative models. First, an oxidative stress model was investigated using H_2O_2 in a human RPE cell line. Second, AAV2-mediated XIAP conferred marked protection on the RPE cells against H_2O_2 induced apoptosis. Third, an *in vivo* analysis using confocal scanning laser ophthalmoscope was applied to the $NaIO_3$ induced retinopathy in two transgenic mice (*NRL-GFP* and $B6^{TGOPN1LW-EGFP}$). However, subretinal injection of AAV2-XIAP did not rescue photoreceptor cells in the $NaIO_3$ -treated animals. Finally, AAV8-XIAP was tested in a *rhodopsin* mutant mouse line with retinal degeneration (the $Rho^{-/-} B6^{TGOPN1LW-EGFP}$ mouse) but did not reveal any protection on cone photoreceptors. Overall the work in this thesis indicates a limited protection of AAV-mediated XIAP on the RPE and photoreceptor cells in the degenerative models used. XIAP based gene therapy may be helpful for RPE preservation in atrophic AMD, but it needs further research.

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List of Abbreviations

AAV: adeno-associated virus

AIF: apoptosis inducing factor

AMD: age-related macular degeneration

BDNF: brain-derived neurotrophic factor

BGH: bovine growth hormone

CAG: cytomegalovirus early enhancer and chicken beta-actin promoter

CFH: complement factor H

CNTF: ciliary neurotrophic factor

cSLO: confocal scanning laser ophthalmoscopy

DCFH-DA: 2',7'-dichlorofluorescein diacetate

EGFP: enhanced green fluorescent protein

ERG: electroretinography

FACS: fluorescence-activated cell sorting

GDNF: glial cell line-derived neurotrophic factor

GFP: green fluorescent protein

IAP: inhibitors of apoptosis proteins

IRES: internal ribosome entry site

ITR: inverted terminal repeat

LCA: Leber's congenital amaurosis

ONL: outer nuclear layer

PARP: poly(ADP-ribose) polymerase

rAAV: recombinant adeno-associated virus

RGC: retinal ganglion cell

ROS: reactive oxygen species

RP: retinitis pigmentosa

RPE 65: retinal pigment epithelium-specific 65-kDa

RPE: retinal pigment epithelium

TdT: terminal deoxynucleotidyl transferase

TUNEL: terminal deoxynucleotidyl-transferase dUTP nick-end labeling

WPRES: Woodchuck post-transcriptional regulatory element

XIAP: X-linked inhibitor of apoptosis

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Chapter 1 General Introduction

1.1 The Retina

1.1.1 Anatomy and physiology of the retina

The human eye is an optical and biological system which detects and converts environmental light into electrophysiological signals. The human retina is a complex light-sensitive tissue lining the inner side of the eye¹. It contains many cell types, including the retinal pigment epithelium (RPE) cell, rod and cone photoreceptor cell, retinal ganglion cell, bipolar cell, amacrine cell and horizontal cell, *et al.* In anatomy, the retina has ten distinct layers under light microscopy². From the inside to the outside are (Figure 1):

- 1) Inner limiting membrane: Müller cell footplates.
- 2) Nerve fiber layer: ganglion cell axons.
- 3) Ganglion cell layer
- 4) Inner plexiform layer: synapse between dendrites of the ganglion cells and the bipolar cell axons.
- 5) Inner nuclear layer: cell bodies composed of the bipolar and horizontal cells.
- 6) Outer plexiform layer: synapses between dendrites of the bipolar cells and projections of rod and cone photoreceptors.
- 7) Outer nuclear layer (ONL): cell bodies of rods and cones.
- 8) External limiting membrane: attachment sites of photoreceptors and Müller cells.
- 9) Photoreceptor layer: inner and outer segments of rods and cones.
- 10) RPE: single layer of pigmented epithelium cells.

The macula is one of the main specific characteristics of human retina³. It locates on the back central area of the retina with a diameter about 5 mm. It features a yellow appearance known as the macula lutea due to high concentration of the xanthophyll carotenoids zeaxanthin and lutein. The macula is a cone-dominant area compared with the peripheral retina which photoreceptor cells are mainly rods. In the centre of macula is the fovea, where cone photoreceptors are concentrated at the largest density with exclusion of the rods in human retina⁴.

In a simplified view of the retina function, rod and cone photoreceptor cells detect light and convert photon stimuli into electrochemical signals⁵. The signals are transmitted to bipolar cells, the retinal ganglion cells and subsequently to the visual cortex of the brain through the visual pathway. There are also interconnections within the phototransduction pathway and other retinal cells such as horizontal cells and amacrine cells, which provide a complex degree of signal processing within the retina⁵.

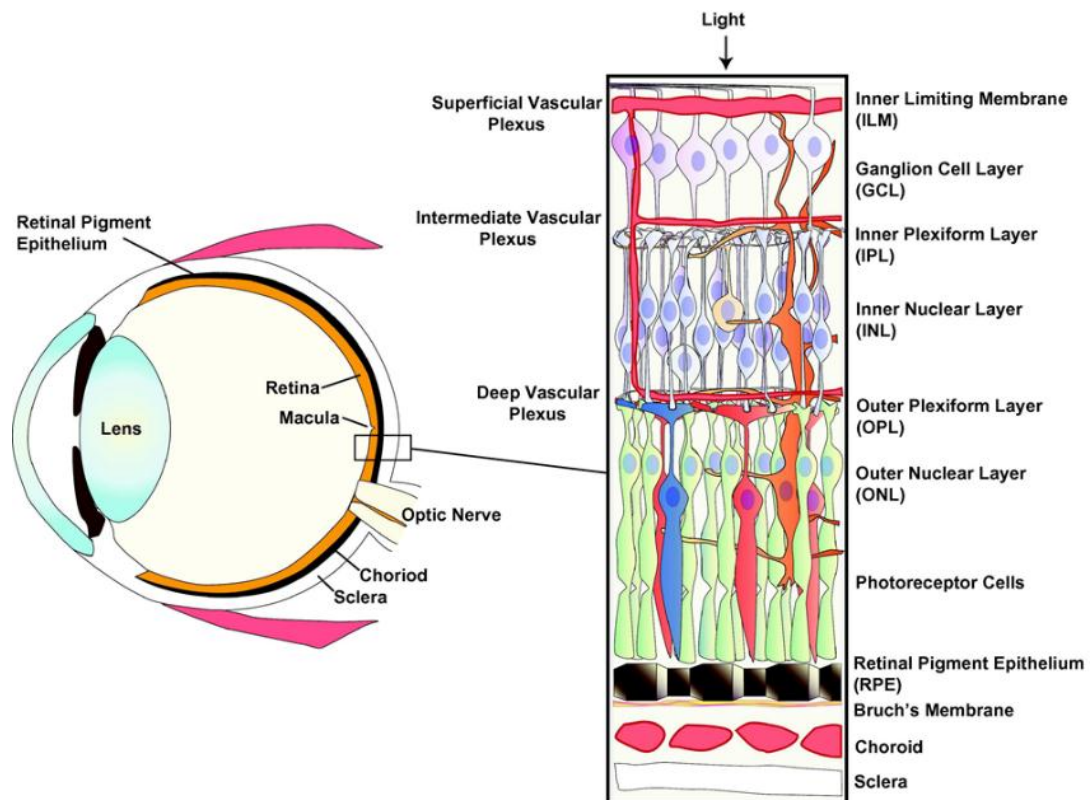


Figure 1 Schematic diagram of human eye (left) and a cross section of the retina (right). Adapted from Jarrett SG and Boulton ME⁶.

1.1.2 RPE

The RPE is composed of a single layer of pigmented epithelium cells between the choroid and the neurosensory retina⁷. It features many functions in maintaining normal retina function. First, the RPE contains dense pigment granules to prevent internal reflections and therefore ensures the retinal photosensitive cells only accept direct light stimuli coming through the cornea and lens⁷. This is critical to create a clear-focused image on the retina. Secondly, it forms a tight-junction epithelium and serves as a part of the blood-retina barrier⁸. Metabolic end products in the subretinal space are selectively transported by the RPE cells to the blood. Thirdly, the RPE features phagocytosis of shed photoreceptor outer segments, which ensures the constant renewal of rods and cones⁹.

Moreover, the RPE synthesizes the essential chemical component 11-*cis* retinol for phototransduction in retinal photoreceptor cells¹⁰. Rhodopsin and opsin are the membranous photoreceptor proteins in rods and cones, respectively¹¹. They are composed with opsin and 11-*cis* retinal in the membrane of outer segment discs. Upon light stimuli, the 11-*cis* retinal absorbs a light photon and is isomerized to the all-trans retinal (leading to retinyl esters). The G-protein is subsequently activated and cation channels changes on the membrane. This process is known as the visual transduction¹². The RPE cells contain the retinal pigment epithelium-specific 65-kDa (RPE 65) protein, which is critical to generate 11-*cis* retinol for recycling into 11-*cis* retinal by isomerizing all-trans retinyl esters¹³. (Figure 2)

Finally, the RPE has been found to produce and secrete various growth factors, such as pigment epithelium-derived factor (PEDF), ciliary neurotrophic factor (CNTF), vascular endothelial growth factor, fibroblast growth factors, transforming

growth factor- β (TGF β) and insulin-like growth factor-1 (IGF-1)^{14, 13, 14}. These factors are important in maintaining retinal cells as well as the retinal choiicapillaris structure¹⁵. PEDF, for example, has been found to protect neurons against glutamate- or hydrogen peroxide induced apoptosis^{16, 17}. It also acts as an antiangiogenic factor, playing an important role in normal eye vascularization and neovascularization development in various ocular disorders¹⁸. Moreover, the RPE supplies nutrients such as glucose and omega-3 fatty acids which are important for photoreceptor cell metabolism¹⁹.

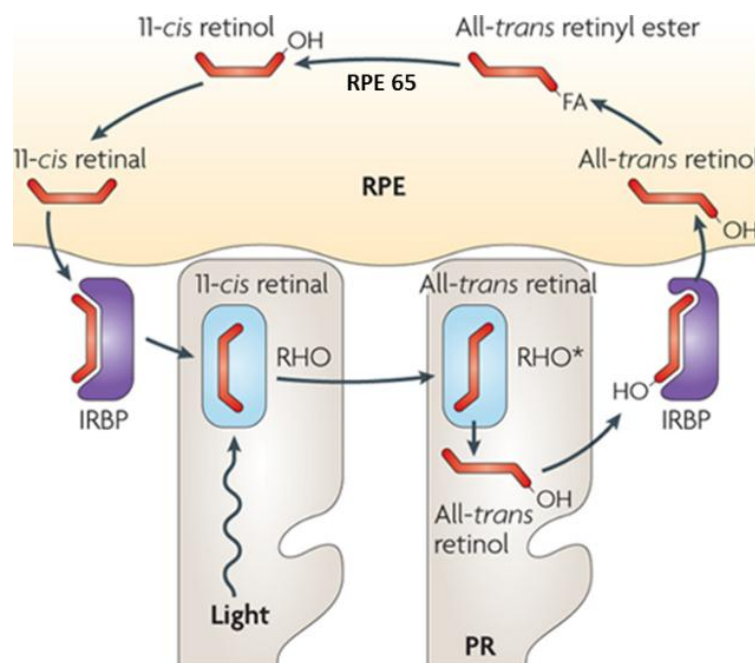


Figure 2 Schematic diagram of the phototransduction. RPE 65: retinal pigment epithelium-specific 65-kDa. RPE: retinal pigment epithelium. PR: photoreceptor. IRBP: interstitial retinol-binding protein. RHO: rhodopsin. FA: fatty acyl. Adapted from Wright AF, Chakarova CF, et al²⁰.

1.1.3 Photoreceptor cells

So far three types of cell have been found photosensitive in the human retina; the rods, cones and photosensitive retinal ganglion cells (RGC)^{11, 21}. In common, they are all specialized types of neuron with transmembrane opsin-based photopigments able to detect light photons and trigger cell membrane potential changes (phototransduction, see previous Figure 2). However, each type is characterized by specific morphology and function.

Rods and cones are the classic photoreceptors located in the outer retina (Figure 3). Rods feature the slim rod-shaped structure with inner and outer segments stretching to the RPE¹¹. They are distributed throughout the extra-foveal retina and are highly sensitive to light. Hence they are responsible for vision under low levels of ambient light (scotopic vision). Cones, on the other hand, are conical-shaped structure with their inner and outer segments situated between rods segments¹¹. They are mainly located in the central retina and dominate the fovea. Cones are relatively less light sensitive than rods, so they mediate vision in higher light levels (photopic vision). Three different types of cones have been found sensitive to blue, green and red light²². They compose the fundamental basis of color discrimination.

The third type of photoreceptors, photosensitive RGC, is relatively rare²¹. They express the photopigment melanopsin which maximally absorbs violet-to-blue light. Unlike rods and cones, the photosensitive RGCs reside in the ganglion cell layer of the inner retina. They have been found responsible for non-image light-sensitive functions, such as circadian rhythm, behavior and the pupillary light reflex/reaction²³.

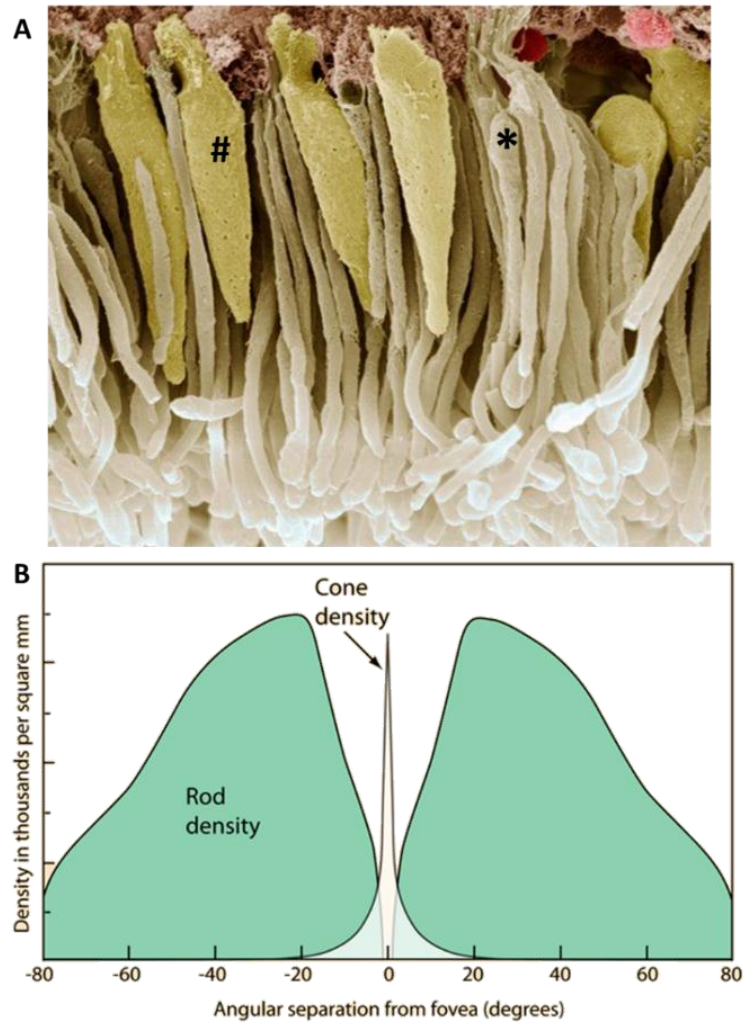


Figure 3 Scanning electron microscope (A) and density distribution (B) of human rod (*) and cone photoreceptor (#). Adapted from Science Photo Library²⁴ and Williamson S J, et al²⁵.

1.2 Retinal degenerative disease

The retinal degenerative disease features a series of retinal disorders with atrophy of retinal cells²⁶. The age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are two main types of retinal degenerative disease and among the major causes of blindness in developed countries^{27, 28}.

1.2.1 AMD

The prevalence of AMD is about 5% in population ≥ 65 years in the UK²⁹. The patients present with decreasing vision acuity, distorted vision and central scotomas. In general, AMD is classified into two forms based on clinical appearance, *e.g.* the “dry” and “wet” AMD³⁰.

The “dry” form of AMD, also known as geographic atrophy (GA) or atrophic AMD, is characterized with progressive RPE atrophy in the macula³¹. The typical yellow deposits called drusen accumulate between the RPE and choroid in the early stage. Later on, geographic hypopigmentation and/or absence of the RPE gradually develop in the macula, leading to vision loss and distortion (Figure 4). The atrophic AMD variant accounts for more than 50% of total AMD cases in the UK²⁹.

The “wet” form of AMD is also known as the neovascular or exudative AMD. It features of choroidal neovascularization through the Bruch’s membrane and retina³². Vision symptoms are mainly caused by associated complications such as bleeding, scarring, exudative RPE detachment and subsequent photoreceptor death (Figure 4). Neovascular AMD is less prevalent than the atrophic form but it leads to more legal blindness in the aged people²⁹.

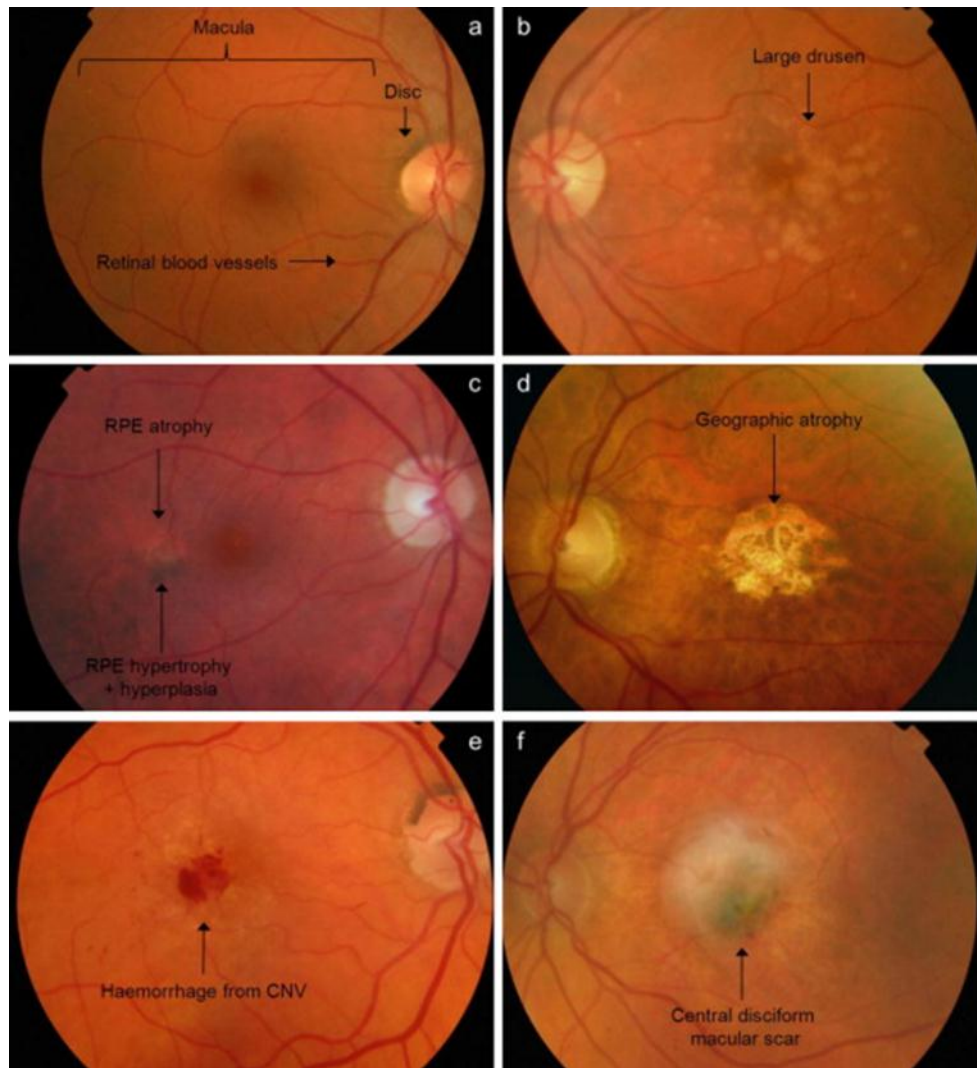


Figure 4 Images of normal fundus (a), early AMD with drusen (b), pigmentary irregularities (c), late “dry” AMD with geographic atrophy (d), and late “wet” AMD with choroidal neovascularization (e), scarring (f). Adapted from Khandhadia SV et al.³³

1.2.2 Oxidative stress in AMD

Oxidative stress is defined as an imbalance that favors the production of reactive oxygen species (ROS) over biological antioxidant defenses³⁴. ROS are chemically-reactive molecules containing oxygen and unpaired electrons, which includes O₂-derived free radicals such as superoxide anion radical (O₂⁻) and the hydroxyl radical (.OH)³⁵. As a natural byproduct of oxygen in normal cellular metabolism, ROS plays important roles in cell signaling and homeostasis. However, ROS levels can increase dramatically under certain conditions³⁶. Such cumulative ROS can cause the progressive modification or degradation of cellular biochemicals and finally lead to cell death.

Oxidative stress is believed to play an important role in AMD pathogenesis³⁷. The strongest risk factors for AMD, *e.g.* age, cigarette smoking and genetic variations, are all related to oxidative stress (Figure 5)³⁸. The human retina is constantly exposed to a strong photo-oxidative environment due to intensive light exposure, oxygen metabolism and a high concentration of polyunsaturated fatty acids³⁹. The RPE is capable of efficient defense against free radicals but gradually loses the anti-oxidant ability with age, whilst ROS accumulates³⁷. Lipofuscin, for instance, is a lipid-protein that acts as a photosensitizer and generates ROS under light irradiation. Intracellular accumulation of lipofuscin can distort cellular architecture and lead to failure of metabolic processes⁴⁰.

In animal studies, the observation and progression of AMD is linked with antioxidant deprivation. Increased oxidative damage in the RPE was found in a mouse model which superoxide dismutase was reduced by delivering ribozyme⁴¹. Their eyes exhibit degeneration of the RPE and photoreceptors, thickening of Bruch's membrane,

and elevated levels of the lipofuscin fluorophore A2E. Rapid degeneration of photoreceptors were also observed in another model with glutathione peroxidase 4 genetically ablated⁴². In human eyes, a diet poor in antioxidants and low plasma levels of antioxidants are shown to favor AMD development to some degree, suggesting the oxidative stress in AMD⁴³.

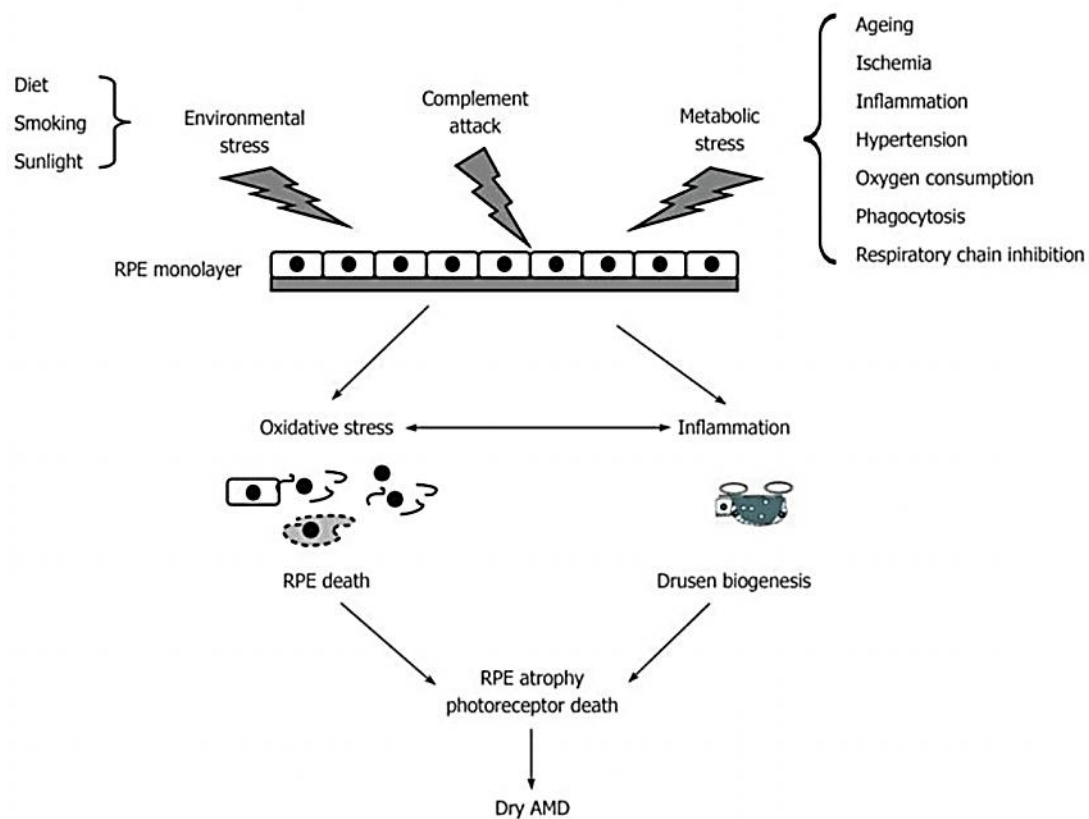


Figure 5 Schematic diagram showing conditions that contribute oxidative stress of RPE cells and subsequent etiology of dry AMD. RPE: Retinal pigment epithelial. AMD: Age-related macular degeneration. Adapted from Qin S⁴⁴.

1.2.3 Retinitis pigmentosa

Retinitis pigmentosa (RP) is the most common form of inherited retinal degeneration, with a worldwide prevalence of 1/4,000⁴⁵. It presents a heterogeneous group of inherited retinal disorders with similar clinical features. Most patients follow typical rod-cone dystrophy, a progressive loss of night vision (nyctalopia) and visual field constriction, which eventually leads to central vision loss⁴⁶. Typical fundus findings are bone spicule-shaped pigment deposits around attenuated vessels and pale optic disc. Less commonly, RP patients display cone-rod dystrophy and present with decreased visual acuity in the early stages followed by loss of peripheral vision⁴⁷. Most cases of RP result from dysfunction and/or degeneration of the RPE or photoreceptors⁴⁵.

So far, mutations have been identified in 225 genes and loci in RP⁴⁸. Based on the inheritance pattern, they can be classified into autosomal dominant (~25%), autosomal recessive (~20%) and X-linked RP (~15%)⁴⁹. Sporadic RP cases are those with no known affected relatives and account for the rest ~40% of RP.

Autosomal dominant RP

Autosomal dominant RP (adRP) describes the RP in which the phenotype is expressed in those who have inherited at least one copy of a particular gene mutation (heterozygotes) on non-sex chromosomes⁵⁰. So far mutations in 22 genes have been identified in adRP⁴⁸. The *RHO* gene, encoding rhodopsin, is the most common mutated gene in adRP. It accounts for approximately 30% of all adRP cases⁵¹. Most *RHO* mutations lead to aberrant rhodopsin protein production with amino acids changed or deleted rather than the total absence of the protein. The *RHO* Pro23His

mutation, for instance, results in the production of a misfolded protein which is subsequently degraded by the ubiquitin proteasome system⁵².

Other commonly seen mutant genes in adRP are *RDS* and *RP1*. The *RDS* gene encodes peripherin-2, a transmembrane glycoprotein in rods and cones⁵³. It may function as an adhesion molecule for stabilization of outer segment discs⁵⁴. Mutations of *RDS* cause photoreceptor outer segment mal-development and dystrophy⁵⁵. The *RP1* gene encodes a photoreceptor-specific protein, which may function as a transcription factor⁵². It may have a role in photoreceptor differentiation and the maintenance of neural retina structure. Most clinical mutations in *RP1* result in truncated protein production and subsequent photoreceptor death.

Autosomal recessive RP

Autosomal recessive RP (arRP) describes RP requiring the presence of two copies of a gene mutation at a particular locus in order to express an observable phenotype, specifically refers to genes on non-sex chromosomes⁵⁰. Mutations have been reported in many genes, such as *RPE65*, *USH2A* and *CRB1*. The *RPE65* encodes retinal pigment epithelium-specific 65 kD protein, essential for 11-*cis*-retinol generation in the visual cycle as described previously⁵⁶. Mutations in *RPE65* cause 11-*cis*-retinol deficiency and photoreceptor dysfunction. It accounts for ~2% of arRP and ~16% of Leber congenital amaurosis (LCA). The *USH2A* encodes usherin, a transmembrane protein expressed specifically in photoreceptors and cochlear hair cells⁵⁷. It is localized distal to the photoreceptor ciliary rootlet and believed to be important for maintaining structural integrity. Mutations in *USH2A* account for ~5% of arRP and cause Usher syndrome (Type II), characterized by hearing loss and retinitis pigmentosa⁵⁸. *CRB1* mutations have been found in 5% of arRP and 10% of

LCA patients. It encodes the Crumbs homolog 1 protein, localized specifically to the sub-apical region at the outer limiting membrane in the retina⁵⁹. It is required to maintain photoreceptor polarization and adhesion during light exposure⁵⁹. Other commonly seen mutant genes in arRP also include *ABCA4* encoding a photoreceptor-specific ABC transmembrane protein⁶⁰, *PDE6α* and *PDE6β* encoding the rod cGMP-specific phospho-diesterase alpha and beta subunits, respectively^{61, 62}.

X-linked RP

X-linked RP refers to the RP caused by a mutation in a gene on the X chromosome (dominant or recessive). It is a severe form of RP and accounts for ~20% of all RP cases⁶³. Patients generally exhibit early onset night blindness and progress to blindness by age 40. It primarily affects males, but female carriers can display mild phenotypes probably due to random X-inactivation.

RPGR and *RP2* are the mostly studied genes in X-linked RP. The *RPGR* gene encodes retinitis pigmentosa GTPase-regulator⁶⁴. It localizes to the transition zone and basal body of photoreceptor cilia, and functions in ciliary and microtubule-associated assembly. Most *RPGR* mutations are detected in alternatively spliced *RPGR*-ORF15 isoform, which associate with microtubule transport proteins and lead to photoreceptor dysfunction⁶⁵. The *RP2* gene encodes a protein that is structurally and functionally homologous to the tubulin-specific chaperone, cofactor C⁶³. *RP2* mutations may cause accumulation of incorrectly folded tubulin isoforms followed by progressive photoreceptor degeneration⁶⁶. Mutations in *RPGR* and *RP2* account for ~70% and ~10% of X-linked RP, respectively⁶³.

1.3 Cell death in retinal degeneration

RPE and/or photoreceptor cell death are the common pathology observed in AMD and RP. In the “dry” form of AMD, the RPE atrophy gradually progresses with aging⁶⁷. In the “wet” AMD, CNV can lead to RPE and photoreceptor death⁶⁸. Although there are various mutant genes in RP, they almost result in aberrant RPE or photoreceptor function which eventually causes photoreceptor degeneration⁶⁹.

1.3.1 Necrosis and apoptosis

Necrosis and apoptosis are the two classic types of cell death according to cellular morphological and biochemical characteristics⁷⁰. Necrosis is typically associated with cell swelling, denaturation of cytoplasmic proteins, membrane rupture and leakage of cell constituents to the extracellular space⁷¹. This cell lysis and release of intracellular content usually leads to local inflammation.

In contrast, apoptosis represents the process of cell death regulated by intracellular programs⁷⁰. It features cell morphological changes including cell shrinkage, chromatin condensation, DNA fragmentation and cellular decomposition into membrane-bound apoptotic bodies destined for phagocytosis⁷². Biochemical features include mitochondrial dysfunction and activation of apoptosis-specific proteins such as the caspases. Apoptosis serves an important role during cell differentiation and development⁷³. It can also be triggered in various pathological stresses, such as RPE and photoreceptor cell death in AMD and RP^{68, 74}.

1.3.2 Caspase in apoptosis

Caspases are a family of cysteine proteases that are critical to many cell apoptotic responses⁷⁵. Based on their roles in apoptosis, they can be divided into the initiator caspases (caspase-2, -8, -9 and -10) and the effector caspases (caspases-3, -6 and -7)⁷⁶. An initiator caspase is characterized by an extended N-terminal region, which comprises one or more adaptor domains that activate effector caspases. Depending on the caspase involvement, apoptosis can be classified into caspase-dependent and -independent pathways⁷⁶.

In the caspase-dependent apoptosis, caspases are activated by extrinsic or intrinsic pathways⁷⁷. The cell death receptors on cell membranes usually trigger the extrinsic pathway. The Fas receptor, for instance, activates initiator caspase-8 when binding with ligands. Activation of caspase-8 cleaves by proteolysis the downstream inactive pro-forms of effector caspase-3, which eventually leads to DNA fragmentation and chromatin condensation⁷⁸. In the intrinsic pathway, the mitochondria release apoptotic protein such as cytochrome-c in response to apoptotic stimuli⁷⁵. Cytochrome-c binds to apoptotic protease activating factor 1 and activates the initiator caspase-9, which subsequently activates caspase-3 and results in cell demise (Figure 6). Other proteins have been found in regulating the caspase-dependent apoptosis. The inhibitor of apoptosis proteins (IAPs), such as X-linked inhibitor of apoptosis (XIAP), can interact with caspases and inhibit cell death⁷⁹. SMAC/DIABLO and HTRA2/OMI are both mitochondrial-located proteins. They can react with the IAPs and therefore promote apoptosis (Figure 6)^{80, 81}.

In contrast, the caspase-independent apoptosis is executed by other proteins instead of caspases. Apoptosis-inducing factor (AIF) is a pro-apoptotic protein localized in mitochondria⁸². It is released to cytoplasm and translocated to the nucleus

in response to cell death stimuli to trigger chromatin condensation independent of caspases (Figure 6). Endonuclease G is an apoptotic DNase released from mitochondria. It can cleave chromatin DNA into nucleosomal fragments independently of caspases⁸³. Calpains are calcium-dependent neutral proteases which normally reside in the cytosol⁸⁴. Activated calpains can induce release of lysosomal cathepsins and subsequent leads to cell death⁸⁵. Cathepsins are a family of proteases mainly found in lysosomes. Cathepsin D has been found to initiate Bax activation and release AIF from the mitochondria, eventually cause cell death⁸⁶.

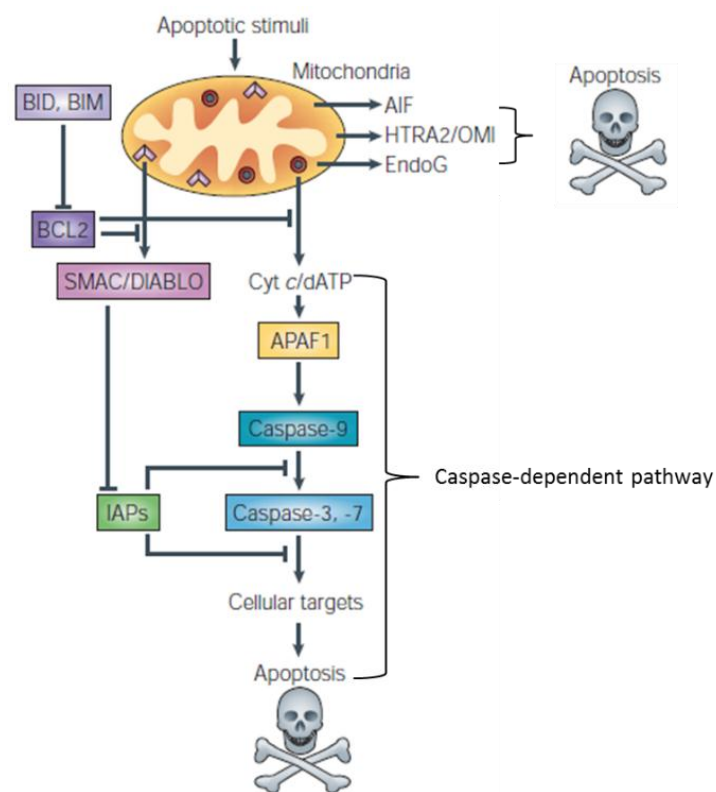


Figure 6 Schematic graph showing the caspase-dependent and -independent apoptosis. AIF: apoptosis-inducing factor. EndoG: endonuclease G. Cyt c: cytochrome-c. dATP: deoxyadenosine triphosphate. APAF1: apoptotic protease activating factor 1. IAPs: inhibitor of apoptosis proteins. Bcl-2: B-cell lymphoma 2. Adapted from Riedl SJ and Shi Y⁷⁵.

1.3.3 Apoptosis in RP and AMD

Photoreceptor death is commonly seen in most types of RP, although they represent a heterogeneous group of affected genes. In the PDE- β 6 mutant *rd* mouse of retinal degeneration, photoreceptor apoptosis was confirmed by DNA fragmentation⁸⁷. Apoptosis in photoreceptors has also been observed in other mouse models of retinal degeneration: peripherin-2 mutation (the *rd*s mouse)⁶⁹, *RHO* mutation⁸⁸, arrestin/rhodopsin kinase-deficiency⁸⁹, *Rpe65* knock-out⁹⁰ and a canine model with *RPGR* ORF15 mutation⁹¹.

It is, however, still in doubt whether caspase-dependent apoptosis is the main form of photoreceptor death in RP. Activation of caspase-3 has been found in *rd* mice⁹²⁻⁹⁴ and the *RHO* mutant rat (s334ter)⁹⁵. Using pan-caspase inhibitors such as Z-VAD-FMK or XIAP, the retinal ONL was preserved in the *rd* mouse⁹⁶, *RHO* mutant rat⁹⁷ and MNU (N-methyl-N-nitrosourea)-induced retinal degeneration rat⁹⁸. All these indicate that caspase-dependent apoptosis is the major form of photoreceptor death in RP. However, the caspase-independent pathway was also reported in some RP models. In *rd* mice, the photoreceptor apoptosis is also reported to be mediated by calpain and AIF^{84, 99}. Activation of calpain and poly(ADP-ribose) polymerase (PARP) were found associated with photoreceptor death in the P23H and S334ter *RHO* mutant rats^{100, 101}. A calpain inhibitor, SNJ-1945, suppressed the MNU-induced photoreceptor cell death in mice¹⁰².

Studies of photoreceptor death are relatively limited in AMD due to the less available animal models and samples. In a post-mortem study, maculas were carefully

examined in 10 AMD patients and 9 normal eye donors¹⁰³. The AMD maculas had significantly more apoptotic cells in the RPE, outer and inner nuclear layers determined by TUNEL assay. In addition, most of the apoptotic RPE and photoreceptor cells appeared near the edge of GA areas. Another study also observed apoptotic RPE cells in the surgical excised CNV membranes from AMD patients¹⁰⁴. A2E was reported to induce apoptosis in primary cultured RPE cells at concentrations found in human retinas¹⁰⁵. Such apoptosis was accompanied by a rise of cytochrome-c and AIF, suggesting both caspase-dependent and -independent pathway may be involved.

1.4 Gene therapy for retinal degeneration

In general, gene therapy represents the treatment involving insertion, alteration, or removal of DNA/RNA oligonucleotides¹⁰⁶. The retina has many unique features as an advantageous target for gene therapy¹⁰⁷. The human eye is small and highly compartmentalized, which enables efficient delivery of vectors. The retina-blood barrier and its immune privilege prevent severe immune systematic responses and inflammation against vectors, whilst minimizing the side effects. Retinal neurons and/or RPE cells can be successfully transduced by current vectors. In addition, techniques such as intraocular and subretinal injections have been well established in clinic practice for vector delivery. Non-invasive imaging and functional examinations, such as confocal scanning laser ophthalmoscopy (cSLO) and electroretinography (ERG), are suitable for assessment of gene therapy. Most importantly, the molecular understanding of retinal degeneration has rapidly developed in recent years. All these make the retina an ideal target for gene therapy.

1.4.1 Vector

A vector is the vehicle to deliver external DNA into the host cell in gene therapy. It can be divided into viral and non-viral vectors¹⁰⁷. Non-viral vectors stand for naked DNA or synthetic oligonucleotides which are delivered by injection, electroporation or agent-mediated transfection (lipoplexes, polyplexes and magnetofection, *et al*). They are mainly used in non-clinical research due to their low transfection efficiency.

In contrast, viral vectors advantage high level of transduction in many cell types¹⁰⁸. Transduction is the abortive infection that introduces functional genetic information expressed from the vectors into the target cell¹⁰⁹. The viral life consists of two distinct phrases, infection and replication. The infection results in the integration of the viral genome and the target cell, which enables the replication phrase characterized by viral regulated gene expression¹⁰⁹. The viral genome comprises viral coding sequences and *cis*-acting gene regulatory sequences. In the case of gene therapy, the viral coding genes are replaced with a therapeutic expression cassette to generate the recombinant viral vector (Figure 7). The *cis*-acting sequences, generally separated into distinct plasmids, are introduced to the viral gene to reconstitute into productive viral by recombination^{109, 110}. The vector concentration is measured as a titer expressed as the concentration of physical particles, transfective particles or the viral genome¹¹¹.

So far there have been many viral vectors developed for gene delivery. The most studied are adenovirus, adeno-associated virus (AAV), lentivirus and herpes

virus¹¹²⁻¹¹⁵. Each type features an inherent set of properties that affect its suitability for gene therapy applications, including the packaging capability, tissue specificity, host immune responses and integrating ability into the host cell genome. Here we mainly focus on AAV.

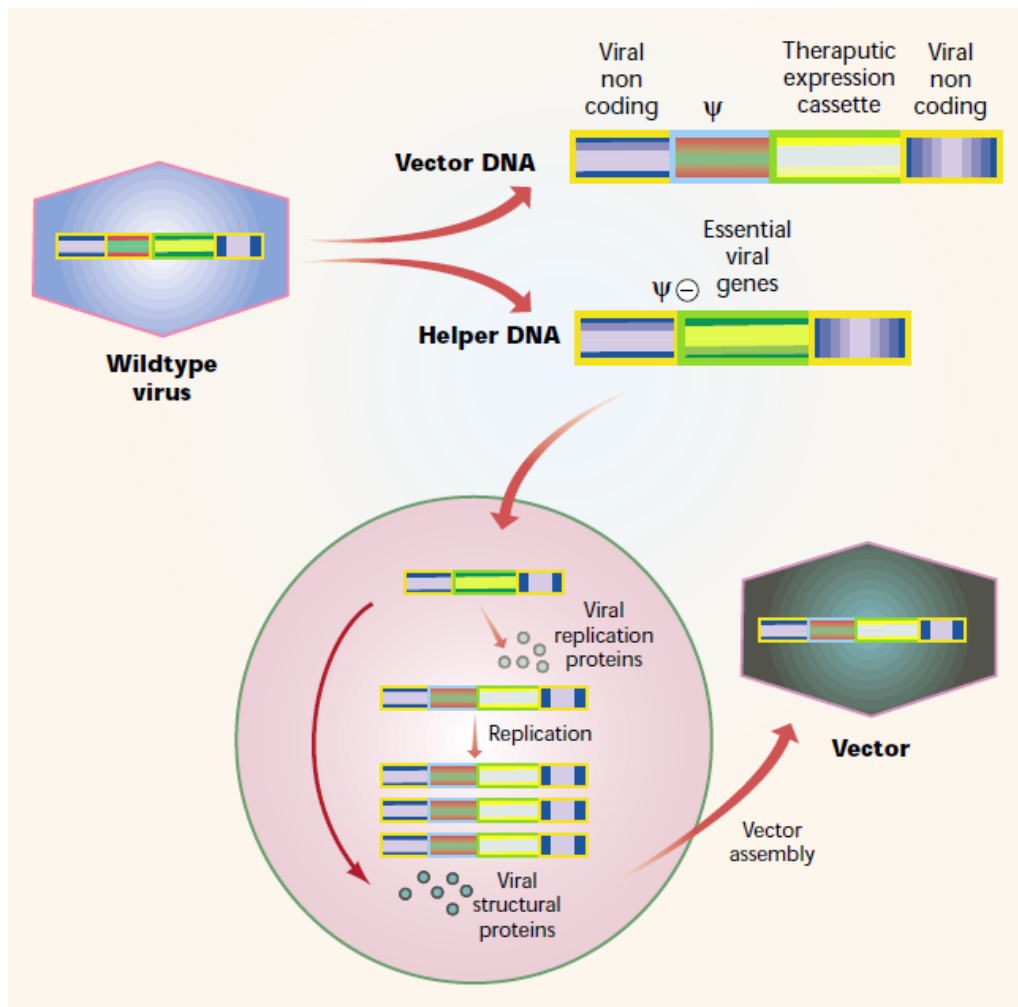


Figure 7 Engineering the viral vector. The vector DNA contains the packaging domain (ψ) and therapeutic expression cassette. The helper DNA contains genes essential for viral replication. The viral proteins required for replication of the vector DNA and viral structure are produced, leading to packaging of the vector genome into a particle. Adapted from Kay, MA, Glorioso, JC, et al¹⁰⁹.

1.4.2 AAV

The wild type AAV life form belongs to human parvovirus that requires a helper virus (such as adenovirus) to mediate replication and spread¹¹⁶. Each AAV consists of the protein capsid and a single strand DNA genome. This genome contains the *rep* and *cap* genes, encoding 4 non-structural proteins essential for replication and 3 structural proteins comprising the viral capsid, respectively. The two genes are flanked by inverted terminal repeats (ITRs), which function as origins of replication¹¹⁶.

So far, at least 12 serotypes of AAV have been reported¹¹⁷. They vary in capsid structure and different tropic properties in retinal cell transduction. Administered by subretinal injection, AAV2 results in transduction of both photoreceptors and RPE cells with a peak of transgene expression at 4 weeks¹¹⁸. In contrast, subretinal delivery of AAV1 and AAV4 mainly infects the RPE¹¹⁹. AAV5 and AAV8 present robust expression in photoreceptor cells whilst exhibiting more rapid expression than AAV2¹²⁰. By intravitreal injection in adult rats, recombinant AAV (rAAV) 1, 2, 3, 4, 5, 6 and 8 all transduced RGCs¹²¹. rAAV6 exhibited more transduction in amacrine or bipolar cells than others, whilst the highest photoreceptor transduction was seen after intravitreal rAAV3 injection¹²¹. Recently, point mutations were developed in surface exposed tyrosine residues in AAV2 and AAV8 capsids (Y444F and Y733F)¹²². They showed an impressive transduction and transgene expressions in the RPE and photoreceptor cells following intravitreal injection, providing an ideal approach for retinal gene therapy.

1.4.3 AAV mediated gene therapy

The rAAV vector has many unique features for retinal gene therapy^{120, 123}. No pathology has been known to humans after AAV transfection. Only a mild immune response is found after AAV administration. rAAV is able to transduce non-dividing retinal cells such as photoreceptor cells and the RPE *via* subretinal injection. In addition, rAAV reduces the potential risk of insertional mutagenesis due to the lack of integration ability into human chromosomes.

Supplementary gene therapy is probably the most studied strategy for retinal degeneration treatment. The basis is to introduce an external functional gene into the host cell¹²⁴. This is ideal for autosomal recessive disorders caused by single-gene mutations. Even in homozygous *rd1* mice with rapid retinal degeneration due to a recessive nonsense mutation of *Pde6b*, subretinal injection of AAV-*Pde6b* may have some effect of preserving photoreceptor morphology and ERG function¹²⁵. AAV mediated long-term *RPE65* expression has been found in various animal models of mouse, rat, dog and non-human primate¹²⁶⁻¹²⁹. The ERG was also improved after AAV-*RPE65* subretinal delivery¹²⁹. In a naturally occurring *RPE65*^{-/-} dog model, AAV2-*RPE65* injection restored 11-*cis*-retinal supply and improved ERG and behavioral vision¹²⁷. AAV safety was well confirmed in non-human primates by clinical examinations, electroretinography, and retinal histopathology¹²⁶.

The robust outcomes of *RPE65* gene therapy in animals eventually lead to 3 separate human clinical trials of LCA¹³⁰⁻¹³². In these trials, recombinant AAV2 vectors carrying human *RPE65* cDNA was subretinally injected in a total of 9 patients. Despite differences in promoter and vector amount delivered, there were no severe complications caused by AAV treatment. In addition, participants reported improved visual sensitivity of the treated eye in dim light. The improvements were further

confirmed by objective examinations (microperimetry, dark-adapted perimetry, pupillometry and dark-adapted full-field sensitivity test) as well as functional test (visual mobility and obstacle navigation). A long-term observation further confirmed the safety and efficiency of AAV mediated gene therapy for LCA¹³³.

Another Phase 3 clinical trial is currently undergoing in Oxford targeting choroideremia. It is an X-linked recessive retinal disorder featuring progressive degeneration of the RPE, photoreceptors and choroicapillaris. Mutations of the *REP-1* gene, which encodes Rab Escort Protein-1, are found to cause choroideremia¹³⁴. Viral delivery of human *REP-1* cDNA to defective cells from choroideremia patients was found to produce the functional protein¹³⁵. In a mouse model of choroideremia, *REP-1* gene therapy rescues the functional molecular defect (prenylation) in the RPE for > 6 months¹³⁶. The ongoing trial will further examine the safety and efficiency of gene therapy in retinal degeneration treatment.

For autosomal dominant RP, a common strategy is to suppress the mutant gene and replace it with modified functional gene¹³⁷. Ribozyme and RNA interference methods have been developed to inhibit mutant gene expression. A ribozyme designed to target rhodopsin mRNA was able to reduce rod opsin protein by 80% in *Rho*^{+/-} mice and 50% in wild-type mice after AAV delivery¹³⁸. AAV5 delivered ribozyme reduced 46% of *RHO* mRNA expression in rat¹³⁹. Subretinal delivery of AAV5 carrying siRNA led to 60% decrease of rhodopsin protein in mice¹⁴⁰. The preferential silencing of the mutant allele alone (P23H) did not prevent retinal degeneration in the rhodopsin mutant rat¹⁴¹. An accepted theory for this is that additional copies of the functional allele may be required apart from the suppression

of the mutant allele. Such “suppress and replace” approach was found to preserve photoreceptors in a dominant mouse model with *RHO* mutation^{142, 143}.

In X-linked RP, AAV mediated gene therapy is found to rescue photoreceptors in dogs with *RPGR* mutations. AAV5-mediated *RPGR* treatment, driven by human *IRBP* or *GRK1* promoters, preserves photoreceptors determined by *in vivo* imaging, ERGs and histopathology¹⁴⁴.

The major limitation of AAV is its packaging capacity <5 kb¹⁴⁵. Some larger but commonly seen RP genes are difficult to package into rAAV, such as *ABCA4* (~7.3 kb) and *USH1B* (~6.6 kb)^{146, 147}. Recently, some studies showed that efficient transgene expression was found in AAV containing larger DNA (up to 8.9 kb)^{148, 149}. It was assumed that the partially packaged transgene may recombine in transduced cells. Such results indicate the AAV-mediated supplementary gene therapy may be possible for those RP variants caused by relatively large genes.

1.5 Neuroprotection therapy for retinal degeneration

In general, neuroprotection refers to the strategy to protect neurons against dysfunction and degeneration following acute injury or in chronic degenerative diseases. In this section, three major types of neuroprotective agents are reviewed, including antioxidants, neurotrophic factors and anti-apoptotic proteins.

1.5.1 Antioxidants

Antioxidants are chemicals which are able to react with free radicals and inhibit oxidative cell apoptosis. Clinical studies have shown that the combination of

high-dose antioxidant vitamins and zinc can reduce the risk of developing advanced AMD by 34% in people at high risk of developing advanced AMD¹⁵⁰. Photoreceptors in RP models are also found to benefit from antioxidant treatment, such as vitamin A¹⁵¹, melatonin¹⁵², N-acetylcysteine¹⁵³, nilvadipine¹⁵⁴, bilirubin and tauroursodeoxycholic acid¹⁵⁵. Vitamin A supplement therapy, for example, slows the rate of photoreceptor degeneration in a rhodopsin mutant mouse model of RP¹⁵¹. A beneficial effect of 15,000 IU/d vitamin A intake was reported in a randomized trial with 601 RP patients¹⁵⁶.

Studies also revealed that a combination of antioxidants may be more beneficial for photoreceptors than the individual¹⁵⁷. In *rd1* mice, daily injections of a mixture of 4 antioxidants (alpha-tocopherol, ascorbic acid, Mn-tetrakis porphyrin, and alpha-lipoic acid) markedly reduced oxidative damage of cones¹⁵⁸. This was accompanied by increasing cone density, opsin mRNA expression and preservation of cone function. Preservation in rods and cones was also observed in the *rd10* mouse and the rhodopsin mutant strain (Q344ter) when they were treated with the same components¹⁵⁹.

Apart from chemicals, antioxidant enzymes or nanoparticles are also reported neuroprotective in RP models. Cerium oxide nanoparticles, a material able to scavenge reactive oxygen intermediates, were reported to prevent vision loss in a rat model of light-induced photoreceptor degeneration¹⁶⁰. Overexpression of glutathione peroxidase 4, an antioxidant enzyme, confers a strong protection of retinal structure and ERG function in three different models of oxidative damage-induced retinal degeneration¹⁶¹. Co-expression of two antioxidant enzymes, the superoxide dismutase

2 and catalase, was found to reduce cone cell death in two mouse models of RP, the *rd1* and *rd10* mouse¹⁶².

1.5.2 Neurotrophic factors

Neurotrophic factors are a family of proteins that are essential for neuron development, maintenance and survival¹⁶³. The most studied neurotrophic factors probably are brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF). Intraocular administration of BDNF has been found protective to photoreceptors in several animal models including the RCS rat model of inherited retinal dystrophy¹⁶⁴, Usher syndrome¹⁶⁵ and rhodopsin mutant mice¹⁶⁶. BDNF acts through TrkB, a receptor with kinase activity expressed in cone photoreceptors and retinal ganglion cells¹⁶⁷. TrkB is found essential for rod, RPE and inner retina development so may have a role in retinal degeneration¹⁶⁸⁻¹⁷⁰.

GDNF is a highly conserved neurotrophic factor first reported to enhance survival of midbrain dopaminergic neurons¹⁷¹. It was found able to reduce photoreceptor death and augment opsin expression in rat retina neuronal cultures *in vitro*¹⁷². GDNF also preserves rods in the *rd1* and *rd2* mouse²²⁶¹⁷³, RCS and S334ter *RHO* mutant rat^{173, 174}. Its protection may be mediated by activation of Müller glial cells^{175, 176}.

CNTF is another neurotrophic factor which has been found to retard photoreceptors degeneration in *rd1* and *RHO* mutant mouse models¹⁷⁷. It can sustain a long-term photoreceptor survival in an autosomal dominant feline model of rod-cone dystrophy (*Rdy cat*)¹⁷⁸. CNTF is found more effective than GDNF for cone

preservation in culturing retinal explant from *RHO* knockout mice¹⁷⁹. In a recent clinical trial, there was some limited evidence that it could slow the progression of vision loss in atrophic AMD patients¹⁸⁰. Its neuroprotection may be mediated through a heterotrimeric complex consisting of CNTF receptor alpha, gp130 and leukemia inhibitory factor receptor beta, which signals the downstream JAK/STAT pathways and inhibit apoptosis¹⁸⁰.

1.5.3 Anti-apoptotic proteins

Anti-apoptotic proteins are those that inhibit cell death by suppression/interfering with the apoptosis pathway. The most studied anti-apoptotic proteins are B-cell lymphoma 2 (Bcl-2) and the inhibitors of apoptosis proteins (IAPs).

Bcl-2

Bcl-2 is known as a potent inhibitor of apoptosis and able to promote neuron survival during cell stress such as growth factor withdrawal and free radical peroxidation¹⁸¹. Down-regulation of bcl-2 is found in cultured retinal photoreceptors under acute oxidative stress and MNU-induced photoreceptor degeneration rats^{182, 183}. Bcl-2 overexpression can protect photoreceptors against light induced oxidative stress¹⁸⁴. It also protects human RPE cells from mitochondrial respiratory dysfunction, mitochondrial DNA damage and promotes cellular survival in oxidative stress induced by H₂O₂¹⁸⁵. Transgenic mice that overexpress bcl-2 in photoreceptors (driven by *rhodopsin* promoter) showed increasing survival of photoreceptors when crossed with various retinal degeneration mouse models (*rd1*, *rds*, *Rho*, and *Pde* mutants) or subjected to light-damage^{181, 186-188}. Adenoviral delivery of bcl-2 also delays photoreceptor cell death in the *rd1* mouse¹⁸⁹.

There are, however, some controversial studies regarding bcl-2 neuroprotection in retinal degeneration. Bcl-2 was found unable to preserve photoreceptors in RCS rats¹⁹⁰. It was reported that bcl-2 overexpression may lead to an increasing level of α -crystallin, a protein associated with cellular stress, thus promoting photoreceptor death¹⁹¹. In addition, bcl-2 directed by the IRBP promoter was found to cause bipolar cell loss¹⁹².

IAP

The inhibitors of apoptosis protein (IAP) is a family of proteins containing baculovirus IAP repeat (BIR) domains which have been found to inhibit caspases¹⁹³. The BIR domain is a zinc-binding fold of approximately 70 amino acid residues. They are classified into type I and type II type based on the presence of a deep peptide-binding groove. Type I BIRs lack this groove and mediate binding to TNFR-associated factor and transforming growth factor-activated kinase binding protein¹⁹⁴. Type II BIR domains contain this groove and predominantly associate with proteins carrying an IAP-binding motif (IBM)¹⁹⁵.

So far 8 IAPs have been identified in human cells. They are XIAP, neuronal inhibitory protein (NAIP), cellular-IAP1 (c-IAP1), c-IAP2, IAP-like protein-2 (ILP-2), melanoma inhibitor of apoptosis protein (ML-IAP), survivin and BIR-containing ubiquitin conjugating enzyme (BRUCE)¹⁹⁶. Among them, XIAP is known as the strongest IAP. It is a 57-kDa protein that is ubiquitously expressed in all human tissues. It consists of three BIR domains and a RING-finger domain. The BIR2 is able to bind motifs of caspase-3 and -7, whilst the BIR3 can bind caspase-9^{197, 198}. The RING domain utilizes E3 ubiquitin ligase activity and enables to catalyze ubiquitination of itself, caspase-3 and caspase-7 as markers for degradation via

proteasome activity¹⁹⁹. XIAP is the only IAP that interacts with both initiator and executor caspases (Figure 8)²⁰⁰. Meanwhile, XIAP activity can be inhibited by mitochondrial releasing proteins including SMAC/DIABLO and HTRA2/Omi^{195, 201}.

XIAP has been found to attenuate neuron death in the mouse models of transient middle cerebral artery ischemia²⁰², seizure²⁰³, neonatal hypoxia-ischemia²⁰⁴ and noise-induced hearing loss²⁰⁵. Growing evidence also shows viral delivery of XIAP can protect photoreceptors, inner retinal neurons or retinal ganglion cells in various retinal degeneration models^{97, 206-213}. Structural and functional rescue of photoreceptors is observed in N-methyl-N-nitrosourea-induced retinal degeneration²¹⁰, P23H and S334ter rhodopsin mutant rat⁹⁷ and retinal detachment²⁰⁶. Adenovirus mediated XIAP overexpression rescue retinal ganglion cells in rats with optic nerve axotomy^{211, 213}. When delivered *via* AAV with a ubiquitous chicken beta-actin (CBA) promoter, XIAP significantly preserves the optic nerve damage in a rat model of glaucoma²¹². Determined by retinal histology and the TUNEL assay, AAV-mediated XIAP treatment preserved retinal inner nuclear layer in rats with transient retinal ischemia²⁰⁸. In addition, XIAP is found beneficial in combination with other gene therapy. Early administration of AAV-XIAP can strengthen the effect of AAV5-PDE6 β treatment in the *rd10* mouse²¹⁴.

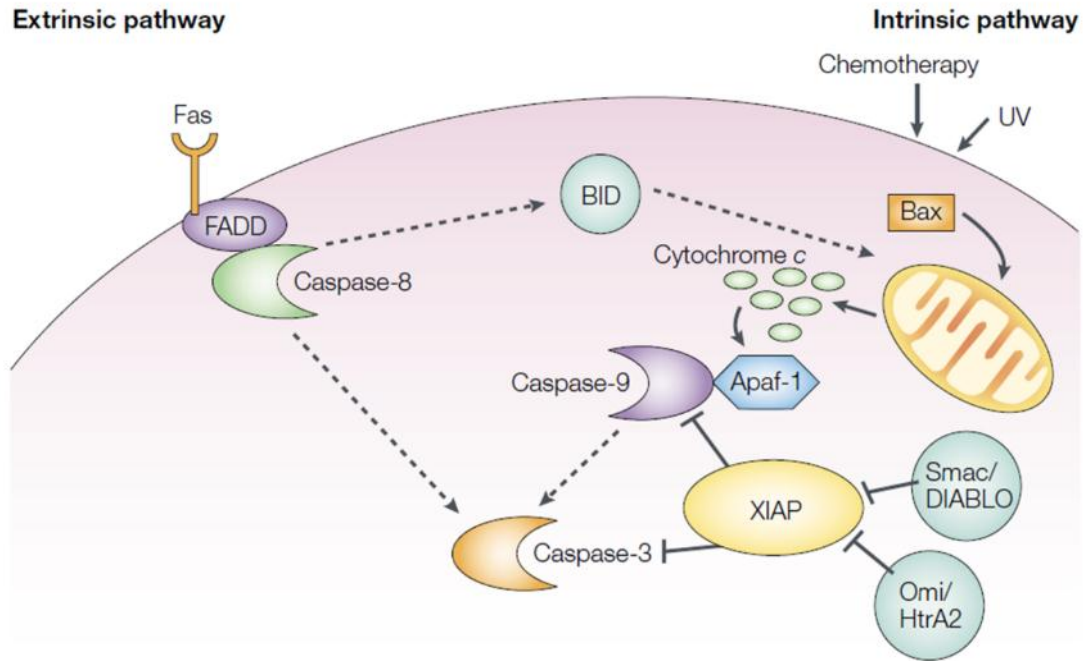


Figure 8 Schematic graph showing XIAP interaction with caspase-3 and -9 in the intrinsic and extrinsic cell-death pathways. FADD: Fas-associated death domain. Adapted from Salvesen GS and Duckett CS²¹⁵.

1.6 Aim of thesis

XIAP is likely a neuroprotective candidate in future gene therapy on retinal degenerative disease. However, studies of AAV-mediated XIAP effect on the RPE and photoreceptor cells are very limited. The aim of this thesis is to investigate AAV-mediated XIAP treatment in several retinal degeneration models, specifically:

- (1) To assess the XIAP effect on human RPE cells in an *ex vivo* oxidative stress model induced by H_2O_2 .
- (2) To investigate the XIAP effect on rod and cone photoreceptors in sodium iodate ($NaIO_3$) induced retinopathy in two transgenic strains of mouse, the *NRL-GFP* and *B6^{TGPN1LW-EGFP}* mouse.
- (3) To investigate the XIAP effect on cone photoreceptors in a mouse model with *RHO* mutation and GFP expressing cones, the *Rho*^{-/-} *B6^{TGPN1LW-EGFP}* mouse.

Chapter 2 Materials and Methods

2.1 Cell Culture

The human RPE-derived cell line (ARPE-19) and the HEK293 cell line were obtained with kind help of Neville Osborne at University of Oxford. The cells were maintained in DMEM: F12 and DMEM, respectively. The culture medium contained 10% fetal bovine serum, 1% L-glutamate, 100 U/mL penicillin G potassium and 0.1 mg/mL streptomycin sulphate (all from Sigma-Aldrich, UK). Cells were maintained in T75 culture flasks in humidified atmosphere of 5% CO₂ at 37 °C.

For sub-culturing, culture medium was removed and briefly rinsed with 0.05% trypsin-0.53 mM EDTA solution once followed by incubation with 5 ml trypsin-EDTA at 37 °C until cells were dispersed (3-10 min). Cells were added 5ml of complete growth medium and centrifuged at 125 g for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in growth medium and transferred to new culture vessels.

2.2 H₂O₂ stress

Fresh H₂O₂ was prepared by dissolving 30% hydrogen peroxide (Sigma-Aldrich, UK) in DMEM-F12 medium. Culture medium was removed and H₂O₂-conditioned medium was added to the APRE-19 cells in 96-well plate (100 ul/well). The cells were incubated at 37 °C for 1 hour and carefully rinsed with 200 ul sterile PBS twice, followed by replacement with 100 ul normal growth medium. The cells were cultured for an additional 24 hours before further MTT and TUNEL assays.

2.3 Cell viability measurement

2.3.1 MTT Assay

The MTT assay is a widely used colorimetric assay for measuring viable cells based on the activity of mitochondrial reductase that reduces yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, UK) to purple formazan as described previously²¹⁶. In brief, solution of MTT (0.5 mg/ml dissolved in DMEM-F12) was added to the ARPE-19 cells of 96-well plate (100 μ l/well). The cells were incubated for 2 hour at 37 °C. The media was discarded and 100 μ l of dimethyl sulphoxide was added, followed by 5 min of gentle agitation. The density of purple formazan was measured using a microplate photometer with a 570 nm test wavelength (Multiskan Ascent, Thermo Scientific). (Figure 9)

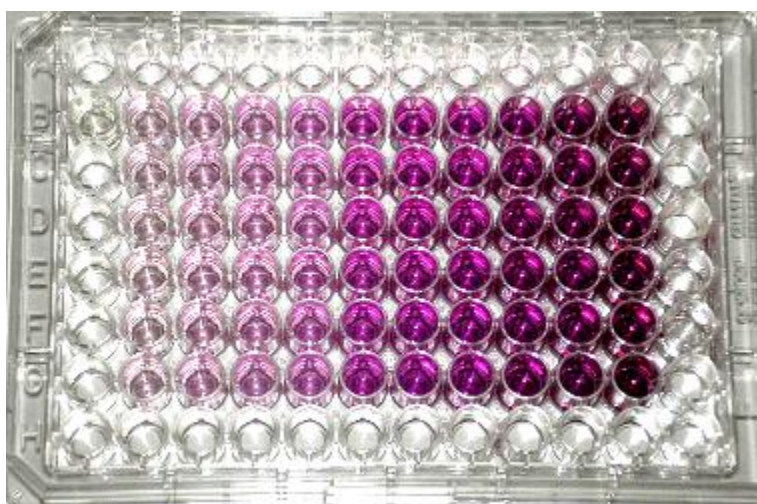


Figure 9 MTT assay showing purple formazan formed after cells were incubated with MTT followed. The gradient color indicates different mitochondrial function due to various H_2O_2 stress.

2.3.2 TUNEL Assay

The terminal deoxynucleotidyl-transferase dUTP nick-end labelling (TUNEL) assay is a common method for detecting DNA fragmentation that results from apoptosis²¹⁷. The assay identifies the presence of DNA nicks by terminal deoxynucleotidyl transferase (TdT). The TdT catalyzes the DNA nick ends with additional dUTPs which are labeled with a detectable marker. A commercial TUNEL kit was used according to the manufacturer's protocol (Roche, UK). Fixed and permeabilized cells was incubated with DNase I recombinant (5000 U/ml in 50 mM Tris-HCl, pH7.5, 1 mg/ml BSA, Sigma-Aldrich, UK) for 10 min at room temperature to induce DNA strand breakset up as a positive control. Apoptotic cells were identified under an inverted fluorescence microscope with an excitation wavelength of 560 nm (Leica, UK). (Figure 10)

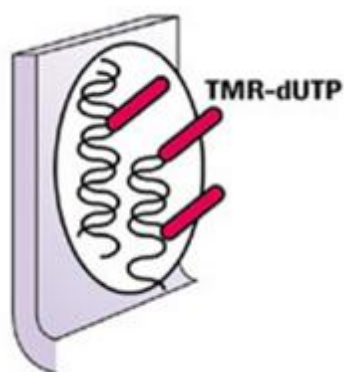


Figure 10 Schematic graph showing the principal of TUNEL assay. Adapted from the Roche Applied Science website (<http://www.roche-applied-science.com>).

2.3.3 ROS Measurement

Intracellular reactive oxygen species (ROS) was measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, UK) as previously reported²¹⁸. DCFH-DA enters cells and is deacetylated to 2',7'-dichlorofluorescein, which reacts with ROS to form the fluorescent product dichlorofluorescein (DCF). Briefly, DCFH-DA dissolved in DMEF-F12 was added to 96-well plate at a final concentration of 5 μ M (100 μ l/well). The cells were incubated for 30 min at 37 $^{\circ}$ C, followed by 8 wells stressed with 0.125, 0.25, 0.5 and 1 mM H_2O_2 as described above. 8 wells of cells were used as the non-stressed control. The fluorescence intensity was read immediately after 1 hour H_2O_2 stress using spectrofluorometer with excitation and emission settings of 485 and 530 nm (Fluoroskan Ascent, Thermo Scientific). (Figure 11)

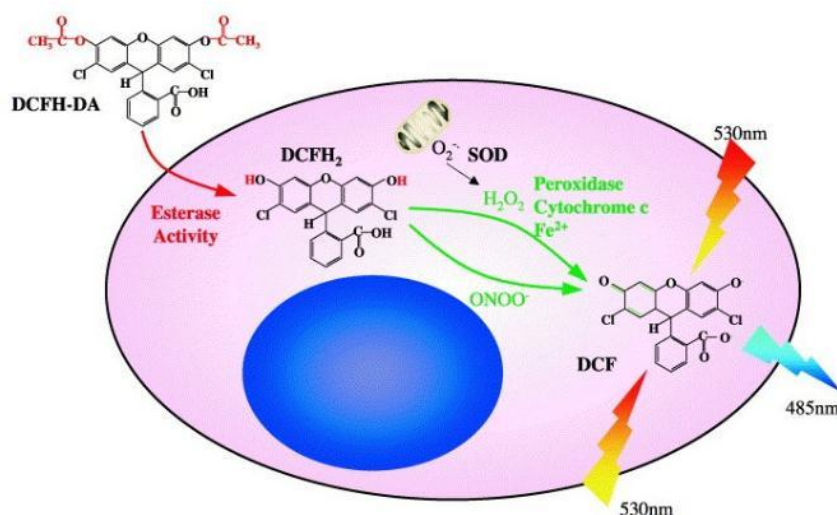


Figure 11 Schematic graph showing the principal of DCFH-DA assay. Adapted from Curtin JF et al²¹⁹.

2.4 Immunostaining

2.4.1 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, followed by 10 min incubation in PBS containing 0.25% Triton X-100 for permeabilization. Non-specific binding of antibodies was blocked by 5% BSA in PBST for 30 min at room temperature. Primary antibodies were diluted in PBST containing 1% BSA and incubated overnight at 4 °C (~ 16 hours). Appropriate secondary antibodies, diluted in 1% BSA-PBST, were incubated for 1 hour at room temperature. The cells were gently washed 3 times with PBS followed by incubation with DAPI solution (0.1 ug/ml in PBS, Sigma-Aldrich, UK) for 5 min. Samples were examined under a fluorescence microscopy (Leica, UK) with appropriate laser source according to the secondary antibodies.

2.4.2 Immunohistochemistry

Slides with eye sections were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by 10 min incubation in PBS containing 0.25% Triton X-100 for permeabilization. Non-specific binding of antibodies was blocked by 5% BSA in PBST for 30 min at room temperature. Primary antibodies were diluted in PBST containing 1% BSA and incubated overnight at 4 °C (~ 16 hours). Appropriate secondary antibodies, diluted in 1% BSA-PBST, were incubated for 1 hour at room temperature. The slides were gently washed 3 times with PBS followed by incubation with DAPI solution (0.1 ug/ml in PBS, Sigma-Aldrich, UK) for 5 min. The slides were mounted with antifade reagent (Invitrogen, UK) and sealed with nail polish oil.

Samples examined under a fluorescence microscopy (Leica, UK) with appropriate laser source according to the secondary antibodies.

2.4.3 Western Blot

The Western blot is a widely used technique to detect specific proteins in cells or tissues²²⁰. Briefly, proteins are extracted from samples using lysis buffer and denatured. Gel electrophoresis is performed to separate native proteins by the length of the polypeptide. The proteins are then transferred to a membrane which is stained with primary antibodies specific to the target protein. Secondary antibodies are applied and associated detection is developed.

Sample preparation: Cells were washed with ice cold PBS and harvested by RIPA Lysis Buffer (Millipore, UK) with freshly mixed proteinase inhibitors (Roche Applied Science, UK), containing 0.5M Tris-HCl (pH 7.4), 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA, 1 mM PMSF, 1 g/mL aprotinin, 1 g/mL leupeptin, 1 g/mL pepstatin, 1 mM Na₃VO₄ and 1 mM NaF. The lysate was transferred into 1.5 ml Eppendorf tubes and ultrasonically homogenized for 5 seconds. The samples were centrifuged at 14,000g for 15 minutes at 4 °C. The supernatant were immediately collected in a fresh tube and stored at -20° C. Protein concentrations were determined at 280 nm using a UV-spectrophotometer (Thermo Scientific, Germany).

For tissues, mice eyes were dissected with clean forceps and placed in pre-chilled microfuge tubes with dry ice. RIPA lysis buffer with freshly mixed protease inhibitors was added in the tube (300 ul/eye). Eyes were mashed with a clean pestle and ultrasonically homogenized for 10s in ice-cold lysis buffer. The samples were

centrifuged and the supernatants were collected and stored at -20°C as described above.

Electrophoresis and transfer: Samples were denatured by mixing with 2X Laemmli buffer (Sigma-Aldrich, UK) and were boiled at 95°C for 5 min before loading into a SDS-PAGE gel (BioRad, UK, 20-40 ug proteins per lane). A molecular weight marker was loaded each time (Thermo Scientific, UK). Electrophoresis was performed in a standard 1X Tris-glycine buffer under 200 constant voltages for ~ 1 hour when the blue dye nearly reached the bottom. The gel was immediately transferred to a nitrocellulose membrane in pre-chilled transfer buffer (1X Tris-glycine with 20% methanol) with 100 constant voltages for ~ 1 hour.

Antibody incubation and development: The transferred membrane was blocked with freshly prepared 5% BSA in TBST for 1 hour at room temperature, followed by primary antibody incubation overnight at 4°C (~ 16 hours). The membrane was washed 3 times with PBST and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature. Finally the membrane was washed again 3 times with PBST followed by enhanced chemiluminescence (ECL) development according to the manufacturer's protocol (Thermo Scientific Pierce, UK).

Quantification: The developed membrane was scanned and imported in ImageJ developed by Wayne Rasband (National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). The intensity of protein bands was measured and analyzed as previously described by Luke Miller (<http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html>). See Table 1 for the antibodies used in immunostaining.

Table 1 Overview of the antibodies used in the immunostaining.

Name	Source	Type	Host	Reactivity	Application
XIAP antibody	Abcam	primary	rabbit	mouse, human	WB (1:1000), ICC/IHC (1:200)
DDK antibody	Origene	primary	mouse	n/a	WB (1:1000) ICC/IHC (1:500)
β -actin antibody	Sigma- Aldrich	primary	rabbit	mouse, human	WB (1:5000)
IgG-HRP	Santa Cruz	secondary	donkey	rabbit	WB (1:5000)
IgG-HRP	Santa Cruz	secondary	donkey	mouse	WB (1:5000)
Alexa Fluor 488	Invitrogen	secondary	goat	rabbit	ICC/IHC (1:2000)
Alexa Fluor 488	Invitrogen	secondary	goat	mouse	ICC/IHC (1:2000)
Alexa Fluor 555	Invitrogen	secondary	goat	rabbit	ICC/IHC (1:2000)
Alexa Fluor 555	Invitrogen	secondary	goat	mouse	ICC/IHC (1:2000)

WB: Western blot, ICC: immunocytochemistry, IHC: immunohistochemistry

2.5 Molecular biology

2.5.1 DNA digestion

The restriction endonucleases were purchased from New England Biolabs (UK). DNA digestion was performed according the manufacturer's protocol. Typically, a 50 μ l reaction is composed of 1 μ g DNA, 10 units of endonuclease, restriction buffer, bovine serum albumin (BSA, add to a final concentration of 100 μ g/ml (1X) and molecular grade water. The mixture was incubated at 37 $^{\circ}$ C for > 1 hour. Endonucleases were inactivated by heating at 65 $^{\circ}$ C for 20 min.

2.5.2 Electrophoresis

Agarose gel electrophoresis was performed to check DNA after restriction. The gel was prepared by dissolving agarose in TAE buffer to a final concentration of 1%. The solution was heated in microwave to boiling, cooled down to 60-70 $^{\circ}$ C and added ethidium bromide (0.5 μ g/ml). Then the mixture was poured into a rack with combs to solidification. DNA samples were loaded with loading dye and DNA ladders (0.1 Kb or 1 Kb, New England Biolabs, UK). The gels were running on constant 120 voltages generally for 15 - 30 min. The gel was exposed to UV light and photos were taken when DNA bands appeared fluorescent.

2.5.3 DNA Ligation

T4 ligase was used according to manufacturer's protocol (New England Biolabs, UK). Briefly, total 100 ng of vector DNA and insert DNA at a molar ratio 1:5 were mixed with T4 DNA ligase, reaction buffer and water. The mixture was

incubated at 16 °C overnight (~16 hours), followed by heat inactivation at 65 °C for 10 min.

2.5.4 Heat Shock Transformation

Plasmids were introduced into XL10 competent bacteria by heat shock technique according to the manufacturer's protocol (Stratagene, Santa Clara). In brief, 100 µl XL10 cells and 2 µl of the ligation mixture were mixed and incubated on ice for 30 min in a pre-chilled 14-ml polypropylene round-bottom tube (BD Falcon, UK). The tube was then quickly put in a 42 °C water bath and warmed for 30 seconds, followed by incubation on ice for additional 2 min. 0.9 ml of preheated S.O.C media (Invitrogen, UK) were added to the mixture and incubated at 37 °C for 1 hour with shaking at 250 rpm. 100 µl of the total mixture were dispersed on a LB agar plate containing 100 mg/L ampicillin. The plate was incubated at 37 °C overnight. Separated growing colonies were carefully picked and added to tubes with 5 ml LB culture media containing 100 mg/L ampicillin. The tubes were further incubated at 37 °C overnight (~16 hours) with shaking at 250 rpm followed by further DNA extraction.

2.5.5 DNA Extraction

DNA extraction from agarose gel was performed using a commercial kit according to manufacturer's protocol (Qiagen, UK). In brief, the gel containing proper DNA fragment was excised with a clean and sharp scalpel. The slice was dissolved in chaotropic agent at 50 °C for 10 minutes followed by adding isopropanol. The solution was applied to special spin-columns and DNA remained in the column after spinning

for 1 minute at 13,000 rpm. The DNA was washed in 70% ethanol and eluted in EB buffer to collect.

Small scale DNA extraction from 5 ml cultured bacteria was performed using a MiniPrep kit according to the manufacturer's protocol (Qiagen, UK). Briefly, the bacteria were harvested by centrifuge and lysed under alkaline conditions. After adding acetate-containing neutralization buffer, protein and large chromosomal DNA precipitated but the small plasmids remained in the solution after centrifuge for 10 min at 13,000 rpm. The solution was poured into special spin columns and plasmid DNA was combined in the columns. The DNA was washed with 70% ethanol and eluted by EB buffer into a clean Eppendorf tube.

For large scale DNA extraction (< 2.5 mg), a MegaPrep kit was performed according to the manufacturer's protocol (Qiagen, UK). Briefly, the bacteria were harvested by centrifuge and lysed in alkaline solutions. After adding acetate-containing neutralization buffer, the mixture was filtered. Filtered solution containing bacteria DNA was washed with Buffer ETR to remove endotoxin, filtered again through special DNA binding columns. The columns were then washed with 70% ethanol and eluted by EB buffer into a clean Eppendorf tube.

For all extracted DNAs, the concentration was determined using a spectrophotometer by calculating the absorbance at 260 nm (Thermo Scientific, Germany). The DNA was restricted by Xma1 to examine the ITRs and XIAP insertion, followed by sequencing to confirm the XIAP coding sequence (Source Bioscience, Oxford). Geneious v5.5 was used to analysis plasmids, design primers and alignment sequenced data.

2.5.6 DNA sequencing

DNA samples were mixed with appropriate primers according to the sequencing provider's protocol (Source BioScience LifeSciences, Oxford). All sequencing data were imported and aligned using Geneious software (Biomatters Ltd, New Zealand).

2.6 rAAV production

2.6.1 Cell transfection and harvest

Two AAV vectors were made in our experiments, the AAV8-XIAP and AAV8-Null. For each vector, HEK293 cells were seeded and cultured for 3 days at 37 °C and 5% CO₂ in 2 Hyperflasks (Sigma). The cells were transfected with a total of 500 µg of the helper and pAAV-ITR plasmids, using polyethylenimine (PEI) methods. The cells were cultured for additional 3 days in DMEM (4.5 g / L glucose) containing 2% FBS, 1% L-glutamate, 100 U/mL penicillin G potassium and 0.1 mg/mL streptomycin sulfate. The cells were harvested by centrifugation at 1000 rpm for 10 min and lysed with 50mM Tris.HCl- 0.15 M NaCl (pH8.5) by freeze-thawed three times. (Table 2)

Table 2 Plasmids used in co-transfection to produce rAAV

Plasmids	AAV2/8-XIAP (µg)	AAV2/8-NUL (µg)
pAdΔ6	288	303
pRep-Cap(2/8) :	108	113
pAAV-ITR-XIAP :	104	
pAAV-ITR		84

2.6.2 AAV purification

The lysate containing AAV vectors were incubated with Benzonase endonuclease (Merck Chemicals, Darmstadt) at 37 °C for 30 min and spun at 3300 g for 20 min at room temperature. The supernatant was carefully transferred to the top of iodixanol gradient layers in an ultra-centrifuge tube (Beckman Quick-Seal Ultra-Clear 25 x 89 mm). The iodixanol gradient centrifuge tube was prepared with 7.2 ml of 15% iodixanol, 4.8 ml of 25% iodixanol, 4 ml of 40% iodixanol and 4 ml of 60% iodixanol (from top to bottom). See Table 3 for iodixanol preparation.

The tube with gradient iodixanol and lysate was then centrifuged at 59000 RPM for 90 min at 20 °C using an ultra-centrifuge (Beckman). At the end of the centrifuge, the tube was pieced with an 18G needle at the interface between the 60% and 40% phases. The 40% phase was carefully collected with a 5 ml syringe. The 40% iodixanol fraction was applied for further concentration. (Figure 12)

Table 3 Preparation of iodixanol gradient centrifuge

Iodixanol concentration	Iodixanol (ml)	5M NaCl (ml)	5X PBS-MK* (ml)	H ₂ O (ml)	Phenol Red (ul)
15%	2	1.6	1.6	2.8	0
25%	2,5	-	1.2	2.3	10
40%	3.35	-	1	0.65	-
60%	5	-	-	-	10

*: 5X PBS-MK (500 ml) : 250 ml PBS 10X (GIBCO), 2,5 ml 1M MgCl₂, 6,25 ml 1M KCl, H₂O qsp 500 ml

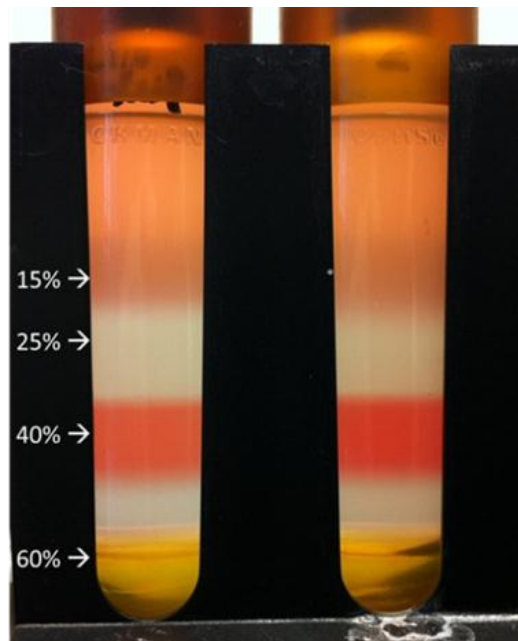


Figure 12 Tubes after iodixanol gradient centrifuge, showing the gradient level of iodixanol based on the percentage.

2.6.3 AAV concentrate

AAV vectors were filtered and concentrated using Amicon Ultra-15 according to the manufacture's protocol (Millipore, UK). In brief, the 40% iodixanol fraction was mixed with 5 ml PBS and centrifuged at 4000 g at 20 °C until the residue was approximately 500 ul. The tube was washed with 15 ml PBS and re-centrifuged for three times until the left over was about 150 ul. Both sides of the filter were washed with the left over solution. This was the concentrated AAV and stored in an autoclaved tube at -80 °C.

2.6.4 AAV titre measurement

The AAV titer was examined by quantitative real-time PCR (Q-PCR) as described previously¹¹¹. Briefly, the AAV sample was 1:10 diluted in PBS and then treated with equal volume of recombinant DNase1 (7500 U/ml, Sigma) at 37 °C for 30 min. DNase was inactivated by heating at 65 °C for 10 min. Q-PCR was performed in a final volume of 20 ul for each reaction, containing 0.8 ul of each primer (0.4 uM), 2 ul of the sample, 10 ul SYBR Green Master Mix and H₂O. Three duplicates were measured for each template and the average C_T value was calculated. Primers were designed to target the cytomegalovirus (CMV) enhancer as following:

CMV Forward (5' -> 3'): TGCCAAGTACGCCCCCTATTGAC

CMV Reverse (5' -> 3'): TGCCAGGCGGGCCATTACC

A standard Q-PCR curve was drawn using pAAV-ITR backbone plasmid of 0.01, 0.1, 1, 10 and 100 pg. It is estimated that 1 pg AAV-ITR plasmid (6889 bp)

contains 1.3×10^5 plasmid molecules. The value from each sample was multiplied by the dilution factor (1:250) and by 500 to obtain the titer per milliliter (2 ul samples). The mass of pAAV-ITR (5.7×10^{-6} pg) was first calculated based on its size (5227 bp) using the formula²²¹: Mass (gram) = genome size (bp) $\times 1.096 \times 10^{-21}$. Thus each of the pAAV-ITR samples contains 1.75×10^3 , 1.75×10^4 , 1.75×10^5 , 1.75×10^6 and 1.75×10^7 DNA molecules, respectively. Regression of the C_T value and DNA molecules was calculated and statistically tested by R^2 and P value. The AAV genome molecules were then calculated based on their C_T value and final dilution (1:200). (Figure 13)

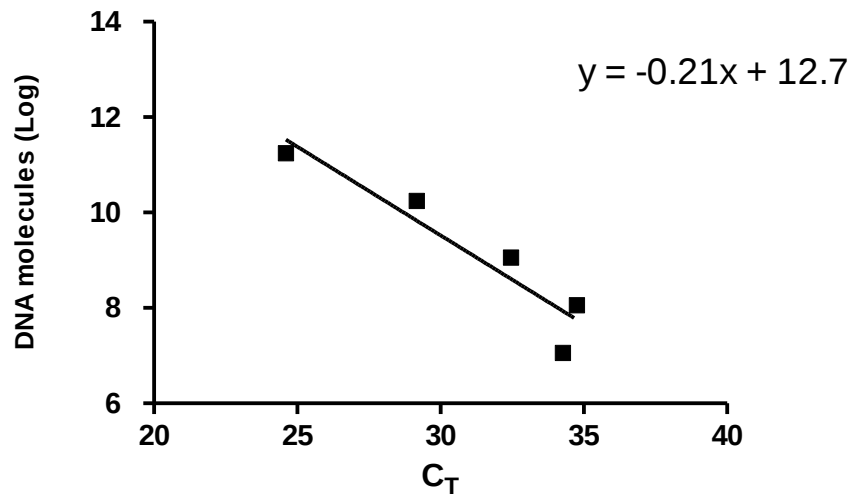


Figure 13 Lineal regression of the DNA molecule and C_T value ($R^2=0.97$).

2.7 In vivo procedures

2.7.1 Mice

All animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and UK Home Office guidelines. Mice were bred and housed in the animal facilities in the Biomedical Sciences Division of the University of Oxford. Animals were kept in a 12-hour light (<100 lux)/12-hour dark cycle, with food and water available. Breeding pairs were established to maintain each strain.

2.7.2 Genotyping

The *Rho*^{-/-} *TgOPN1LW-EGFP*^{+/-} mouse was examined by genotyping the *Rho*^{-/-} and C57BL/6J^{TgOPN1LW-EGFP85933Hue} as described previously^{222, 223}. Briefly, primers were designed as following (5' → 3'):

Rho^{-/-} oligo a: TTCAAGCCCAAGCTTTCGCG

Rho^{-/-} oligo b: AGGTTAGAGCTGGAGGACTG

Rho^{-/-} oligo c: TAAGACTGATTGGACCATTC

C57BL/6J^{TgOPN1LW-EGFP85933Hue} oligo d: CAATTAAGAGATCAGGTAGTGT

C57BL/6J^{TgOPN1LW-EGFP85933Hue} oligo e: AGTTCACCTTGATGCCGTTCTT

For *Rho*^{-/-} genotyping, three *Rho*^{-/-} oligonucleotide primers (a, b, c) were used together in the following 25 ul amplification reaction: 100 ng DNA, 50 pmol each of

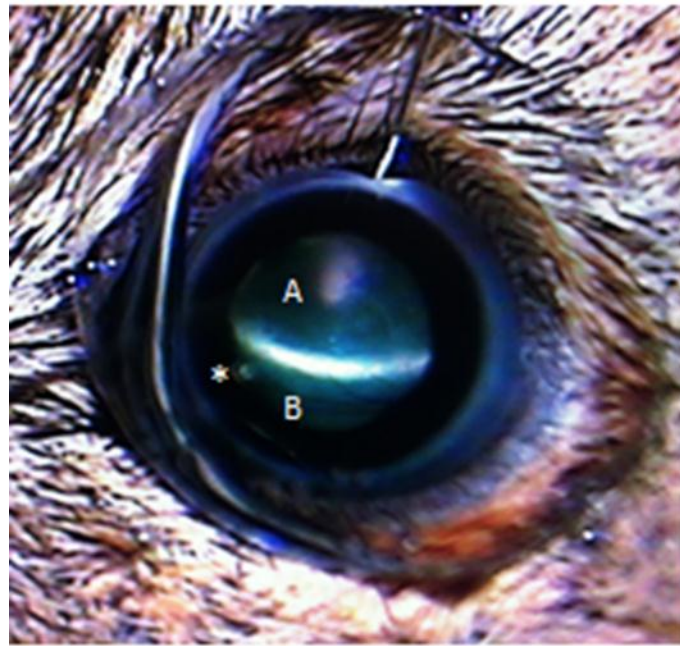
oligo b and c, 25 pmol of oligo a and 12.5 ul ImmoMix RedTM (Bioline Reagents, UK), containing dGTP, dATP, dTTP and dCTP, Taq polymerase and PCR buffer. PCR was performed on a MultiGeneTM OptiMax Thermal Cycler (Labnet International, NJ) with the following thermal cycling profiles: 1 cycle of 10 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1.2 min at 72 °C, and 1 cycle of 10 min at 72 °C. PCR products were resolved on a 1% agarose gel. Fragments of 139 and 394 bp were diagnostic of the Rho^{-/-} and wild-type respectively.

For C57BL/6J^{TgOPN1LW-EGFP85933Hue} genotyping, the primers d and e (50 pmol) were added to the same PCR reaction mix as described above. PCR was performed on a MultiGeneTM OptiMax Thermal Cycler (Labnet International, NJ) with the following thermal cycling profiles: 1 cycle of 10 min at 95 °C, 35 cycles of 1 min at 92 °C, 50 s at 53 °C and 1 min at 72 °C, and 1 cycle of 10 min at 72 °C. The Rho^{-/-} ^{TgOPN1LW-EGFP+/-} and B6^{TgOPN1LW-EGFP} mice showed 641 bp PCR product, whilst the wild type showed no products.

2.7.3 Subretinal injection

Mice were anesthetized by intraperitoneal injection of 1 mg/kg medetomidine (Dormitor 1 mg/mL; Pfizer, UK) and 60 mg/kg ketamine (Ketaset 100 mg/kg; Fort Dodge, UK). Pupils were dilated with 1% mydriaticum and 2.5% phenylephrine hydrochloride eye drops (Bausch & Lomb, UK). The mouse was positioned with its left eye (or right) up towards the microscope. A drop of carbomer eye gel (Bausch & Lomb, UK) was placed on the cornea. A ø15 mm coverslip was placed on the gel and adjusted to view the fundus clearly.

For sub-retinal injection, a 34 gauge needle with micro-syringe (Hamilton, Switzerland) was manipulated to puncture the superior sclera near the equator of the eyeball until the tip of needle was clearly seen in the subretinal space under the microscope. Each eye was injected a total 2 μ l of component, including 1 μ l drug and 1 μ l air. A retinal bleb was immediately noted following successful subretinal injection. The needle was left in the subretinal space for about 60 seconds to equilibrate with the intraocular pressure and then removed. The mouse was kept on a 37 $^{\circ}$ C heating chamber until anesthesia was reversed by intraperitoneal injection of 1 mg/kg atipamezole (Pfizer, UK). (Figure 14)



*Figure 14 Sub-retinal injection in mice. A: detached retina after injection. The needle tip was underneath the retina. B: attached retina in un-injected areas. *: optic disc*

2.7.4 Confocal scanning laser ophthalmoscope (cSLO)

cSLO imaging was performed with the Spectralis HRA device commercially available for human fundus imaging (Heidelberg Engineering, Germany, Figure 15) and was previously reported²²⁴. Briefly, three modes were used including the near-infrared reflectance mode with a 820 nm laser source (820 nm NIR), the fundus autofluorescence (AF) using 790 nm laser (790 nm AF) and the fundus autofluorescence using 488 nm laser (488 nm AF) mode.

To acquire cSLO images, mice were anesthetized and pupils were dilated as described in the subretinal injection part. A PMMA mouse contact lens was carefully placed on cornea (Cantor and Nissel, UK). The 0.3% hypromellose eye drops (Matindale Pharmaceuticals, UK) were used as lubricating reagent between the lens and cornea. To focus and align the camera, a confocal reference plane of high reflectivity could be identified in the outer retina using the 820 nm NIR mode with slight overexposure, as signals beyond the range of detector were highlighted as white pixels by the software²²⁵. This assisted the user in detecting the areas of highest reflectivity and the confocal plane with the largest overexposed area. The dioptric focus was then adjusted to align the camera and obtain paracentral high fundus NIR reflectivity. The detector sensitivity was adjusted between 31 and 107 to acquire a proper exposure images, depending on different modes and the images brightness. Images were usually recorded using the equipped 55 °lens and the automatic real-time (ART) mode, which was able to track slight movements of the fundus caused by respiration and improved the signal-to-noise ratio. Single-averaged images were

recorded with an intensity resolution of 8 bits/pixel, a high-resolution mode (1536 X 1536 pixels), and a frame rate of 4.8 frames/s. (Figure 16)

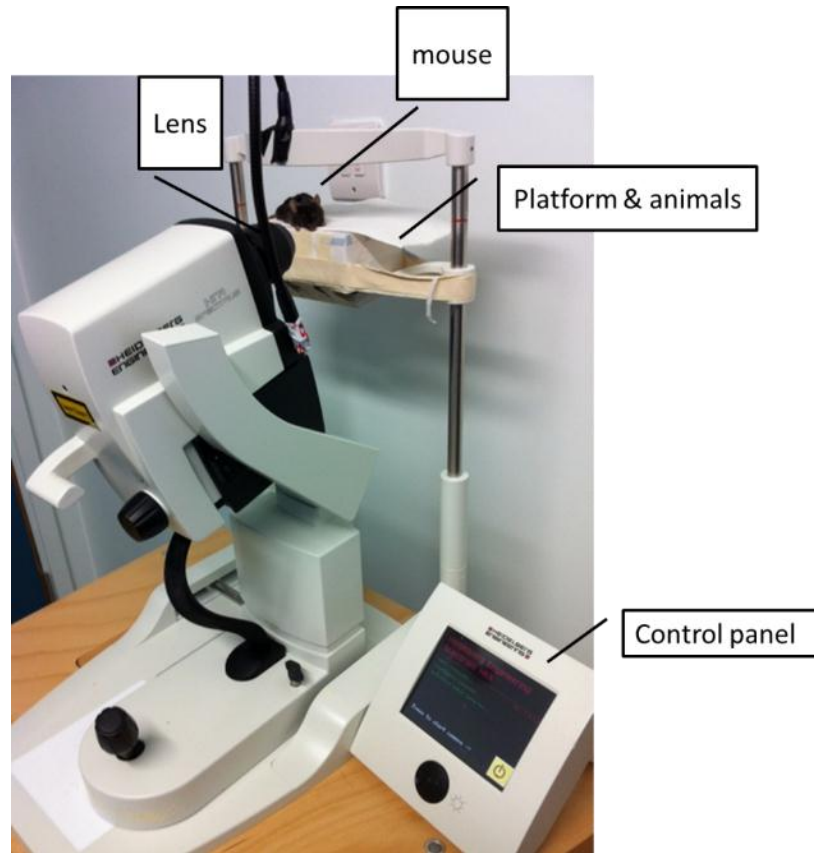


Figure 15 The Spectralis HRA confocal scanning laser ophthalmoscope system.

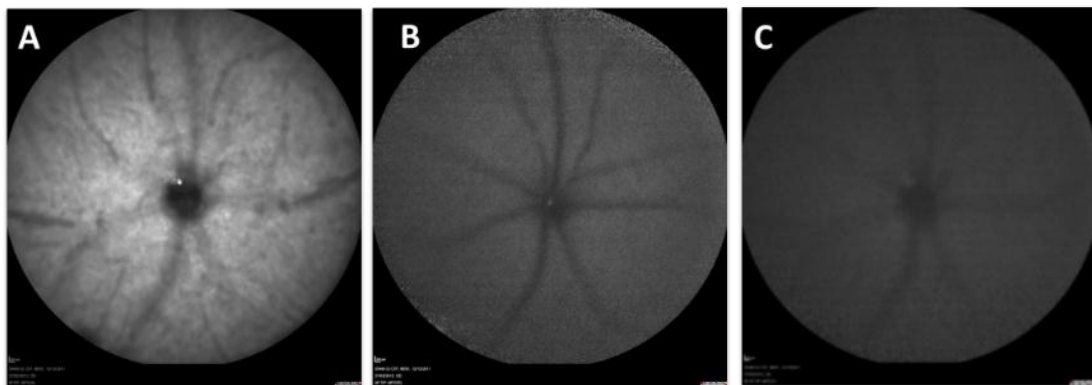


Figure 16 cSLO images in C57BL/6 mice. A: 820 nm near-infrared reflectance mode. B: 790 nm autofluorescence mode. C: 488 nm autofluorescence mode.

2.7.5 Electroretinography (ERG)

The ERG was performed to measure photoreceptor and RPE function and was previously reported²²⁶. In brief, the mice were dark adapted >2 hours before the ERG procedure under dim red illumination. The mice were anesthetized and pupils were dilated as described above. ERGs were recorded using an electroretinography console equipped with the light stimulus (Espion E2, Diagnosys LLC, UK, Figure 17). The active electrode was positioned carefully to the central surface of cornea using a micromanipulator. Custom-made contact lens and 0.5% hypromellose eye drops were used to maintain corneal moisture and provide good electrical contact. A reference electrode was placed in the scruff subcutaneously. An identical ground electrode was positioned at the base of the tail. Signals were differentially amplified and digitized at a rate of 5 kHz.

For scotopic ERG, single-flash stimuli (6500k white) were generated in a Ganzfeld dome with the intensity increasing from -6 to $2 \log \text{cd} \cdot \text{s/m}^2$ in 8 steps. The averaged response in each step was obtained from 4 to 25 repeats with an interstimulus interval (ISI) of 5 to 20 seconds. For photopic ERG, the mice were light adapted for 10 min under $30 \text{ cd} \cdot \text{s/m}^2$. Single-flash stimuli were delivered with the intensity from 0.25 to $25 \text{ cd} \cdot \text{s/m}^2$ in 5 steps. The averaged response in each step was obtained from 25 repeats. Photopic flicker ERGs were also obtained with 15 Hz and 30 Hz $10 \text{ cd} \cdot \text{s/m}^2$ stimuli. The amplitude of the b-wave was measured from the trough of the a-wave to the following peak of the b-wave (Figure 18). See Table 4 for details of the protocol for scotopic and photopic ERG.

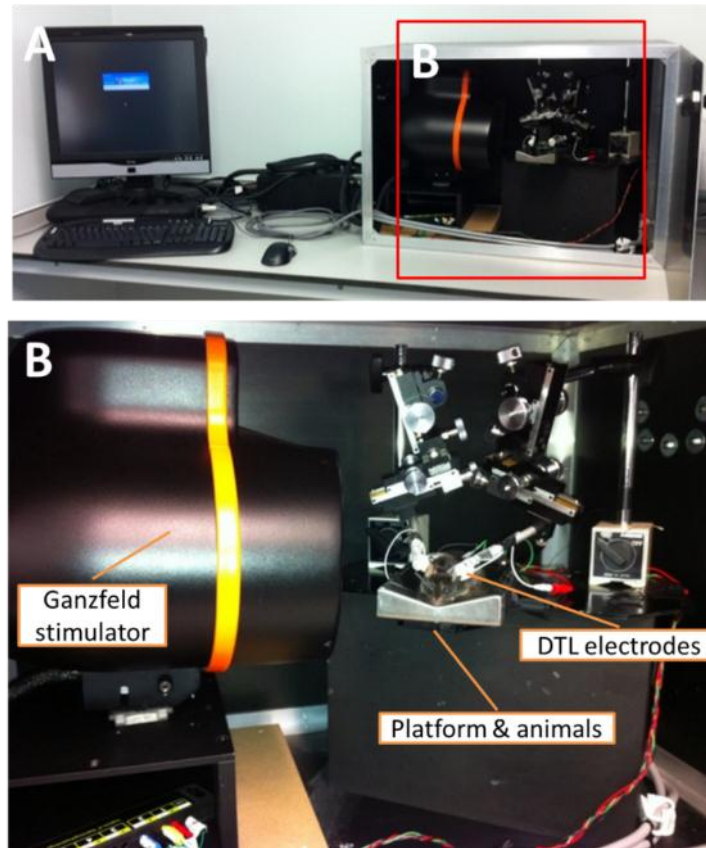


Figure 17 Electrophysiology system (A) and the stimulator (B)

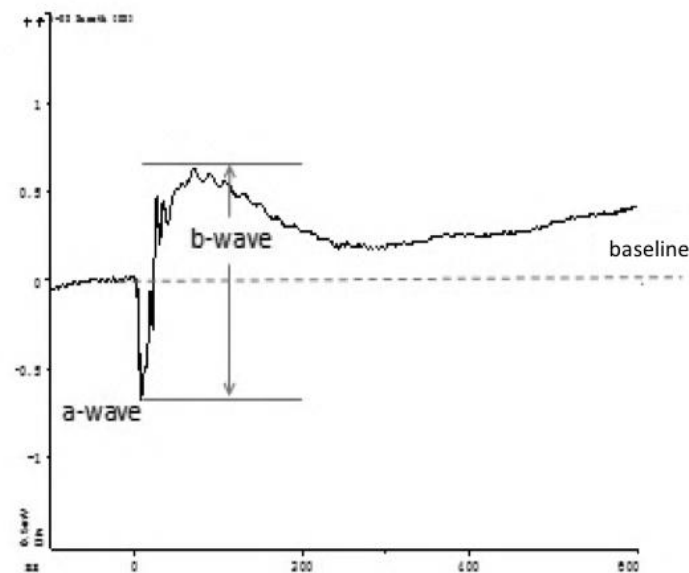


Figure 18 ERG components and amplitude measurement, showing the a- and b-wave.

The b-wave amplitude was measured from the trough of the a-wave to the following peak of the b-wave.

Table 4 Protocol of the scotopic and photopic ERG

		Step	Stimulus (cd s/m ²)	Repeats
Scotopic ERG	Single-flash	1	1 X 10 ⁻⁶	25
		2	1 X 10 ⁻⁵	25
		3	1 X 10 ⁻⁴	16
		4	1 X 10 ⁻³	16
		5	0.01	9
		6	0.1	9
		7	1	4
		8	10	4
Light adaption (30 cd/m2 for 10min)				
Photopic ERG	Single-flash	9	0.25	25
		10	1	25
		11	2.5	25
		12	10	25
		13	25	25
	15 Hz Flicker	14	10	25
	30 Hz Flicker	15	10	25

2.7.6 Eye section

Eyes were harvested following cervical dislocation of the mouse. The cornea and lens were removed carefully. The rest of the eye was fixed for 30 min in 4% paraformaldehyde (Fisher Scientific, UK). The eye was transferred to 30% sucrose and incubated until the eye sunk to the bottom of the tube. The sample was embedded in O.C.T compound (VWR International, UK) in pre-chilled foil tube and quickly frozen in dry ice mixed with ethanol. Samples were generally cut at 16 μm using Cryostat (Leica, Germany). Slides were stored at $-80\text{ }^{\circ}\text{C}$ until staining.

2.8 Statistical Analysis

Un-paired t-test was performed to compare two independent groups. One-Way ANOVA with Bonferroni post test was used in comparison among multiple groups > 2. Data were analyzed and graphed by PASW Statistics 18 or Graph Prism. All data were presented as mean $\pm$ SEM in graphs. The level of significance was set at 0.05.

**Chapter 3 *An Ex Vivo* Model of Oxidative Induced
Apoptosis in Human RPE Cells**

3.1 Introduction

Hydrogen peroxide (H₂O₂) is a classical oxidant widely used to induce oxidative stress *in vitro*. It is formed by dismutation of •O₂⁻ and easily diffuses across cell membranes. Cells treated with H₂O₂ show the typical morphological and biochemical features of apoptosis, as determined by electron microscopy and TUNEL assays in previous studies²²⁷⁻²²⁹.

The APRE-19 cells are a human RPE cell line characterized with typical morphological and biochemical markers of RPE cells *in vivo*⁷. They exhibit hexagonal appearance with packing of pigmented, polarized epithelia after differentiation. Moreover, they present RPE specific markers CRALBP and RPE65 in RT-PCR and Western blot. Compared to primary culture, this cell line can be easily handled, maintained and stored for experiments. Thus they have been widely in human RPE studies as an appropriate model *in vitro*²³⁰⁻²³³.

H₂O₂ has been applied to the APRE-19 cells to induce oxidative stress in previous studies²³²⁻²³⁸. The mechanism of H₂O₂ stimulated cell death may involve the caspase pathway. Activation of caspase-3 is found in ARPE-19 cells exposed to H₂O₂²³³. Other types of cells, such as Jurkat cells²³⁹, eosinophils²⁴⁰, chondrocyte²⁴¹ and HL-60²⁴² cells, also show increasing expression of caspase-3 after H₂O₂ treatment. Furthermore, release of cytochrome-c is found associated with H₂O₂ treated ARPE-19 cells. The Smac/Diablo released by mitochondrial is reported to up regulated after H₂O₂ stress²⁴⁴. These evidences reveal that caspases may play a crucial role in H₂O₂

induced cell death, suggesting an ideal model to assess XIAP neuroprotection for oxidative stress in human RPE cells.

However, there seems no “standard” set-up for H₂O₂ stress in previous studies. They vary among each other with regard to H₂O₂ concentration, treatment time, dissolving media and releasing agents. A wide range of concentrations have been applied from 0.1 to 1 mM H₂O₂. The treatment time varies from 10 min to 24 hours. Different types of media are used to dissolve H₂O₂, such as MEM, DMEM, DMEM:F12 or PBS, with or without serum. H₂O₂ is given as single pulse or using glucose oxidase to achieve a continuous releasing system.

Furthermore, previous studies have shown that these factors can affect H₂O₂ toxicity on the ARPE-19 cells. Kim *et al* found that apoptosis occurs at lower concentrations of H₂O₂ (0.4, 0.5, 0.6 mM), whereas higher concentrations of H₂O₂ induces necrosis rather than apoptosis²³³. When added as a single pulse to ARPE-19 cultures, H₂O₂ is found rapidly depleted within 2 hours²³². Therefore, the H₂O₂ stress needs to be carefully examined and optimized based on our experiment before further assessment of XIAP neuroprotection.

3.2 Aims

The aim of this chapter is to investigate H₂O₂ induced oxidative stress in the differentiated ARPE-19 cells, specifically:

- H₂O₂ dose-toxicity relations
- Mitochondrial function
- Cell apoptosis
- Intracellular ROS

See Figure 19 for an overview of process and methods in this chapter.

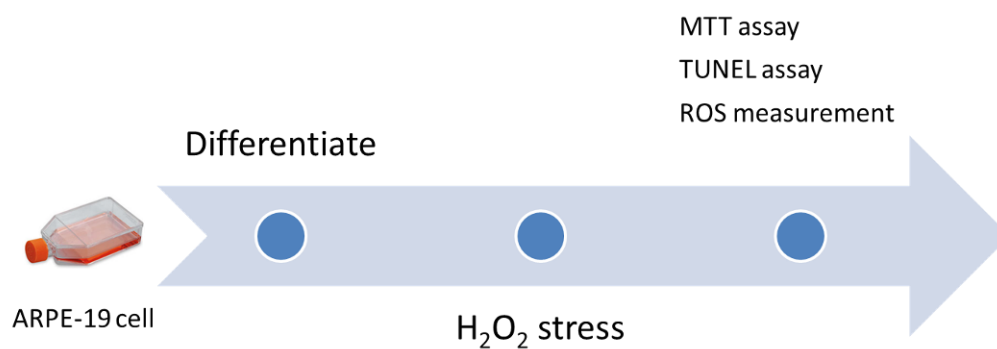


Figure 19 Schematic diagram showing the main steps and methods in Chapter 3.

3.3 Materials and methods

Cell Culture

The ARPE-19 cells were maintained in DMEM: F12 containing 10% fetal bovine serum. For differentiation, the cells were grown to confluence and maintained in DMEM-F12 culture media with reduced 1% serum for at least 4 weeks. See Chapter 2 for general information of cell culture.

H₂O₂ stress

The differentiated ARPE-19 cells were seeded into 96-well plates for H₂O₂ stress. Fresh H₂O₂ was prepared at concentration of 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4 and 8mM by dissolving 30% H₂O₂ in DMEM-F12 medium. See Chapter 2 for general information of H₂O₂ stress.

MTT Assay

The MTT assay was performed in 8 wells of non-stressed cells in 96-well plate, and 8 wells of stressed cells with each concentration of H₂O₂ as described above, respectively. See Chapter 2 for more details of MTT assay.

TUNEL Assay

The MTT assay was performed in 3 wells of positive controls in 96-well plate, non-stressed cells and stressed cells with 0.125, 0.25, 0.5 and 1 mM H₂O₂, respectively. The number of apoptotic cells was counted in x200 magnification field

in each group and repeated in 2 separate fields. See Chapter 2 for more details of TUNEL assay.

ROS Measurement

The DCFH-DA assay was performed in 8 wells of non-stressed cells in 96-well plate, and 8 wells of stressed cells with 0.125, 0.25, 0.5 and 1 mM H₂O₂, respectively. See Chapter 2 for the more information of DCFH-DA assay.

3.4 Results

3.4.1 ARPE-19 cell differentiation

Grown to confluence and incubated for 4–6 weeks, the ARPE-19 cells exhibited typical RPE hexagonal appearance, as previously demonstrated by Dunn *et al*⁷. (Figure 20)

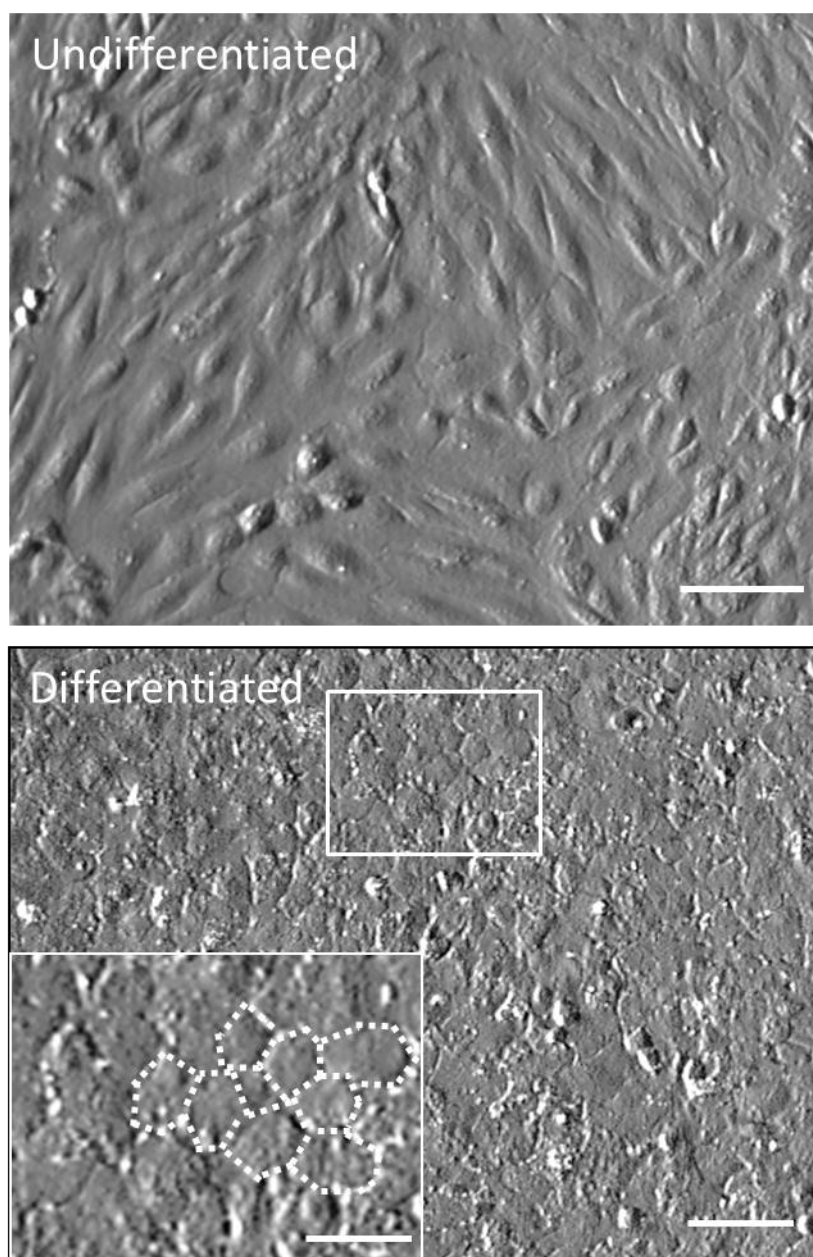


Figure 20 Undifferentiated ARPE-19 cells showed irregular size and spindle-like shape. After differentiation, the ARPE-19 cells exhibited typical RPE hexagonal appearance (e.g. the outlined areas in the embedded figure). Scale bar=50 μ m or 25 μ m (embedded figure).

3.4.2 Mitochondrial function and cell viability

The MTT results showed that H₂O₂ toxicity was dose-dependent ($F=80$, $p<0.0001$, One-way ANOVA). With 1 hour exposure, the ARPE-19 cells treated with 0.25 mM or higher dose of H₂O₂ had a significant reduction of mitochondrial function compared with non-stressed controls ($p<0.001$, One-way ANOVA with Bonferroni Post test, Figure 21), whilst there was no significant change with 0.125mM or lower concentration of H₂O₂ ($p>0.05$, One-way ANOVA with Bonferroni Post test, Figure 21). For instance, the cell viability of 0.125 mM H₂O₂ treatment and non-stressed controls was 0.46 ± 0.05 vs. 0.54 ± 0.02 ($t=2.3$, $p=0.17$). The cell viability of 0.25 mM H₂O₂ treatment and controls was $0.34 \pm 0.03\%$ vs. $0.54 \pm 0.02\%$ ($t=5.3$, $p=0.00003$).

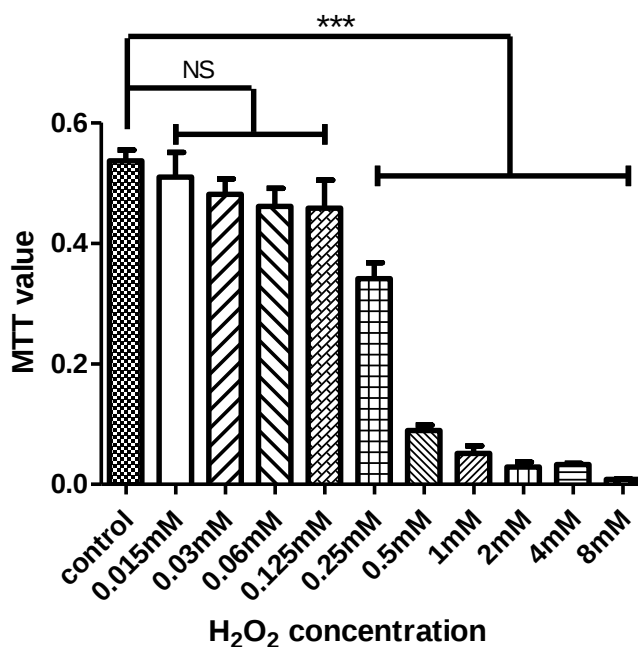


Figure 21 MTT assay of ARPE-19 cells subjected to H₂O₂ stress. NS: no significance,

***: $p<0.001$. $F=80$, One-way ANOVA with Bonferroni's multiple test, $n=6$.

3.4.3 Cell apoptosis

To determine if H₂O₂ induced cell toxicity via apoptosis, the TUNEL assay was performed in the non-stressed cells and cells treated by 0.125 mM, 0.25 mM, 0.5 mM and 1mM H₂O₂, respectively. Treatment with 0.125 mM H₂O₂ did not cause a significant increase of apoptotic cells compared to non-stressed controls (2.4 ± 0.9 vs. 1.8 ± 0.4 , $p=0.55$). However, cells treated with 0.25 mM or higher concentration of H₂O₂ showed significantly more apoptosis than non-stressed controls. For example, the number of apoptotic cells treated with 0.25 mM H₂O₂ and controls was 18 ± 1.0 vs. 1.8 ± 0.4 ($p=0.000006$) (Figure 22).

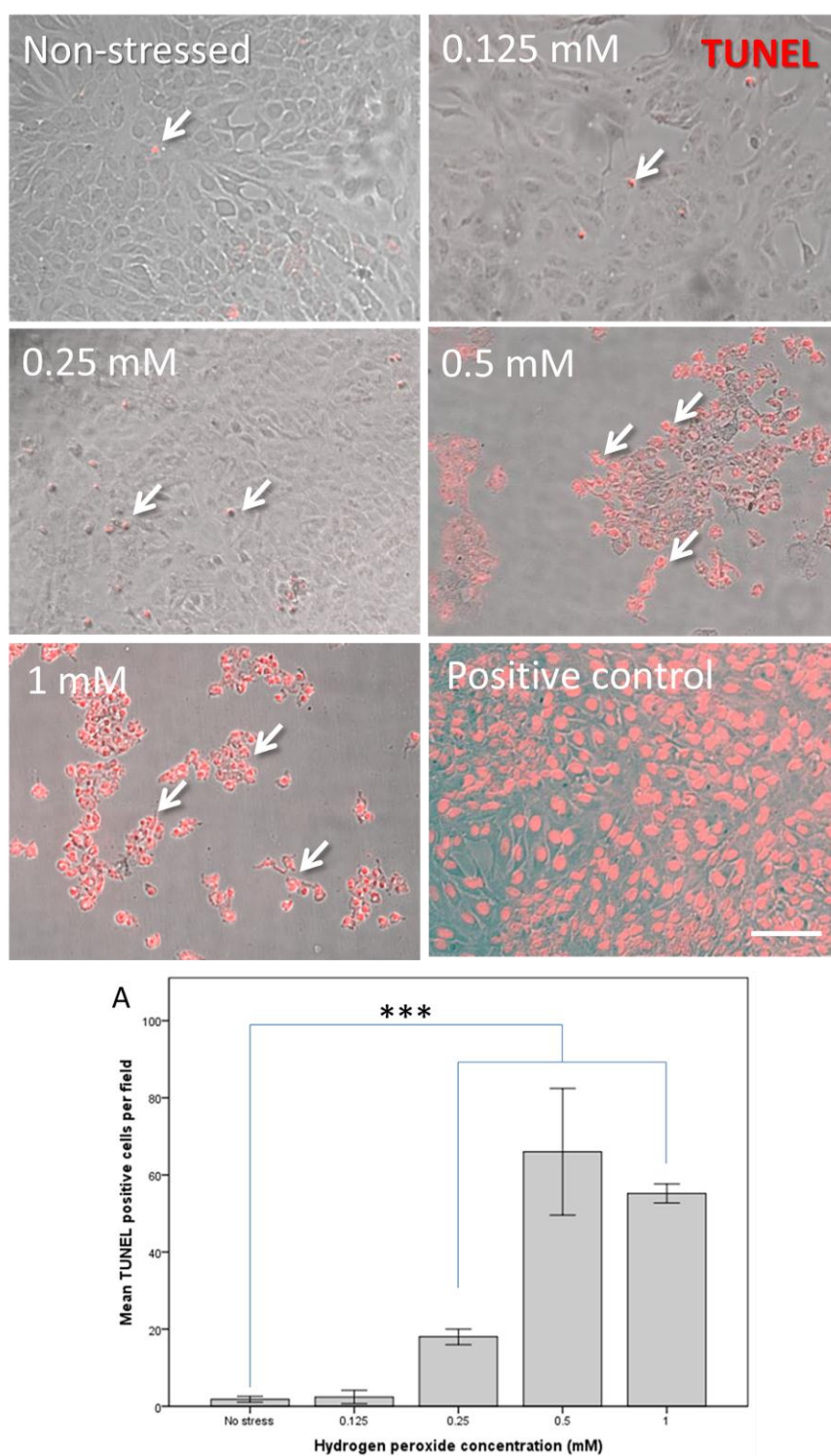


Figure 22 TUNEL assay in the ARPE-19 cells. The non-stressed and 0.125 mM H₂O₂ treated cells showed few apoptotic cells (arrows), whilst the 0.25, 0.5 and 1 mM H₂O₂ treated cells showed significantly more apoptotic cells (A). Scale bar=100 μ m. ***: $p < 0.001$, One-way ANOVA with Bonferroni's multiple test, $n = 6$.

3.4.4 Intracellular ROS

To investigate if such H₂O₂ induced apoptosis involved oxidative stress, the DCFH-DA assay was performed to measure intracellular ROS. Normalized by the non-stressed cells, ROS of the cells treated with 0.125 mM, 0.25 mM, 0.5 mM and 1mM H₂O₂ was 165 ±20%, 207 ±46%, 220 ±11% and 353 ±32%, respectively. There was an increasing trend of ROS production along with H₂O₂ concentration. The ROS of all stressed groups were significantly higher than the non-stressed controls ($p<0.001$) (Figure 23).

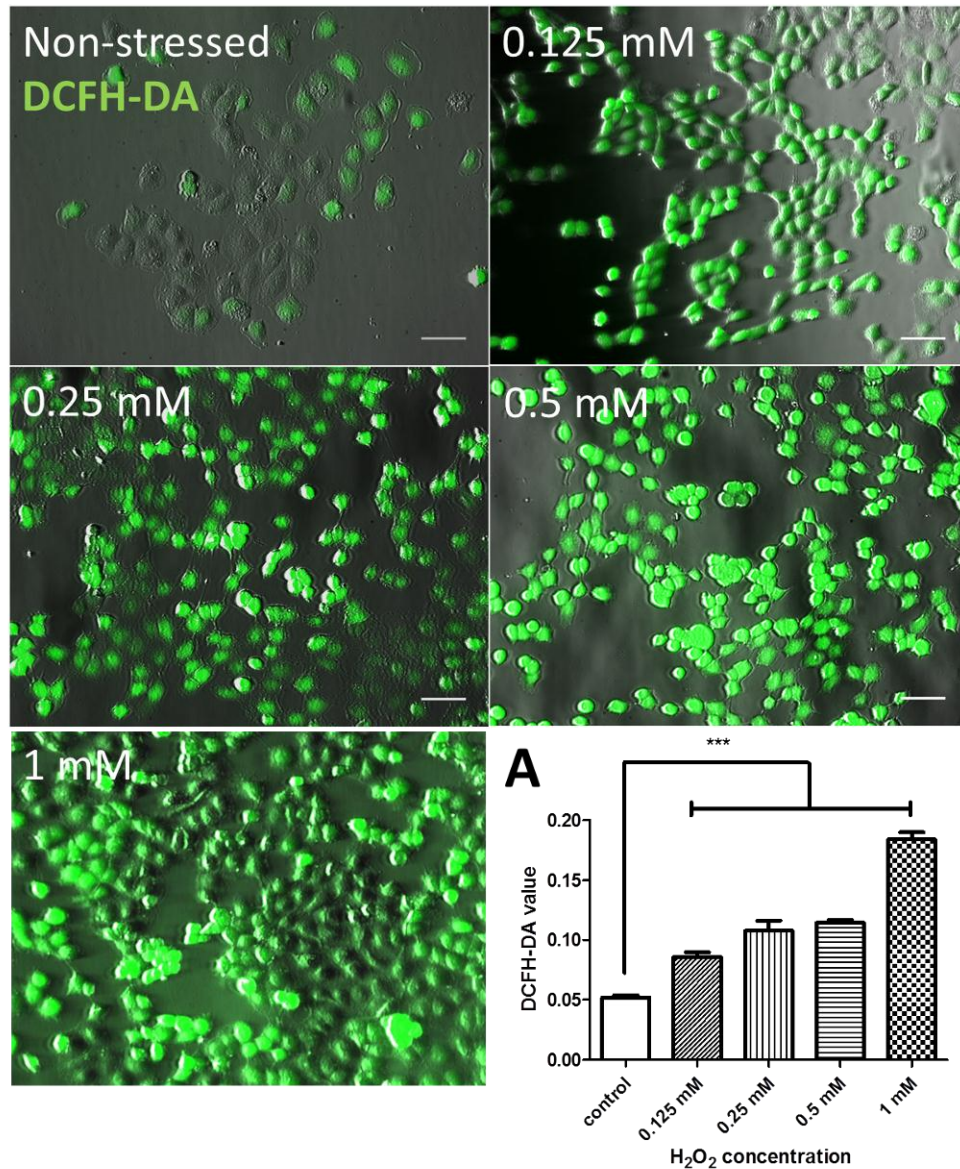


Figure 23 ROS measurement in the ARPE-19 cells. Compared to the non-stressed cells, the H₂O₂ treated cells (0.125, 0.25, 0.5 and 1 mM) showed significantly more fluorescence intensity derived from DCFH-DA, suggesting more generation of ROS (A). ***: $p < 0.001$, One-way ANOVA with Bonferroni's multiple test, $n=6$. Scale bar=50 μ m.

3.5 Discussion

In this chapter, cell viability, apoptosis and ROS production were examined in the H₂O₂ treated ARPE-19 cells. We found 0.25 mM or higher concentration of H₂O₂ can cause a significant oxidative cell death after 1 hour incubation. The marginal H₂O₂ stress, *e.g.* 0.25 mM H₂O₂ for 1 hour, is identified as an acceptable and reproducible model to assess neuroprotective agents in cultured human RPE cells.

The MTT assay shows that H₂O₂ toxicity in the ARPE-19 cells is dose-dependent with 1 hour incubation. This finding corresponds to other H₂O₂ studies. Several factors can affect H₂O₂ toxicity on RPE cells, including H₂O₂ concentration, exposure time, temperature and media types^{232, 233, 243, 244}. In previous studies, a wide range of H₂O₂ have been used to induce oxidation, from 0.1-1 mM in concentration and 10 min to 24 hours in treatment time^{232, 233}. Different types of media have been applied to dissolve H₂O₂, such as MEM, DMEM, DMEM:F12 or PBS, with or without serum^{233, 243, 244}. Therefore, H₂O₂ stress needs to be carefully examined and optimized based on our experiment set up before further implication.

A short incubation time of H₂O₂ (1 hour) is chosen in this study, despite various exposure times have been reported in previous studies^{232, 233}. When added as a single pulse, H₂O₂ is found rapidly depleted within 2 hours incubation with cultured ARPE-19 cells. For instance, H₂O₂ concentration decreases 10-fold at 2 hours after 0.2 mM H₂O₂ is incubated with ARPE-19 cells²³². A continuous H₂O₂ release system is developed using glucose oxidase to maintain stable H₂O₂ concentration²³². However, it produces complex outcomes as H₂O₂ level depends on generation, depletion and cell toxicity. Thus in our study, a single pulse of H₂O₂ with a short incubation is an easy

but reliable way to deliver oxidative stress, instead of long time treatment or continuous H₂O₂ generation system.

In this study, the MTT and TUNEL assay are performed together to examine upstream and downstream changes of H₂O₂ toxicity on the RPE cells. The mechanism of H₂O₂ stimulated cell apoptosis involves caspase-dependent pathway. H₂O₂ can damage mitochondrial and release cytochrome-C^{233, 243}. Activation of caspase-3 is found in many cell lines exposed to H₂O₂, including the ARPE-19 cells²³³. The MTT assay is based on mitochondria function to cleave the yellow tetrazolium salt MTT to purple formazan. It is sensitive to detect mitochondria damage at an early stage of apoptosis. The TUNEL assay detects DNA fragmentation by labelling the terminal end of nucleic acids with terminal deoxynucleotidyl transferase and dUTPs. DNA fragmentation is regarded as a late stage landmark of apoptosis. Therefore, the MTT and TUNEL assay provide a picture of the pathway in H₂O₂ induced apoptosis *in vitro*.

Using a marginal H₂O₂ stress, e.g. 0.25 mM H₂O₂ for 1 hour, is particularly important for XIAP assessment in order to assess its neuroprotective role in the RPE against oxidative stress. This stress can produce a significant increase of oxidative cell death, as determined by the MTT and TUNEL assays in our experiments. Any possible rescue effect of neuroprotective agents, e.g. XIAP, is likely to be more marked at marginal stress rates, when typically 50% of cells survive cell toxicity. An overtly strong stress, e.g. H₂O₂ >0.25 mM, would most likely damage most of the cells through mechanisms of necrosis²³³, which would confound any assessment specifically of neuroprotection. Hence we first identified the marginal, or ‘tipping point’ for oxidative stress and then confirmed the mechanism by apoptosis-specific TUNEL staining in the stressed cells.

In conclusion, H₂O₂ can cause oxidative apoptosis in cultured human RPE cells. The marginal stress, *e.g.* 1 hour treatment of 0.25 mM H₂O₂, provides an ideal and reproducible model *in vitro* to assess XIAP neuroprotection in human RPE cells.

Chapter 4 XIAP effect on ARPE-19 Cells in H₂O₂
Induced Oxidative Cell Death

4.1 Introduction

Oxidative stress is thought to play a critical role in the pathogenesis of AMD. RPE cells may be particularly susceptible due to accumulation of lipid molecules secondary to the phagocytosis of photoreceptor outer segments, high oxygen tension, light illumination and other types of stress such as complement activation. All of these generate reactive oxygen species (ROS)³⁷. On the other hand, anti-oxidative enzymes in RPE cells decreases with age, potentially allowing ROS to cause mitochondrial DNA damage and ultimately leading to the apoptosis of RPE cells³⁷. Therefore, an approach to delay oxidative RPE cell death could be helpful for preventing the progression of AMD.

XIAP is an inhibitor of apoptosis protein featured with regulating caspase-3, 7 and 9 and inhibiting caspase-dependent apoptosis²⁴⁵. Studies in vivo have shown that XIAP mediated by viral vectors can preserve photoreceptors and retinal ganglion cells in animal models of retinal detachment²⁰⁶, glaucoma²¹², optic nerve trauma²¹³, rhodopsin mutant retinal degeneration⁹⁷ and N-methyl-N-nitrosourea induced retinal degeneration²¹⁰. Despite these promising results, however, there is little data on the role of XIAP in neuroprotection of the RPE undergoing oxidative stress, which arguably would be highly relevant to a potential treatment strategy for AMD.

4.2 Aims

The aim of this chapter is to assess AAV-mediated XIAP neuroprotection in ARPE-19 cells subject to H_2O_2 induced oxidative stress, specifically:

- AAV2 transduction in ARPE-19 cells
- AAV-mediated XIAP overexpression in ARPE-19 cells
- Cell apoptosis
- Mitochondrial function

See Figure 24 for an overview of process and methods in this chapter.

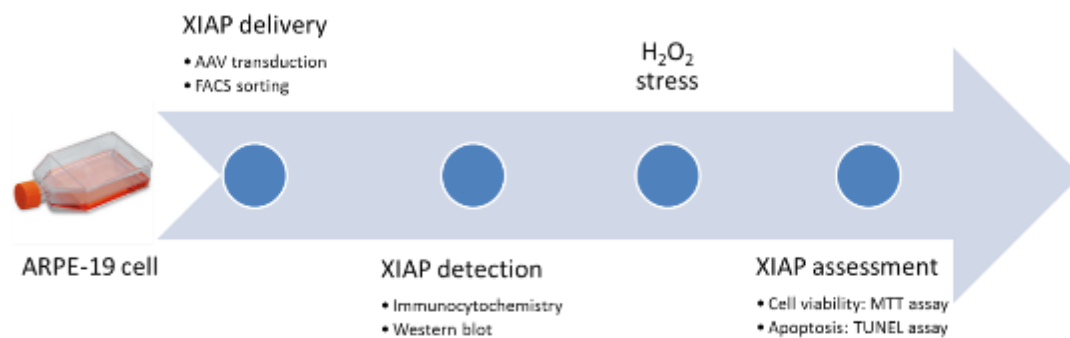


Figure 24 Schematic diagram showing the main steps and methods in Chapter 4.

4.3 Materials and methods (Figure 24)

Cell Culture

The ARPE-19 cells were maintained and differentiated in DMEM-F12 culture medium. See Chapter 2 and 3 for more details.

Vector

The vectors were designed and cloned by Professor Robert MacLaren (the project supervisor) and commercially packaged into AAV2 by GeneDetect in Auckland, New Zealand (now part of Roche). In brief, a plasmid encoding the human XIAP coding sequence was commercially purchased (Origene, Rockville) and packaged into a serotype 2 AAV vector. This plasmid contained the chicken beta actin promoter and the GFP coding sequence fused downstream to XIAP, expressed bicistronically through an internal ribosome entry site sequence (IRES) as previously described²⁴⁶. Hence in this construct (AAV-XIAP), the GFP reporter gene is co-expressed with XIAP in cells, but the proteins are not fused. A null vector, containing identical regulatory sequences including GFP but without XIAP, was used as a control (AAV-NULL). Both vectors were purified against immobilized heparin sulfate proteoglycan and the final concentrations were diluted to an identical titre of 1.0×10^{12} genomic particles/ml. The schematic drawing of AAV vectors is shown in Figure 25.

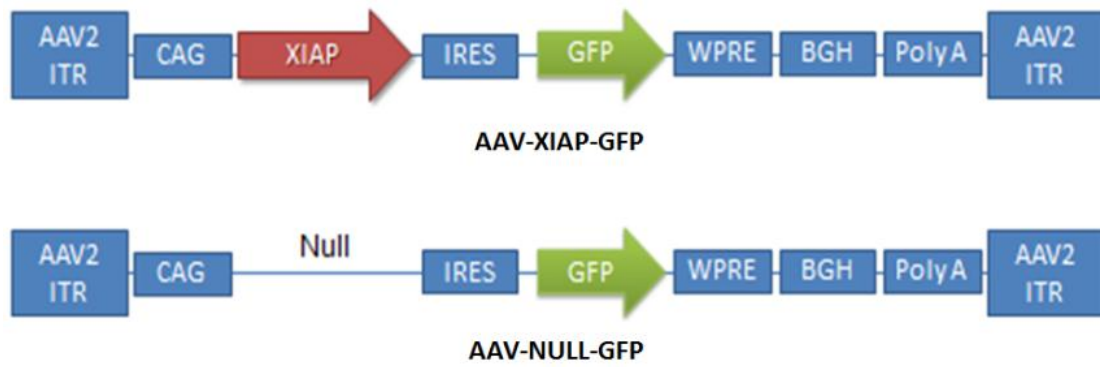


Figure 25 Schematic graph of the AAV vectors. Please also see the list of abbreviations.

Transduction

The differentiated cells were seeded into 6-well plate and reached ~ 80% confluence. The AAV-XIAP and AAV-NUL vector were diluted with pre-warmed growth medium to a final titre of 5×10^9 genomic particles/ml. The culture medium was removed and 2 ml medium containing vectors was added to each well to reach a multiplicity of infection of 1×10^4 genomic particles per cell. 3 wells of cells were transduced by the AAV-XIAP and AAV-NUL, respectively. The cells were incubated for additional 3 days without changing the culture medium before further fluorescence-activated cell sorting.

Fluorescence-activated cell sorting (FACS)

The transduced cells were carefully washed by PBS and detached by 5 min incubation with 0.05% Trypsin- 0.53 mM EDTA solution. The cells were centrifuged at 125 g for 5 minutes and re-suspended in PBS containing 1% FBS. Flow sorting was performed using Beckman Coulter Legacy MoFlo MLS High Speed Cell Sorter with kind help of Andrew Worth at University of Oxford. A blue diode (488nm) was used to sort cells based on the GFP expression due to AAV transduction. The sorted populations were plated in pre-warmed culture medium and seeded at a density of 2×10^5 /ml in 96-well plate for XIAP immunostaining and H_2O_2 stress (2×10^4 /well) or in 6-well plate for XIAP Western blot (2×10^5 /well).

Immunocytochemistry

XIAP immunocytochemistry was performed in 2 wells of unsorted AAV-XIAP and AAV-NUL transduced cells in 96-well plate, respectively. The cells were

incubated with rabbit anti-mouse XIAP antibody (Abcam, Cambridge, 1:200 diluted in PBST) overnight at 4° C (~ 16 hours). Goat anti-rabbit fluorescence conjugated antibody was applied for 1 hour at room temperature (Alexa Fluor 555, Invitrogen, US, 1:2000 dilution in PBST). See Chapter 2 for more details.

Western Blot

Samples were obtained from 2 wells of non-transduced cells, sorted AAV-XIAP and AAV-NULL transduced cells in 6-well plate, respectively. 30 ul of each group were loaded per lane of a 10% SDS-PAGE gel (4 lanes/sample). The nitrocellulose blots were incubated with rabbit anti-mouse XIAP antibody (Abcam, Cambridge, 1:1000 diluted in TBST) overnight at 4° C. Donkey anti-rabbit HRP-conjugated antibody (Santa Cruz, US, 1:5000 diluted in TBST) were applied for 1 hour at room temperature. For quantification, the intensity of appropriate bands in each lane was measured three times and the average was applied for analyses. See Chapter 2 for more details.

H₂O₂ stress

All cells were exposed to freshly made 0.25 mM H₂O₂ for 1 hour at 37 °C. See Chapter 2 for more details.

MTT Assay

The MTT assay was performed in 8 wells of non-stressed cells in 96-well plate, H₂O₂ treated non-transduced, AAV-XIAP and AAV-NULL transduced cells, respectively. See Chapter 2 for the general information.

TUNEL Assay

The assay was performed in 3 wells of non-stressed cells in 96-well plate, H₂O₂ treated non-transduced, AAV-XIAP and AAV-NULL transduced cells, respectively. See Chapter 2 for the general information.

4.4 Results

4.4.1 Transduction and FACS sorting

The GFP-expressing populations were counted and isolated using FACS in AAV-XIAP and AAV-NULL transduced cells. Based on GFP percentage of total cells sorted, the transduction rate of the AAV-XIAP group was 55% and the AAV-NULL was 58%. This confirmed that the XIAP and null vectors had similar properties with regard to GFP expression following transduction. (Figure 26)

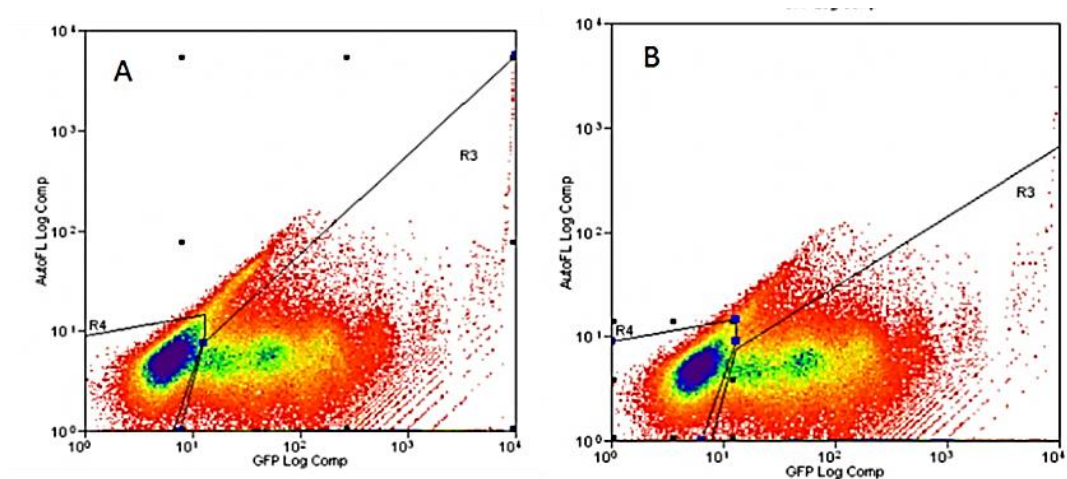


Figure 26 Fluorescence-activated cell sorting of the AAV-XIAP (A) and AAV-NULL transduced ARPE-19 cells (B).

4.4.2 XIAP immunostaining

To further explore XIAP expression after AAV transduction, immunocytochemistry was performed in the transduced ARPE-19 cells. We detected markedly increased staining of intracellular XIAP in the AAV-XIAP transduced cells transduced, which was identified by GFP co-expression. However, there was a much lower level of endogenous XIAP immunostaining in the AAV-NULL transduced controls. (Figure 27)

To quantify the level of XIAP protein expression, Western blotting was performed on the GFP-positive sorted cells from AAV-XIAP and AAV-NULL transduced groups. The AAV-XIAP transfected cells had approximately 11-fold higher XIAP expression compared to the AAV-NULL controls ($1300 \pm 130\%$ vs. $120 \pm 10\%$, $p=0.0006$). Compared to mRNA measurement such as QPCR, the Western blot results clearly demonstrated that XIAP protein overexpression can be achieved with a bicstronic expression sequence delivered by an AAV vector. In addition, the magnitude of XIAP overexpression driven by the vector (over one log unit increase in individual ARPE-19 cells) might be predicted to act broadly within the physiological range. Certainly at this level, the cytoplasmic distribution of the over-expressed XIAP protein has a very similar pattern to that seen in the AAV-NULL controls. (Figure 28)

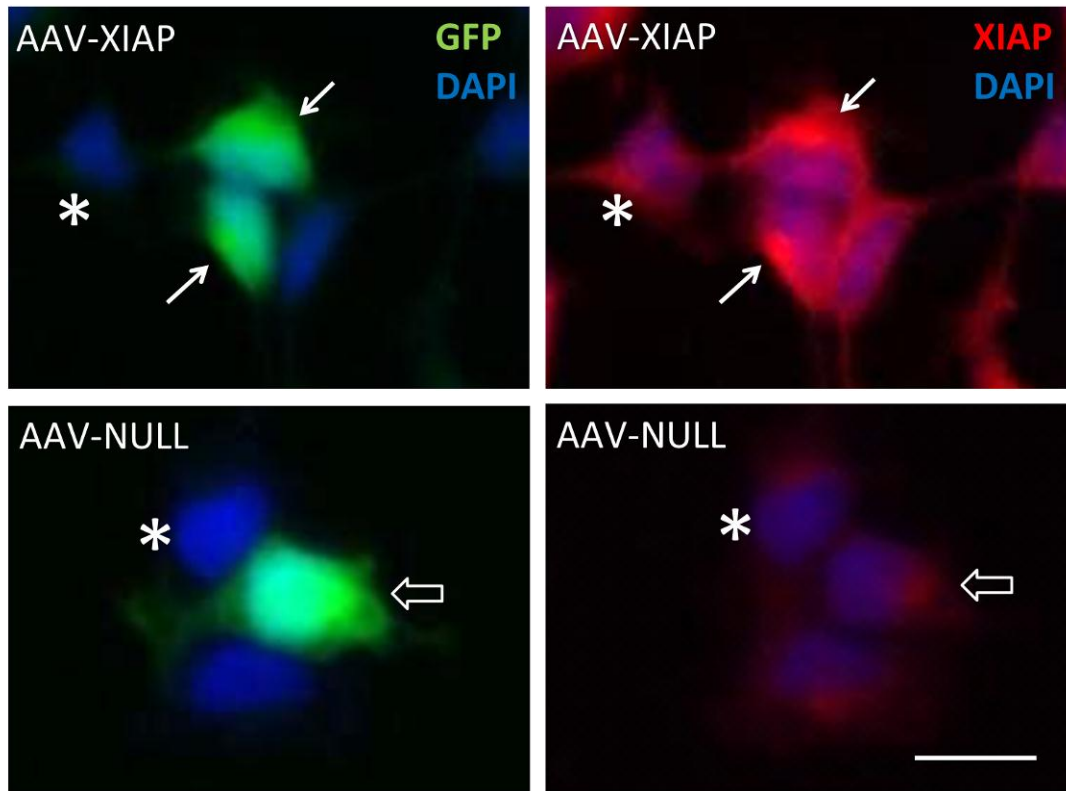


Figure 27 XIAP immunostaining in AAV transduction ARPE-19 cells. Compared to the non-transduced cells (asterisk), the AAV-XIAP transduced cells showed increasing XIAP staining along with downstream GFP expression (solid arrows), whilst a similar XIAP immunostaining was observed in the AAV-NULL controls (hollow arrow). Scale bar=25 μ m.

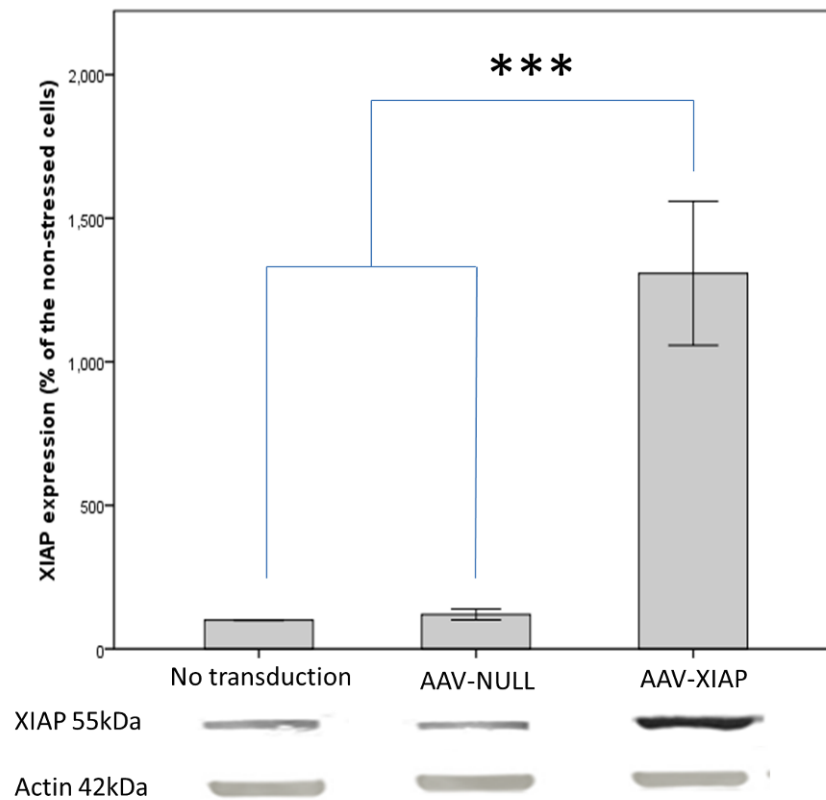


Figure 28 Western blot of XIAP in the ARPE-19 cells. The AAV-XIAP transduced cells showed a stronger XIAP immunostaining band (the far right lane) and significantly more XIAP protein expression compared with the non-transduced (the far left lane) and AAV-NULL controls (the middle lane). Quantification was analyzed based on band gray intensity, normalized by the actin expression. See Chapter 2 for more details. ***: $p < 0.001$, One-way ANOVA with Bonferroni's multiple test, $n = 4$.

4.4.3 Cell apoptosis

The H₂O₂ induced RPE cell apoptosis was determined by the TUNEL assay. A significant improvement of cell survival was found in the AAV-XIAP transduced cells after H₂O₂ stress. The AAV-XIAP transduced population had significantly less apoptotic cells compared to the AAV-NULL controls (3.2 ± 0.6 vs. 19 ± 1.3 cells per x200 field, $p=4 \times 10^{-6}$), or the non-transduced cells (3.2 ± 0.6 vs. 18 ± 1 , $p=1 \times 10^{-6}$). In fact, the number of TUNEL positive cells in the AAV-XIAP transduced group was reduced almost to the same level as in non-stressed cells (3.2 ± 0.6 vs. 1.8 ± 0.4 , $p=0.08$), whilst the AAV-NULL transduced population had significantly more apoptotic cells than the non-stressed controls (19 ± 1.3 vs. 1.8 ± 0.4 , $p=0.000001$, Figure 29).

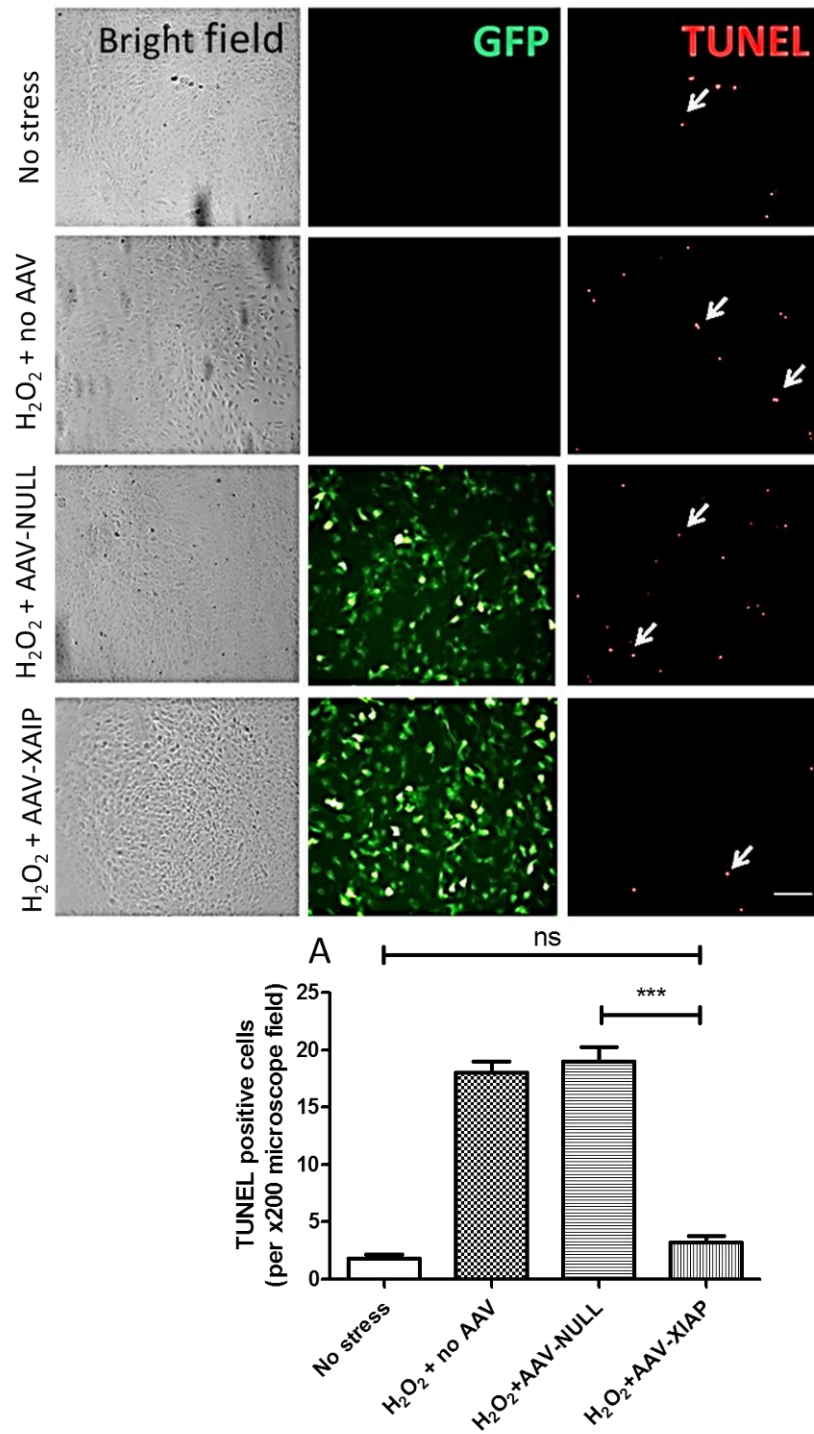


Figure 29 TUNEL assay of ARPE-19 cells. Subject to H₂O₂, the AAV-XIAP transduced cells showed significantly less apoptotic cells compare to the AAV-NULL control, which was similar to the non-stressed cells. The GFP fluorescence indicated successful transduction of AAVs. ns: no significance, ***: $p < 0.001$, One-way ANOVA with Bonferroni's multiple test, $n = 6$. Scale bar = 50 μm .

4.4.4 Mitochondrial function and cell viability

Mitochondrial function was measured by the MTT assay to determine cell viability. In contrast to the TUNEL assay, cell viability attenuated in both the AAV-XIAP transduced cells and the AAV-NULL controls when subjected to 1 hour treatment of 0.25 mM H_2O_2 ($P=0.60$, $F=13$, One-way ANOVA, Figure 30). The AAV-XIAP transduced cells had a significantly impaired mitochondrial function compared to non-stressed cells (0.4 ± 0.02 vs. 0.5 ± 0.02 , $t=4.3$, $p=0.002$, One-way ANOVA with Bonferroni's post test, Figure 30). There was no significant difference of the cell viability between the AAV-XIAP and non-transduced cells following H_2O_2 exposure (0.4 ± 0.02 vs. 0.3 ± 0.03 , $t=1.5$, $p=0.15$, One-way ANOVA with Bonferroni's post test, Figure 30).

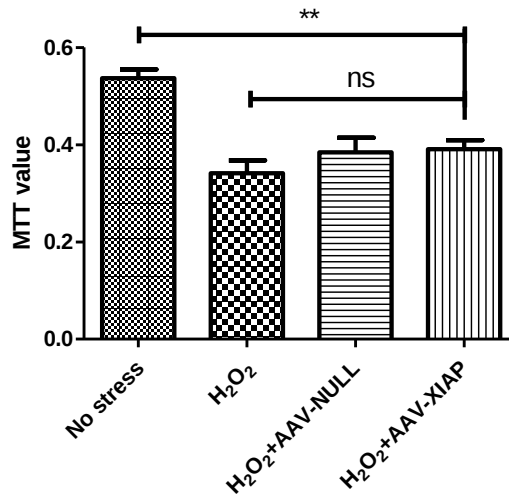


Figure 30 MTT assay of ARPE-19 cells. The cell viability, determined by the MTT value, was significantly lower in AAV-XIAP transduced cells compare to the non-stressed cells and similar to the H_2O_2 -treated cells. ns: no significance, **: $p<0.01$, One-way ANOVA with Bonferroni's multiple test, $n=8$.

4.5 Discussion

In this chapter, XIAP is successfully over expressed by the AAV-XIAP transduction in a human RPE cell line. The TUNEL results demonstrate a significant neuroprotective effect of XIAP on H₂O₂ induced apoptosis. Mitochondrial function, however, was not preserved by the XIAP treatment according to the MTT assay.

A recent study examined the molecular changes following H₂O₂ induced oxidative stress in the ARPE-19 cell line²³⁶. The study showed that stress induces the translocation of serine protease HtrA2/Omi from the mitochondria to the cytoplasm, which in turn degrades endogenous XIAP and activate apoptosis via a caspase-3 dependent mechanism. The investigators also showed a modest reduction in apoptosis when cells were also exposed to the HtrA2/Omi inhibitor, UCF-101. In our study however, we have gone a step further, because AAV mediated gene delivery does not just prevent the degradation of endogenous XIAP, it results in overexpression of XIAP (approximately one log unit increase in XIAP expression in individual ARPE-19 cells). In a study in transgenic mice, overexpression of XIAP was achieved in retinal bipolar and cerebellar Purkinje cells through use of the L7 promoter and resulted paradoxically in neuronal degeneration²⁰⁷. It may be difficult to extrapolate this directly, because caspase-3 has a key role in neuronal development, particularly with regard to long-term depression and NMDA synapse modeling²⁴⁷. Hence overexpression of XIAP during development might lead to secondary neuronal death through mechanisms of developmental failure that would not be relevant to the classic apoptotic pathway. Nevertheless we were keen to quantify XIAP levels as accurately as possible and the 11-fold higher expression we observed compared to the AAV-NULL controls was clearly neuroprotective. In this model however, an upper limit of

XIAP protein expression above which it might become pro-apoptotic was not investigated.

Another interesting observation from our study was that whilst the TUNEL assay outcomes were significantly altered with XIAP, the MTT assay did not show a statistically significant protective effect of XIAP after H₂O₂ treatment. A plausible explanation for this is evident if one considers the mechanism of the assays and the action of XIAP. In our RPE oxidative stress system, one mechanism of XIAP action is to inhibit activity of caspase-3, 7 and 9, which are activated following mitochondria changes but upstream to DNA fragmentation in the apoptotic pathway²⁴⁸. The MTT assay measures cell viability indirectly through mitochondrial function, by quantifying the reduction of a bromide salt (MTT) by mitochondrial succinate dehydrogenase²⁴⁹. The mitochondria acts upstream and releases pro-apoptotic proteins such as cytochrome-c, which activates caspases and induces apoptosis pathway²⁵⁰. The TUNEL assay, however, detects DNA fragmentation and represents one of the final steps in apoptosis after caspase activation²¹⁷. Hence since XIAP acts largely downstream of the mitochondria, it might be predicted to have much less effect on mitochondrial stress (MTT) than on DNA fragmentation (TUNEL), which is directly influenced by caspase activity.

The AAV-NULL serves as a precise control vector in this study. The lack of any detectable protective effect following AAV-NULL transduction confirms that the beneficial effects are specific from XIAP and not secondary to GFP expression or generalized effects of AAV transduction on cell homeostasis. Furthermore, apoptosis and cell viability with the control vector was similar to non-transduced controls following H₂O₂ stress. It demonstrates that the mechanism of cellular transduction

with AAV does not itself appear to accelerate cell death in this model of human RPE oxidative cell death. This, together with other studies and clinical trials, supports the safety of AAV treatment in human eyes¹³⁰⁻¹³².

Theoretically, inhibiting apoptosis at the late effector steps of caspases may be more effective than targeting upstream mitochondrial mechanisms. An advantage of targeting caspases is that they represent a late common effector of several apoptotic pathways²⁵¹. Here we have observed that XIAP has a marked effect in preventing DNA fragmentation despite mitochondrial stress, at least in the short term (24 hours after the H₂O₂ stress). Others have shown a long term effect of XIAP neuroprotection in rodent monogenic degeneration models⁹⁷.

However, a disadvantage of XIAP therefore might be that it could act too far downstream to influence many of the upstream cellular stress responses. Our MTT results indicate that mitochondria damages due to H₂O₂ stress cannot be rescued by XIAP. This might result in the preservation of cells that remain highly stressed and have no useful function. This raises the question, as to how to restore a cell that undergoes mitochondrial stress but not apoptosis due to XIAP-mediated caspase inhibition. Surviving but completely non-functional cone photoreceptor cells have been observed in patients with end-stage retinitis pigmentosa²⁵²⁻²⁵⁴. There is evidence that these cells may improve morphologically in animal models when stimulated with ciliary neurotrophic factor which signals via the JAK/STAT pathway upstream of mitochondria²⁵⁵. Therefore, a combined treatment with XIAP and neurotrophic factors may be helpful for retinal degenerative diseases.

**Chapter 5 *In vivo* Analysis of Sodium Iodate
Induced Retinopathy in Mice**

5.1 Introduction

Sodium iodate (NaIO₃) is an oxidizing agent and has been found specific toxicity on retinal pigment epithelium (RPE) after systematic administration since 1920s²⁵⁶. Disrupted RPE and reduction of c-wave have been described in various animal models of NaIO₃ induced retinopathy including rabbit^{257, 258}, dog²⁵⁹, rat²⁶⁰ and mouse^{261, 262}. Degeneration of photoreceptor cells is also observed and believed as a secondary consequence of RPE death^{261, 263}. The model has been widely used for assessment of neuroprotective agents and stem cell therapy²⁶⁴⁻²⁶⁷. However, the established method of studying morphological changes of RPE and photoreceptors is by histology, whilst an *in vivo* method would be ideal to track these changes on a longitude scale and significantly reduce the amount of animals needed for experiments.

The confocal scanning laser ophthalmoscope (cSLO) is a retinal imaging technique which has been widely used at human clinical practice^{268, 269}. Fundus autofluorescence (AF) in cSLO allows non-invasive assessment of retinal disease. With near-infrared light using a 790 nm laser source, the AF signal appears to mainly originate from melanin in the RPE and choroid²⁷⁰⁻²⁷². A 488 nm laser has also been used in cSLO. The main fluorophore at this wavelength is lipofuscin in human retina²⁷³. Coincidentally, this mode is also able to detect cone photoreceptors expressing green fluorescence protein (GFP) due to the identical excitation wavelength of GFP at 488 nm wavelength²⁷⁴. Meanwhile, transgenic engineering techniques have enabled selective GFP expression in rod photoreceptors driven by the rod-specific Nr1

promoter^{275, 276}. Therefore, the cSLO imaging may provide an *in vivo* technique to evaluate NaIO₃-induced RPE and photoreceptor degeneration.

In this study, the sSLO findings in the NaIO₃ treated wide type and two transgenic strains of mouse (the *NRL-GFP* and C57BL/6J^{TgOPN1LW-EGFP85933Hue}) are characterised. This unique method provides an easy and repeatable way to monitor RPE and photoreceptor degeneration *in vivo*.

5.2 Aims

The aim of this chapter is to investigate NaIO₃ induced retinopathy in the *NRL-GFP* and B6^{TgOPN1LW-EGFP} transgenic mouse, specifically:

- cSLO imaging
- ERGs
- Histology changes

See Figure 31 for an overview of the process and methods in this chapter.

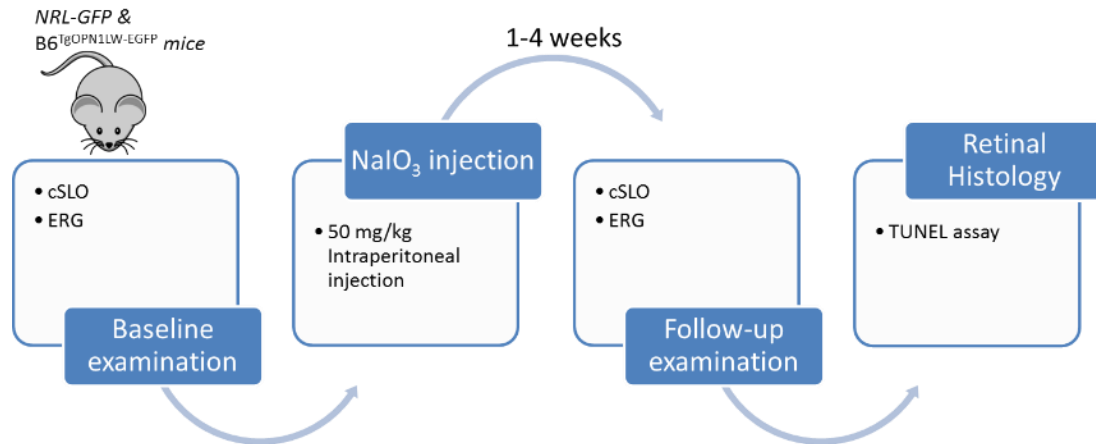


Figure 31 Schematic diagram showing the main steps and methods used in Chapter 5

5.3 Materials and methods

Mice

The wild type (wt) mouse, C57BL/6, was obtained from the Biomedical Sciences Division of the University of Oxford. The *NRL-GFP* mouse was bred by Robert MacLaren (University of Oxford) and has been previously described²⁷⁶. It is characterized with EGFP expression specifically to rod photoreceptors, driven by a 2.5-kb *Nrl* promoter segment²⁷⁵. The C57BL/6J^{TgOPN1LW-EGFP85933Hue} mouse (referred as B6^{TgOPN1LW-EGFP}) was obtained from the Mutant Mouse Regional Resource Centres, National Institutes of Health (000043-MU *Opn1.gfp*). It features EGFP expression in long/middle (LM) wavelength cones, driven by human LM-opsin²²³. Founders of this colony were kindly provided by Rachel Pearson (University College London Institute of Ophthalmology, London, UK). See Chapter 2 for general information of mice breeding and maintenance.

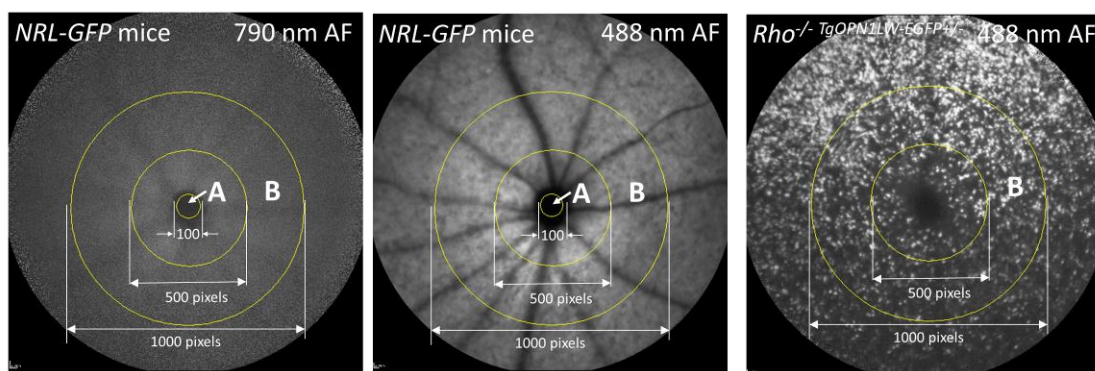
Sodium iodate delivery

Sodium iodate solution (Sigma-Aldrich, UK) was freshly prepared by dissolving in sterile PBS for a 0.5% solution. Mice were carefully weighted and intraperitoneally injected with the NaIO₃ solution using a 29 G insulin syringe (50 mg/kg). A same volume of sterile PBS was used as the control.

cSLO imaging and analysis

Fundus images were obtained from 6 mice of each strain at baseline, 1 and 4 weeks after intraperitoneal injection, respectively. For quantification, three concentric

circles were drawn on each image with 100, 500 and 1000 pixels diameters, respectively (Figure 32). For the images obtained on 790 nm AF mode, the grey value of the optic disc (A in Figure 32) and the area between the other two circles (B in Figure 32) were measured in Image J (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). The grey value of same areas was measured to quantify fundus AF on 488 nm AF mode in the *NRL-GFP* mice (Figure 32). The grey value of the area b was normalized to that of the optic disc and each baseline for comparison between the NaIO₃ and PBS treated eyes. For the images obtained in the *B6^{TgOPN1LW-EGFP}* mice on 488 nm AF mode, the bright individual AF dots were counted in the areas as described above to quantify the GFP expressing LM-cones (Figure 32). The LM-cone number was normalized to each baseline for comparison between the NaIO₃ and PBS groups.



*Figure 32 sSLO imaging and its analysis. Three concentric circles (100, 500 and 1000 pixels diameters, respectively) were drawn on each image using Image J. In NRL-GFP mice, the grey value of the optic disc area (A) and the area of interest (B) were measured using Image J. In the *B6^{TgOPN1LW-EGFP}* mice, the individual bright dots were counted in the area B to quantify the GFP expressing LM-cones. Images were acquired under proper sensitivity using 488 nm and 790 nm laser source. See Chapter 2 for more details on cSLO technique.*

ERG

ERGs were recorded at baseline, 1 and 7 days after intraperitoneal injection. Scotopic ERGs were performed in 6 *NRL-GFP* mice. Photopic and flicker ERGs were applied in 6 B6^{TgOPN1LW-EGFP} mice. The b- and c-wave amplitude were measured and normalized to the baseline before comparison between the PBS and NaIO₃ injected mice. See Chapter 2 for general information of the ERG technique.

Retinal histology

6 eyes of *NRL-GFP* mice were collected and sectioned at 1, 3, 7, 14 and 28 days post NaIO₃ injection, respectively. Another 6 PBS injected eyes were collected at 28 days post injection. 6 eyes of B6^{TgOPN1LW-EGFP} mice were collected at 28 days post NaIO₃ and PBS injection, respectively. See Chapter 2 for general information of eye sectioning.

TUNEL assay

The TUNEL assay was performed in least 1 slide of the *NRL-GFP* eyes at the time points described above. Apoptotic cells of the retinal ONL were counted in 3 eye rings of each slide and the average was compared. See Chapter 2 for general information of the TUNEL assay.

5.4 Results

5.4.1 cSLO imaging on 790 nm AF mode

At baseline, there was weak fundus AF on cSLO images of the wt, *NRL-GFP* and B6^{TGPN1LW-EGFP} mice. However, high diffused AF areas were observed from 1 week post NaIO₃ injection in all three strains (Figure 33-Figure 35). Determined by the grey value, the normalized fundus AF significantly increased at 4 weeks post NaIO₃ injection in the wt (120±4% vs. 100±3%, $p=0.01$, Figure 33), *NRL-GFP* (160±20% vs. 100±8%, $p=0.01$, Figure 34) and B6^{TGPN1LW-EGFP} mice (120±6% vs. 100±4%, $p=0.006$, Figure 35) when compared to the PBS controls. These cSLO findings appeared strongly correlated to the RPE damage associated with the NaIO₃ toxicity on retina.

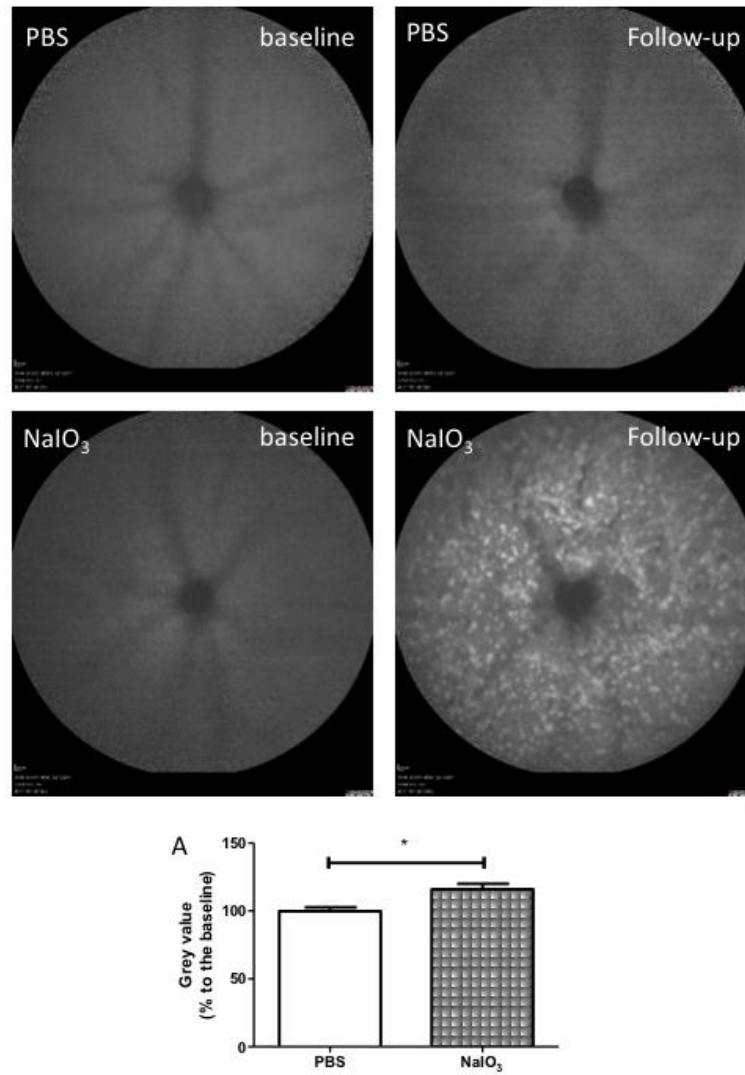


Figure 33 cSLO images of C57BL/6 mice on 790 nm AF mode. Compare to the PBS controls, the NaIO₃ treated group showed significantly high autofluorescence lesions when normalized to each baseline (A). The lesions were irregular and diffused distributed, appeared at 1 week post NaIO₃ treatment. *: $P < 0.05$, Unpaired *t*-test, $n = 6$.

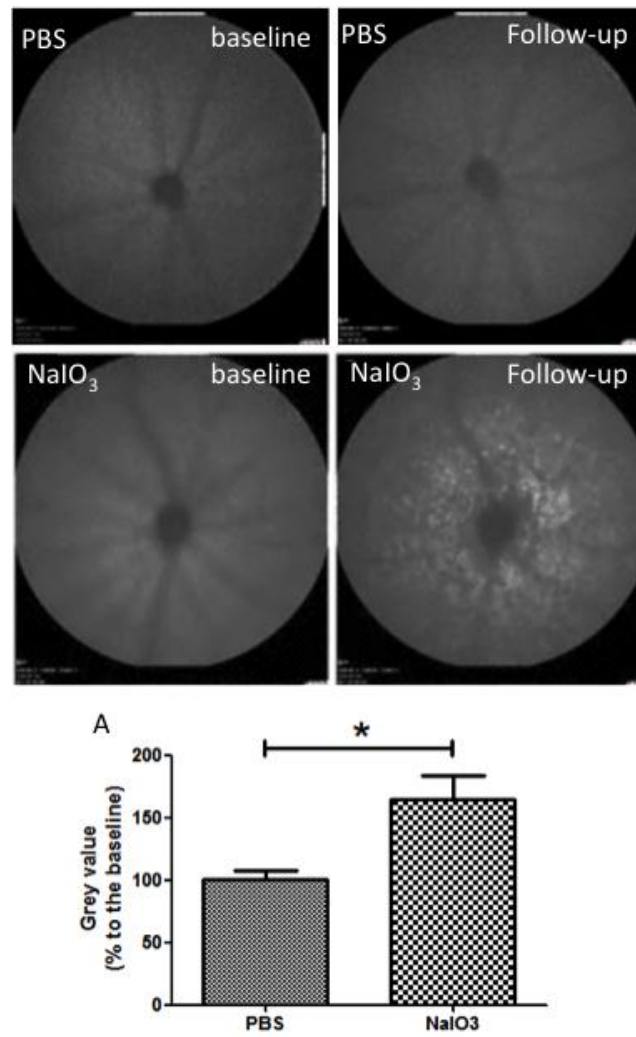


Figure 34 cSLO images of NRL-GFP mice on 790 nm AF mode. Compare to the PBS controls, the NaIO₃ treated group showed high autofluorescent areas at 4 weeks post PBS treatment. Determined by the grey value, the autofluorescence of NaIO₃ treated group was significantly higher than the PBS controls when normalized to each baseline (A). *: $P < 0.05$, Unpaired *t*-test, $n = 6$.

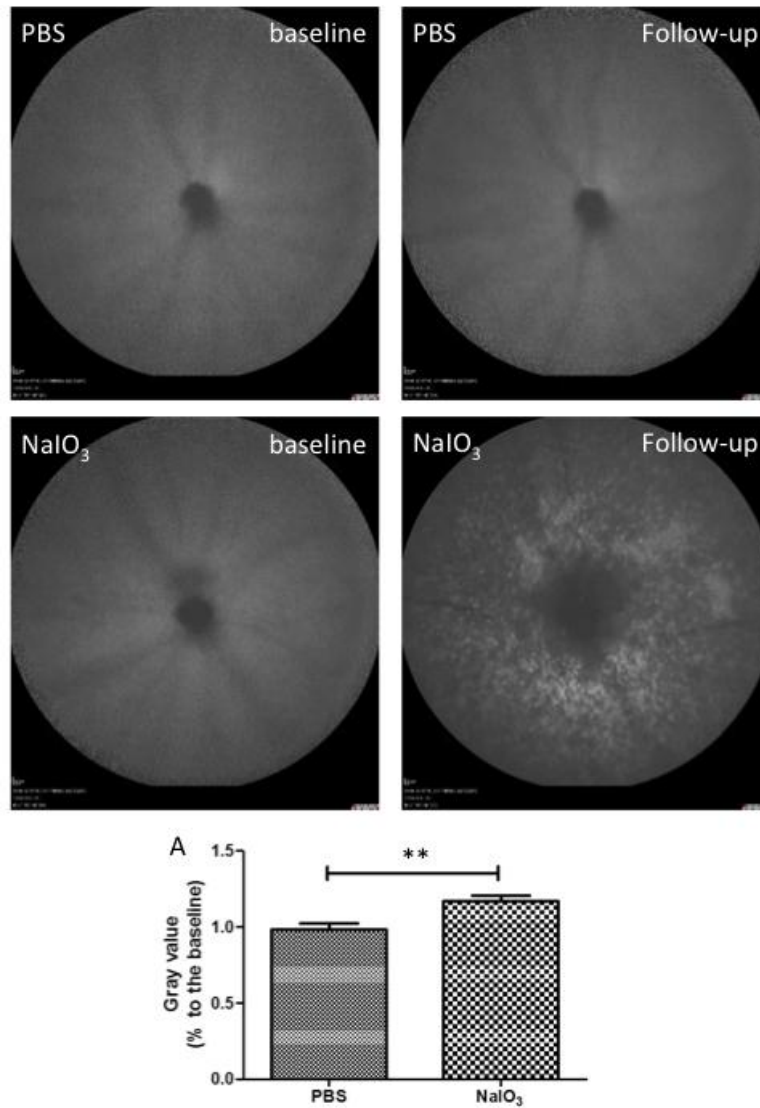


Figure 35 cSLO images of $B6^{TGOPN1LW-EGFP}$ mice on 790 nm AF mode. Compare to the PBS controls, the NaIO₃ treated group showed high autofluorescent areas at 4 weeks post PBS treatment. Determined by the grey value, the autofluorescence of NaIO₃ treated group was significantly higher than the PBS controls when normalized to each baseline (A). **: $P < 0.01$, Unpaired t-test, $n = 6$.

5.4.2 cSLO imaging on 488 nm AF mode

Because of the similar excitation wavelength, rods and cones expressing GFP were detectable using a 488 nm laser source of cSLO imaging in our transgenic strains. In the *NRL-GFP* mice, an overwhelming distribution of bright fundus AF was observed due to the dominant number of rods (PBS panel in Figure 36). In the NaIO₃ treated mice, however, scattered areas of reduced AF were noted at 4 weeks post treatment. Determined by the grey value, the baseline-normalized AF was significantly decreased in the NaIO₃ treated mice than the PBS controls (0.7 ± 0.2 vs. 2 ± 0.8 $p=0.04$, Figure 36), suggesting a reduction of rods after NaIO₃ treatment.

In the B6^{TGPN1LW-EGFP} mice, the GFP expressing LM-cones appeared individual bright dots on cSLO images of 488 nm AF mode (Figure 37). Compared to each baseline, a gradual loss of cone number was observed in both PBS (850 ± 60 vs. 1100 ± 50 , $p=0.004$) or NaIO₃ (580 ± 50 vs. 1100 ± 60 , $p=0.0003$) treated mice at 4 weeks post treatment. When normalized to each baseline, the survival LM-cones were significantly less in the NaIO₃ treated eyes than the PBS controls ($50 \pm 3\%$ vs. $70 \pm 2\%$, $p=0.0006$, Figure 37), suggesting that NaIO₃ treatment accelerated the LM-cone loss in this strain.

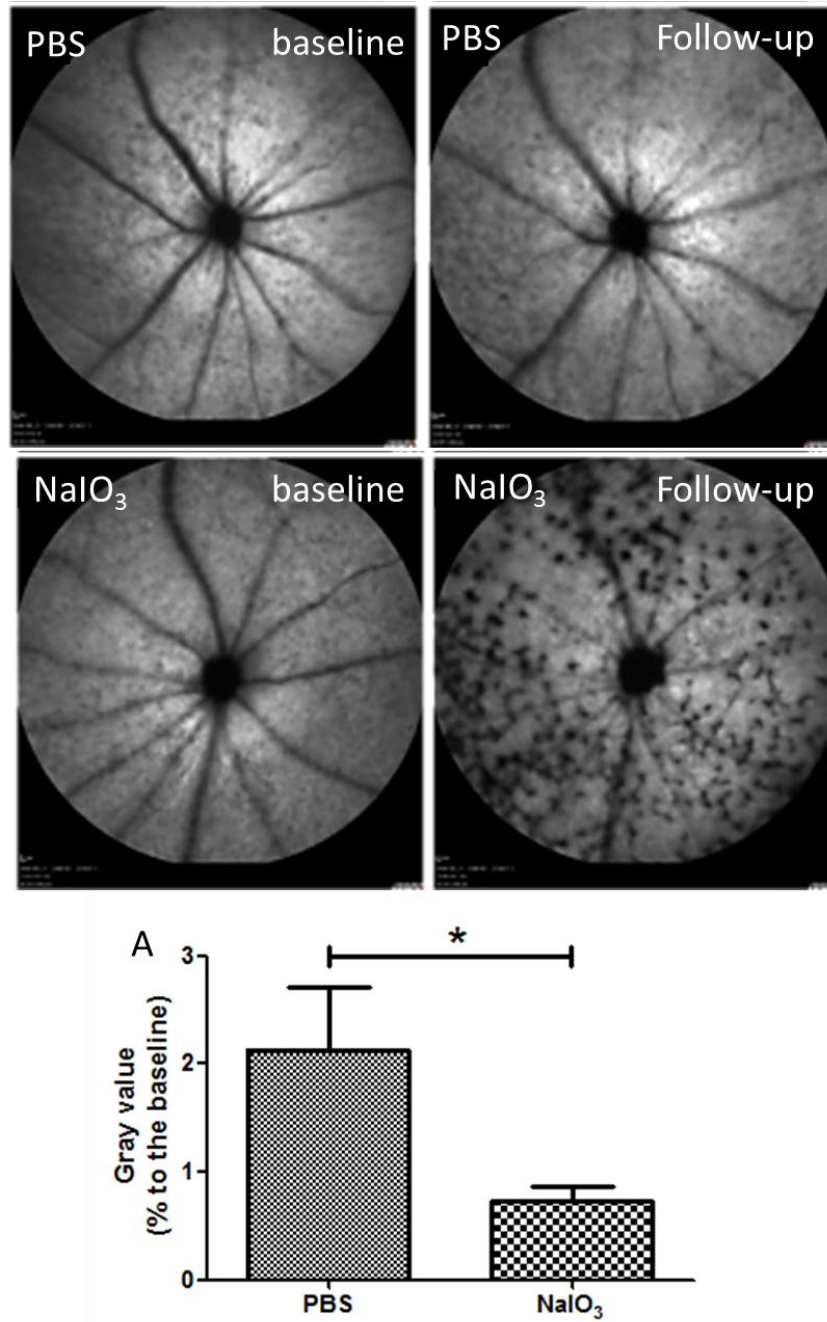


Figure 36 cSLO images of NRL-GFP mice on 488 nm AF mode. Compare to the PBS controls, the NaIO₃ treated group showed dark autofluorescent areas at 4 weeks post PBS treatment. Determined by the grey value, the autofluorescence of NaIO₃ treated group was significantly lower than the PBS controls when normalized to each baseline (A). *: $P < 0.05$, Unpaired *t*-test, $n = 6$.

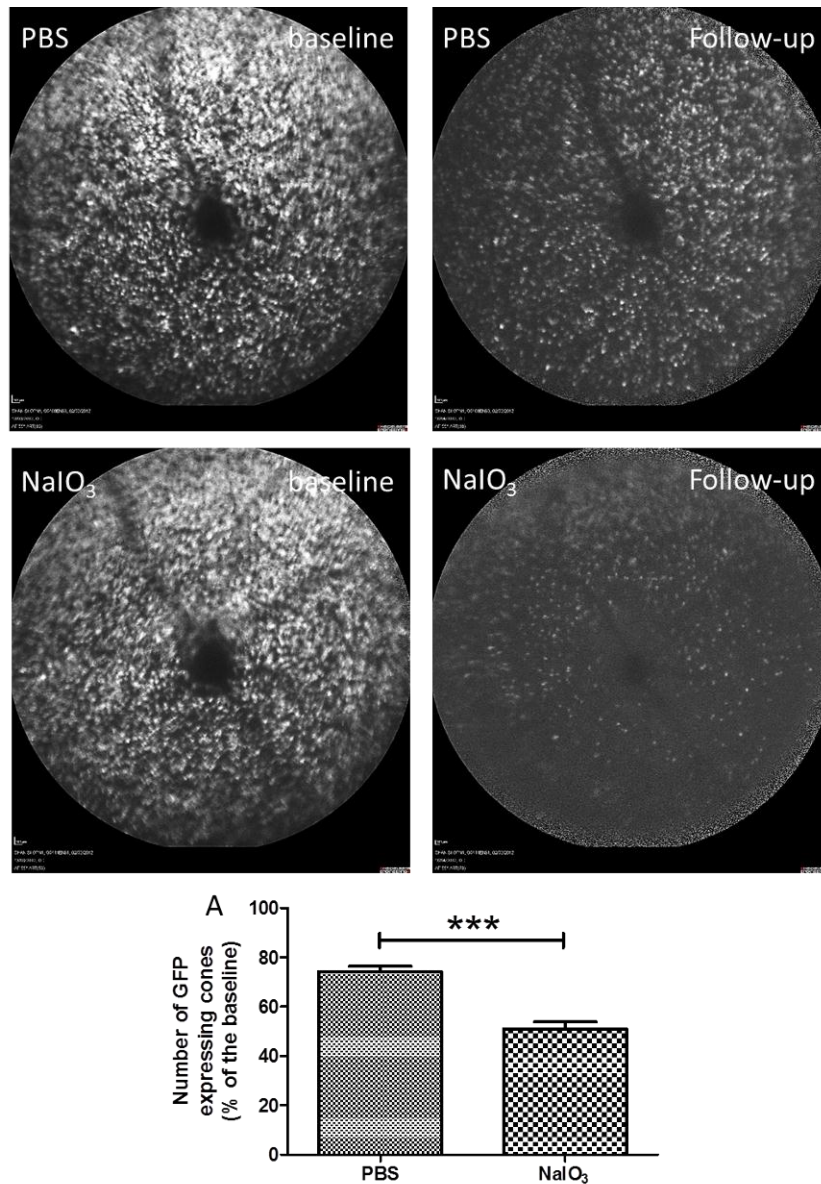


Figure 37 cSLO images of *B6^{TGOPN1LW-EGFP}* mice on 488 nm AF mode. The cones appear individual bright dots on the images due to GFP expression with opsin promoter in this transgenic strain. Compare to the PBS controls, the NaIO₃ treated group showed significantly more reduction of cones when normalized to each baseline (A). ***: $p < 0.001$, Unpaired *t*-test, $n = 6$.

5.4.3 Correlation of 790 nm and 488 nm cSLO images

To see if there is any correlation of the cSLO images on 790 nm and 488 nm modes, the AF changes on each mode were compared in the *NRL-GFP* mice treated with NaIO₃. The reduced AF areas on 488 nm AF mode were outlined and then compared with the high AF areas on 790 nm AF mode. The “matched areas” were defined when the two kinds of areas were overlapped at most. Otherwise they were regarded as the “unmatched areas” (Figure 38). The percentage of each category was calculated and compared. The “matched areas” were found significantly fewer than the “un-matched areas” in the NaIO₃ treated *NRL-GFP* mice ($35 \pm 3\%$ vs. $65 \pm 3\%$, $p=0.02$), suggesting that the AF changes on 790 nm and 488 nm modes may not totally interact with each other.

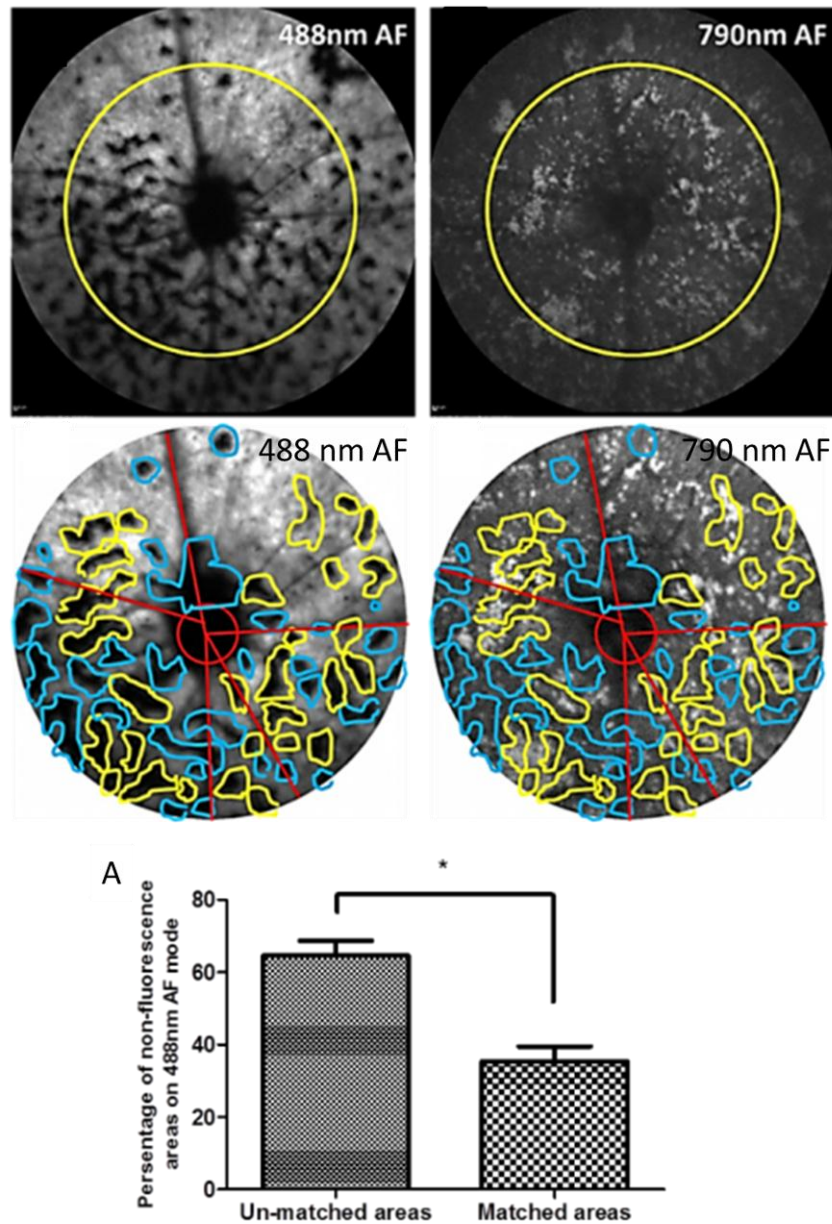


Figure 38 Comparison of the cSLO images on 488 nm and 790 nm mode in the NaIO₃ injected NRL-GFP mice. The yellow outlined areas indicate the dark autofluorescent areas on 488 nm AF mode where “abnormal” high autofluorescent areas on 790 nm mode exist, defined as “matched areas”. The blue outlined areas are “un-matched areas” where the two kinds of autofluorescent areas did not overlapped. The matched areas were significantly less than the un-matched areas. *: $p < 0.05$, Paired t-test, $n = 6$.

5.4.4 ERGs

ERGs were recorded in the *NRL-GFP* and B6^{TGPN1LW-EGFP} mice to measure the photoreceptor function changes before and after NaIO₃ treatment (Figure 39, Figure 41). In ERG, the a-wave originates from the closure of cGMP-gated channels on the out segments of photoreceptors and mainly reflects the current change of photoreceptors under light. The b-wave is mainly derived from the post-synaptic second retinal neurons. Since rod dominates the mouse retina photoreceptors (~97%), the b-wave amplitude, determined by the trough of a-wave and the peak of b-wave, mainly reflects the rod function in the dark-adapted *NRL-GFP* mice (i.e. Scotopic ERG). In the light-adapted B6^{TGPN1LW-EGFP} mice, the b-wave mainly indicates the cone function as the rods are suppressed by the light (i.e. Photopic ERG).

According to our ERGs, the b-wave of dark-adapted *NRL-GFP* mice decreased significantly at 1 week post NaIO₃ treatment under the various stimuli in our protocol, suggesting a loss of rod function after NaIO₃ treatment (Figure 39). Under the stimulus of 0.1 cd.s/m², for instance, the b-wave of the NaIO₃ and PBS treated *NRL-GFP* mice was 3 ± 1% and 70 ± 4% of each baseline, respectively ($p < 0.0001$, Figure 40). The b-wave of light-adapted B6^{TGPN1LW-EGFP} mice, reflecting the cone function, also decreased significantly at 1-week post NaIO₃ injection (A-D in Figure 41). Under the single flash stimulus of 10 cd.s/m², for instance, the b-wave of the NaIO₃ and PBS group was 30 ± 10% and 105 ± 10% to each baseline, respectively ($p = 0.002$, E in Figure 41). In the flicker ERGs, the b-wave was 30 ± 3% vs. 160 ± 7% at the 15 Hz stimulus ($p < 0.0001$) and 50 ± 8% vs. 160 ± 10% at the 30 Hz stimulus ($p = 0.0001$, F in Figure 41).

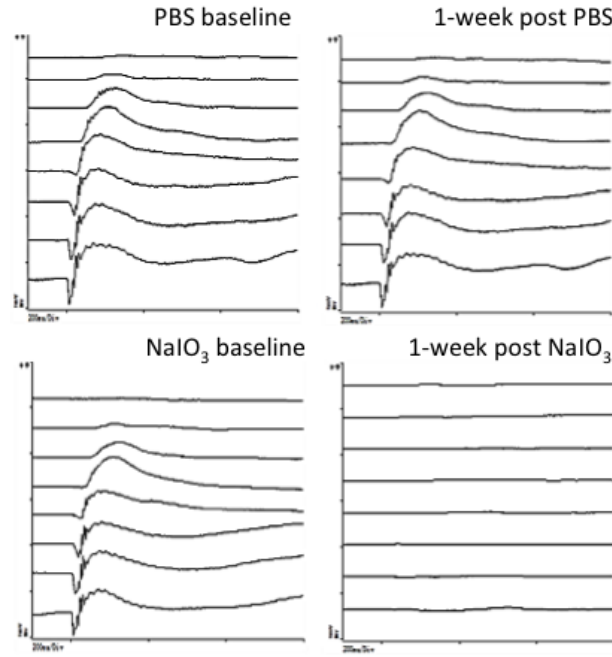


Figure 39 Scotopic ERGs of the NRL-GFP mice. Compare to the PBS controls, the b-wave of NaIO₃ treated group decreased significantly at 1-week post treatment. This highly indicates the NaIO₃ toxicity on rods.

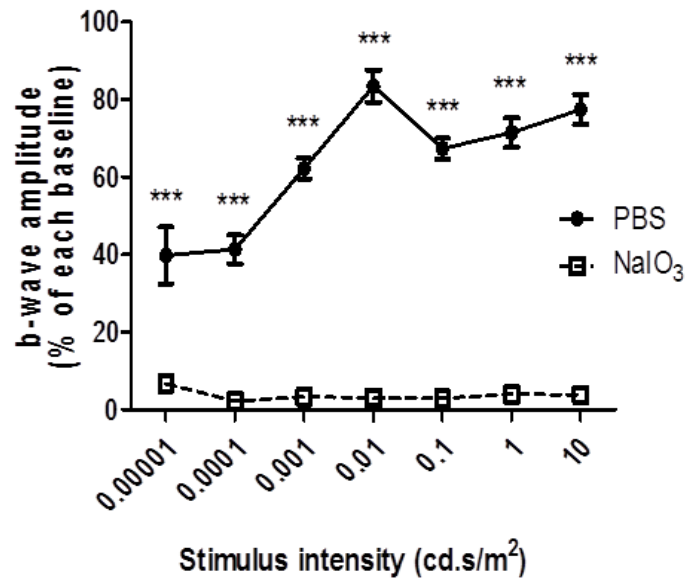


Figure 40 Comparison of b-wave in the PBS and NaIO₃ treated group at 1-week post treatment. Data were normalized to each baseline. ***: $p < 0.001$, Two-way ANOVA with Bonferroni post-tests, $n = 6$.

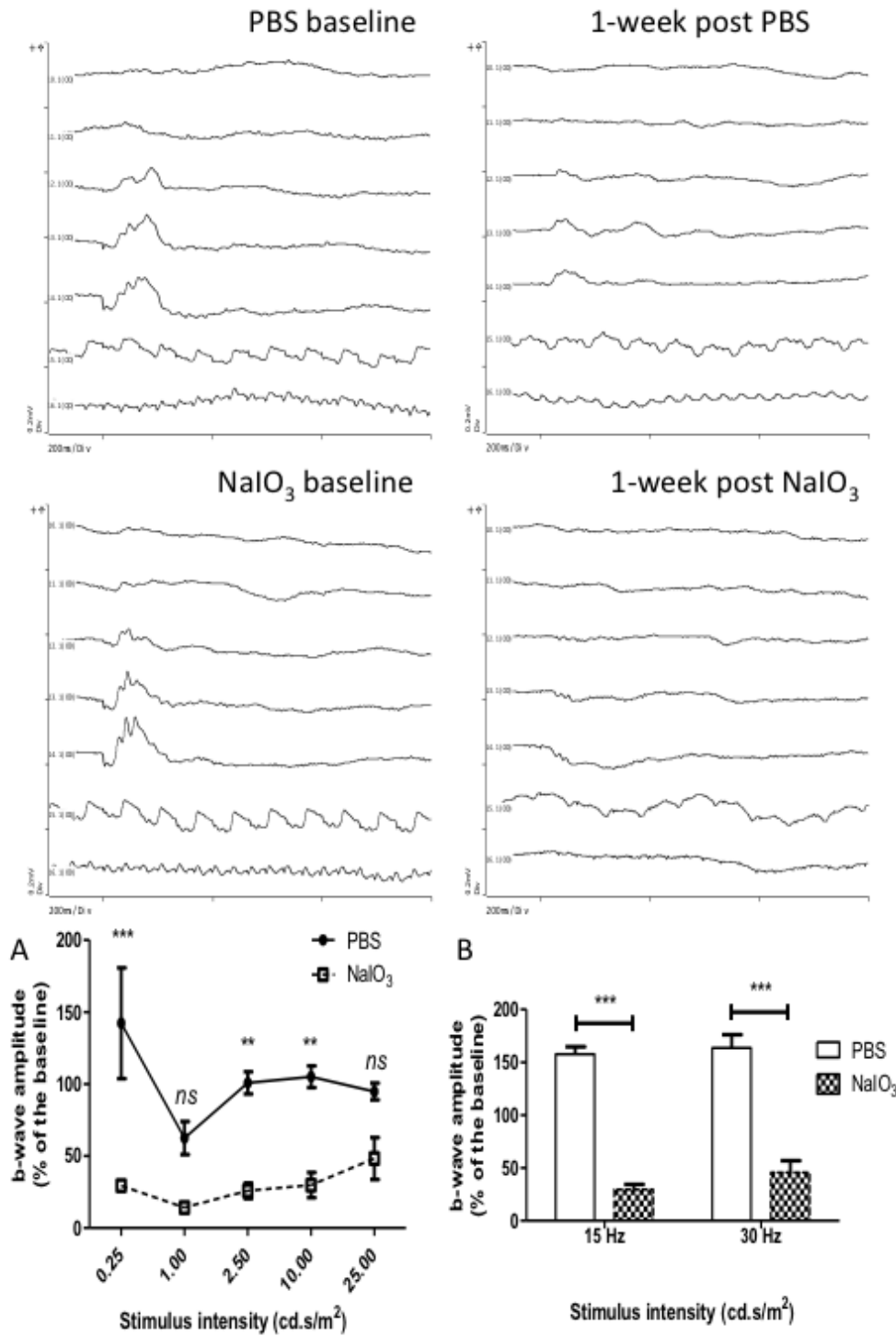


Figure 41 Photopic ERGs of the B6^{TGPN1LW-EGFP} mice. Compare to the PBS controls, the b-wave of NaIO₃ treated mice reduced significantly. Normalized to each baseline, the b-wave decreased both under single flash stimuli of 0.25-25 cd.s/m² (A) and 15/30 Hz “flicker” ERGs (B). ns: no significance, **: $p < 0.01$, ***: $p < 0.001$, Two-way ANOVA with Bonferroni post-tests (E) or unpaired t-test (F), $n = 6$.

5.4.5 Retinal histology

Compared with the PBS controls, disrupted RPE layer was observed in the NaIO₃ injected *NRL-GFP* mice at 1 day post injection. RPE cells were found immigrated in the inner retinal layers at 4 weeks post injection. The ONL appeared apparent disruption and distortion from 3 days post injection, which progressed to fragmented ONL at 4 weeks post injection. The GFP fluorescence was partially lost in the ONL and inner segments at 4 weeks post injection (Figure 42). In the sections undergoing TUNEL assay, apoptotic cells were mainly observed in the ONL at 1 and 3 days post NaIO₃ injection. There were significantly more apoptotic cells at 3 days than 1 day post NaIO₃ injection (37 ± 4 vs. 25 ± 2 , $p=0.005$, Figure 42). In the eye sections of B6^{TGPN1LW-EGFP} mice, similarly, RPE and ONL appeared severely disrupted at 4 weeks post NaIO₃ injection, whilst they remained the organized structure in the eye sections of PBS controls (Figure 44).

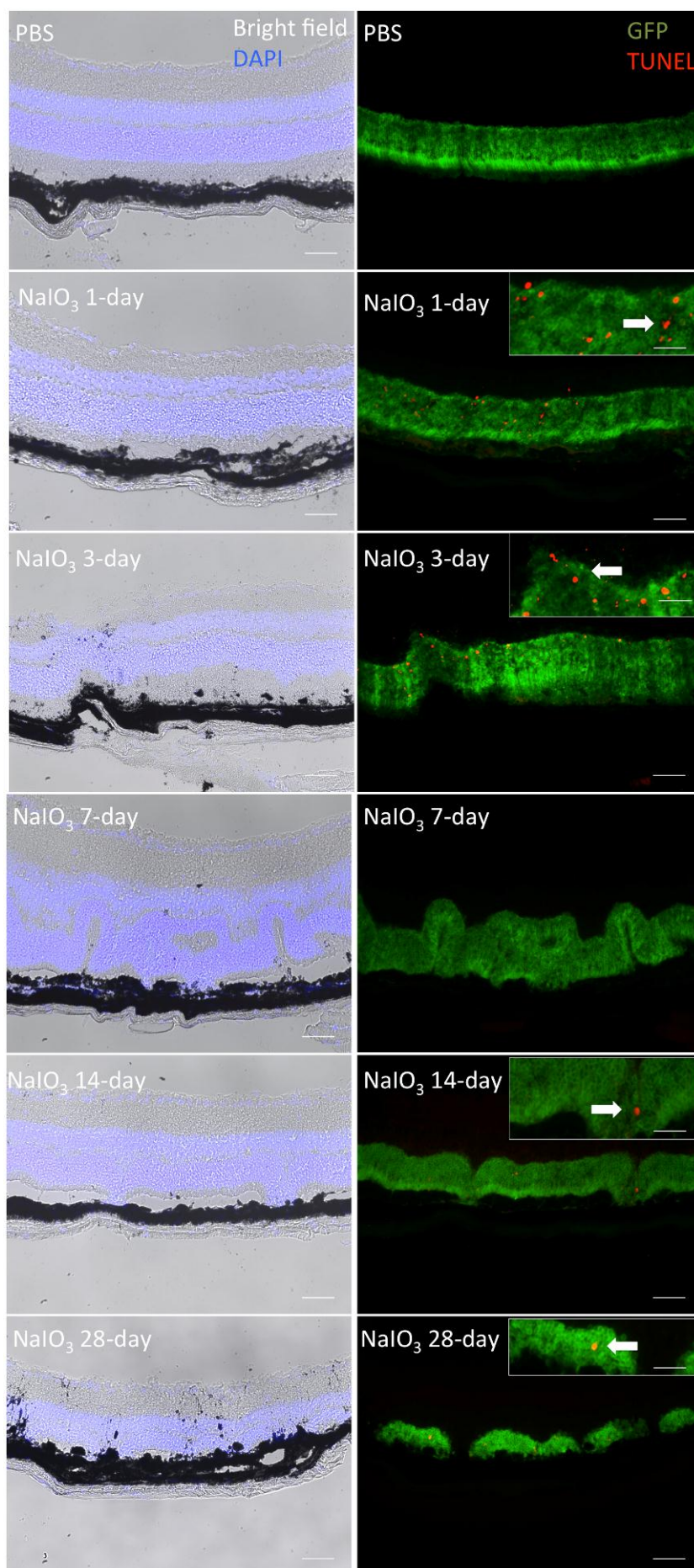


Figure 42 (Previous page) Histology and TUNEL assay of the NRL-GFP mice.

Compared to the PBS controls, the NaIO₃ treated mice began to show disrupted RPE at 1-day post treatment. The outer nuclear layer appeared distorted at 3 days post treatment and progressed to fragment at late time points. The apoptotic cells were seen in most time points but mainly on Day 1 and 3 post NaIO₃ treatment (arrows in the embedded figures). Scale bar=50 μ m or 25 μ m (embedded figure).

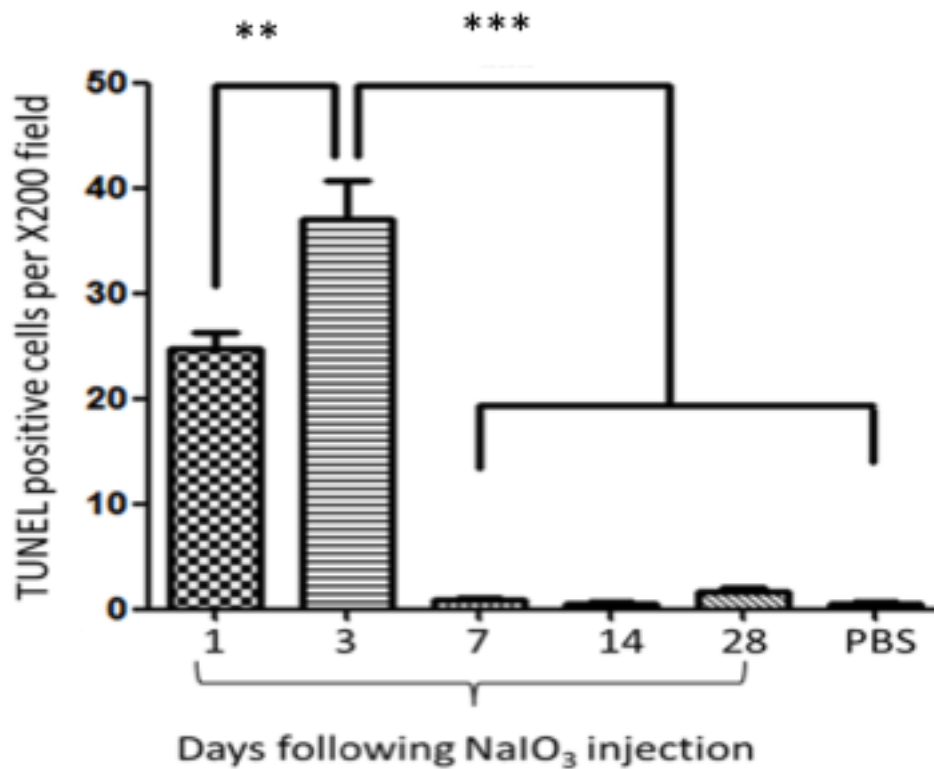


Figure 43 Comparison of the TUNEL assay in the NRL-GFP mice. The apoptotic cells at 3 days post NaIO₃ treatment were significantly more than those at other time points and the PBS controls. **: $p < 0.01$, ***: $p < 0.001$, One-way ANOVA with Bonferroni post-tests, $n = 6$.

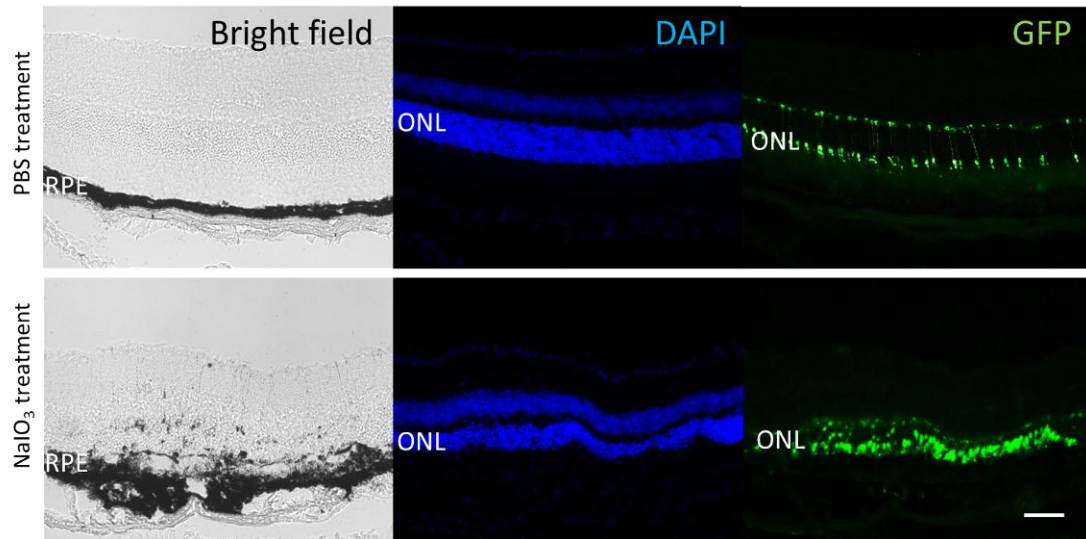


Figure 44 Histology of the B6^{TGOPN1LW-EGFP} mice. Compared to the PBS controls, the NaIO₃ treated mice showed disrupted RPE and ONL at 14 days post treatment. RPE: retinal pigment epithelium, ONL: outer nuclear layer. Scale bar=50 μ m.

5.5 Discussion

In the study we mainly described the cSLO findings in the NaIO₃ treated wide type and two transgenic strains of mouse. High diffused AF areas were observed in all animals on 790 nm mode. On 488 nm mode, scattered areas with reduced AF were found in the *NRL-GFP* mice whilst loss of GFP expressing cones was in the B6^{TGPN1LW-EGFP} mice. The ERGs and retinal histology further confirmed the NaIO₃ toxicity on these mice.

The present results, for the first time, revealed the fundus AF changes on 790 nm imaging mode in NaIO₃ treated mice. The AF signal at this wavelength seems to originate from melanin, a major component of the RPE and choroid^{270, 271}. The RPE is well known to be specifically damaged after NaIO₃ administration^{256, 261, 277}. NaIO₃ can lead to destruction of the RPE basal membrane, necrosis of the RPE cells and a quick loss of the c-wave in ERGs²⁷⁸⁻²⁸⁰. It was reported that NaIO₃ increases the ability of melanin to convert glycine to glyoxylate in mouse retina, highly indicating a chemical reaction between NaIO₃ and melanin²⁸¹. NaIO₃ is supposed to induce oxidation with melanin that releases toxic products and disrupt RPE cells²⁸¹. Oxidized melanin, a compound close to melanin, has been found to contribute to the near-infrared autofluorescence²⁷⁰. Such reaction between NaIO₃ and melanin may partially, if not at all, explain the AF changes on 790 nm AF images.

Another explanation of the fundus AF changes might be the ocular inflammation caused by NaIO₃ toxicity. RPE necrosis, which has been previously reported in NaIO₃ treated animals, is very likely to induce local inflammation

macrophage migration²⁶³. Inflammation cells such as retinal perivascular macrophages and microglia cells have been found to emit autofluorescence due to their phagocytosis of rod outer segments and cytoplasmic inclusions containing protein-bound oxidized lipids (ROSs)^{282, 283}. Moreover, oxidation of ROSs, which may well happen after NaIO₃ treatment, results in their faster phagocytosis and higher levels of autofluorescence²⁸². In the present study, the high autofluorescent lesions appeared a diffused pattern on 790 nm AF mode of cSLO. This might be the local accumulation of autofluorescent subretinal macrophages due to NaIO₃ toxicity on RPE and photoreceptors. However, it was reported that the fundus autofluorescence on 488 nm AF mode also altered due to macrophages changes. In the present study, we did not observe the similar pattern of autofluorescence changes on 488 nm AF mode compared to the appearance on 790 nm AF mode. Therefore specific immunostaining related to macrophage and microglia may be helpful to further determine their role in the abnormal autofluorescence appearance in NaIO₃ treated mice.

Apart from RPE toxicity, NaIO₃ is also known to induce photoreceptors degeneration^{277, 280}. Recently cSLO has been reported to track GFP expressing retinal ganglion cells²⁸⁴, microglia²⁸⁵ or photoreceptors in transgenic mice²⁷⁴. In the present study, similarly, the 488 nm imaging mode was successfully performed to detect the GFP expressing rods and LM-cones in the *NRL-GFP* and B6^{TGPN1LW-EGFP} mice, respectively. Determined by the GFP intensity or the number of individual cell, cSLO revealed the degeneration of rod and cone photoreceptors in the NaIO₃ induced retinopathy. To the best of our knowledge, this is the first report to describe the cSLO technique to longitudinal monitor photoreceptor degeneration in this model.

Recently the outer retinal degeneration was studied by ultrahigh resolution optical coherence tomography (UHR-OCT) in NaIO₃ treated rats²⁸⁶. Compared with the UHR-OCT which provides cross-sectional morphologic tomograms of the retina, the cSLO features layer-based morphologic imaging of the retina. It confers a global overview of the photoreceptor changes instead of a special retina section in the UHR-OCT. Moreover, the different wavelength in cSLO enables the detection of potential NaIO₃-melanin reaction in RPE and choroidal. Both techniques, however, provide a unique combination of non-invasive optic imaging way to assess morphological changes in NaIO₃ induced retinopathy. Results of this study could potentially benefit the understanding and translational research of retinal degenerative diseases affecting primarily the RPE, such as AMD and Stargardt disease.

**Chapter 6 XIAP Effect on Photoreceptors of
Transgenic Reporter Mouse with Sodium Iodate Induced
Retinopathy**

6.1 Introduction

As described in the previous chapter, the cSLO provides a very useful technique to examine the NaIO₃ toxicity on RPE and photoreceptor cells *in vivo* in the *NRL-GFP* and B6^{TgOPN1LW-EGFP} mice. On 790 nm AF mode, the high fundus autofluorescence may result from the melanin changes of the RPE cells by NaIO₃ oxidation. In addition, the GFP expressing rods and cones are detectable using a 488 nm laser source. This enables the measurement of the photoreceptor survival *in vivo* based on their GFP fluorescence changes in the cSLO.

The underlying mechanism of NaIO₃ induced photoreceptor death still remains unclear. Predominant apoptotic cells have been found in the ONL after NaIO₃ treatment, suggesting that the photoreceptors may die via apoptosis instead of necrosis^{263, 287}. NaIO₃ is known to specifically damage the RPE prior to photoreceptor cell death²⁶¹. Thus photoreceptor apoptosis may be a result secondary to the loss of RPE structure or function. Another possible reason may be direct NaIO₃ oxidative damage to photoreceptors. As a strong oxidizing agent, NaIO₃ is found to react with melanin in the RPE cells after systematic delivery²⁸¹. Therefore it is likely that photoreceptors may somehow die due to a number of mechanisms following NaIO₃ induced oxidative stress.

The previous chapter documented that AAV-mediated XIAP overexpression could rescue cultured RPE cells against H₂O₂ induced oxidative apoptosis. The NaIO₃ induced retinopathy, together with the cSLO technique, seems to be a useful oxidative

stress model to assess XIAP neuroprotection *in vivo*. However, there is no report regarding XIAP neuroprotection in NaIO₃ induced retinopathy to date.

6.2 Aims

The aim of this chapter is to assess the effect of AAV-mediated XIAP on rod and cone photoreceptors in NaIO₃ induced retinopathy of the *NRL-GFP* and B6^{TgOPN1LW-EGFP} transgenic mice, respectively. The XIAP neuroprotection will be investigated specifically by:

- *In vivo* analysis of rod and cone survival using cSLO imaging
- Functional rescue of rod and cone using ERGs

See Figure 45 for an overview of this chapter.

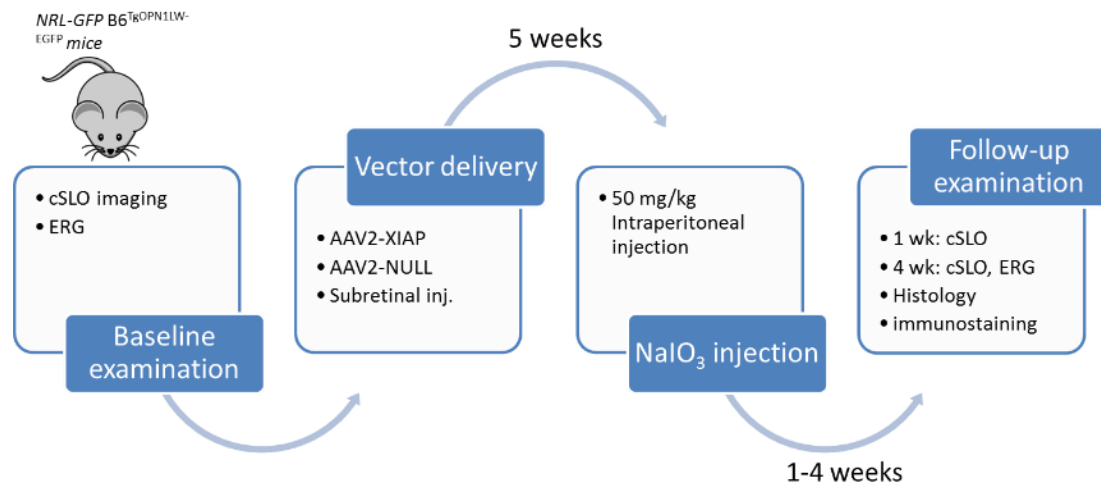


Figure 45 Schematic diagram showing the main steps and methods used in Chapter 6

6.1 Materials and methods

Mice

The *NRL-GFP* and B6^{TgOPN1LW-EGFP} mice were obtained as described in Chapter 5. Also see Chapter 2 for general information on mouse breeding and maintenance.

Vectors and subretinal delivery

The vectors were designed by Professor Robert MacLaren and commercially packaged (see Chapter 2). Briefly, a human XIAP coding sequence was packaged into AAV2 (AAV-XIAP). An identical vector without XIAP was constructed as the control (AAV-NULL). Neither of these vectors contained the GFP reporter gene. Both vectors were purified against immobilized heparin sulfate proteoglycan and the final concentrations were diluted to 1.0×10^{13} genomic particles/ml.

The vectors were injected into the superior subretinal space of the *NRL-GFP* and B6^{TgOPN1LW-EGFP} mice (9 mice each strain). The AAV-XIAP and AAV-NULL were injected into the right and left eye, respectively. The mice were bred for an additional 5 weeks at which point they underwent NaIO₃ injection (50 mg/kg, 6 mice each strain) or Western blot (3 mice each strain). See Chapter 2 for general information on the subretinal injection technique.

cSLO imaging

The baseline images were obtained before vector injection in both strains (6 mice in each strain). The follow-up imaging was performed at 4 weeks post NaIO₃

injection. cSLO was performed and analyzed as described in Chapter 5. However, only the superior half of the quantification area was analyzed, in order to represent the subretinal injection area exposed to the vectors. Also see Chapter 2 for general information of the cSLO technique.

ERG

Scotopic ERGs were recorded in 6 *NRL-GFP* mice at baseline and 4 weeks post NaIO₃ injection. Photopic ERGs were performed in 6 B6^{TgOPN1LW-EGFP} mice at baseline and 4 weeks post NaIO₃ injection. See Chapter 2 and 5 for more information of ERG technique and analysis.

Immunohistochemistry

The eyes of the *NRL-GFP* and B6^{TgOPN1LW-EGFP} mice were harvested and underwent sectioning after the follow-up examination of cSLO and ERG at 4 weeks post NaIO₃ injection. XIAP immunohistochemistry was performed in at least 3 slides of each vector in each strain. The slides were incubated with XIAP antibody (Abcam, 1:200) overnight at 4 °C. An appropriate secondary antibody (Alexa Fluor 555, Invitrogen) was applied to the slides followed by DAPI staining. See Chapter 2 for general information of eye sectioning, immunohistochemistry and DAPI staining.

Western blot

The samples were collected in the *NRL-GFP* and B6^{TgOPN1LW-EGFP} mice at 5 weeks post vector injection (3 eye samples each vector in each strain). A total amount of 30 ul samples were loaded in each lane of 10% SDS-PAGE gels (2 lanes per sample). The nitrocellulose blots were incubated with XIAP antibody (Abcam, 1:1000) overnight at 4° C, followed by incubation of appropriate HRP-conjugated

secondary antibody (Santa Cruz, 1:5000) at room temperature. See Chapter 2 for general information and quantification of Western blot technique.

6.2 Results

6.2.1 cSLO imaging on 790 nm AF mode

In the *NRL-GFP* mice, the fundus autofluorescence was significantly increased at 4 weeks post NaIO₃ injection compared to baseline in both the AAV-XIAP treated eyes (0.8 ± 0.06 vs. 0.6 ± 0.04 , $p=0.04$) and the AAV-NULL control (0.9 ± 0.07 vs. 0.6 ± 0.02 , $p=0.01$, Figure 46). Normalized to each baseline, there was no significant difference of the fundus autofluorescence between the AAV-XIAP and AAV-NULL groups ($130 \pm 11\%$ vs. $130 \pm 13\%$, $p=0.8$, Figure 46).

In the B6^{Tg^{OPN1LW}-EGFP} mice, similarly, the fundus autofluorescence increased at 4 weeks post NaIO₃ injection compared to the baseline in the AAV-XIAP treated eyes (0.8 ± 0.04 vs. 0.7 ± 0.02 , $p=0.004$) and the AAV-NULL controls (0.9 ± 0.02 vs. 0.7 ± 0.02 , $p=0.0002$, Figure 47). Normalized to the baseline, there was no significant difference of the fundus autofluorescence between the AAV-XIAP and AAV-NULL eyes ($120 \pm 10\%$ vs. $120 \pm 4\%$, $p=0.7$, Figure 47).

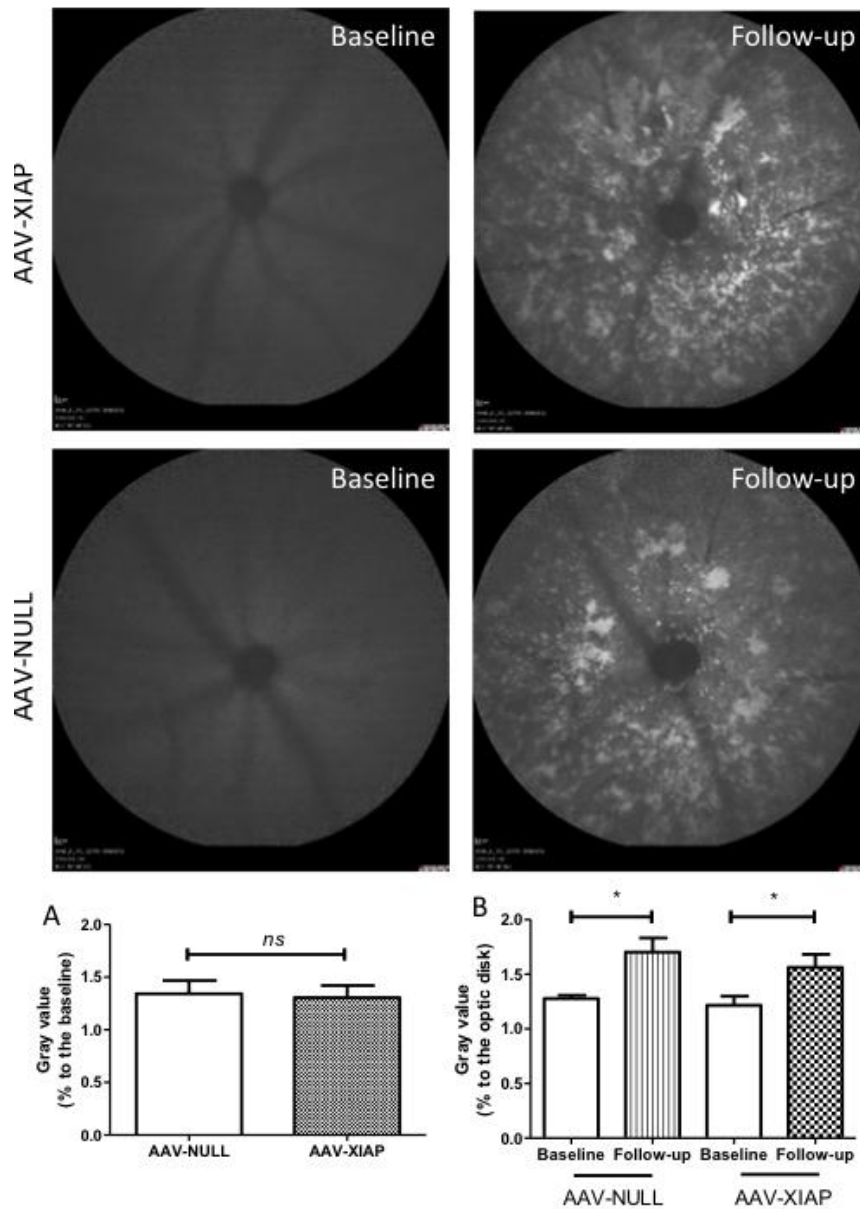


Figure 46 cSLO images on 790 nm AF mode in the AAV injected NRL-GFP mice.

Compared to the AAV-NULL injected controls, the AAV-XIAP injected mice showed a similar AF after NaIO₃ treatment (A: Unpaired t-test, n=6). Both groups showed a significant increase of AF after NaIO₃ treatment, possibly due to melanin oxidation or local inflammation (B, Two-way ANOVA with Bonferroni's post-hoc test, n=6). ns: no significance. *: p<0.05

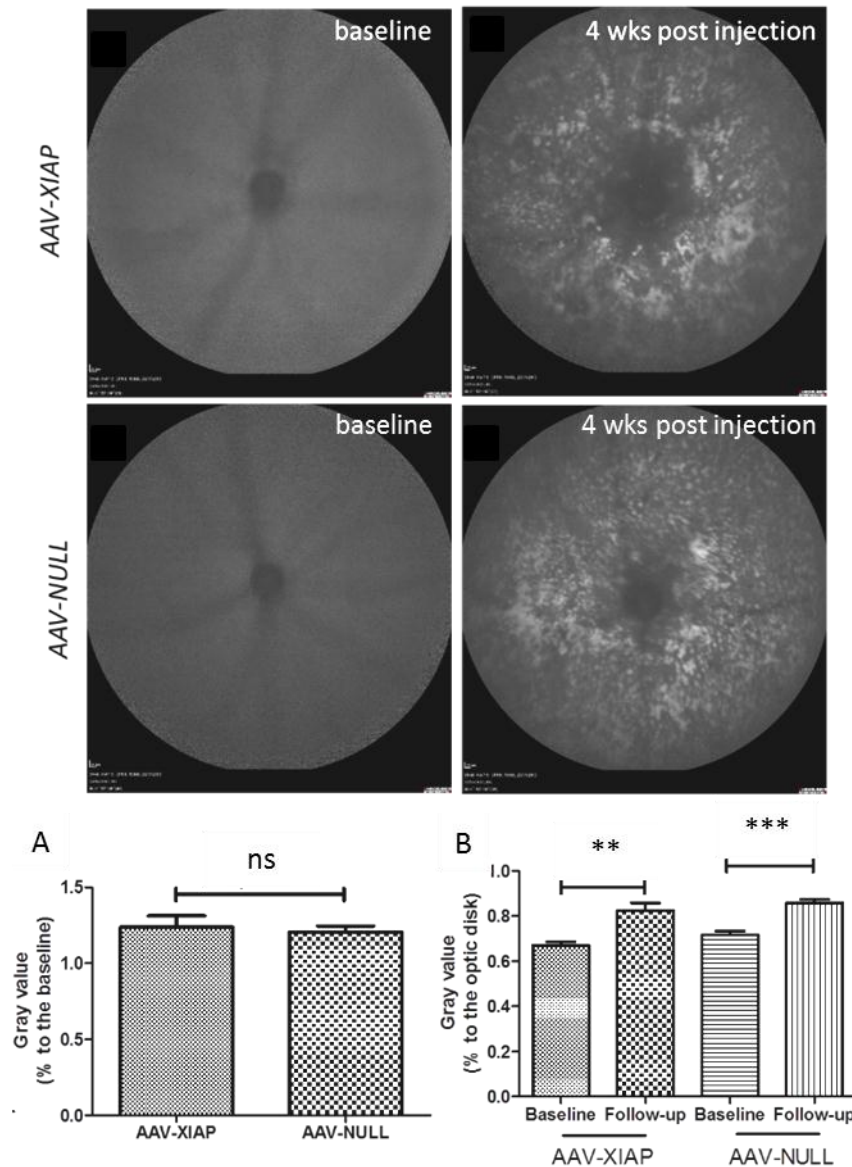


Figure 47 cSLO images on 790 nm AF mode in the AAV injected $B6^{TgOPN1LW-EGFP}$ mice. Compared to the AAV-NULL injected controls, the AAV-XIAP injected mice showed a similar AF after NaIO₃ treatment (A, Unpaired *t*-test, *n*=6). Both groups showed a significant increase of AF after NaIO₃ treatment, possibly due to melanin oxidation or local inflammation (B, Two-way ANOVA with Bonferroni's post-hoc test, *n*=6). ns: no significance, **: *p*<0.01, ***: *p*<0.001.

6.2.2 cSLO imaging on 488 nm AF mode

In the *NRL-GFP* mice, the fundus autofluorescence reduced significantly at 4 weeks post NaIO₃ injection compared to the baseline of the AAV-XIAP treated eyes (16 ± 2 vs. 10 ± 1 , $p=0.04$) and the AAV-NULL controls (17 ± 3 vs. 10 ± 1 , $p=0.05$, Figure 48). Normalized to each baseline, there was no significant difference of the residual fundus autofluorescence between the AAV-XIAP and AAV-NULL treated eyes, suggesting the AAV-XIAP subretinal injection did not rescue the GFP expressing rods in this strain ($63 \pm 10\%$ vs. $61 \pm 10\%$, $p=0.89$, Figure 48).

In the B6^{TgOPN1LW-EGFP} mice, the GFP expressing LM-cones reduced significantly at 4 weeks post NaIO₃ injection compared to the baseline of the AAV-XIAP treated eyes (200 ± 20 vs. 560 ± 40 , $p=0.0003$) and the AAV-NULL controls (250 ± 20 vs. 530 ± 40 , $p=0.001$, Figure 49). Normalized to the baseline, there was no significant difference of the survival LM-cones between the AAV-XIAP and AAV-NULL treated eyes, suggesting the AAV-XIAP subretinal injection did not rescue the GFP expressing LM-cones in this strain ($37 \pm 4\%$ vs. $47 \pm 5\%$, $p=0.12$, Figure 49).

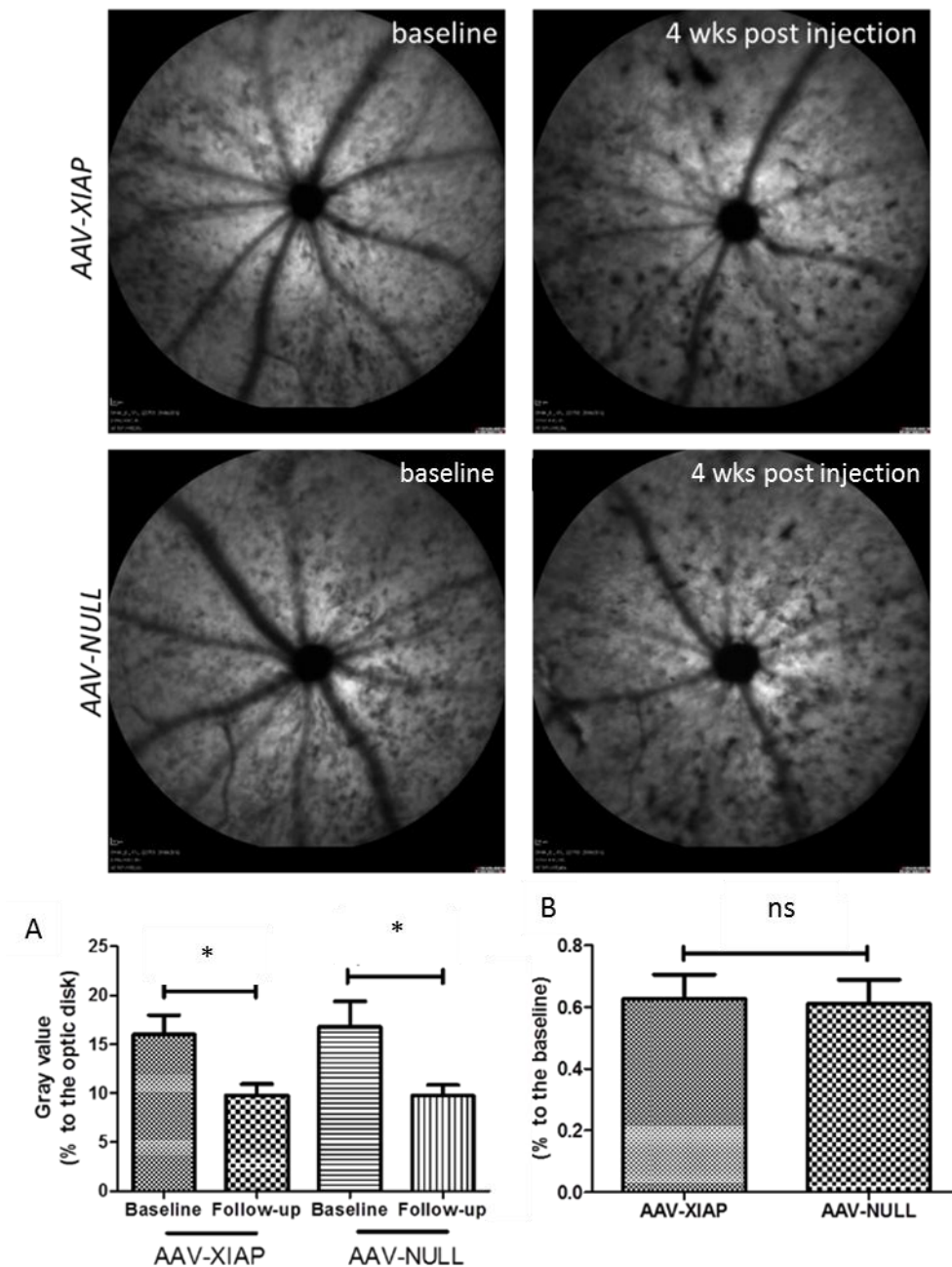


Figure 48 cSLO images on 488 nm AF mode in the AAV injected NRL-GFP mice. The high AF originates from the GFP expressing rods driven by NRL promoter. The rods, determined by the grey value of AF, decreased significantly after NaIO₃ treatment in both the AAV-NULL and AAV-XIAP injected group (A, Two-way ANOVA with Bonferroni's post-hoc test, $n=6$). There was no significant difference of residual rods between these two groups (B, Unpaired t -test, $n=6$). ns: no significance, *: $p<0.05$.

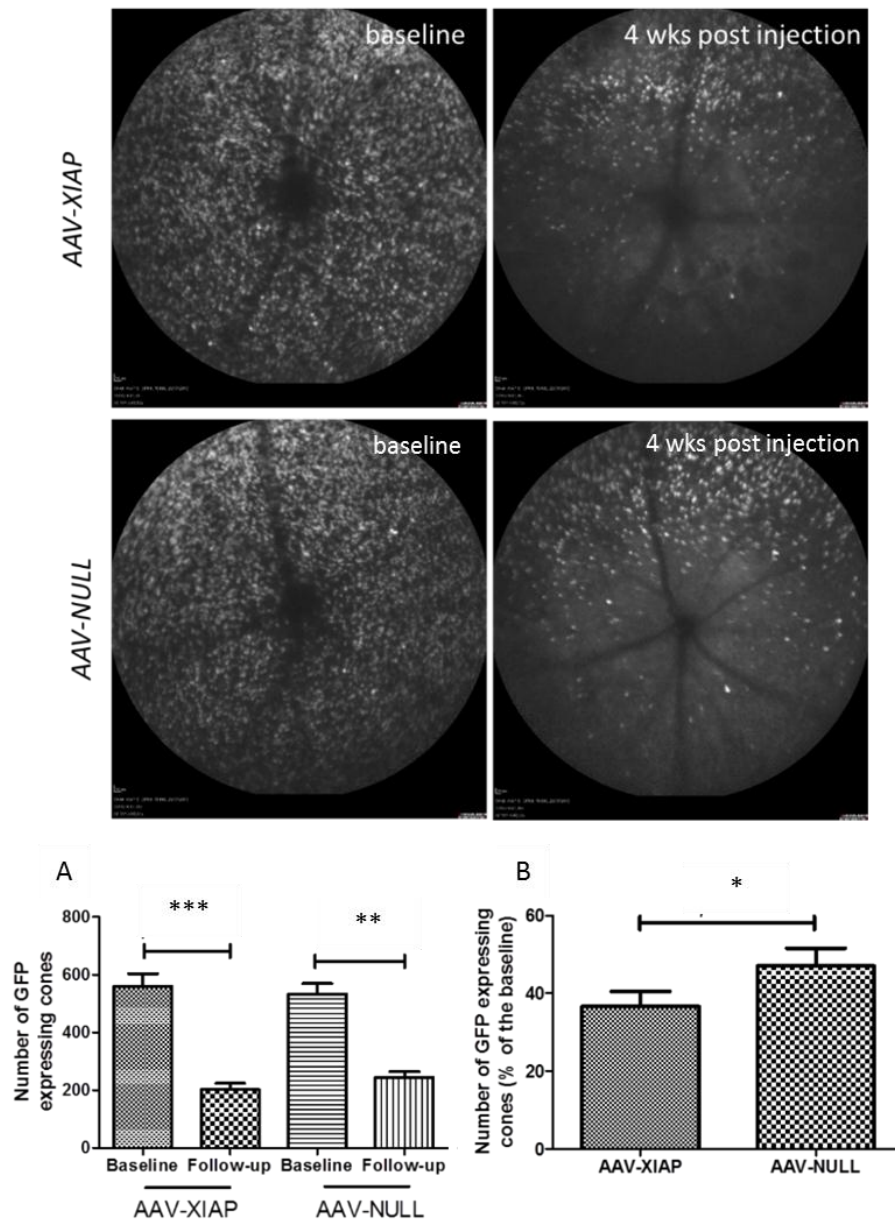


Figure 49 cSLO images on 488 nm AF mode in the AAV injected B6^{TgOPN1LW-EGFP} mice. The high AF dots represent the GFP expressing cones driven by LW- opsin promoter. The cones, determined by the grey value of AF, decreased significantly after NaIO₃ treatment in both the AAV-NULL and AAV-XIAP injected group (A, Two-way ANOVA with Bonferroni's post-hoc test, $n=6$). The residual cones were significantly less in the AAV-XIAP treated mice compared to the AAV-NULL controls (B, Unpaired t -test, $n=6$). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

6.2.3 ERG

Scotopic ERGs were performed to examine the rod function in the *NRL-GFP* mice. Compared to the baseline, the b-wave amplitude of follow-up ERGs was significantly reduced at 1-day post NaIO₃ treatment and nearly undetectable at 4 weeks post treatment (Figure 50). In the AAV-NULL injected eyes, the follow-up and baseline b-wave amplitude at 4 weeks post treatment was 9 ± 7 vs. 64 ± 10 ($p=0.01$), 1 ± 7 vs. 160 ± 20 ($p=0.001$), -1 ± 5 vs. 470 ± 20 ($p<0.001$), 24 ± 10 vs. 770 ± 15 ($p<0.001$), 15 ± 8 vs. 780 ± 60 ($p<0.001$), 8 ± 8 vs. 980 ± 50 ($p<0.001$), 3 ± 15 vs. 1000 ± 100 ($p<0.001$) and 21 ± 9 vs. 1100 ± 120 ($p<0.001$) with the various stimuli in our protocol (see Chapter 2). Reduction of b-wave was similarly observed in the AAV-XIAP injected eyes. Normalized with each baseline, there was no significant difference of b-wave reduction between the AAV-NULL and AAV-XIAP treated eyes (Figure 51 and 52).

In the B6^{TgOPN1LW-EGFP} mice, photopic ERGs were performed to examine the cone function. With single flash stimuli, significant reduction of b-wave was found in both AAV-NULL and AAV-XIAP treated eyes after NaIO₃ injection (Figure 53). Normalized with each baseline, however, there was no significant difference of b-wave reduction between the two groups at 1-day and 4 weeks post NaIO₃ treatment at all stimuli, except at $1 \text{ cd} \cdot \text{s/m}^2$ where the b-wave was even more reduced in the AAV-XIAP group compared to the AAV-NULL controls (-25 ± 5 vs. -8 ± 2 , $p=0.02$, Figure 54). With 15 and 30 Hz “flicker” stimuli, the b-wave reduction was also similarly reduced in both groups (Figure 55).

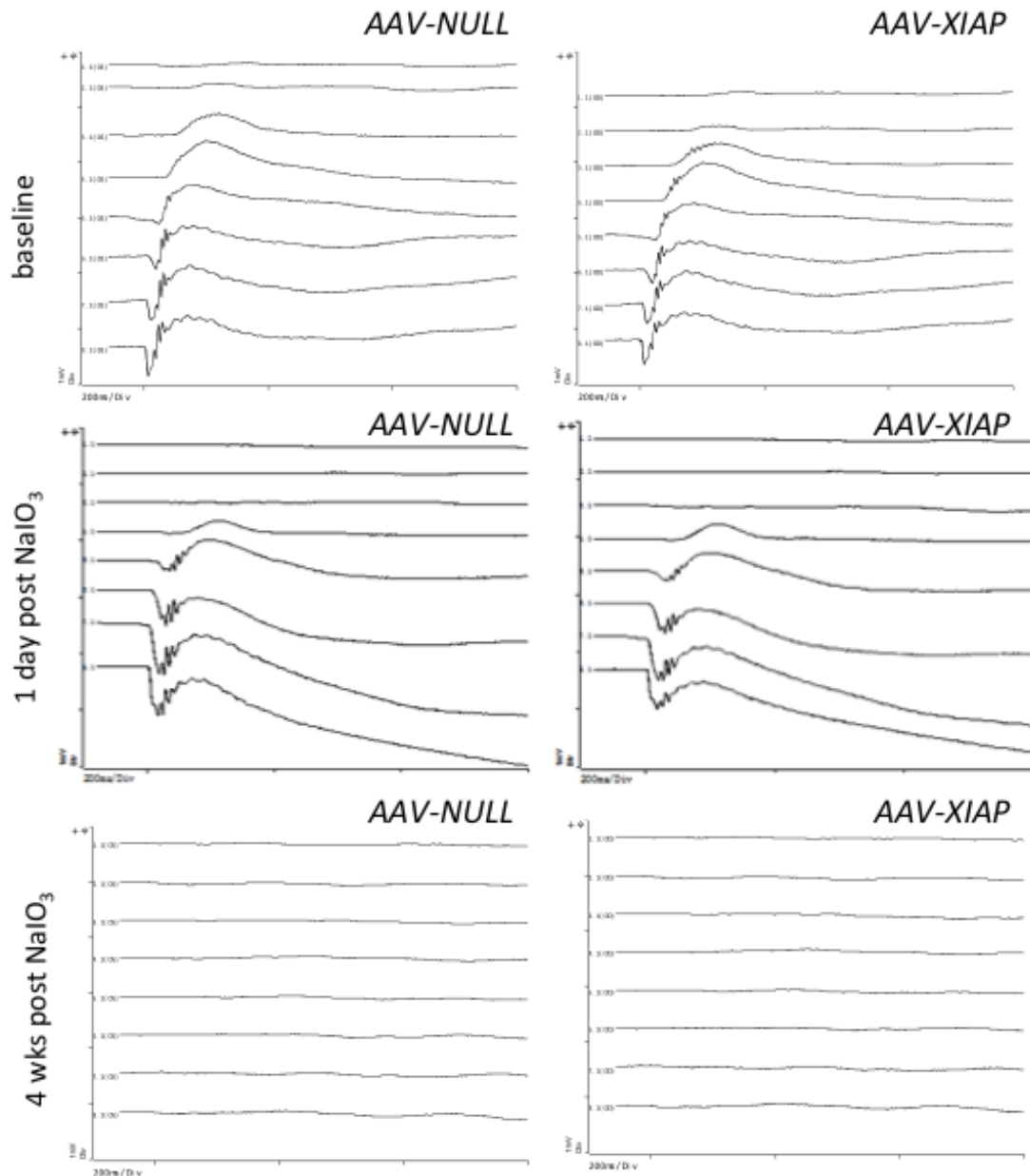


Figure 50 Scotopic ERGs of the AAV injected NRL-GFP mice. Compared to the baseline, the b-wave of both AAV-NULL and AAV-XIAP injected eyes decreased at 1 day post NaIO₃ treatment. Both b-wave became nearly undetectable at 4 weeks post NaIO₃ treatment, suggesting the AAV-XIAP injection did not preserve photoreceptor function against NaIO₃ toxicity.

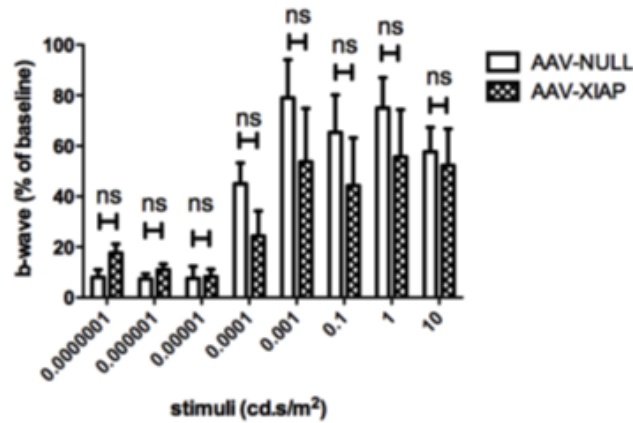


Figure 51 Scotopic ERGs of the AAV injected NRL-GFP mice at 1-day post NaIO₃ treatment. Compared to the AAV-NULL controls, there was no significant improvement of b-wave of the AAV-XIAP injected eyes under the various stimuli in our ERG protocol. ns: no significance. Two-way ANOVA with Bonferroni post-tests, $n=6$.

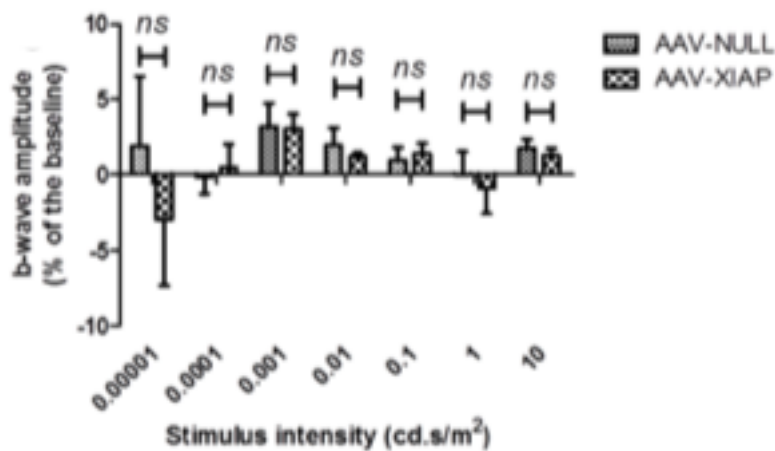


Figure 52 Scotopic ERGs of the AAV injected NRL-GFP mice at 4 weeks post NaIO₃ treatment. Compared to the AAV-NULL controls, there was no significant improvement of b-wave of the AAV-XIAP injected eyes under the various stimuli in our ERG protocol. ns: no significance. Two-way ANOVA with Bonferroni post-tests, $n=6$.

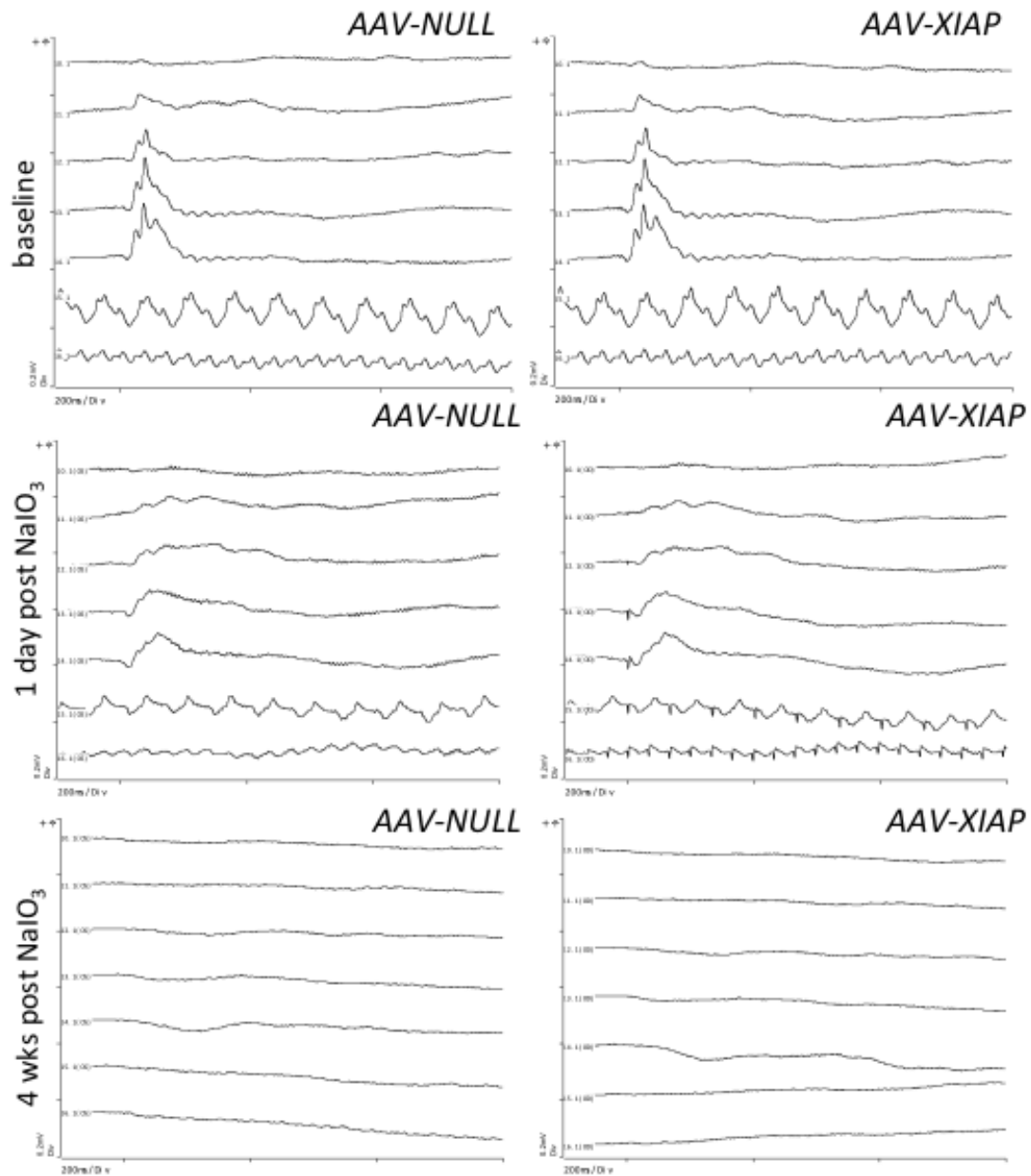


Figure 53 Photopic ERGs of the AAV injected $B6^{TgOPN1LW-EGFP}$ mice. Compared to the baseline, the b-wave of both AAV-NULL and AAV-XIAP injected eyes decreased at 1 day post NaIO₃ treatment. Both b-wave became nearly undetectable at 4 weeks post NaIO₃ treatment, suggesting the AAV-XIAP injection did not preserve photoreceptor function against NaIO₃ toxicity.

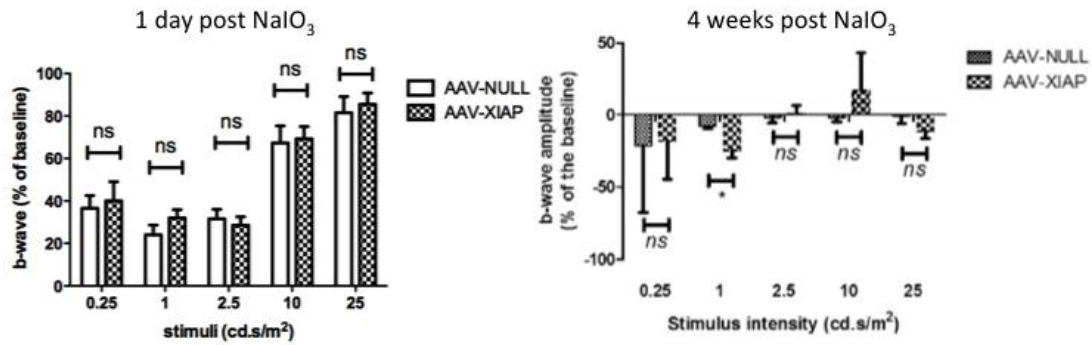


Figure 54 Photopic ERGs of the AAV injected B6^{TgOPN1LW-EGFP} mice at 1-day and 4 weeks post NaIO₃ treatment. Compared to the AAV-NULL controls, there was no significant improvement of b-wave in the AAV-XIAP treated group under the various stimuli in our ERG protocol. This suggests the AAV-XIAP treatment did not reserve photoreceptor function under the NaIO₃ toxicity in our settings. ns: no significance. *: $p < 0.05$. Two-way ANOVA with Bonferroni post-tests, $n = 6$.

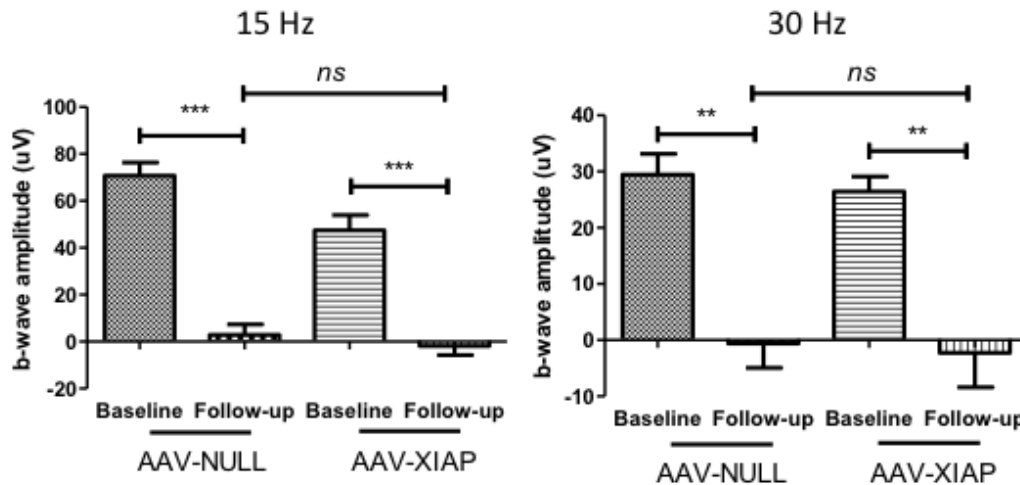


Figure 55 Flicker ERGs of AAV injected B6^{TgOPN1LW-EGFP} mice at 15 Hz and 30 Hz stimuli. The b-wave of AAV-NULL and AAV-XIAP group reduced significantly after NaIO₃ treatment, while there was no significant difference between the b-wave of both groups. **: $p < 0.01$, ***: $p < 0.001$, ns: no significance, Two-way ANOVA with Bonferroni post-tests, $n = 6$.

6.2.4 Retinal histology

At 4 weeks post NaIO₃ injection, disrupted RPE and ONL were observed in both the AAV-XIAP and AAV-NULL treated eyes of the *NRL-GFP* (Figure 56) and B6^{TGOPN1LW-EGFP} mice (Figure 57 *Retinal histology of the AAV injected eyes in the B6TgOPN1LW-EGFP mice. The AAV-XIAP injected eyes showed stronger XIAP immunostaining localized in the outer nuclear layer (ONL) and GFP-tagged cones in the injected subretinal space (arrow in the embedded figure, scale bar=10 um), whilst the AAV-NULL control only appeared diffused background XIAP immunostaining. Both eyes showed distorted RPE and ONL layer, suggesting the AAV-XIAP treatment did not reduce NaIO3 toxicity in this strain. Scale bar: 25 um.*). The GFP expressing rods and LM-cones were similarly disorganized in the eye sections treated with both vectors. Thus the AAV-XIAP treatment did not appear to preserve RPE and ONL normal structure compared with the AAV-NULL controls.

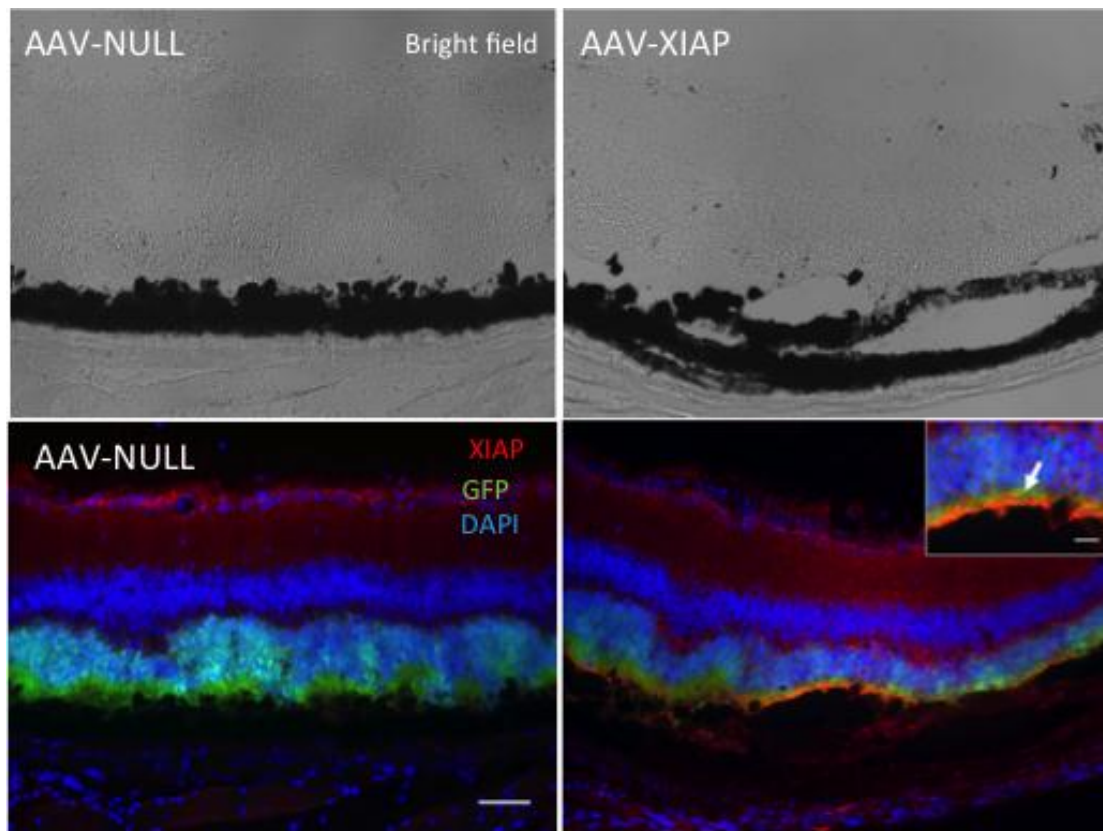


Figure 56 Retinal histology of the AAV injected eyes in the NRL-GFP mice. The AAV-XIAP injected eyes showed stronger XIAP immunostaining localized in the outer nuclear layer (ONL) of injected subretinal space (arrow in the embedded figure, scale bar=10 μ m), whilst the AAV-NULL control only appeared diffused background XIAP immunostaining. Both eyes showed distorted RPE and ONL layer, suggesting the AAV-XIAP treatment did not reduce NaIO₃ toxicity in this strain. Scale bar: 25 μ m.

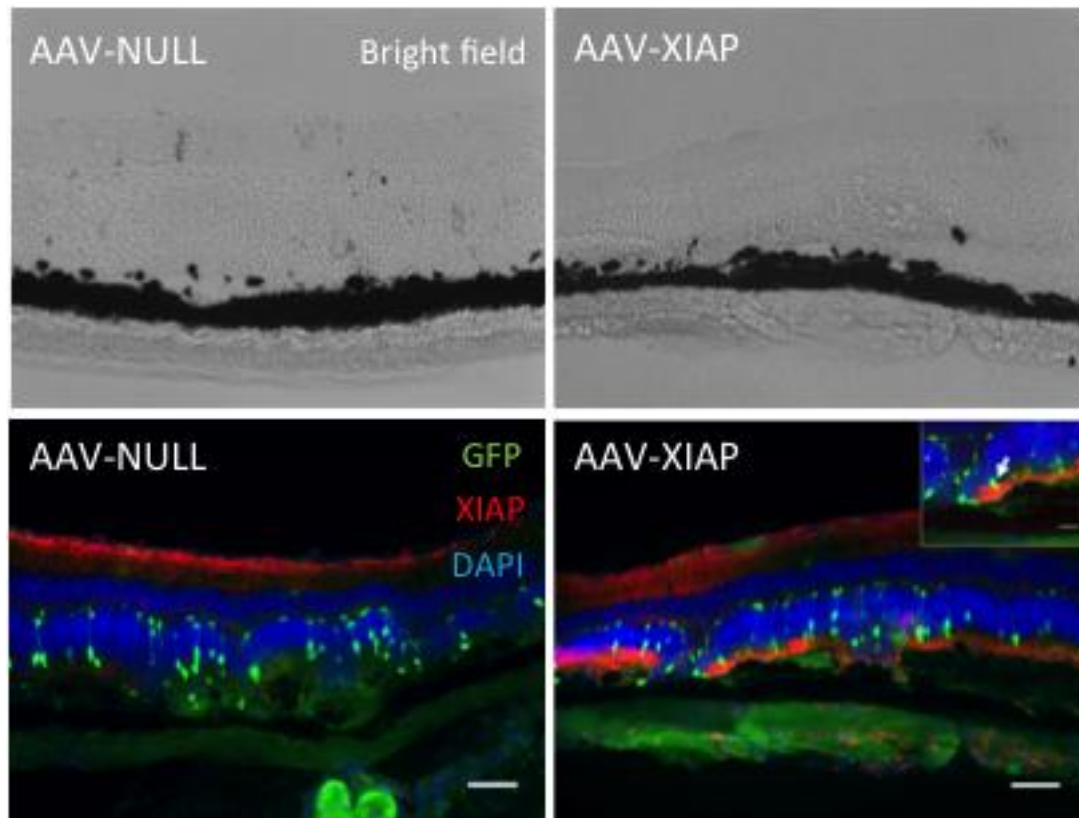


Figure 57 Retinal histology of the AAV injected eyes in the $B6^{TgOPN1LW-EGFP}$ mice. The AAV-XIAP injected eyes showed stronger XIAP immunostaining localized in the outer nuclear layer (ONL) and GFP-tagged cones in the injected subretinal space (arrow in the embedded figure, scale bar=10 μ m), whilst the AAV-NULL control only appeared diffused background XIAP immunostaining. Both eyes showed distorted RPE and ONL layer, suggesting the AAV-XIAP treatment did not reduce NaIO₃ toxicity in this strain. Scale bar: 25 μ m.

6.2.1 XIAP immunostaining

In the eye sections undergoing immunohistochemistry, some photoreceptors expressing strong XIAP immunostaining were observed in the AAV-XIAP treated eyes, whilst there was only weak background staining of endogenous XIAP expression in the AAV-NULL treated eyes in both stains (*Figure 56 and 57 on the previous page*). The XIAP staining in photoreceptors could be differentiated from RPE staining in areas where the retina had detached. The photoreceptors overexpressing XIAP were mainly located in the outer 1-2 layers of the ONL. In the RPE layer, however, there was no apparent XIAP staining in either strain at 4 weeks post NaIO₃ injection.

To quantify XIAP expression, the Western blot was further performed in the eyes at 5 weeks post vector delivery. The AAV-XIAP treated eyes showed a significant XIAP overexpression compared with the AAV-NULL controls in both the *NRL-GFP* mice (3 ± 1 vs. 1.2 ± 0.5 , $p=0.005$, *Figure 58*) and B6^{TgOPN1LW-EGFP} mice (2.9 ± 1 vs. 1.6 ± 0.6 , $p=0.02$, *Figure 59*). Hence although there might be up-regulation of endogenous XIAP as a result of the oxidative stress stimulus, over the whole eye cup there were still increased levels detectable compared to controls, confirming that the failure of neuroprotection was not due to impaired transgene expression from the vectors.

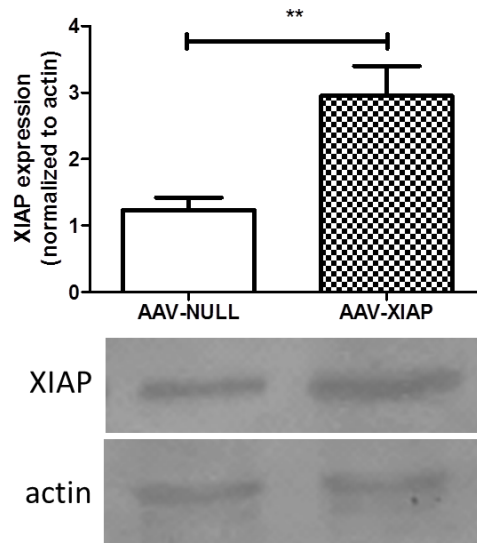


Figure 58 Western blot of the AAV-NULL and AAV-XIAP injected eyes in the NRL-GFP mice. **: $p < 0.01$, Unpaired t -test, $n = 6$.

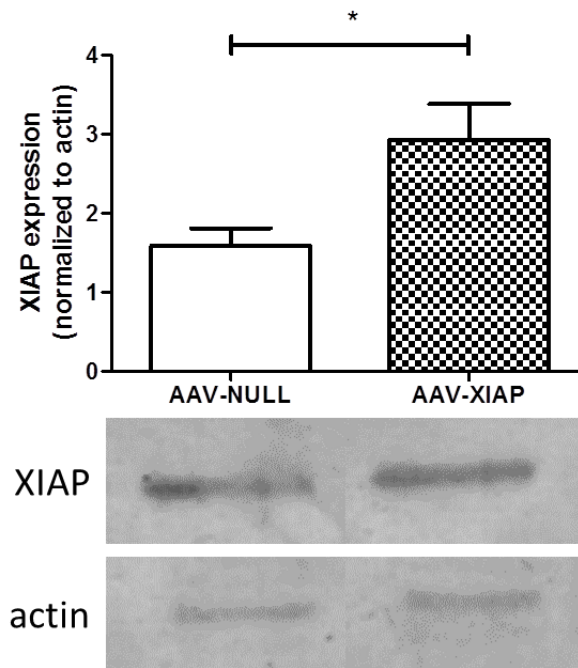


Figure 59 Western blot of the AAV-NULL and AAV-XIAP injected eyes in the B6^{TgOPN1LW-EGFP} mice. *: $p < 0.05$, Unpaired t -test, $n = 6$.

6.3 Discussion

In this chapter, the AAV-XIAP was subretinally delivered in the *NRL-GFP* and B6^{TgOPN1LW-EGFP} mice, followed by systematic injection of NaIO₃. The follow-up cSLO and ERG revealed that XIAP did not rescue photoreceptors in the NaIO₃ induced retinopathy, anatomically or functionally to any significant degree.

The *NRL-GFP* and B6^{TgOPN1LW-EGFP} transgenic mice used in this study are characterized by selective GFP expression in rods and LM-cones, respectively. The GFP coding sequence was under the control of NRL or LW-opsin promoter, which results in the specific GFP expression in the photoreceptors^{223, 275}. This unique feature enables the cSLO to image the photoreceptors *in vivo* with a 488 nm laser source, as described in the previous chapter. The fact that AAV-mediated XIAP did not show any effect on the photoreceptor survival in cSLO was further confirmed by the ERGs. The b-wave, derived from retinal cells post-synaptic to the photoreceptors, is widely used to evaluate rod and cone function under particular scotopic and photopic ERG protocols²⁸⁸. In this study, the AAV-XIAP treatment did not show any functional preservation of b-wave compared with the controls, suggesting that XIAP did not rescue functional photoreceptors or post-synaptic neurons that are enough to produce significant ERG changes.

It is possible that XIAP may have rescued a small amount of photoreceptors but that beyond the sensitivity of ERG, as it records the whole retinal response which may not be altered by a small region of surviving photoreceptors. In the present study, however, the superior retinal areas were measured in the cSLO. This limits the comparison only between the subretinal injection areas instead of the whole retina.

Therefore the cSLO, together with ERG, provides a sensitive and accurate way to investigate the photoreceptor survival in anatomy and function. On the other hand, the XIAP application is still very limited at clinic even if it may rescue a few photoreceptors beyond such sensitivity.

Previous studies have shown that XIAP is neuroprotective on photoreceptors in *RHO* mutant RP rats⁹⁷, N-methyl-N-nitrosourea (MNU)-induced retinal degeneration²¹⁰ and retinal detachment²⁰⁶. However, AAV-mediated XIAP did not preserve photoreceptors in our study. This indicates the photoreceptor death of NaIO₃ induced retinopathy may be mainly in a caspase-independent way. In the NaIO₃ induced retinopathy, the photoreceptor apoptosis has been previously confirmed but the role of caspases in this model remains elusive^{263, 287}. Both caspase-dependent and -independent pathways have been described in other retinal degeneration models^{95, 289, 290}. The caspase molecule may be difficult to be detected by immunolabelling due to its small molecule size. The active (cleaved) form of caspase-3 or -7 is 17 kDa, and the cleaved caspase-9 is 25 kDa. So its inhibitor such as Z-VAD-FMK is often used to explore its role in apoptosis²⁹¹. Compared to the synthetic caspase inhibitors, XIAP is an endogenous expressing protein and can be packaged into AAV vectors as a possible neuroprotective candidate for gene therapy. Our results, for the first time, suggest that caspases may not play an important role in the photoreceptor death of NaIO₃ induced retinopathy.

Another explanation of the negative outcome is that the toxicity of NaIO₃ may be too strong for XIAP to rescue any photoreceptors. In previous studies, mice were intraperitoneally administered 100 mg/kg NaIO₃²⁶³ or i.v. injected 15-70 mg/kg NaIO₃^{261, 292}. It is found that NaIO₃-induced photoreceptor degeneration was dose and

post-injection time dependent, with the peak of photoreceptor apoptosis on the 3rd day post injection^{261, 287}. Hereby a lower dose of NaIO₃ (50 mg/kg intraperitoneally injection) and a long follow-up time (4 weeks) were chosen as a preliminary start to assess XIAP. Although its toxicity on photoreceptors has been proved in the previous chapter, it might be helpful to re-evaluate XIAP in an even lower dose <50 mg/kg. However, a better model would be transgenic animals/mice with disrupted genes that lead to human RP, such as the *RHO* mutant mouse.

**Chapter 7 XIAP Effect on Cone Photoreceptors
in a Novel Mouse Model of Retinal Degeneration**

7.1 Introduction

A great challenge in the treatment of retinal degenerative disease is to rescue and restore cone photoreceptors²⁹³. In most cases of RP and AMD, cone death results from RPE or rod atrophy and eventually leads to blindness. Rod-cone dystrophy, for instance, is the most common type of RP characterized with primary rod death/dysfunction and secondary cone loss due to rod-specific gene mutations²⁹⁴. In AMD, development of RPE atrophy (the “dry” type) or choroidal neovascularization (the “wet” type) is generally associated with secondary fovea cone death⁶⁸. In some cases, cones are the primary affected photoreceptors due to cone-specific gene mutations that cause loss of function rather than cell death. For instance, mutations in *CNGB3* the gene encoding the β -subunit of the cone photoreceptor cGMP-gated channel are responsible for achromatopsia, an autosomal recessive disorder featuring total color blindness and reduced visual acuity²⁹⁵.

To investigate cone cell death there are many naturally occurring and genetically modified animal models of retinal degeneration currently available for cone neuroprotection research^{179, 296-301}. Among them, the *Rho*^{-/-} *TgOPN1LW-EGFP*^{+/-} mouse is a recently developed strain with some novel features. It was generated by crossing of the C57BL/6J^{TgOPN1LW-EGFP85933Hue} mouse (refer herein as B6^{TgOPN1LW-EGFP}) with the C57B/6.129 *Rho*^{tm1Phm} mouse (refers to *Rho*^{-/-}), followed by backcrossing of F1 progeny (*Rho*^{+/-} *TgOPN1LW-EGFP*^{+/-}) to the parental *Rho*^{-/-} line¹⁷⁹. The B6^{TgOPN1LW-EGFP} mouse is genetic modified with inserted enhanced GFP (EGFP) driven by long wavelength (LW) opsin promoter²²³. It is characterized by EGFP specific expression targeted to the LM-cones. The *Rho*^{-/-} mouse carries a targeted disruption in the

rhodopsin gene²²². It does not elaborate rod outer segments at birth and loses almost all photoreceptors over 3 months. No significant cone ERG response is detectable beyond 8 weeks. Therefore, the cross-bred *Rho*^{-/-} *TgOPN1LW-EGFP*^{+/-} mouse features rod atrophy followed by secondary fluorescent cone degeneration which is detectable *in vivo* using a 488 nm laser source on cSLO imaging³⁰².

These features enable the *Rho*^{-/-} *TgOPN1LW-EGFP*^{+/-} mouse an ideal model for cone rescue study in retinal degeneration. The *RHO* mutation represents the most common form of human autosomal dominant RP³⁰³. The cSLO detectable LM-cones hugely limit the number of animals to be sacrificed and applied specific immunostaining to distinguish cones. More importantly, errors are avoided when comparing two time points among variant individuals and quality of sectioning and immunostaining.

In the previous chapter, AAV-mediated XIAP was investigated in the NaIO₃ induced retinopathy but no neuroprotective effect on photoreceptors was observed. The *Rho*^{-/-} *TgOPN1LW-EGFP*^{+/-} mouse is different from the previous chemical induced retinopathy model and may be a more representative RP model. Currently little is known about XIAP neuroprotection on cones in retinal degeneration. This is particularly important when applying gene therapy in clinic practice as cones are responsible for daylight visual acuity and may remain a certain amount at the time of diagnosis.

7.2 Aims

The aim of this chapter is to investigate the AAV-mediated XIAP effect on preserving cone photoreceptors in the $Rho^{-/-}$ $TgOPN1LW-EGFP^{+/-}$ mouse, specifically:

- XIAP is delivered by recombinant serotype 8 AAV (AAV8) which has a higher efficiency in photoreceptor transduction than AAV2³⁰⁴.
- To measure and compare cone survival using cSLO imaging *in vivo*.
- To measure and compare cone function using photopic ERGs.

See Figure 60 for an overview of the process and methods in this chapter.

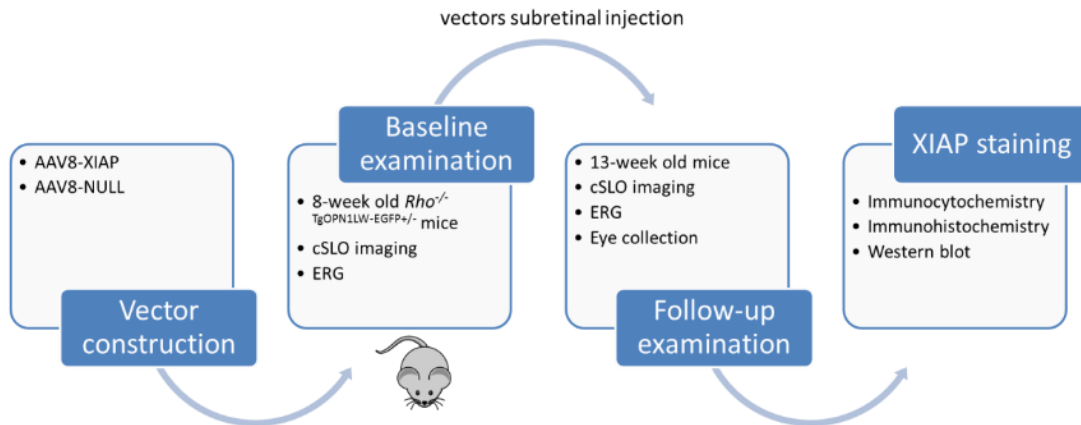


Figure 60 Schematic diagram showing the main steps and methods used in Chapter 7.

7.3 Materials and methods

Mice

The $Rho^{-/-}$ $TgOPN1LW-EGFP^{+/-}$ mouse was originally bred by Mr. Edward Lee FRCOphth PhD at University College London and has been previously described¹⁷⁹. Briefly, they were created through crossing of $B6^{TgOPN1LW-EGFP^{+/+}}$ mice with $Rho^{-/-}$, followed by backcrossing of F1 progeny ($Rho^{+/-}$ $TgOPN1LW-EGFP^{+/-}$) to the parental $Rho^{-/-}$ line. The parental $Rho^{-/-}$ mouse was a kind gift obtained from Jane Farrar (Trinity College, Dublin, Ireland) and has been previously described²²². The parental $B6^{TgOPN1LW-EGFP}$ mouse has been described in the previous Chapter 5. The cross-bred $Rho^{-/-}$ $TgOPN1LW-EGFP^{+/-}$ mouse was genotyped at weaning by PCR as previously reported^{222, 223} (Figure 61 and Figure 62). See Chapter 2 for general information of mice breeding and maintenance.

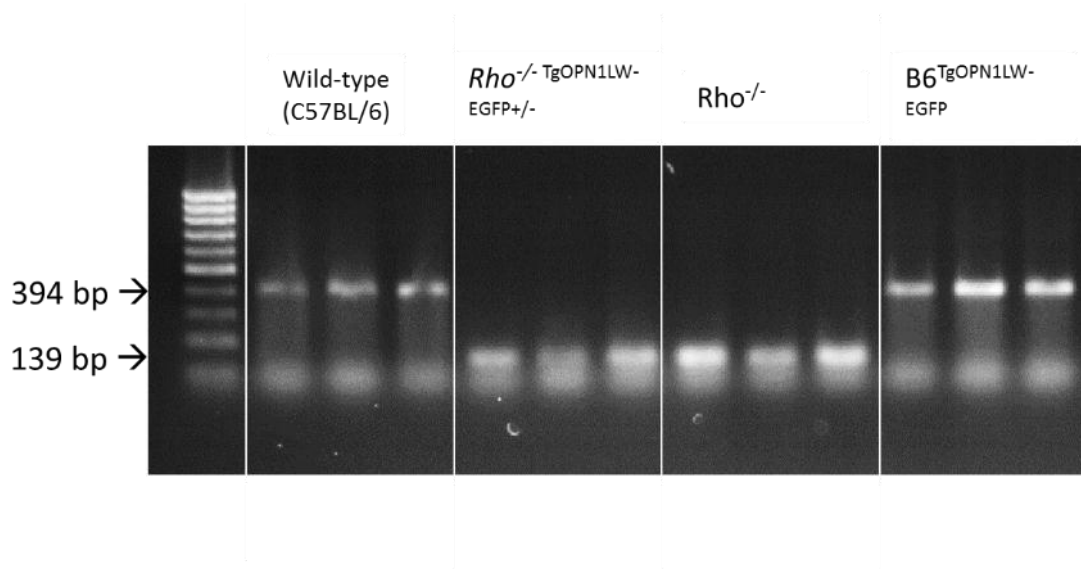


Figure 61 Genotyping $Rho^{-/-}$ $TgOPN1LW-EGFP^{+/-}$ mice with primers detecting $Rho^{-/-}$. The $Rho^{-/-}$ $TgOPN1LW-EGFP^{+/-}$ and $Rho^{-/-}$ samples showed one PCR product (139 bp), whilst the wild type and $B6^{TgOPN1LW-EGFP}$ samples showed two products (139 bp and 394 bp).

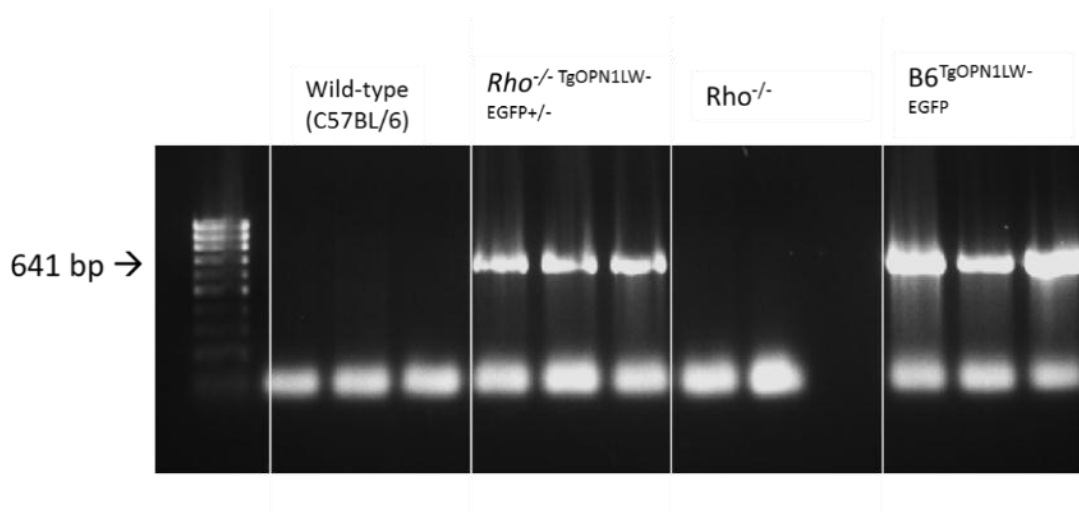


Figure 62 Genotyping $Rho^{-/-}$ $TgOPN1LW-EGFP^{+/-}$ mice with primers detecting $B6^{TgOPN1LW-EGFP}$. The $Rho^{-/-}$ $TgOPN1LW-EGFP^{+/-}$ and $B6^{TgOPN1LW-EGFP}$ mice showed one PCR product (641 bp), whilst the wild type and $Rho^{-/-}$ showed no 641 bp product.

Vector

The human XIAP coding sequence was restriction digested from a commercial plasmid (Origene, Rockville) using Sac1 and Pma1. A downstream in-frame DDK tag was included in the isolated XIAP fragment to discriminate the vector driven XIAP from any endogenous XIAP present. The null AAV transgene plasmid (pAAV-ITR) was kindly provided by Dr Matthew Wood at University of Oxford. The plasmid was digested with Sac1 and Pma1 and ligated with XIAP fragment using T4 ligase (NEB, Ipswich). The ligation products were introduced into XL10 competent bacteria (Stratagene, Santa Clara) by heat shock transformation. The ligated plasmid was checked by digestion (Sac1 and Pma1, Figure 63) and sequencing (Figure 64). The presence of ITRs was confirmed by Xma1 digestion (Figure 65). The pAAV-ITR-XIAP and pAAV-ITR were used to produce AAV8-XIAP and AAV8-NUL, respectively. The final vectors were examined by SDS-PAGE electrophoresis followed by Coomassie staining (Figure 66). The titre was 5×10^{12} and 1.5×10^{12} genomic particle/ml, respectively. See Chapter 2 for more information of the molecular biology and rAAV production techniques.

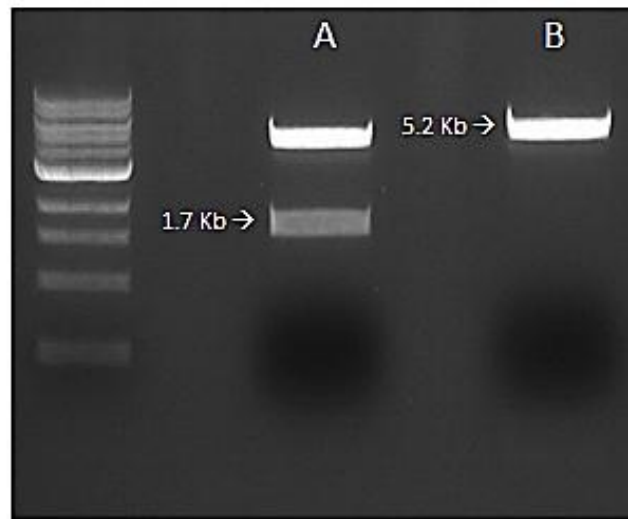


Figure 63 Electrophoresis of pAAV-ITR-XIAP (A) and pAAV-ITR (B) restricted by *Sac*I and *Pma*I. XIAP: ~1.7 kb. pAAV-ITR: ~5.2 kb.

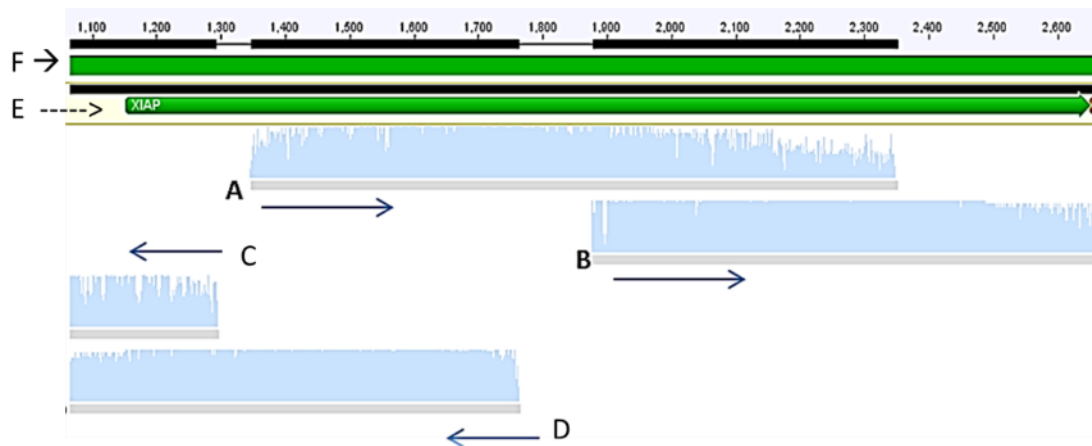


Figure 64 The sequencing results of pAAV-ITR-XIAP and alignment. A-D: sequencing results. E: human XIAP sequence (GeneBank). F: alignment, green=100% alignment. Hence the AAV.XIAP used expresses the same mRNA sequence as the human XIAP gene.

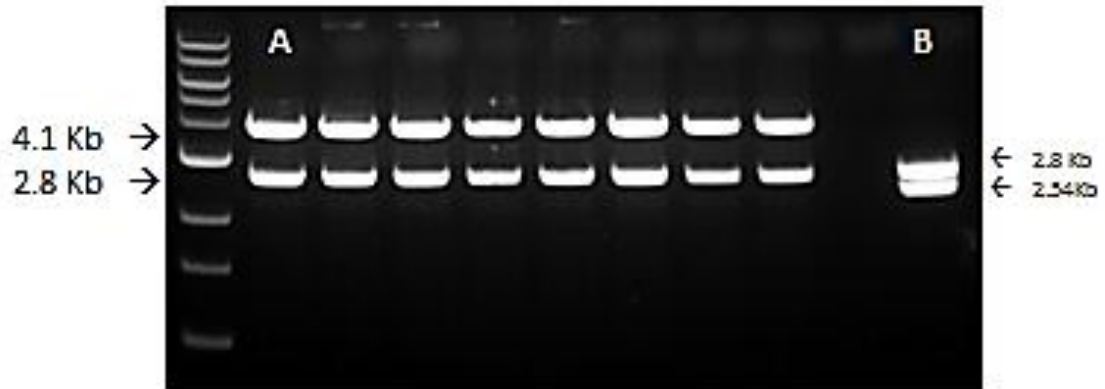


Figure 65 Electrophoresis of the *Xma1* digested pAAV-XIAP-ITR (A) and pAAV-ITR as the positive control (B).

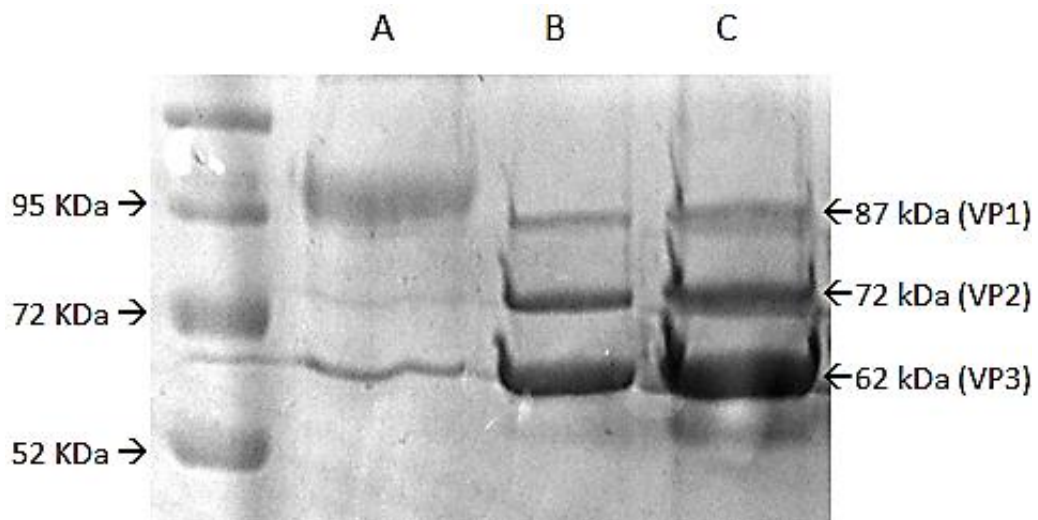


Figure 66 Coomassie staining of the AAV5-GFP (as the positive control, lane A), AAV-NULL (lane B) and AAV-XIAP (lane C) after SDS-PAGE electrophoresis.

Subretinal injection

The vectors were prepared to a titre of 1.5×10^{12} genomic particles/ml and subretinally injected to the superior retinas of six 8-week old *Rho*^{-/-} *TgOPN1LW-EGFP*^{+/-} mice. A total volume of 1 μ l AAV8-XIAP and AAV8-NUL were injected into the right and left eye, respectively. See Chapter 2 for general information of subretinal injection.

cSLO

cSLO imaging was performed at baseline before the vector injection and 5 weeks post injection (6 eyes each vector). The fundus was imaged on 820 nm, 488 nm and 790 nm mode. GFP expressing LM-cones in the superior retina on 488 nm images were counted as described in the previous Chapter 5 & 6. Also see Chapter 2 for general information of cSLO technique.

ERG

Photopic ERGs were recorded in 6 eyes of each vector group at baseline before the vector injection and 5 weeks post injection. See Chapter 2 for general information of ERG technique.

Immunostaining

Immunostaining was performed in the HEK293 cells (transfected by the pAAV-ITR-XIAP and the pAAV-ITR controls) and slides with frozen sections of the AAV8-XIAP and AAV8-NUL injected eye. The samples were incubated in XIAP antibody (Abcam, Cambridge, 1:200 in PBST) or DDK antibody (Origene, Rockville,

1:500 in PBST) overnight at 4 °C. Appropriate secondary antibody (Alexa Fluor 488, Invitrogen) were applied and followed by DAPI staining. See Chapter 2 for general information of immunocytochemistry, immunohistochemistry and DAPI staining.

Western blot

The AAV8-XIAP and AAV8-NUL injected eyes were harvested and 30 ul samples per lane were loaded to the 10% SDS-PAGE gels. The nitrocellulose membrane blots were incubated with XIAP antibody (Abcam, Cambridge, 1:1000 in TBST) or DDK antibody (Origene, Rockville, 1:1000 in TBST) overnight at 4° C. Appropriate HRP-conjugated secondary antibodies (Santa Cruz, US, 1:5000 in TBST) were applied to the blots for 1 hour at room temperature. See Chapter 2 for general information of Western blot and its quantification.

7.4 Results

7.4.1 cSLO imaging

With a 488 nm laser source, the GFP expressing LM-cones were identified on the fundus images using cSLO. Compared with each baseline, the number of LM-cones was significantly reduced at 5 weeks post AAV8-XIAP injection (90 ± 17 vs. 240 ± 24 , $p=0.0002$, Figure 67) or AAV8-NULL (110 ± 14 vs. 280 ± 25 , $p=0.0003$, Figure 67). When comparing the survival of LM-cones between the AAV8-XIAP and AAV8-NULL injected eyes, there was no significant difference between these two vector treatments (90 ± 17 vs. 110 ± 14 , $p=0.2$, Figure 67).

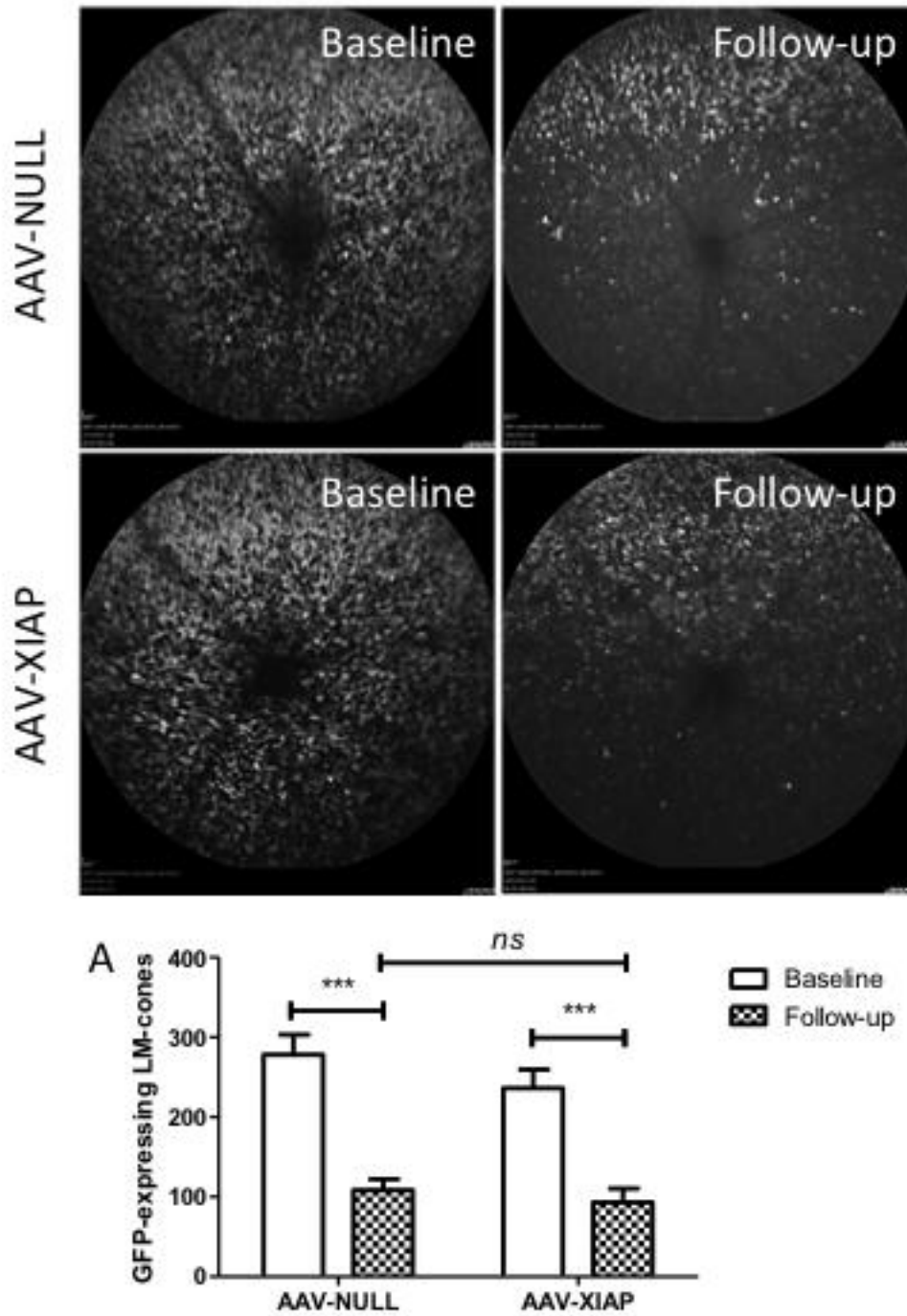


Figure 67 cSLO images of AAV injected $RHO^{-/-}$ $TGPN1LW-EGFP^{+/-}$ mice at baseline and 5 weeks post injection. Both AAV8-NULL and AAV8-XIAP injected eyes showed significant reduction of GFP expressing cones at follow up, whilst there was no significant difference of residual cones between both groups (A). ns: no significance, ***: $p < 0.001$, Two-way ANOVA with Bonferroni post-tests, $n = 6$.

7.4.2 ERG

Flash and flicker photopic ERGs were recorded to examine the cone function (Figure 68). In the follow-up flash ERGs, the b-wave amplitude was significantly lower than the baseline in the AAV8-XIAP injected eyes under the single flash stimuli of 2.5 (20 ± 9 vs. 80 ± 16 μ V, $p=0.02$), 10 (10 ± 2 vs. 130 ± 20 μ V, $p=0.0006$) and 25 cd s/m² (20 ± 5 vs. 160 ± 30 μ V, $p=0.0005$, Figure 69). A similar reduction was also observed in the AAV8-NUL injected controls (Figure 69). Normalized to the baseline, there was no significant difference of the follow-up b-wave amplitude between the AAV8-XIAP and AAV8-NUL injected eyes under all stimuli ($p=0.7$, Two-way ANOVA, Figure 70).

In the 15 flicker ERGs, a significant reduction of b-wave amplitude was observed at 5 weeks post AAV8-XIAP or AAV8-NUL injected eyes compared with the baseline (24 ± 7 vs. 140 ± 20 μ V, $p=0.02$ and 20 ± 7 vs. 160 ± 20 μ V, $p=0.005$, respectively). A similar reduction of b-wave amplitude was also observed in the 30 Hz ERGs of both groups (20 ± 4 vs. 50 ± 6 μ V, $p=0.04$ and 14 ± 5 vs. 50 ± 5 μ V, $p=0.008$, respectively). There was however no significant difference of the follow-up b-wave amplitudes between the vector injected eyes in the 15 or 30 Hz flicker ERGs ($p=0.6$ and 0.5 , respectively, Two-way ANOVA, Figure 71).

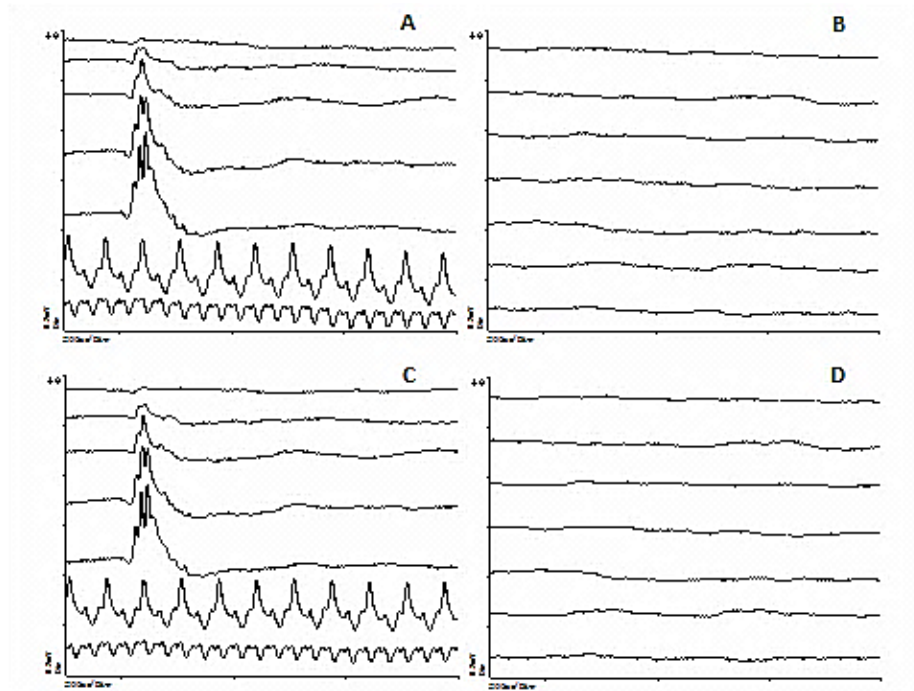


Figure 68 Photopic ERGs in the AAV8-XIAP (A, B) and AAV8-NULL (C, D) injected $RHO^{-/-}$ $TGPN1LW-EGFP^{+/-}$ mice at baseline and 5 weeks post injection, respectively. The sweep in each graph represents ERG records under flash stimuli of 0.25, 1, 2.5, 10, 25 $cd\ s/m^2$, 15 Hz and 30 Hz flicker stimuli of 10 $cd\ s/m^2$ (from top to bottom).

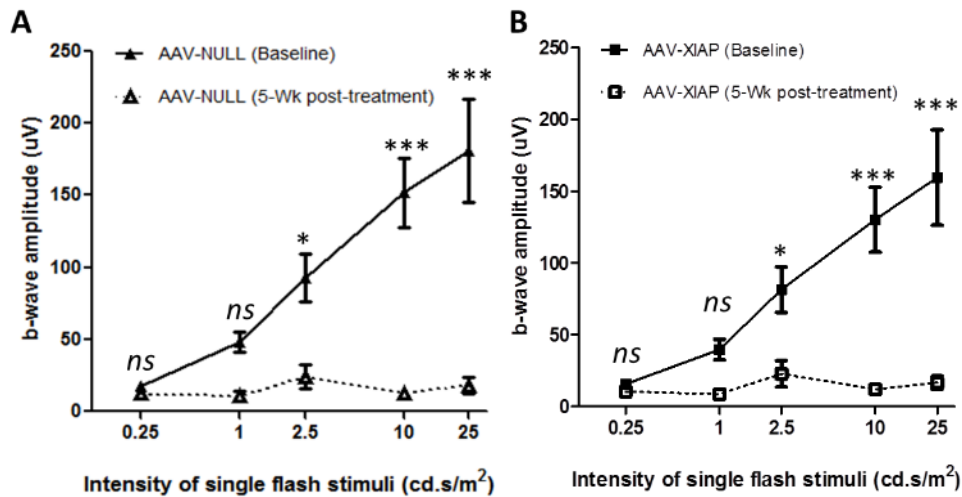


Figure 69 Comparison of the b-wave amplitude in flash ERGs of the AAV8-NULL (A) and AAV8-XIAP (B) injected eyes. ns: no significance, *: $p < 0.05$, ***: $p < 0.001$, Two-way ANOVA with Bonferroni post-tests, $n = 6$.

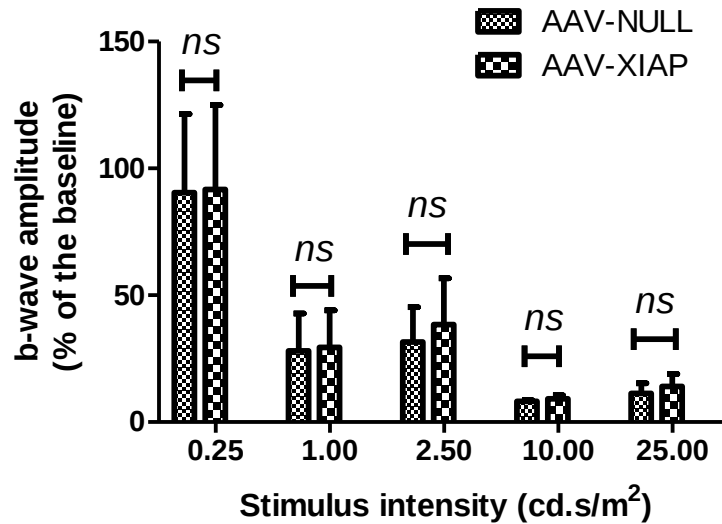


Figure 70 Comparison of the follow-up b-wave amplitude in flash ERGs between the AAV8-XIAP and AAV8-NULL injected eyes. Data were normalized to each baseline. Two-way ANOVA with Bonferroni post-tests, $n=6$.

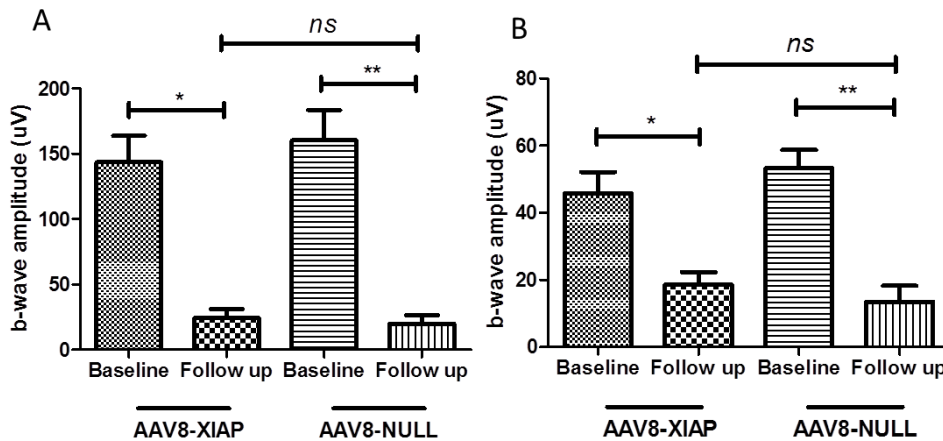


Figure 71 Comparison of b-wave amplitude in 15 Hz (A) and 30 Hz (B) flicker ERGs of AAV8-XIAP and AAV8-NULL injected eyes. *: $p<0.05$, **: $p<0.01$, ns: no significance, Two-way ANOVA with Bonferroni post-tests, $n=6$

7.4.3 XIAP expression

The AAV8-mediated XIAP overexpression was first examined in HEK293 cells transfected with the AAV8-XIAP backbone plasmid pAAV-ITR-XIAP. A strong XIAP immunostaining was clearly observed in the cytoplasm of pAAV-ITR-XIAP transfected cells compared with the pAAV-ITR controls (Figure 72). Meanwhile, a DDK antibody was applied to the cells as the DDK was fused downstream with the XIAP. Not surprisingly, the pAAV-ITR-XIAP transfected cells revealed a stronger DDK staining in the cytoplasm compared with the pAAV-ITR controls (Figure 73). Moreover, the distribution of DDK expression was similar to that in the XIAP immunostaining, demonstrating the DDK tag as a readily detectable signal for successful XIAP overexpression.

Thereafter the DDK immunostaining was applied to the eye sections to identify the viral driven XIAP from any endogenous XIAP present. Strong DDK staining was seen in parts of the AAV8-XIAP injected eye rings, presumably representing the injected area (Figure 74). The DDK signal was localized mainly in the RPE layer and some photoreceptor cells in the ONL. The DDK staining was absent on the opposite side of retinal section, which likely indicates the un-injected and unaffected retina. No DDK staining was found in the AAV8-NUL injected eye sections. It was also observed that most cells in ONL had disappeared due to degeneration in both group sections.

Western blotting was performed to further explore XIAP and DDK expression. Normalized to the endogenous β -actin expression, the AAV8-XIAP injected eyes had nearly 2-fold higher XIAP overexpression than the AAV8-NUL controls (1.5 ± 0.5 vs.

0.7 ± 0.3, $p=0.02$, Figure 75). Although the DDK was merely detected in the AAV8-NULL controls, the Western showed the AAV8-XIAP injected eyes had almost 11-fold higher DDK expression than the controls in quantification (1.1 ± 0.005 vs. 0.1 ± 0.005 , $p=0.01$, Figure 76).

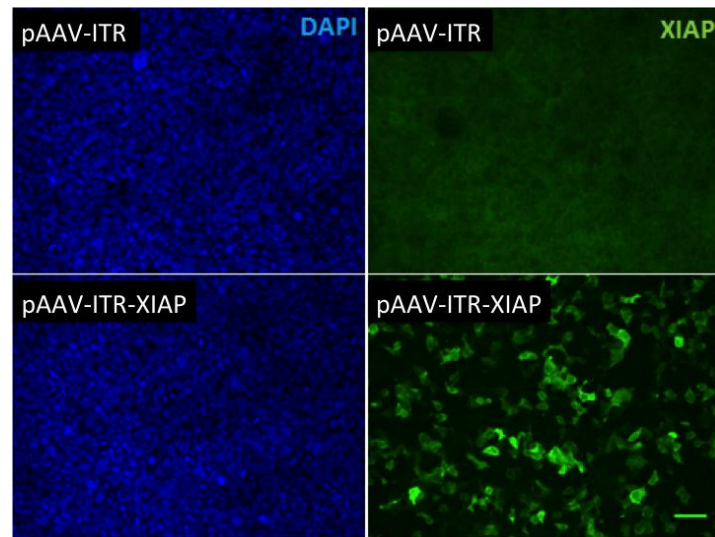


Figure 72 Immunostaining of the pAAV-ITR and pAAV-ITR-XIAP transfected HEK293 cells. Compared to the pAAV-ITR controls, the pAAV-ITR-XIAP transfected cells showed strong XIAP expression in the cytoplasm, suggesting the XIAP overexpression in the cells after transfection. Scale bar: 50 μ m.

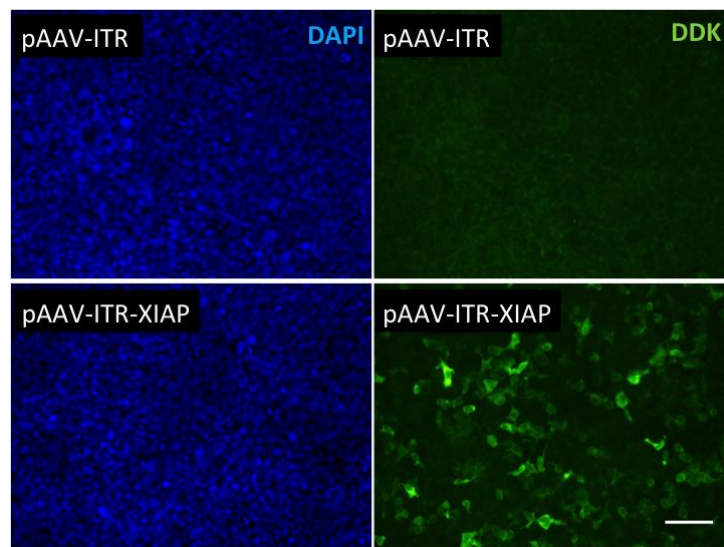


Figure 73 Immunostaining of the pAAV-ITR and pAAV-ITR-XIAP transfected HEK293 cells. Compared to the pAAV-ITR controls, the pAAV-ITR-XIAP transfected cells showed strong DDK expression in the similar pattern of XIAP (See Fig 71), suggesting the successful downstream overexpression of DDK after transfection. Scale bar: 50 μ m.

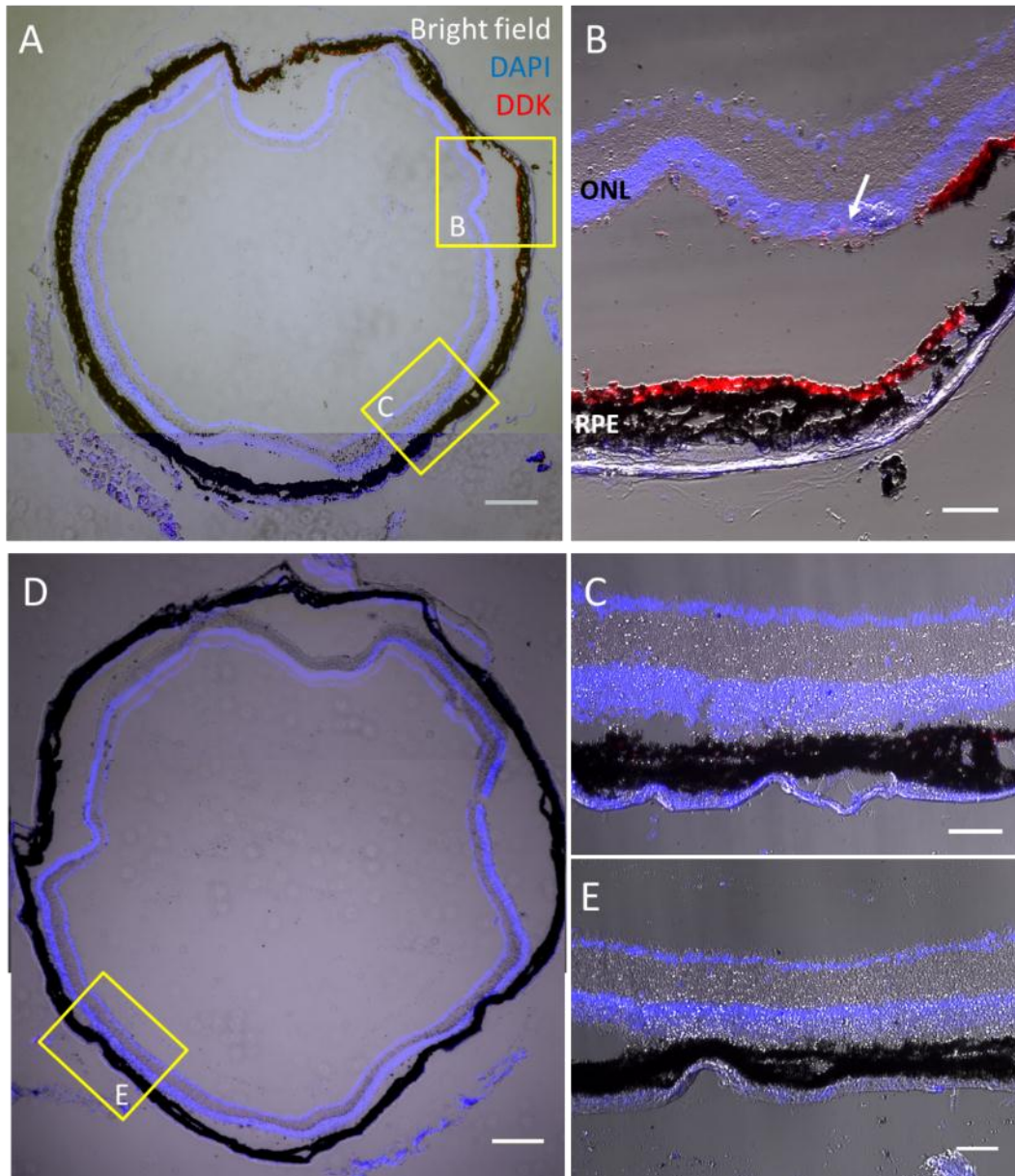


Figure 74 Immunostaining in the AAV injected eyes. In the AAV8-XIAP injected eyes (A), strong DDK staining was observed in the RPE and some photoreceptor cells (arrow) localized in the injected area (B), whilst no DDK was seen in the un-injected area of the same eye (C). No DDK was observed in the AAV8-NUL injected eyes (D, E). There were merely outer nuclear layers in both eyes, suggesting the AAV8-XIAP did not preserve photoreceptors in the $RHO^{-/-}$ $TGPN1LW-EGFP^{+/-}$ mice. Scale bar=200 μ m (A, D) or 50 μ m (B, C, E).

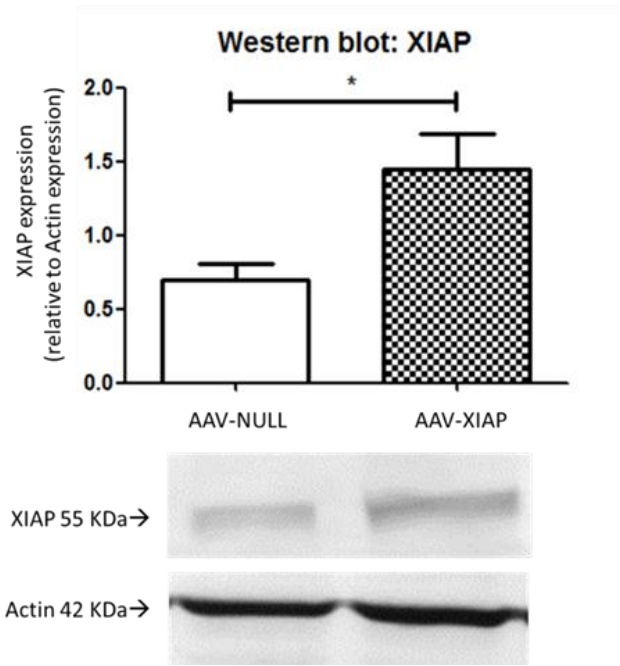


Figure 75 Western blot of XIAP in AAV8-XIAP and AAV8-NULL injected eyes. *: $p < 0.05$, Unpaired t -test, $n = 8$

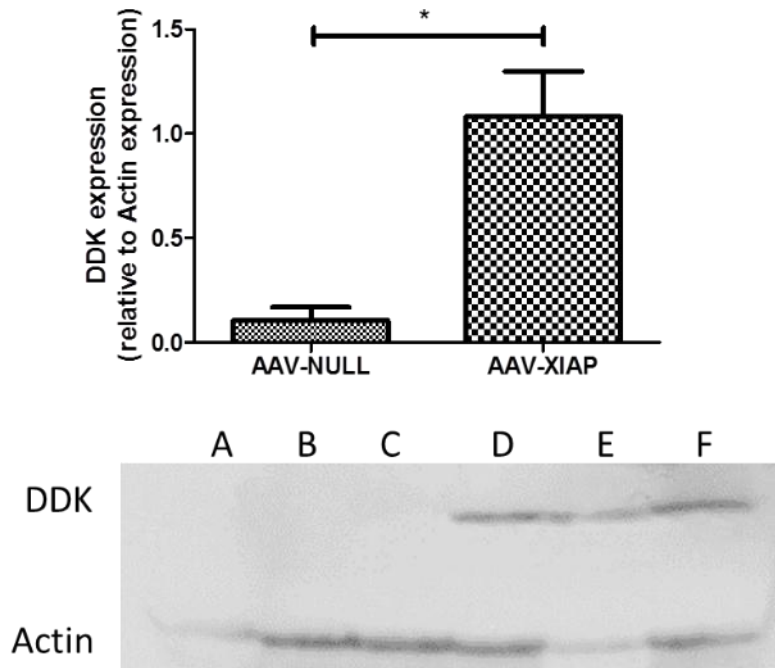


Figure 76 Western blot of DDK in vector injected eyes. A-C: AAV8-NULL injected eyes. D-F: AAV8-XIAP injected eyes. *: $p < 0.05$, Unpaired t -test, $n = 8$

7.5 Discussion

In this chapter, the XIAP coding sequence was package into AAV8 and subretinally injected into the *RHO*^{-/-} *TGPN1LW-EGFP*^{+/-} mouse. The viral driven XIAP was successfully overexpressed in the retina. However, the cSLO and ERG results did not show any preservation of cone photoreceptors at 5 weeks post vector treatment.

One possible explanation is that the cone death in *RHO*^{-/-} *TGPN1LW-EGFP*^{+/-} mice may not be caspase-dependent. Thus the cones cannot be rescued by the XIAP which inhibits apoptosis by regulating the caspases. Studies indicate that apoptosis is the common fate of cones in retinal degeneration, but the role of caspases remains elusive^{305, 306}. Activation of caspase-3 and -7 is found during the photoreceptor degeneration of the *rd* mouse and two lines of transgenic rats with *rhodopsin* mutation s334ter and P23H^{92, 95, 307}. Meanwhile, caspase-3 inhibitors (Z-DEVD-FMK, Ac-DEVD-CHO and XIAP) can significantly reduce photoreceptor death in these models, suggesting that caspases play an important role in it^{96, 97, 308}. On the other hand, the caspase-independent pathway is also reported in the photoreceptor death of the *rd* mouse, N-methyl-N-nitrosourea and light induced retinal degeneration^{290, 309}. In the present study, however, the lack of any detectable cone protection by XIAP overexpression is consistent with a mechanism of cone death in *RHO*^{-/-} *TGPN1LW-EGFP*^{+/-} mice may be caused via the caspase-independent pathway.

Another reason might be the XIAP vector injection was a bit too late. In this study, the AAV8-XIAP was delivered into the 8-week old *RHO*^{-/-} *TGPN1LW-EGFP*^{+/-} mouse. At the time of vector injection, the mouse has lost more than 50% ONL compared to the 24-day old mouse as reported previously²²². It is found that cone survival may require other retinal cells, particularly rods which can secrete

neurotrophic factors such as the rod-derived cone viability factor (RdCVF)^{254, 310, 311}. Therefore, the rest of rods (less than 50%) may not be able to provide enough RdCVF to maintain cone viability, even when XIAP is overexpressed in the cones. This also suggests that some neurotrophic factors may be essential for XIAP neuroprotection on cones. The combination of XIAP and neurotrophic factors such as RdCVF may be helpful for cones survival in RP.

It is arguable that an earlier delivery of XIAP into younger *RHO*^{-/-} *TGPN1LW-EGFP*^{+/-} mice might rescue rod and subsequently preserve cones. Recently XIAP is reported to increase survival of transplanted rod precursors in the host *rd9* mouse³¹². However, XIAP is unlikely to rescue rods in this model. According to the retinal histology, there was only a single layer of ONL at 5 weeks post AAV8-XIAP injection, compared to the nearly 50% of ONL before the injection. AAV8 is known to transduce photoreceptors efficiently and quickly, leading to the earliest transgene expression after 1 week post injection³¹³. Thus the AAV8-mediated XIAP did not prevent rod loss during the follow up 5 weeks, suggesting an early vector administration may have a very limited effect on the rods as well as the cones in this model.

Compared to the early vector administration, the design and outcome of this study may be more significant to clinical practice in the future gene therapy for RP. most RP patients are diagnosed after having developed night blindness and losing rods to some extent²⁷. The stage of degeneration for patients at the time of treatment may therefore be similar to the time when the *RHO*^{-/-} *TGPN1LW-EGFP*^{+/-} mouse has nearly 50% of ONL and was injected with vectors in this study (age of 8-weeks). In addition, cones dominate the human macula area and are critical for the most

important aspects of human vision. Therefore, if our results show that cone cell death cannot be slowed by a XIAP-caspase dependent process, it may limit the role of AAV-mediated XIAP in neuroprotective gene therapy for human retinal degeneration.

Chapter 8 General Discussion

This thesis is mainly to investigate the neuroprotection of XIAP on RPE and photoreceptor cells in retinal degeneration. The human XIAP coding sequence was packaged into AAV vectors and assessed in various retinal degeneration models. In an *ex vivo* model of H₂O₂ induced oxidative stress, AAV2 mediated XIAP overexpression showed a marked neuroprotective effect on ARPE-19 a human RPE cell line against the oxidative apoptosis. AAV2-XIAP was subretinally injected into *NRL-GFP* and B6^{TgOPN1LW-EGFP} mice undergoing NaIO₃ induced retinopathy to induce photoreceptor apoptosis secondary to RPE cell death. However, cSLO and ERG did not reveal any preservation of rod and cone photoreceptors by XIAP treatment in this model. Lastly, XIAP was packaged into AAV8 and subretinally delivered into *RHO*^{-/-} *TGOPN1LW-EGFP*^{+/-} mice featured with disrupted *rhodopsin* gene and GFP expressing LM-cones causing cone cell death secondary to rod loss. AAV8-XIAP treatment did not rescue cone degeneration either in this model, as determined by cSLO and ERG. These results imply that the positive effects of XIAP seen *in vitro* may be limited *in vivo*, possibly due to the complexity of other parallel apoptotic pathways that may bypass the XIAP specific pathway, such as the complement system and other cell-mediated responses. Alternatively the effects of XIAP may be cell specific and more tuned to the RPE than photoreceptors, as in the *in vivo* model the RPE cell death was largely by necrosis induced by sodium iodate. Further experiments with rodent models of RPE apoptosis may therefore be the logical follow on from this work.

8.1 XIAP neuroprotection on RPE cells against oxidative stress

One of the key findings in the current work is that AAV2-mediated XIAP overexpression strongly protects ARPE-19 cells against H₂O₂ induced oxidative apoptosis. Previous studies have demonstrated an increasing activation of caspase-3 in ARPE-19 cells exposed to H₂O₂^{233, 243}. Since caspase-3 plays a critical role in caspase-dependent apoptosis, it would be predicted that XIAP can markedly reduce ARPE-19 cell death in this model.

Apart from XIAP, other substances have also been reported to protect RPE cells against H₂O₂ stress in previous studies, including paeoniflorin³¹⁴, melanosomes³¹⁵, clusterin²³⁵, pigment epithelium-derived factor (PEDF)¹⁶, flavonoids³¹⁶ and quercetin³¹⁷. XIAP, however, has the unique feature that it is an endogenous protein expressed ubiquitously in most cells, which might make it more biocompatible compared with synthetic chemicals. In addition, the coding sequence of XIAP is 1494 bp, which can be efficiently packaged into AAV vectors. In the past two decades, rAAV-based gene therapy has become the most promising treatment for retinal degeneration. rAAV can efficiently transduce RPE and photoreceptor cells, which are the main targeted cells in AMD or RP treatment¹¹⁸. The transgene sequence packaged in rAAV can be long-lasting expressed within cells and delivered with tissue-specific promoters such as RPE65 and rhodopsin kinase promoters, for RPE and photoreceptors respectively^{318, 319}. Furthermore, the eye is an ideal organ for gene therapy due to its highly compartmentalized structure, minimizing systemic immune responses and side effects¹⁰⁷. The safety and efficiency of AAV-based gene therapy has been well established in a series of clinical trials on human patients^{131, 133, 320, 321}.

On the other hand, alternative strategies targeting mitochondrial oxidants have not so far been particularly effective in clinical trials aimed at preventing RPE cell loss in AMD. In the AREDS study, geographic atrophy continuously progressed despite of a long-term combined therapy of anti-oxidant agents^{322, 323}. Our *in vitro* findings indicate however that XIAP may be a good neuroprotective candidate to consider in future gene therapy approaches directed against oxidative stress in retinal degenerative disease in the RPE, such as AMD.

The results of XIAP neuroprotection on RPE cells, however, should be carefully interpreted due to some limitations in the present study. First, ARPE-19 is a spontaneously arising RPE cell line which is not exactly as same as the post-mitotic RPE cells *in vivo*, despite it sharing features of the RPE in terms of appearance and molecular markers⁷. Results from primary culturing of human RPE cells may be more convincing, but it involves human tissues and is difficult to carry out due to potential ethical problems. Secondly, XIAP neuroprotection was only tested on cells exposed to a “marginal” dose of H₂O₂, *e.g.* 0.25 mM, which may not representative as the actual level of oxidative stress in retinal degenerative disease. The oxidative stress *in vivo* is far more complicated than the H₂O₂ model *in vitro*. It involves other ROS such as superoxide and peroxynitrite, and the balance between oxidants and anti-oxidant agents³²⁴⁻³²⁶. Nevertheless, our results for the first time proved that AAV-mediated XIAP overexpression can rescue human RPE cells against oxidative stress, providing a therapeutic approach for AMD treatment.

8.2 Non-invasive imaging in retinal degeneration research

Another key finding of this thesis is that the cSLO can be a useful non-invasive tool to visualize NaIO₃ toxicity on RPE and photoreceptor cells *in vivo*. As discussed in previous chapters, the fundus autofluorescence (FAF) on 790 nm laser source originates from the RPE and choroid to some degree²⁷⁰. Oxidized melanin and/or compounds closely associated with melanin are believed to contribute substantially to this FAF^{270, 327}. In previous studies, confocal laser scanning microscopy was performed to examine retinal tissues and its autofluorescence *in vitro*^{261, 328}. The cSLO imaging, however, moves a step forward by visualizing pathologic features of the RPE and the choroid *in vivo*, as the high fundus FAF observed after NaIO₃ treatment in this thesis. In AMD patients, the fovea exhibits higher 790 nm FAF than the perifovea area²⁷⁰. Higher FAF was found in hyperpigmentation and low FAF was observed in geographic atrophy. The ratio of foveal to perifoveal FAF decreased with age. Another study reveals that confluent patches of reduced 790 nm FAF were frequently seen in patients with ABCA4-associated retinal dystrophy³²⁷. The 790 nm FAF may precede 488 nm FAF alterations during the degenerative process³²⁷.

The 488 nm laser source in cSLO, on the other hand, is mainly used to detect lipofuscin-related FAF in retinal disorders such as AMD and RP^{268, 329}. In AMD patients with GA, diffuse areas of elevated autofluorescence are recorded along with existing GA areas enlargement and new GF developing over time³³⁰. Additionally, the distinct FAF patterns are found to have an impact on atrophic AMD progression and may serve as prognostic determinants³³¹. In a group of retinal dystrophies confirmed

by molecular genetic analysis, FAF abnormalities was observed in all examined eyes with Stargardt disease (*ABCA4* mutation), Best vitelliform macular dystrophy (*VMD2* gene) and retinal dystrophies associated with mutations in the *peripherin/RDS* gene³³². Focally increased FAF was the most common finding, frequently accompanied by areas of decreased or absent FAF³³².

In the present study, however, the 488 nm mode was performed to examine the FAF caused by EGFP expression in photoreceptors. The cSLO was first reported by Beck SL *et al.* to identify GFP expressing cones in the B6^{TgOPN1LW-EGFP} mouse²⁷⁴. cSLO was also applied for longitudinal monitoring of cone survival in crossbreds of B6^{TgOPN1LW-EGFP} mice with *cpfl1* and *Rpe65*^{-/-} mutant mice^{274, 333}. By using transgenic mice expressing GFP under the transcriptional control of the smooth muscle type alpha-actin promoter, the cSLO imaging allowed quantification of retinal vessel outer diameters *in vivo*³³⁴. In this thesis, the cSLO was successfully applied to detect NaIO₃ induced rod and cone photoreceptor loss in the *NRL-GFP* and B6^{TgOPN1LW-EGFP} mouse, respectively. The cSLO imaging, together with transgenic animals with selective GFP expressing, constitutes a useful tool to identify retinal structure.

Apart from the cSLO, other imaging techniques such as optical coherence tomography (OCT) and adaptive optics SLO have been fast developed and successfully applied in retina clinic and/or research^{2, 335, 336}. There are obviously many benefits of the non-invasive imaging techniques. First of all, it greatly reduces the number of animals sacrificed for tissue samples and its associated costs. This is particularly helpful for large animal studies such as in dogs and non-human primates when animal availability is limited, or in human clinical studies when tissue samples are difficult to obtain. Secondly, the *in vivo* method avoids any variation caused by

tissue processing such as eye sectioning and immunostaining. Moreover, it allows longitude observation in the same animal at different time points. Hence there is no individual difference when comparing between the baseline and follow-up examinations. Lastly, it provides an overview of retinal pathology rather than local histological changes in a limited field of eye sections. Although retinal histology may still be required as the “golden standard” evidence in many studies, these retinal imaging techniques have offered an easy and promising approach for retina analysis.

8.3 Caspase-independent mechanism of photoreceptor death in retinal degeneration

It is perhaps unexpected that the AAV-mediated XIAP did not rescue rod and cone photoreceptors in the NaIO₃ induced retinopathy. Neither did it rescue LM-cones in the *RHO*^{-/-} *TGPN1LW-EGFP*^{+/-} mouse, even though XIAP was overexpressed by AAV delivery as determined by immunostaining and Western blotting.

Such an outcome is different from some previous studies, which found XIAP neuroprotection on photoreceptors in other models of retinal degeneration^{97, 206, 210}. The difference may be mainly due to the variant animal models and the potential mechanism of photoreceptor death, *i.e.* whether it is caspase-dependent or -independent pathway. *N*-methyl-*N*-nitrosourea (MNU), for example, is another widely used chemical to induce photoreceptor death²¹⁰. It is an alkylating agent that causes DNA methylation, leading to GC→AT transition mutations, caspase-3/-9 activation and photoreceptor apoptosis^{183, 337, 338}. AAV2-mediated XIAP treatment was found to reduce apoptotic cells in the ONL and preserve ERGs in the MNU-treated rats²¹⁰. In

contrast, NaIO_3 is a strong oxidizing agent. It causes RPE and photoreceptor toxicity presumably due to its oxidative damage thus is a good model to assess XIAP neuroprotection against oxidative stress *in vivo*. There is no report so far about the caspase role in NaIO_3 induced RPE or photoreceptor damage. According to our findings, it is highly possible that their death is mainly due to a caspase-independent pathway, which XIAP has little effect on.

Compared to the model with chemical induced retinopathy, human RP is more faithfully reproduced in transgenic animals which contain mutant genes found in human cases, such as the $RHO^{-/-} \text{ TGOPN1LW-EGFP}^{+/-}$ mouse. In contrast to our results, Leonard *et al.* reported the AAV-mediated XIAP neuroprotection on photoreceptors in two other *rhodopsin* mutant animals, the P23H and S334ter rat⁹⁷. These three strains share some similar genetic background in homozygous mutant/disrupted *rhodopsin* gene. The $RHO^{-/-} \text{ TGOPN1LW-EGFP}^{+/-}$ mouse in this thesis has a disrupted exon 2 of the rhodopsin gene *RHO*. The P23H rat in Leonard *et al.* report features a histidine substituted for proline in position 23 of the *RHO*^{339, 340}. The S334ter rat contains a termination codon at residue 334 of the *RHO*, which results in the incomplete form of protein³⁴¹. All these *RHO* mutations/disruptions result in a gradual apoptotic loss of photoreceptors in retina. In addition, both studies used the human XIAP coding sequence tagged with either DDK or hemagglutinin for distinguish immunostaining.

The difference of two studies may be due to the type of photoreceptors targeted in each study and its possible mechanism of cell death. Leonard *et al.* examined the structure of ONL which are mainly the rod photoreceptors (~97%). They are the primarily affected cells due to *RHO* mutation/disruption. In the $RHO^{-/-}$

TGPN1LW-EGFP^{+/-} mouse, however, the cone photoreceptors were studied. Their death may be secondary to the rod loss due to *RHO* disruption, but also an independent result of transgenic insertion. Recently a dominantly inherited cone dystrophy was reported in its parental B6^{TgOPN1LW-EGFP^{+/+}} 342. The transgene insertion of EGFP was mapped to a specific region on mouse chromosome 10 orthologous with loci for progressive bifocal chorioretinal atrophy and North Carolina macular dystrophy on human chromosome 6.

Unfortunately, little is known about the molecular mechanism of cone death in the *RHO^{-/-} TGPN1LW-EGFP^{+/-}* mouse. The fact that XIAP overexpression did not rescue cones highly suggests that it is mainly caspase-independent. In contrast, studies have showed increasing activation of caspases in the P23H and S334ter rat^{95, 100, 307}. Activation of caspases-3 and -9 were observed in the S334ter rat^{95, 100}. The photoreceptor level of cleaved caspase-7 was much higher in the P23H than the wild-type rat³⁰⁷. Mislocalized rhodopsin is proposed to activate G proteins (such as transducing) and adenylate cyclase, which eventually leads to caspase-3 activation³⁴³. Hence the XIAP may well have a neuroprotective effect on photoreceptors in the P23H and S334ter rat, shown as the preservation of ONL thickness in Leonard *et al.* paper.

In Leonard *et al.* study, however, no preservation of a- or b-wave was identified in the ERGs of S334ter rats up to 28 weeks post XIAP treatment⁹⁷. Meanwhile, the P23H rats did not appear any significant preservation of a- or b-wave until at ~20 weeks post XIAP treatment. No additional function or structure examinations were provided in the paper. It seems obviously that the XIAP treatment did not show a strong neuroprotection on photoreceptor function, suggesting the

caspase-independent mechanism may well also exist in these two *RHO* mutant animals. Indeed, activation of calpain and PARP, two pro-apoptotic proteins regarded as caspase-independent pathway, has been found in the P23H and S334ter rats¹⁰⁰.

Inevitably, there are some limitations in this part of thesis. First of all, the animal models used in this thesis are by no means representative as all types of retinal degenerative diseases. There are many other gene mutations that cause dysfunction of RPE and photoreceptor cells and lead to retinal dystrophy. The mechanism of photoreceptor cell death is far more complicated than we thought and may differ with each other. Secondly, the *RHO*^{-/-} *TGOPN1LW-EGFP*^{+/-} mouse may contain two mechanisms independently leading to cone death as discussed above. This may limit XIAP neuroprotection, if any, and affect the final conclusion. In addition, the NaIO₃ dose in the current model may cause an intense toxicity than XIAP neuroprotection. A lower dose may be helpful for XIAP assessment. Lastly, caspase activation pathways were not examined due to the limits of our technique. Exploring the XIAP effects on the caspase pathway may be helpful to better understand why XIAP showed limited efficacy in the current models.

8.4 Further work

Although encouraging outcomes have gained in the recent gene therapy clinical trials of *RPE65*-related LCA, the majority of RP is still incurable and remains the leading cause of inherited blindness in developed countries. Neuroprotective agents, such as XIAP, may offer a common treatment strategy in RP treatment despite of their various pathogeneses. The experiments here have provided a comprehensive

understanding of XIAP neuroprotection on the RPE and photoreceptor cells in several retinal degeneration models, including H₂O₂ induced oxidative stress, NaIO₃ induced retinopathy in the *NRL-GFP* and B6^{TgOPN1LW-EGFP} mouse, and the transgenic *RHO*^{-/-} *TGOPN1LW-EGFP*^{+/-} mouse. In future, it may be helpful to address on XIAP effect against oxidative stress and a component in combined therapy as a translational approach for retinal degeneration treatment.

According to the present study, AAV-mediated XIAP is very likely a potential therapeutic approach for atrophic AMD, which presents gradual RPE loss presumably due to oxidative stress to some degree. However, XIAP protection of the RPE needs to be assessed further in human AMD models rather than the H₂O₂ model. This is particularly important as the current study reveals that XIAP did not rescue photoreceptor cells in the NaIO₃ induced retinopathy model, which is presumably caused by NaIO₃ oxidation and RPE loss. So far there are a few transgenic animal models available for human AMD studies. Ambati J, *et al.* reported that mice deficient either in monocyte chemoattractant protein-1 (Ccl-2) or its cognate C-C chemokine receptor-2 (Ccr-2) develop features of AMD, including accumulation of lipofuscin and drusen beneath the RPE, photoreceptor atrophy and CNV³⁴⁴. A further double knock-out *Ccl2/Cx3cr1*-deficient mouse line is found to develop similar AMD-like retinal lesions at an early age (6 weeks old)³⁴⁵. In addition, high omega-3 long-chain polyunsaturated fatty acid, a well-known anti-oxidant agent, reversed such AMD lesions³⁴⁵. Rakoczy PE, *et al.* reported a transgenic *mcd/mcd* mouse line expressing a mutated inactive form of cathepsin D and impairing the RPE processing of phagocytosed photoreceptor outer segments³⁴⁶. RPE atrophy and an accelerated photoreceptor cell death were detected at 12 months old age. Superoxide dismutase 1 (SOD1) is one of three types of superoxide dismutases responsible for cleaning free

superoxide radicals in the body³⁴⁷. In *SOD1*^{-/-} mice, oxidative damage was observed in the RPE accompanied by AMD pathologies such as drusen and CNV³⁴⁸. Provided the availability of these AMD models, it may worth trying AAV-mediated XIAP treatment as this may directly answer the question if XIAP is beneficial for the RPE and/or photoreceptor cells under oxidative stress in AMD.

Another strategy is to combine XIAP with other therapeutic approaches in AMD or RP treatment. In a recent study of rod precursor cell transplantation, the AAV-XIAP pretreatment significantly increased the survival of integrated donor cells in the *Rd9* mouse, a model of X-linked retinal degeneration³⁴⁹. The *rd10* mouse is another retinal degeneration strain caused by mutation in the rod cyclic guanosine monophosphate phosphodiesterase β -subunit gene (*PDE β*). Co-injection with AAV5-XIAP and AAV5-PDE β was found to improve the preservation of retinal ONL structure and scotopic b-wave response compared with the AAV5-PDE β treatment along with the AAV5-GFP control²¹⁴. In a rat model of optic nerve transection, Straten G, *et al.* found a joint injection of adenoviral XIAP and glial cell line-derived neurotrophic factor (GDNF) significantly rescued more retinal ganglion cells from degeneration compared with their separate injection²¹¹. As these preliminary studies proved, XIAP may be a helpful adjunction to the cell-replacement, gene supplement and neurotrophic factor treatment of retinal degeneration.

On the other hand, the future studies also need to draw an attention on the potential malignancy risk of XIAP. Multiple studies in cultured cells and clinical samples support XIAP overexpression as an event in carcinogenesis³⁵⁰⁻³⁵³. It is also demonstrated that XIAP overexpression confers resistance of cultured cells to multiagent chemotherapy³⁵⁴⁻³⁵⁶. Gene therapy to the outer retina only requires small

doses of AAV compared to other organs. It has very limited systemic spread due to the compartment structure of eye and blood-retina barrier. However, it might be judicious to limit XIAP transgene expression by using a specific serotype (such as AAV4 for the RPE) or an inducible promoter (such as the RPE65 promoter for the RPE or rhodopsin kinase promoter for rods)^{119, 131, 319}.

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