



Developing Treatments for Rhodopsin-Related Dominant Retinitis Pigmentosa

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

I declare that this thesis is my own work except where otherwise indicated. All figures are original unless otherwise stated. Where figures or tables have been adapted from work previously published by others, this has been clearly stated and appropriately referenced. At the time of submission, no material from this thesis has been published elsewhere. Material from Chapter 3 is currently under peer review for consideration of publication. I was the sole producer of material for this paper, with co-authors having an advisory role. Plasmids that formed the basis for the cloning presented herein were either acquired from commercial manufacturers or provided as kind gifts from other researchers. In all cases, these sources have been acknowledged. All DNA sequencing was subcontracted to Source Biosciences, UK. The word count (excluding table of contents, lists of figures and tables, figure legends and references) is approximately 45,430. No material presented here been previously submitted to the University of Oxford, or to any other academic institution, for another degree or qualification.

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Abstract

Developing treatments for rhodopsin-related dominant retinitis pigmentosa

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Mutations in the rhodopsin gene are one of the most common causes of autosomal dominant retinitis pigmentosa (ADRP) and there is at present no treatment available. In this thesis, three potential treatments were developed and tested in a *Rho*^{P23H/+} knock-in mouse model.

In the first, animals were housed in red cages which acted as long-pass light filters. This resulted in a significant slowing in the rate of both rod and cone photoreceptor degeneration as determined anatomically using non-invasive *in vivo* imaging and immunohistochemistry, and functionally by dark- and light-adapted electroretinography.

In the second, efficient adeno-associated viral (AAV) vectors expressing wild type human rhodopsin were manufactured and injected subretinally in *Rho*^{P23H/+} mice to test the hypothesis that overexpression of the normal protein might overcome the effect of the P23H mutant. No structural or functional rescue effect was however observed.

The final treatment strategy involved development of artificial mirtrons to suppress expression of endogenous rhodopsin. Mirtrons are novel RNA interference effectors that are spliced from mRNA transcripts. Both efficient mirtrons and a codon modified mirtron-resistant version of the human rhodopsin coding sequence were designed and validated *in vitro*. These elements were then combined under the control of a single rhodopsin promoter within AAVs as knockdown/replacement gene therapy vectors for ADRP. Subretinal injection in the *Rho*^{P23H/+} mouse resulted in mouse-to-human rhodopsin mRNA replacement, and this was associated with a modest rescue effect when injected at a dose of 2×10^8 gc. This represents the first use of an artificial mirtron to induce gene knockdown *in vivo*.

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List of Abbreviations

A:	Adenine
AAV:	Adeno-associated virus
AD:	Autosomal dominant
ADRP:	Autosomal dominant retinitis pigmentosa
ANOVA:	Analysis of variance
AR:	Autosomal recessive
ART:	Automatic real-time
BAF:	Blue autofluorescence
BM:	Bruch's membrane
Bp:	Base pairs
BSA:	Bovine serum albumin
C:	Cytosine
cDNA:	Complementary DNA
CDS:	Coding sequence
cGMP:	Cyclic guanosine monophosphate
CNTF:	Ciliary neurotrophic factor
CRISPR:	Clustered regulatory interspersed short palindromic repeats
cSLO:	Confocal scanning laser ophthalmoscopy
CSNB:	Congenital stationary night blindness
C_t:	Cycle threshold
DMEM:	Dulbecco's Modified Eagle Medium
DNA:	Deoxyribonucleic acid
EBSS:	Earle's balanced salt solution
EDTA:	Ethylenediaminetetraacetic acid

eGFP:	Enhanced green fluorescent protein
ELM:	External limiting membrane
ER:	Endoplasmic reticulum
ESE:	Exonic splice enhancer
ETDRS:	Early Treatment Diabetic Retinopathy Study
EZ:	Ellipsoid zone
FACS:	Fluorescence-activated cell sorting
FBS:	Fetal bovine serum
FDA:	Food and Drug Administration (USA)
FIPA:	Fluorescence immunoprecipitation assay
G:	Guanine
GCL:	Ganglion cell layer
GFP:	Green fluorescent protein
GPCR:	G-protein-coupled receptor
HD:	High dose
HEK293:	Human Embryonic Kidney 293
ICC:	Immunocytochemistry
ICGA:	Indocyanine green angiography
IHC:	Immunohistochemistry
ILM:	Internal limiting membrane
INL:	Inner nuclear layer
IPL:	Inner plexiform layer
IRC:	Irradiance-response curve
IRD:	Inherited retinal disease
IS:	Inner segments
ITR:	Inverted terminal repeat

IVC:	Individually ventilated cage
kb:	Kilobase pairs
kDA:	Kilodaltons
LB:	Luria's broth
LCA:	Leber congenital amaurosis
LD:	Low dose
MCS:	Multiple cloning site
miRNA:	Micro ribonucleic acid
MOI:	Multiplicity of infection
mRNA:	Messenger ribonucleic acid
MZ:	Myoid zone
NDS:	Normal donkey serum
NFL:	Nerve fibre layer
NIR:	Near infrared
NRL:	Neural retina-specific leucine zipper
ns:	Not significant
NTC:	No template control
OCTC:	Optimal cutting temperature compound
OD:	Oculus dexter (right eye)
ONL:	Outer nuclear layer
OPL:	Outer plexiform layer
ORF:	Open reading frame
OS:	Oculus sinister (left eye)
OS:	Outer segments
PBS(T):	Phosphate buffered saline (Tween-20)
PC:	PsiCHECK2 vector

PCR:	Polymerase chain reaction
PDE:	Phosphodiesterase
PEI:	Polyethylenimine
PFA:	Paraformaldehyde
PI:	Protease inhibitor
PND:	Post-natal day
PNW:	Post-natal week
PRL:	Photoreceptor layer
PVDF:	Polyvinylidene difluoride
R:	Purine (adenine or guanine) nucleotide
RdCVF:	Rod-derived cone viability factor
Rho:	Mouse rhodopsin
RHO:	Human rhodopsin
RHOp:	Human rhodopsin promoter
RIPA:	Radioimmunoprecipitation assay
RISC:	RNA-induced silencing complex
RNA:	Ribonucleic acid
RNAi:	Ribonucleic acid interference
RP:	Retinitis pigmentosa
RPE:	Retinal pigment epithelium
RPGR:	Retinitis pigmentosa GTPase regulator
SD-OCT:	Spectral domain ocular coherence tomography
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM:	Standard error of the mean
shRNA:	Short hairpin ribonucleic acid
SNP:	Single nucleotide polymorphism

T:	Thymine
TAE:	Tris base/acetate/EDTA
T_m:	Melting temperature
U:	Uracil
UPR:	Unfolded protein response
UTR:	Untranslated region
UV:	Ultraviolet
WPRE:	Woodchuck hepatitis virus post-transcriptional regulatory element
WT:	Wild type
XL:	X-linked
ZFN:	Zinc finger nuclease

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1. General Introduction

1.1. The Retina

1.1.1. Anatomy

The mammalian retina is a laminated neural structure located within the posterior segment of the eye that is responsible for detection of light and the initial processing of visual stimuli. It develops from a neuroectodermal outpouching of the lateral forebrain known as the optic vesicle. Ten layers of the neural retina can be distinguished histologically. These are, from innermost to outermost: internal limiting membrane (ILM), nerve fibre layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), external limiting membrane (ELM), inner segments (IS), and outer segments (OS) (Figure 1.1). The neural retina sits above a pigmented monolayer of supporting cells also of neuroectodermal origin known as the retinal pigment epithelium (RPE). The RPE is itself located on Bruch's membrane, an acellular layer that separates it from the vascular choroid beneath. The neural retina is made up of seven classes of cell: rods, cones, bipolar cells, ganglion cells, Müller glia, amacrine cells and horizontal cells. Light focussed by the refractive media of the eye is detected in a process known as phototransduction by photoreceptors located in the outer retina: rods and cones. These specialised cells whose nuclei constitute the ONL, synapse with bipolar cells in the OPL which in turn synapse with ganglion cells in the IPL. The visual signal passes along the axons of ganglion cells which together form the NFL (Figure 1.1). These axons converge to form the optic nerve which projects via the optic chiasm and tracts to the lateral geniculate nucleus of the thalamus. Central neurones then convey the visual signal to the primary visual cortex located at the posterior pole of the occipital lobe of the brain. Müller cells are specialised glia that maintain the structural and functional integrity of the neural retina. Their projections run perpendicularly through the retina whilst

their inner and outer footplates form the ILM and ELM respectively. Horizontal and amacrine cells are neuronal elements involved in lateral processing within the retina.

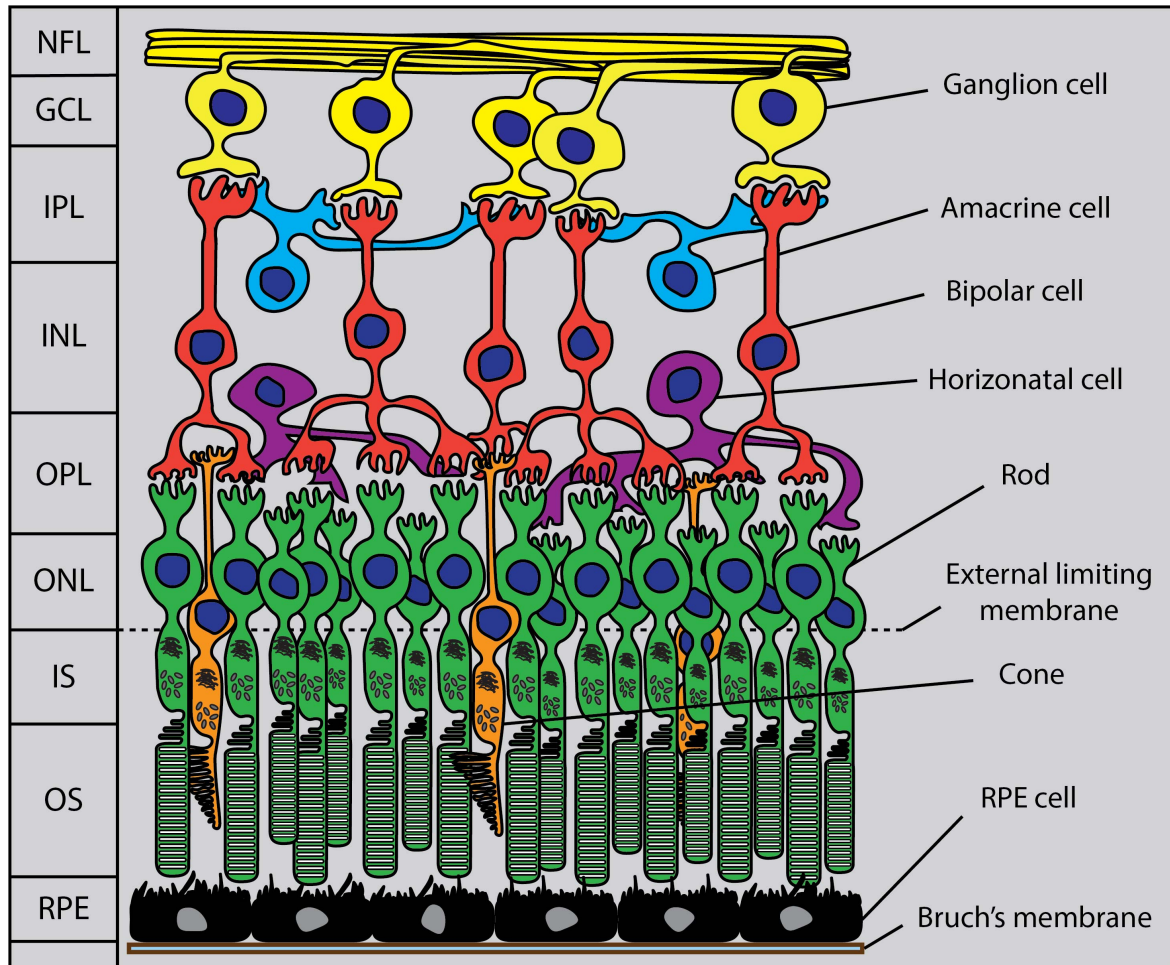


Figure 1.1. Anatomy of the mammalian retina. Schematic representation of the neural cells that constitute the laminar structure of the retina. NFL: nerve fibre layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; IS: inner segments; OS: outer segments; RPE: retinal pigment epithelium. The external limiting membrane is represented by a horizontal dotted line. Illustration not to scale.

The process of phototransduction takes place in the OS of photoreceptors. These specialised organelles are constituted of densely packed membranous discs containing seven-transmembrane receptor proteins known as opsins. OS attach to IS via connecting microtubular

cilia. The inner segment consists of two discernible regions: an outer ellipsoid area which is densely packed with mitochondria, and an inner myoid area containing endoplasmic reticulum and Golgi apparatus. The human retina contains approximately 92 million rods and around 4.6 million cones¹. Rods are exquisitely sensitive to light, capable of detecting a single photon^{2,3}. They function in low luminance (scotopic) conditions to facilitate night vision but do not contribute in brighter (photopic) environments. Fine acuity and colour vision in photopic conditions are mediated by cones. The human retina contains three distinct classes of cone which have different but overlapping spectral sensitivities: a short-wavelength (S)-cone which is maximally sensitive to light in the blue part of the spectrum, a medium-wavelength (M)-cone maximally sensitive to green light, and a long-wavelength (L)-cone maximally sensitive to red light. Non-primate land mammals such as mice are dichromatic, having two classes of cone referred to as S- and L/M-cones.

The macroscopic structure of the human retina as seen by fundoscopy is shown in Figure 1.2. Fixation directs light onto a central specialised region of avascular retina known as the fovea. This forms the centre of the macula, the region of the retina between the vascular arcades responsible for the central 17° of visual field⁴. The inner layers of the retina are outwardly displaced at the fovea which thus forms a pit in cross section. This can be visualised as a light reflex fundoscopically (Figure 1.2). The centre of the fovea is devoid of rod cells with the ONL in this region consisting entirely of cone nuclei. Cone density decreases with increasing distance from the fovea whilst rod density increases rapidly to a maximum in the perifoveal macula before falling gradually with increasing eccentricity^{1,5}.

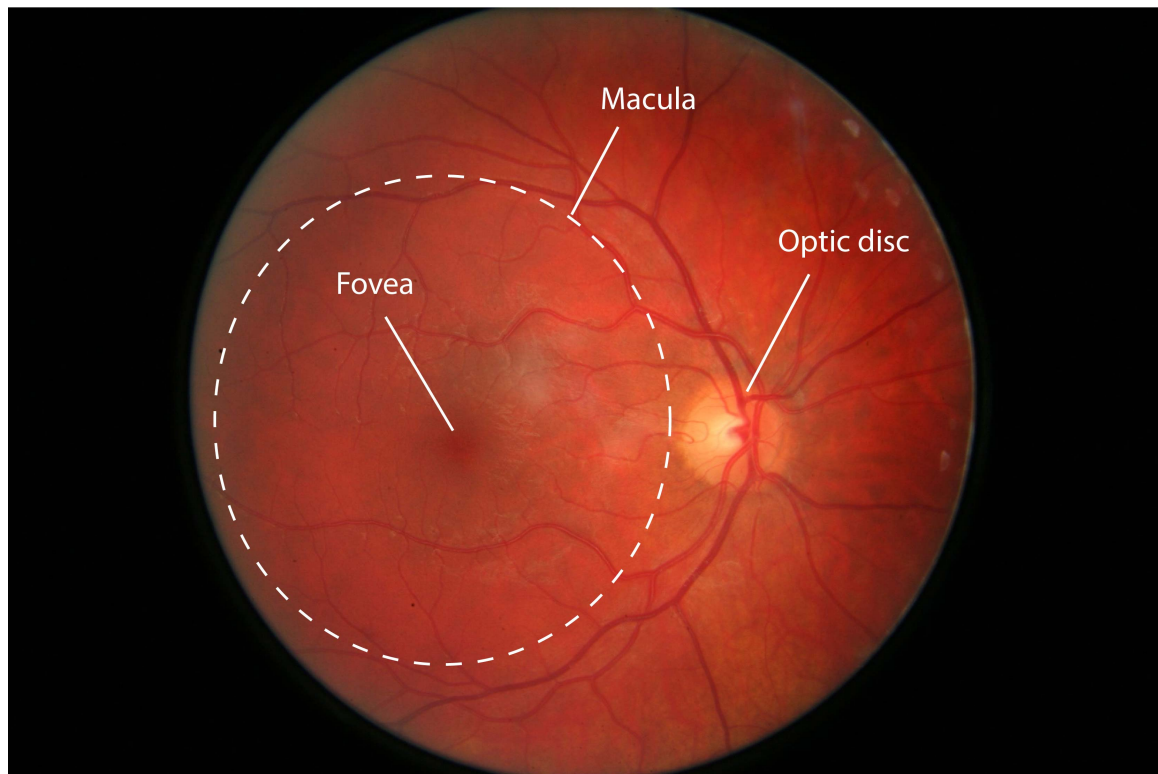


Figure 1.2. The human fundus. Colour fundus photograph of the right eye of the author. The optic nerve head (optic disc), fovea and macular area are indicated.

Non-invasive cross-sectional imaging of the retina is commonly performed in clinical practice using spectral-domain ocular coherence tomography (SD-OCT). This revolutionary technology uses infrared interferometry to construct high resolution cross-sectional images from which retinal layers corresponding to those seen histologically may be readily identified *in vivo* (Figure 1.3). The precise anatomical features that determine the highly reflective bands generated by photoreceptors and the RPE within the outer retina of SD-OCT scans are still debated. The nomenclature adopted herein is that proposed by the International Nomenclature for Optical Coherence Tomography (INOCT) panel, 2014⁶.

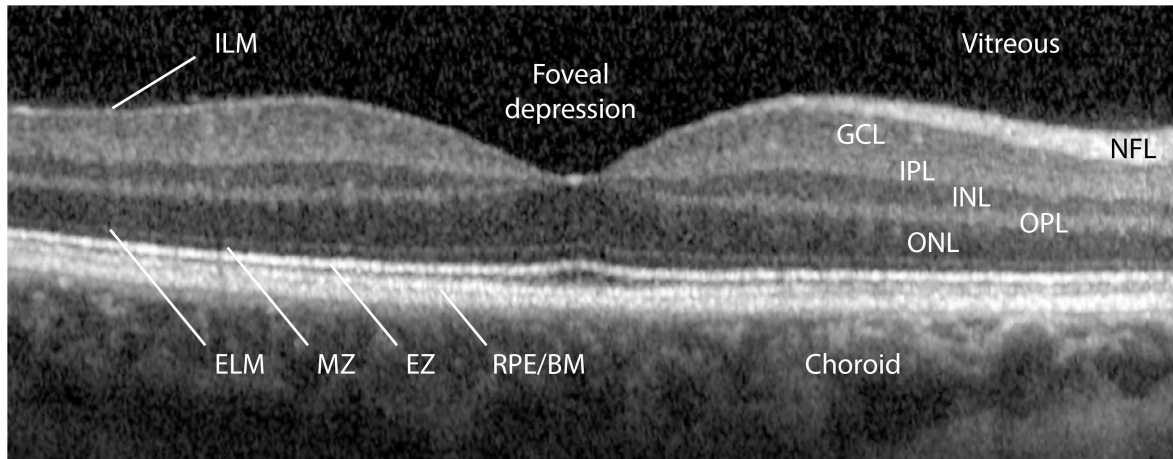


Figure 1.3. Spectral domain optical coherence tomography (SD-OCT) of the normal human retina. A horizontal SD-OCT scan taken through the fovea of the right eye of the author is shown. ILM: internal limiting membrane; NFL: nerve fibre layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; ELM: external limiting membrane; MZ: myoid zone; EZ: ellipsoid zone; RPE: retinal pigment epithelium; BM: Bruch's membrane.

The house mouse *Mus musculus* represents a useful system in which retinal disease may be modelled, but it is important to note how murine retinal anatomy differs from that of the human. Mice are nocturnal mammals with laterally-placed eyes. They lack a fovea and as such rods and cones are differently distributed throughout the retina⁷, with cone density appearing to increase in the dorso-ventral direction⁸. The murine retina is arranged with a centrally placed optic nerve from which the retinal vasculature emanates. The retinal striations seen histologically and by SD-OCT imaging in the mouse retina are equivalent to those observed in humans⁹.

1.1.2. Physiology

Light is detected in rods and cones by rhodopsin and cone opsins respectively. These membrane proteins are members of the seven-transmembrane G-protein-coupled receptor (GPCR)

superfamily which bind to the light-sensitive vitamin A derivative 11-cis-retinal. When this chromophore absorbs a photon of light it isomerizes to form all-trans-retinal. This induces structural changes in the associated opsin converting it through a series of unstable intermediates to metarhodopsin II^{10,11}. This then activates a G-protein known as transducin whose liberated alpha subunit then in turn activates phosphodiesterase 6 (PDE6), which itself catalyses the hydrolyzation of cyclic guanosine monophosphate (cGMP) to 5'-guanosine monophosphate (5'-GMP). As a result, cyclic nucleotide-gated sodium ion channels in the disc membrane close leading to hyperpolarization of the cell and closure of membrane-bound voltage-gated calcium channels¹². The subsequent drop in intracellular calcium inhibits release of glutamate at the photoreceptor synaptic terminal causing depolarization (activation) of on-centre bipolar cells and hyperpolarization (deactivation) of off-centre bipolar cells. Low intracellular calcium simultaneously activates rhodopsin kinase, an enzyme which phosphorylates metarhodopsin II. An inactivating protein called arrestin then binds to phosphorylated metarhodopsin II, thus terminating the phototransduction cascade^{13,14}.

Following photoisomerization, all-trans-retinal is reduced by all-trans-retinol dehydrogenase to all-trans retinol. This is transported to the RPE, where through a series of reactions catalysed by the enzymes lecithin retinol acyltransferase (LRAT), RPE65 and retinol dehydrogenase 5 (RDH5), it is converted back to 11-cis-retinal for use by photoreceptors (Figure 1.4).

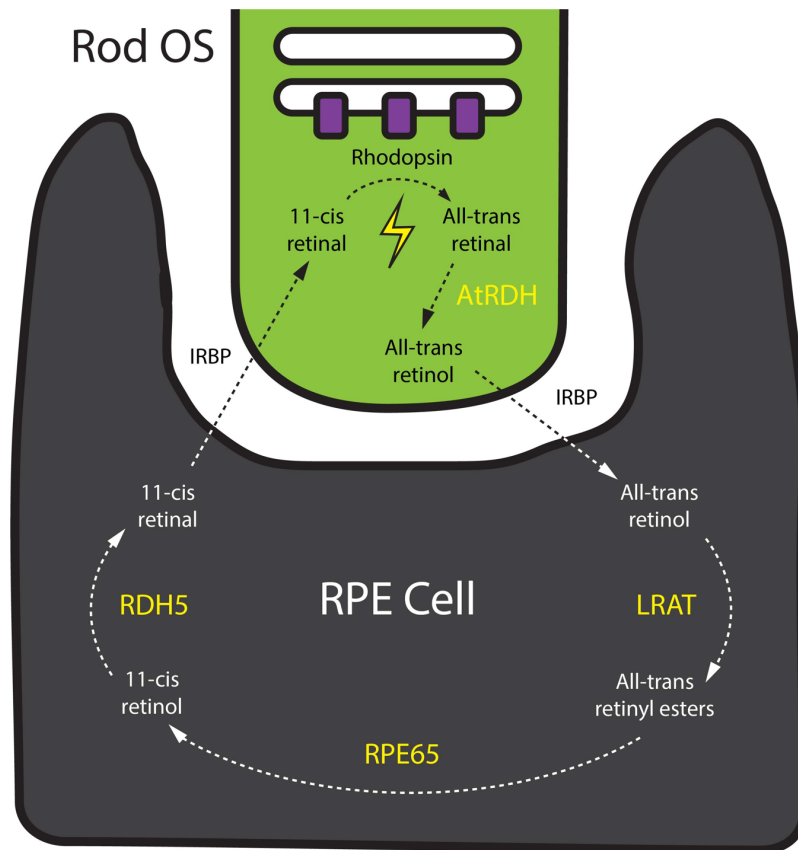


Figure 1.4. The retinoid cycle. Light catalyses the photoisomerization of opsin-bound 11-cis-retinal to all-trans-retinal. This is reduced by the enzyme all-trans-retinol dehydrogenase (AtRDH) to all-trans-retinol which diffuses across the interphotoreceptor matrix from the rod outer segment to the RPE in association with interphotoreceptor binding protein (IRBP). Within cells of the RPE, all-trans-retinol is esterified to form all-trans retinyl esters by lecithin retinol acyltransferase (LRAT) which are themselves converted to 11-cis-retinol by the enzyme RPE65. Finally, 11-cis-retinol is oxidised to 11-cis-retinal by retinol dehydrogenase 5 (RDH5) and is shuttled back into rod outer segments bound to IRBP. Mutations affecting key enzymes of the retinoid cycle are frequently associated with Leber congenital amaurosis (LCA) or retinitis pigmentosa (RP).

Retinal function may be quantitatively assessed in human subjects in a number of ways. Reading charts are typically used under standard conditions for the measurement of central visual acuity and contrast sensitivity, whilst dynamic and static perimetry allow visual field extent and sensitivity to be mapped. Whilst such techniques are essential for determining the effect of an

intervention in the setting of a clinical trial, they cannot be so easily applied to animal models in which novel therapeutics may be initially developed. Full field electroretinography (ERG) provides an objective measure of retinal function that is more appropriate to animal models. ERG measures changes in the electrical potential across the retina that occur in response to flashes of light. These electrical potentials are measured using electrodes placed on the cornea. Following a flash of light, an initial negative deflection is observed which is referred to as the a-wave. This signal is the result of light-induced hyperpolarization of photoreceptors. This is followed by a larger positive deflection known as the b-wave which derives primarily from the depolarization of on-centre bipolar cells. In the dark-adapted state, dim flashes that are below the cone sensitivity threshold elicit a pure rod response. As flash intensity increases, the signal from rods becomes greater and cones increasingly contribute to the recorded signal. In the light-adapted state, the response to bright flashes of light is derived exclusively from cones. ERG can thus be used to objectively assess the function of the rod and cone systems.

1.2. Retinitis Pigmentosa

Retinitis pigmentosa (RP) describes a heterogeneous group of inherited retinal diseases (IRDs) characterised by the dysfunction and the gradual death of rods. This is followed by the death of cone photoreceptors by a secondary mechanism. The term ‘retinitis pigmentosa’ itself, first used by Donders in 1857¹⁵, is somewhat misleading as it implies that the disease process is primarily inflammatory rather than degenerative in nature. More recently, the term ‘rod-cone dystrophy’ has been applied to the RP phenotype which more accurately reflects its cellular basis as being primarily driven by rod loss. Patients typically present with early onset reduced night vision (nyctalopia) due to impaired rod function. As cones degenerate, the visual field progressively constricts leading to tunnel vision. In many cases this is followed by a reduction in central visual acuity and near-complete or complete blindness as the cones undergo a secondary degeneration once rods are lost.

1.2.1. Epidemiology & modes of inheritance

Affecting over 1.5 million people worldwide, RP is one of the most common causes of visual impairment in the young and working age population¹⁶. The prevalence of RP has been estimated at between 1:3000 and 1:4000¹⁷, although this figure depends much upon the population studied^{18,19}. Indeed, a prevalence as high as 1:750 has been recorded in rural central India²⁰. Autosomal dominant (AD), autosomal recessive (AR) and X-linked (XL) modes of inheritance have been reported accounting for approximately 33%, 21% and 13% of cases respectively²¹. The remaining third represent those cases that are clinically isolated (that is with no family history) in whom no genetic diagnosis has been made. Non-Mendelian inheritance has been reported but such instances are rare²². To date, 84 genes have been identified as causative for non-syndromic RP¹⁶. Further AD, AR and XL loci have been mapped by linkage analysis but the causative genes have yet to be identified²³.

1.2.2. Clinical presentation & management

RP may be broadly classified as syndromic, where it occurs as part of a multi-system inherited disease, and non-syndromic where the retinal dystrophy is present in isolation. Over 30 distinct clinical syndromes that include RP as a feature have been described, the most common being Usher syndrome in which RP is associated with hearing loss, and Bardet-Biedl syndrome in which RP is present alongside cognitive impairment, polydactyly, obesity and hypogonadism. The clinical presentation of RP is highly variable due to the large number of genetic mutations that may be responsible. Furthermore, the degree of expressivity within any given pedigree can vary substantially. Typically, nyctalopia and slow dark adaptation kinetics are present from early adolescence. The onset of visual field loss is highly variable: defects may be noted in childhood in more severe forms such as *RPGR*-associated XLRP²⁴, whilst in other cases such as RP caused by mutations in the *TOPORS* or *MAK* genes, degeneration may not commence until the second

to fifth decades of life^{25,26}. Macular involvement occurs late in the natural history of classical RP meaning that the majority of patients retain good central acuity until the latter stages of the disease course. In other instances such as the recessive form of *PROM1*-associated RP, the macula is affected from an early stage with reduced visual acuity being a prominent feature²⁷. Scotomas which may be mapped by perimetry usually appear first in the mid-periphery. These later coalesce to form ring scotomas which gradually expand both peripherally and centrally. Sector RP in which scotomas develop initially in the superior hemifield has been described in association with a subset of RP-causing mutations^{28–30}.

The classical fundoscopic features of RP include thinning of the neural retina, ‘bone spicule’ pigmentary changes, attenuation of the retinal vasculature and so called ‘waxy pallor’ of the optic disc (Figure 1.5). The retinal thinning seen clinically is caused by loss of photoreceptors from the ONL. SD-OCT imaging through these atrophic areas typically reveals thinning of this layer with loss of the ELM and ellipsoid zone (EZ) bands³¹, whilst reduced or absent sensitivity is apparent in corresponding regions of the visual field when tested by perimetry. Bone spicule pigmentation occurs in atrophic areas of retina, most commonly in the mid-periphery^{16,31}. Histologically, bone spicules are composed of RPE cells that have migrated into the inner retina after photoreceptors have degenerated. These cells appear to cluster around both the ILM and the inner retinal capillaries and venules so creating the bone spicule pattern of pigmentation which characterises the disease³². Retinal vascular attenuation and disc pallor occur in the latter stages of RP due to autoregulatory changes in vessel calibre and blood flow. Following ONL degeneration, the inner retina rests in close proximity to the choriocapillaris which provides additional tissue oxygenation. The autoregulated vessels of the central retinal circulation detect the increase in inner retinal oxygen tension and in response undergo vasoconstriction³³.

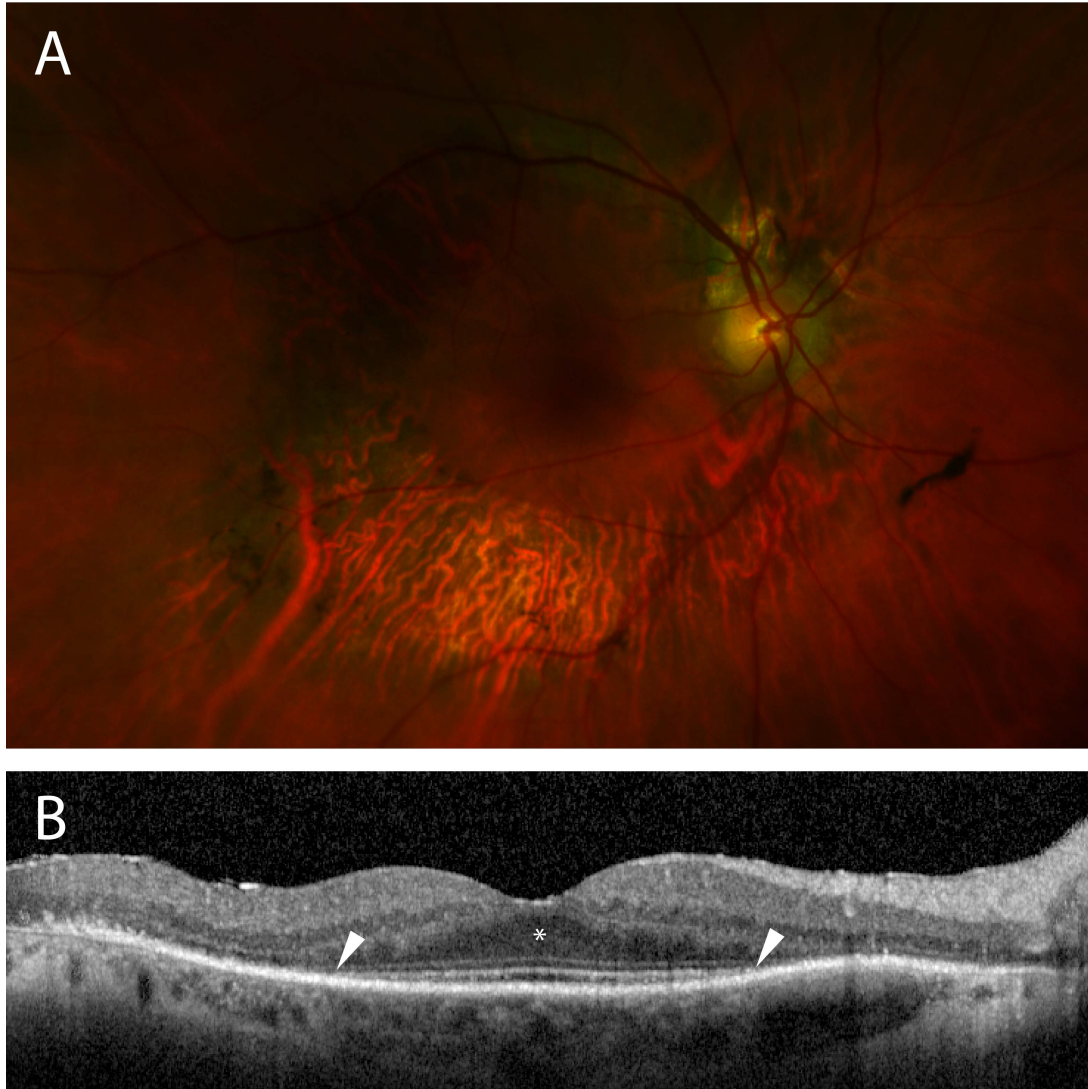


Figure 1.5. Clinical appearance of the human retina in a case of dominant retinitis pigmentosa caused by *RHO*^{P171S/+} mutation. (A) Colour fundus photograph of the right eye showing areas of retinal atrophy and bone spicule pigmentation. **(B)** Horizontal SD-OCT section of the same eye taken through the fovea. Note the typical findings of absence of the external limiting membrane (ELM) and ellipsoid zone (EZ) bands in the most severely affected areas of retina. The peripheral limits of the surviving ELM/EZ bands are indicated by white arrows. These changes are accompanied by thinning of the low signal intensity ONL band in the periphery with normal thickness at the fovea (*).

There are currently few treatment options for RP, although many promising therapies are currently in clinical trial. Current management is largely centred upon the provision of

ophthalmic and genetic counselling as well as social support and visual rehabilitation. Achieving a molecular diagnosis is important both for visual prognostication and to determine heritability, but is not possible in all cases. Although newer molecular genetic techniques such as next generation and whole exome sequencing provide a diagnosis in the majority of cases, these methods may fail to detect structural rearrangements, mutations within non-coding regions of the genome and mutations within regions of repetitive DNA which are inherently difficult to sequence. Regular follow-up for RP patients is advisable. Serial fundus and SD-OCT imaging along with regular perimetry can determine the rate of visual decline for an individual patient, which can in turn aid refinement of visual prognosis. Furthermore, regular clinical follow-up can allow ocular pathologies that are known to complicate RP such as cataract and cystoid macular oedema to be detected and treated in a timely fashion.

1.2.3. Molecular & cellular basis

The genes responsible for RP encode proteins that are either essential for normal functioning of the phototransduction cascade or retinoid cycle, or are required to maintain the structural integrity of photoreceptors. Ten genes involved in the phototransduction cascade have been identified as causative for RP¹⁶: *RHO*^{34,35} (rhodopsin), *CNGA1*³⁶ & *CNGB1*^{37,38} (subunits of the rod cyclic nucleotide gated sodium ion channel), *DHDDS*³⁹ (an enzyme involved in post-translational modification of opsins), *GNAT1*⁴⁰ (a subunit of transducin), *GUCY1B*⁴¹ (an activator of guanylate cyclases), *PDE6A*, *PDE6B* & *PDE6G*^{42,43} (subunits of the rod cGMP phosphodiesterase), and *SAG*⁴⁴ (rod arrestin). A further seven genes involved in the retinoid cycle and 35 involved in ciliary structure and transport are additionally associated with non-syndromic RP¹⁶. Mutations in many pre-mRNA splicing factor genes (including *PRPF3*, *PRPF4*, *PRPF6*, *PRPF8* & *PRPF31*) are also causative for dominant RP. The mechanisms by which mutations in these ubiquitously expressed splicing factors cause non-syndromic disease remain unclear⁴⁵.

The symptoms of visual field constriction and eventual reduction in central acuity that develop in patients with RP are attributable to the death of cone photoreceptors. This occurs as a secondary phenomenon in all forms of RP regardless of the gene involved and several hypotheses as to the underlying mechanisms have been proposed. One possible explanation is that rod-derived toxins are released into the extracellular space upon cell death⁴⁶ or pass directly to cones via gap junctions⁴⁷. This is however unlikely to be the main causal factor as cone death only occurs in RP after the vast majority of rods have already degenerated. Furthermore, mouse models of RP that have been cross-bred to be deficient in connexin 36, an essential component of the cone-rod gap junction, showed a similar rate of cone degeneration to their connexin 36-positive RP littermates⁴⁸.

Léveillard and colleagues identified a trophic factor released by rods which they named rod-derived cone viability factor (RdCVF), and hypothesised that lack of this survival factor may at least in part explain the secondary death of cones in RP⁴⁹. Indeed, reduced expression of RdCVF has been demonstrated in rodent models of RP, and supplementation is associated with improved cone survival in these animals^{49,50}. Knockout of the RdCVF gene *Nxn1* in mice was associated with suppression of rod- and cone-derived ERG responses and a reduction in both ONL thickness and cone density⁵¹.

A gene expression study in four murine models of RP demonstrated an upregulation of genes associated with the insulin/mTOR intracellular metabolic pathway around the time of maximal cone degeneration⁵². The authors further demonstrated that activation of this pathway with insulin lead to improved cone survival and postulated that starvation of cones may be a factor contributing to their secondary degeneration in RP.

Finally, oxidative stress has been proposed as a potential mechanism for the observed loss of cones. Housing of mice in hyperoxic conditions induced photoreceptor death, particularly in the central retina where choroidal blood flow and thus oxygen delivery to the neural retina is greatest⁵³. The choriocapillaris which supplies oxygen to the outer retina is not autoregulated, so it has been suggested that as the ONL thins with progressive rod cell death, the oxygen tension in the surviving tissue increases⁵⁴. This leads to rising levels of superoxide and other free radicals in cones which are known to induce damage to DNA, lipids and proteins, and may trigger apoptotic cell death⁵⁵. Oxidative damage has been demonstrated in a pig model of rhodopsin-related ADRP⁵⁶ and supplementation with antioxidants appears to slow the rate of cone degeneration in animal models^{57,58}. Absence of RdCVF appears to further increase cone susceptibility to oxidative stress⁵⁹.

1.3. Rhodopsin

Rhodopsin was discovered in 1876 by the German physiologist Franz Boll who noted that the outer segments of rods within the frog retina appeared red, and that this colour faded quickly upon exposure to light⁶⁰. The word rhodopsin originates from the Greek 'rhódon' meaning 'rose' and 'ópsis' meaning 'sight' and the protein is sometimes referred to as 'visual purple'. The rhodopsin gene is located on the long arm of chromosome 3 in humans and on chromosome 6 in mice. Its coding sequence is 1047bp in length and is divided between five exons.

1.3.1. Structure, synthesis and trafficking

Rhodopsin is a 39kDa protein composed of 348 amino acid residues and was the first member of the GPCR superfamily to have its structure elucidated by x-ray crystallography (Figure 1.6). The protein consists of seven transmembrane alpha helices with an extracellular (intradiscal) NH₂-terminus and an intracellular (extradiscal) COOH-terminus⁶¹. The 11-cis-retinal

chromophore covalently binds to a lysine residue at position 296 via a protonated Schiff base linkage within the membrane lipid bilayer⁶². A salt bridge between Arg135 of helix III and Glu247 of helix VI acts as an ‘ionic lock’ which effectively blocks the G-protein binding site of rhodopsin in the inactive state. Photoisomerization of the chromophore causes helix VI to shift away from the transmembrane core of the protein which opens the ionic lock and allows activation of transducin^{63–65}. Rhodopsin molecules appear to be arranged as dimers within the disc membrane⁶⁶ and multimeric forms of the protein are typically seen on western blot⁶⁷.

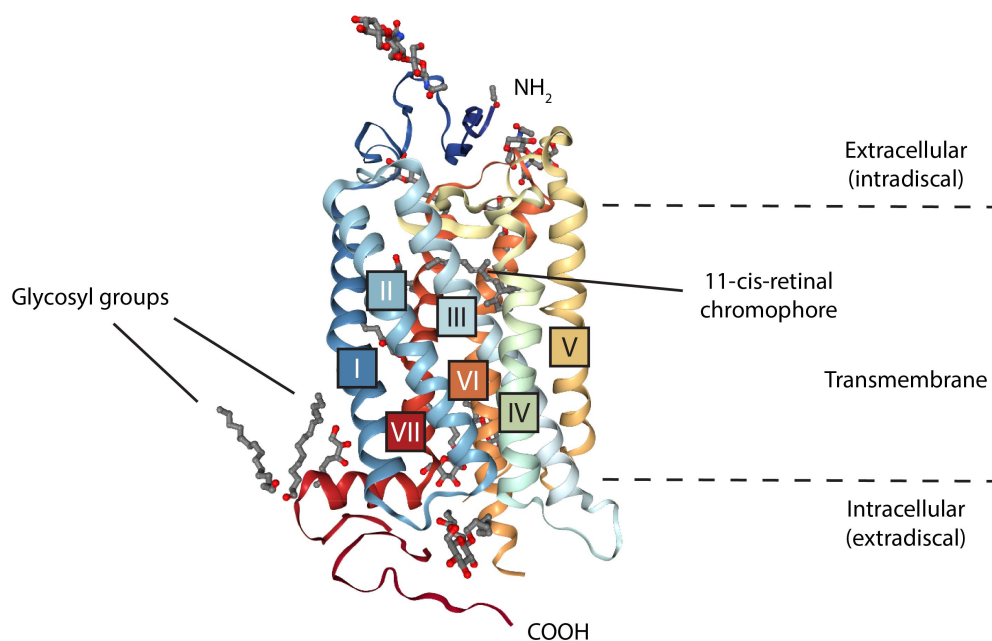


Figure 1.6. Crystal structure of bovine rhodopsin. Amino and carboxyl terminals are indicated by ‘NH₂’ and ‘COOH’ respectively whilst the seven transmembrane domains of the protein are labelled with roman numerals. The chromophore is displayed within its binding pocket inside the ring of helices that span the plasma membrane. Structure based on that described by Teller et al.⁶⁸ and constructed using the Protein Data Bank (PDB)-101 online portal (www.rcsb.org).

Rhodopsin protein is synthesised in the endoplasmic reticulum located within the myoid area of the rod inner segment. The protein is glycosylated within the Golgi apparatus from whence

it is exported to the OS within the membrane of vesicles via the connecting cilium. Rhodopsin is highly expressed accounting for over 90% of total protein within the rod OS. Each OS consists of between 900 and 2000 membranous discs depending on species examined⁶⁹. Each disc provides a membrane surface area of approximately $1.27\mu\text{m}^2$ that is packed with rhodopsin at a density of over 24,000 molecules/ μm^2 ⁷⁰. Microvilli extend from the apical surface of RPE cells and interdigitate with photoreceptor OS. Every day, around 10% of discs are shed from the tips of the OS and are phagocytosed by the RPE⁷¹. This is balanced by the synthesis of new discs from the region of the connecting cilium. To maintain this equilibrium, new rhodopsin molecules must be synthesised and trafficked in large quantities, a process that is tightly regulated by photoreceptors. Indeed, mutations that interfere with rhodopsin trafficking are a frequent cause of RP⁷².

Additionally, rhodopsin plays a key structural role in the rod OS. *Rho*^{-/-} mice completely lack OS⁷³, whilst within the retinas of *Rho*^{+/-} heterozygotes these structures appear shortened and disorganised^{69,74}. By contrast, rods in which rhodopsin has been overexpressed display elongated OS and undergo degeneration in a dose-dependent fashion^{75,76}.

1.3.2. Mutations

Mutations in the rhodopsin gene were the first to be identified as causative for any form of RP⁷⁷. Over 150 different mutations have since been identified which together account for 30-40% of all ADRP^{23,72}. Although the vast majority of mutations in *RHO* result in dominant disease, two missense mutations (p.E150K^{78,79} and p.M253I⁸⁰) and two nonsense mutations (p.W161ter⁸¹ and p.E249ter⁸²) have been described in association with recessive patterns of inheritance. Further, five missense mutations (p.G90D^{83,84}, p.T94I⁸⁵, p.E113K⁸⁶, p.A292E⁸⁷ and p.A295V⁸⁸) appear to cause congenital stationary night blindness (CSNB), an inherited syndrome characterised by nyctalopia but without overt photoreceptor degeneration as seen in

RP. Some degree of overlap is likely to exist between these syndromes. Both ADRP and CSNB phenotypes related to monoallelic p.E113K mutation have been reported within the same pedigree⁸⁶. Additionally, mild degeneration has been observed in older patients with the CSNB-associated RHO p.G90D mutation, suggesting that this may in fact represent a very slowly progressive form of ADRP⁸⁴. The p.P23H missense mutation in RHO was the first to be described and has been most widely studied. Although this is frequently cited as the most common single mutation to cause ADRP, it is observed almost exclusively in North America where it is likely to have arisen as a result of a founder effect. Even within the USA, this genotype only accounts for perhaps 10% of RHO-related ADRP with numerous other pathogenic mutations having been reported throughout the protein⁸⁹.

Dominantly inherited mutations may cause cellular dysfunction and death by one or more of three possible mechanisms. Haploinsufficiency refers to a situation whereby the dominantly inherited allele is not expressed (i.e. a null mutation), or is expressed but is functionally inactive, and the quantity of protein derived from the other wild type allele is inadequate to maintain normal physiology. Given that null alleles appear to cause RP only when present in the homozygous state^{81,82} and that *Rho*^{+/-} heterozygous mice do not show evidence of marked retinal degeneration^{73,90}, it is unlikely that haploinsufficiency is the mechanism by which rhodopsin mutations cause ADRP.

Dominant negative mutations are those in which the resultant protein interferes with its wild type counterpart⁹¹. Typically, dominant negative effects are seen in proteins that form homo- or hetero-multimeric complexes. Since rhodopsin forms dimers within the disc membranes of OS⁶⁶, it is plausible that such a mechanism may at least partly contribute to the observed degeneration for a proportion of mutant alleles. Indeed, evidence of abnormal trafficking of

wild type rhodopsin has been observed in cell culture systems in which P23H, K296E and G188R mutant proteins have been co-expressed^{92,93}.

Finally, gain of function mutations are those that directly exert a toxic effect on the cell. This may be due to protein aggregation, mislocalization or acquisition of novel detrimental functionality. The precise molecular mechanisms of toxicity attributable to rhodopsin missense and truncation mutations have been widely studied in both cell culture and animal models. This has led to a classification system, initially proposed by Mendes and colleagues, in which mutations are grouped into seven classes (Table 1.1)^{72,89}. Class I mutations describe those that are mistrafficked within the cell and primarily involve the C-terminus of the protein. Substitutions or truncations in this highly conserved region such as P347S or Q334ter are associated with a more severe clinical course⁹⁴. Several hypotheses have been proposed for the mechanism by which mistrafficking of rhodopsin might induce cell death in rods. Ectopic accumulation of rhodopsin within the IS, plasma membrane and synaptic terminal might disrupt cell function through direct toxicity, disruption of synaptic transmission or by overwhelming the proteostatic machinery in these locations^{95,96}. Class II mutations such as P23H form the largest single mechanistic group. Mutations falling into this class cluster within the transmembrane and intradiscal domains and cause the protein to misfold and accumulate in the ER. This has been demonstrated for P23H and other Class II mutations in both cell culture expression systems⁹⁷⁻⁹⁹ and in animal models^{67,100-102}. Accumulation of mutant protein induces ER stress which is known to trigger the so-called unfolded protein response (UPR). This cytosolic signalling cascade initially results in the degradation of abnormal protein sequestered within the ER in an attempt to restore intracellular physiology¹⁰³. When normal proteostasis cannot be achieved however, the UPR may trigger cell death¹⁰⁴. A small proportion of Class II mutant protein does however reach the OS¹⁰⁵⁻¹⁰⁷ where it may aggregate and lead to disc instability^{108,109}. This may contribute to the mechanism of cell death as for Class IV mutations

(see below). The retinal dystrophy associated with Class II mutations appears to be exacerbated by light^{109–113}, and rearing of animal models harbouring such mutations in darkness results in a slowing of the observed rate of outer retinal degeneration^{106,111}. Substitutions of the 135th amino acid residue specifically disrupt vesicular trafficking and have been attributed as Class III mutations. These mutant proteins fold normally and are transported out of the ER, but are readily phosphorylated and bind to arrestin with high affinity even in the absence of chromophore¹¹⁴. Class IV mutant rhodopsins are unstable as a result of aberrant post-translational modification. The naturally occurring *Rho*^{T4R/+} dog and the p.T17M transgenic *Xenopus laevis* both display early onset severe RP that is exacerbated by light^{107,115}. These amino acid residues are critical for the normal post-translational glycosylation of rhodopsin which is necessary for efficient activation of transducin⁹⁷. It has been postulated that light may induce destabilization of these opsins within the OS causing aggregation and disruption of disc membranes⁸⁹. Class V rhodopsin mutants are structurally similar to the wild type protein but display enhanced transducin activation kinetics¹¹⁶. Class VI mutations occur around the chromophore binding pocket. These mutant rhodopsins constitutively activate the phototransduction cascade even in the absence of 11-cis-retinal or light stimulation and may cause either CSNB^{84,85,87} or ADRP¹¹⁷. Finally, Class VII mutations are those associated with impaired dimerization¹¹⁸. These mutations do not necessarily segregate with RP within pedigrees however, so the extent to which they may be considered pathogenic remains unclear^{89,119}.

Class	Mechanism of Cell Death	Mutations
I	Mistrafficking	L328P, T342M, Q344R/P/ter, V345L/M, A346P, P347A/R/Q/L/S/T, ter349/Q/E
II	Misfolding/ER retention	N15S, T17M, V20G, P23A/H/L, Q28H, G51R/V, P53R, T58R/M, V87D, G89D, G106R/W, C110F/R/S/Y, E113K, L125R, W161R, A164E/V, C167R/W, P171Q/L/S, Y178N/D/C, E181K, G182S/V, C185R, C187G/Y, G188R/E, D190N/G/Y, H211R/P, C222R, P267R/L, S270R, K296N/E/M
III	Disrupted vesicular trafficking	R135G/L/P/W
IV	Altered post-translational modification/Reduced stability	T4K, T17M, M39R, N55K, G90V
V	Altered transducin activation	M44T, V137M
VI	Constitutive activation	G90D, T94I, A292E, A295V
VII	Dimerization deficiency	F45L, V209M, F220C
Unclassified	Unknown	P12R, R21C, Q28H, L40R, L46R, L47R, F52Y, F56Y, L57R, Y60ter, Q64ter, R69H, N78I, L79P, V87L, L88P, T92I, T97I, V104F, G109R, G114D/V, E122G, W126L/ter, S127F, L131P, Y136ter, C140S, T160T, M163T, A169P, P170H/R, S176F, P180A/S, Q184P, S186P/W, Y191C, T193M, M207R/K, V210F, I214N, P215L/T, M216R/L/K, R252P, T289P, S297R, A298D, K311E, N315ter, E341K, S343C

Table 1.1. Classification of rhodopsin mutations causing ADRP and CSNB by proposed molecular mechanism. Classification system as described in the Human Gene Mutation Database (HGMD; <http://www.hgmd.cf.ac.uk/>). Note that some mutations may fall into more than one class.

Table adapted from Athanasiou et al. 2017⁸⁹.

1.3.3. Mouse models of rhodopsin-related RP

Rhodopsin-related RP has been modelled in a large variety of species including fruit fly, frog, mouse, rat, pig and dog. Murine models are useful mammalian systems for the development of novel treatments for IRDs owing to their ready availability, relative ease of genetic manipulation

and short gestational period. Further, their retinal lamellar architecture and physiology is strikingly similar to that of humans. A rhodopsin null mouse- which may be considered a model of ARRP- was generated through targeted disruption of the endogenous rhodopsin open reading frame (ORF)⁷³. The retinas of these mice completely lack OS and the ONL degenerates rapidly over a period of approximately three months. The rod-derived ERG response is entirely absent in *Rho*^{-/-} animals with no dark-adapted a-wave being detectable at any flash intensity. Whilst RP secondary to biallelic null mutation in rhodopsin is uncommon^{81,82}, this mouse represents a useful model in which expression levels, trafficking and functionality of transgenic rhodopsin protein may be validated.

Dominant rhodopsin mutations have been modelled in both transgenic and knock-in mice. Several transgenic p.P23H mouse lines were generated by Olsson and colleagues through germline insertion of the mutant human transgene¹⁰². The authors found that the rate of degeneration in each line reflected the expression levels of the abnormal human opsin relative to that of endogenous murine protein. Lines in which mouse: mutant human mRNA expression ratios were 1:3 or 1:1 showed severe retinal degeneration, whilst a line with a ratio of 6:1 was found to have a moderate degenerative phenotype. In the same study, the authors generated further transgenic lines through insertion of wild type human rhodopsin. When mouse: wild type human expression ratio was 1:1, no degeneration was evident but severe RP was observed in a line with an expression ratio of 1:5. Transgenic models of dominant RP are thus complicated by the fact that expression levels vary between lines and that at least a proportion of any observed degeneration may be attributable to overexpression of total rhodopsin^{75,76,102} rather than a specific effect of the transgene. Some researchers have attempted to better model the human situation by using human transgenic mouse lines on an endogenous *Rho*^{-/-} background^{120,121}, but the extent to which such models mimic human expression levels is unclear. More recently, a knock-in *Rho*^{P23H/+} mouse has been generated where the native mouse

rhodopsin locus carries the CCC to CAC single nucleotide transversion within its 23rd codon¹⁰⁵. The retina of this mouse expresses equivalent quantities of wild type and mutant mRNA making this a more faithful model of the human disease at the molecular level. The authors found that mutant rhodopsin did not accumulate in the ER of this model, but instead was localised to outer segments which were found by electron microscopy to be disorganised with abnormally angulated discs¹⁰⁵.

In addition to P23H, human transgenic mouse models have been generated for T17M¹²² (Class II/IV) and P347S¹²³ (Class I) mutant rhodopsins. Mice expressing murine mutant rhodopsin transgenes for G90D¹²⁴ (Class VI), Q334ter¹²⁵ (Class I), and the combination of V20G, P23H, and P27L¹²⁶ (VPP; Class II) have also been developed. The E150K¹²⁷ (responsible for ARRP) and D190N¹²⁸ (Class II) dominant mutations have additionally been modelled in knock-in mice.

1.4. Gene Therapy

Gene therapy may be defined as a modality of treatment in which nucleic acid is delivered to a target tissue to affect a change in protein expression. It holds particular promise for the treatment of monogenic Mendelian disease where the aim is to correct the underlying defect and restore normal functionality. As the genes responsible for IRDs have been increasingly identified and the underlying molecular mechanisms elucidated²³, a greater number of research studies have focussed on the development of novel genetic therapeutics. This work led to landmark first-in-man retinal gene therapy clinical trials from which initial phase I data was published in 2008^{129–131}. These trials involved delivery of working copies of *RPE65* in patients with Leber congenital amaurosis type 2 (LCA2) caused by biallelic loss of function/null mutations in this gene. Following demonstration of efficacy in a phase III trial¹³², one such product, voretigene neparvovec, gained regulatory approval from the Food & Drug Administration (FDA) in the USA and has since been marketed by Spark Therapeutics under

the trade name LuxturnaTM. At the time of writing, 27 clinical trials are underway in patients with IRDs¹³³.

In its simplest form, gene therapy involves the delivery of DNA encoding wild type protein in cases of biallelic null or nonsense mutations for the treatment of a recessively inherited disease. More sophisticated strategies are necessary for the treatment of dominant disease where knockdown or gene editing of harmful gain of function or dominant negative mutations may be attempted. The delivery systems used to package DNA or RNA for gene therapy are known as vectors and these may be classified as either viral or non-viral. Viral vectors represent an attractive mode of gene delivery as these intracellular parasites have evolved over many millions of years to efficiently convey their nucleic acid cargo to a host cell. The idea that this could be harnessed for therapeutic delivery of genetic material was first proposed in 1968¹³⁴. Two particular viruses have now been adapted for this purpose and are utilised in current clinical trials: lentivirus and adeno-associated virus (AAV). Lentiviruses are enveloped retroviruses containing RNA. Upon cellular transduction, this is reverse transcribed and integrated into the host genome. These vectors may package relatively large quantities of RNA which makes them better suited for the delivery of larger genes which exceed the 4.7kb carrying capacity of AAV. The lentiviral envelope is however known to be immunogenic and may thus induce inflammation upon delivery¹³⁵. Further, integration of the reverse transcribed viral genome into that of the host cell has the potential to cause insertional mutagenesis with resulting cytotoxicity or oncogenicity^{136–138}. Two retinal gene therapy clinical trials which employ lentiviral vectors are currently underway for the treatment of Stargardt disease caused by *ABCA4* mutations and Usher disease Type 1B caused by mutations in *MYO7A*^{133,139}. Recombinant AAVs do not suffer from these safety concerns to the same extent and have thus been more widely adopted for clinical application.

1.4.1. Adeno-associated virus

AAV is a small icosahedral non-enveloped parvovirus of approximately 20nm in diameter^{140,141}. The virus consists of a protein coat known as a capsid which surrounds a linear single-stranded DNA genome of 4675 nucleotides in length (Figure 1.7A)¹⁴². The capsid is composed of 60 protein monomers of three types designated VP1 (87kDa), VP2 (72kDa) and VP3 (62kDa) which are present in a ratio of 1:1:10^{143,144}. AAV belongs to the genus *Dependoparvovirus*, so called as these viruses are usually dependent upon coinfection with a secondary helper virus, such as adenovirus or herpes virus, for their infectivity. Infection with wild type AAV in itself is not known to cause pathology¹⁴⁵, and the virus is only weakly immunogenic¹⁴⁶. The native AAV genome consists of two ORFs encoding the genes *rep* and *cap* which are flanked by hairpin DNA sequences known as inverted terminal repeats (ITRs; Figure 1.7B). The *rep* ORF encodes four overlapping genes designated *Rep78*, *Rep68*, *Rep52* and *Rep40* required for viral replication and for the directed integration of the viral genome into a specific target locus on the long arm of chromosome 19^{141,147}. The *cap* ORF also contains four overlapping genes: three encode the VP capsid proteins whilst the fourth encodes a protein known as assembly-activating protein (AAP) which is required for capsid construction¹⁴⁸. The ITR sequences of wild type AAV are each 145bp in length¹⁴². These sequences are essential for second-strand DNA synthesis which occurs following viral entry into the host cell, as well as for genomic integration and virion assembly^{149–151}.

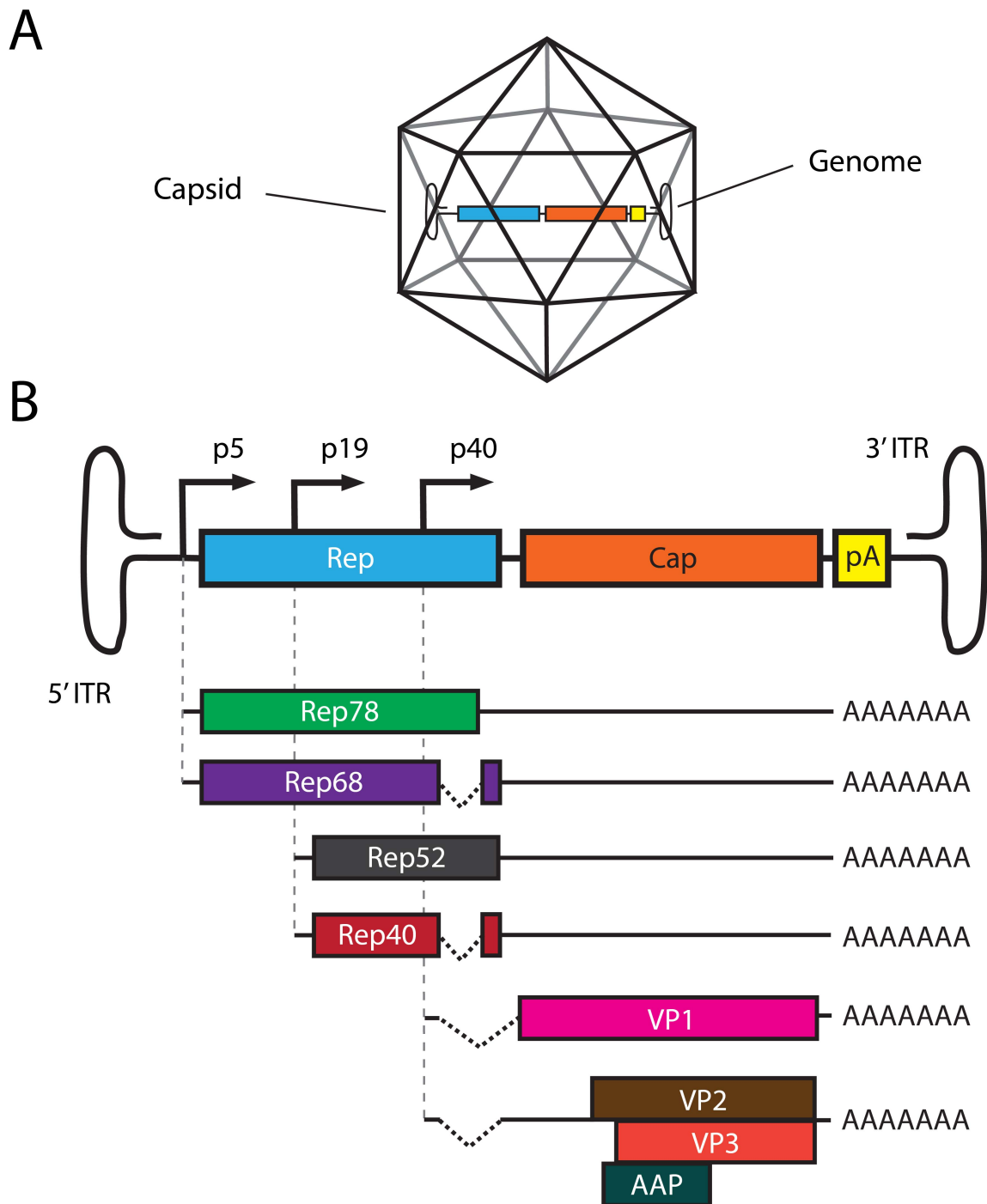


Figure 1.7. Structure of wild type adeno-associated virus (AAV). **(A)** The AAV virion consists of a 4.7kb single-stranded DNA genome encased within an icosahedral protein shell composed of VP1, VP2 and VP3 proteins in a ratio of 1:1:10. **(B)** The genome of wild type AAV includes the *Rep* and *Cap* ORFs together with a downstream polyA signal sequence (pA) all flanked by inverted terminal repeat (ITR) sequences. Approximate positions of the three promoters p5, p19 and p40 are indicated on the genome map by arrows. The mRNAs transcribed from the wild type AAV genome are illustrated beneath the

map. Translated regions are indicated by coloured boxes, untranslated regions by horizontal black lines and introns by angulated dotted lines. Rep78 and Rep68 are expressed from the p5 promoter whilst Rep 52 and 40 are expressed from the p19 promoter. VP1-3 and assembly-activating protein (AAP) are translated from mRNA transcribed from the p40 promoter. VP2 and VP3 are translated from alternative start codons within the same mRNA transcript, whilst AAP is generated from yet another start codon in a different reading frame.

Although 11 distinct AAV serotypes have been described, serotype 2 has been most widely studied and is the serotype most commonly used in retinal gene therapy clinical trials. The virus initially gains access to a target cell through binding to heparan sulphate proteoglycan on the cell surface¹⁵². Co-receptors such as fibroblast growth factor receptor-1, hepatocyte growth factor receptor, laminin receptor, and $\alpha_v\beta_5$ integrin also appear to play an important role¹⁵³. The virion is then internalised by the host cell through endocytosis. The VP1 capsid protein possesses a phospholipase A₂ domain which is thought to facilitate escape from endosomes into the cytoplasm^{154,155}. From here, the virus traffics to the nucleus where it is uncoated. Second strand synthesis occurs in the nucleus through base pair complementarity using the ITR as a primer, a step that is thought to be rate-limiting in the AAV transduction process¹⁴⁹. In the presence of helper virus, the double-stranded AAV genome may replicate and form new virions, following the so-called lytic pathway. In the absence of helper virus, viral gene expression is suppressed and latency is established. This occurs either through integration of viral double-stranded DNA into the host cell genome at the preferred locus on chromosome 19, or alternatively through concatemerization whereby the double-stranded DNA forms circular episomes that exist independently within the cell nucleus. Recombinant AAVs (rAAVs) engineered for therapeutic gene delivery are manufactured by replacing the *rep* and *cap* genes of the AAV genome with transgenic DNA which is inserted between the two ITRs. In the absence of *rep* and *cap* genes, the rAAV is unable to replicate or synthesise new capsid proteins, even in

the presence of coinfection with a helper virus, and the rate of integration is markedly reduced to less than 0.1%^{156,157}. Indeed, malignant transformation due to insertional mutagenesis has never been reported as a result of rAAV transduction in any animal system^{158,159}. Recombinant AAV vectors are therefore thought to act purely as vehicles for transgene delivery giving them a favourable safety profile. Following viral transduction, rAAV-derived transgenic DNA is thought to persist for the lifetime of the host cell in an episomal state. One significant limitation of rAAVs for therapeutic gene delivery is their 4.7kb maximum packaging capacity, meaning that their use is generally restricted to the delivery of smaller transgenes.

1.4.2. Design of rAAV vectors for the treatment of inherited retinal disease

A number of factors must be considered in the design of a rAAV to ensure both efficient transduction of the target tissue and cellular specificity of transgene expression. These include the choice of capsid serotype and use of engineered capsid mutations, the choice of promoter, the addition of DNA regulatory elements and the use of self-complementary AAV genomes.

1.4.2.1. *Capsid serotype and targeted mutation*

In the production of AAV vectors, the ITR sequence of one AAV serotype may be used with the capsid of another, a strategy referred to as pseudotyping. The advantage of this approach is that an effective set of ITRs may be combined with an AAV capsid with a predilection (tropism) for the intended target tissue. AAV2/2 (where the first number denotes the ITR serotype and the second the capsid serotype) was the first such vector to be tested in the retina¹⁶⁰. A single subretinal injection of this virus induced transduction of both the RPE, and to a lesser extent, photoreceptors in the mouse eye. Neutralizing antibodies directed against AAV2 epitopes are however prevalent in the human population, raising concerns of immune response to this capsid serotype which may impair transduction and/or trigger unwelcome inflammatory responses¹⁶¹. This led researchers to develop novel pseudotyped vectors which have since been shown to be

less immunogenic¹⁶². Indeed, a whole plethora of AAV pseudotypes have now been tested in the retina of a wide variety of species¹⁶³. AAV serotypes 2/5, 2/7, 2/8 and 2/9 appear to provide improved photoreceptor tropism compared to AAV2/2^{164–166}, whilst enhanced tropism for bipolar and ganglion cells has been demonstrated for AAV serotypes 2/1, 2/2, 2/8, 2/9 and 2/10¹⁶⁷. AAV2/8 appears to be the one of the most effective serotypes for the transduction of photoreceptors, with robust expression having been demonstrated in mice¹⁶⁴, pigs¹⁶⁸, dogs¹⁶⁹ and non-human primates¹⁷⁰.

Degradation of AAV virions between endocytosis and nuclear entry is thought to occur as a result of phosphorylation of exposed capsid tyrosine residues, leading to ubiquitination and destruction by the proteasome^{171,172}. In an attempt to minimize this effect and thus improve transduction efficiency, various novel capsid serotypes have been engineered in which one or more surface tyrosine (Y) residues have been replaced by phenylalanine (F)¹⁷³. Improved transduction efficiency of the order of ten-fold has been demonstrated in the retina with AAV2/2^{Y733F}, AAV2/8^{Y733F} and AAV2/9^{Y733F} serotypes, each of which include a single Y-T substitution, as well as the AAV2/2^{QuadY-F} serotype, which has four such mutations^{174–177}.

The AAV2/8^{Y733F} mutant serotype would thus be expected to favourably transduce rods with high efficiency, and so was selected for use in this project.

1.4.2.2. *Promoter*

Cellular specificity and level of transgene expression will critically depend upon the choice of promoter included within the rAAV construct. Whilst ubiquitous promoters such as the CMV promoter¹⁷⁸ and CAG promoter¹⁷⁹ typically drive high levels of gene expression, they will necessarily result in expression of transgenic protein in cell types other than those at which the therapy is targeted. Such ectopic protein expression may result in toxicity and thus act to

accelerate retinal degeneration following delivery of gene therapy. Specific transgene expression may be achieved through the use of cell-specific promoters such as the rhodopsin or PDE6B promoters in the case of rods. The human rhodopsin promoter is highly effective at driving high levels of AAV-derived gene expression specifically in rods of the murine retina¹⁶⁴. Additional transgene expression in cones has however been reported when the mouse version of the rhodopsin promoter has been used in this capacity¹⁸⁰.

1.4.2.3. *Regulatory elements*

Inclusion of non-coding *cis*-acting regulatory elements within the rAAV genome may have a significant impact on final levels of transgene expression. One such sequence, known as the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) has recently been shown to substantially increase the level of AAV-derived transgene expression in both the mouse and human retina¹⁸¹. The WPRE has also been included within the REP1-AAV expression cassette used in gene therapy clinical trials for choroideremia with no safety concerns having been reported^{182,183}. The addition of an intron placed between the promoter and the transgene within the rAAV genome also appears to enhance downstream gene expression^{184–186}. For this reason, many commonly employed ubiquitous promoters such as CAG include an intron within their sequence.

1.4.2.4. *Self-complementary vectors*

Second-strand synthesis appears to be one of the key rate-limiting steps in the process of AAV transduction¹⁴⁹. This can be circumvented through the use of self-complementary AAV genomes in which both forward and reverse strands are packaged within a single capsid joined by a central mutated ITR¹⁸⁷. The DNA from these vectors thus folds into a double-stranded molecule upon delivery from the capsid by complementary base pairing. Increased transgene expression along with a more rapid onset has been demonstrated in the retina for self-

complementary AAVs when compared with their single-stranded counterparts^{188,189}. This however comes at the expense of packaging capacity, which is effectively halved from 4.7kb for the wild type AAV genome to around 2.2kb for that of self-complementary AAV¹⁸⁷. Given that the cDNA sequence for rhodopsin is just 1047bp in length, the gene together with its promoter and downstream polyA signal just fit within the packaging limit of self-complementary AAV. Such a vector would however have no further coding capacity for additional sequences such as regulatory elements, introns or effectors of RNA interference.

1.4.3. Gene therapy strategies for the treatment of rhodopsin-related dominant RP

Since the rhodopsin gene fits comfortably within the packaging capacity of AAV, much research has focussed on the development AAV-base gene therapy strategies for the treatment of related dominant RP. As discussed in Section 1.3.2, mutations in rhodopsin may cause rod cell toxicity by dominant negative and/or toxic gain of function effects. In the first instance, simple supplementation with wild type protein might be expected to slow the rate of degeneration as the wild type protein would ‘outcompete’ the mutant form and preferentially form stable dimers within the rod disc membrane^{66,190}. For toxic gain of function mutations, gene augmentation may in itself be insufficient to slow the degenerative process and a strategy to limit expression of the mutant allele may be required. Given that monoallelic nonsense/null mutations in the rhodopsin gene do not appear to cause RP, specific knockdown of a toxic gain of function allele should have a beneficial effect in cases of dominant RP. The large degree of mutational heterogeneity that exists for rhodopsin-related RP means that this approach would however be unfeasible in that specific blocking therapies would need to be designed and validated for each individual mutation. Some such therapies may ultimately only be applicable to a single pedigree. Many researchers have therefore focussed on ‘knockdown/replacement’ gene therapy strategies whereby mutant and wild type alleles are indiscriminately targeted and this is combined with

supplementation of wild type cDNA which has been modified to render it resistant to the blocking mechanism. Such approaches are thus potentially mutation-independent¹⁹⁰.

1.4.3.1. *Gene supplementation*

The rate of retinal degeneration observed in mice expressing the human RHO^{P23H} transgene appears to be heavily influenced by the expression level of wild type protein. Frederick et al. assessed the rate of retinal degeneration in mouse lines expressing a human transgene bearing the P23H mutation on $Rho^{-/-}$, $Rho^{+/-}$, and $Rho^{+/+}$ backgrounds¹⁰¹. The authors found that the most rapid loss of photoreceptors occurred in those mice expressing no wild type protein. Mice with two copies of Rho exhibited the slowest rate of degeneration, whilst the ONL of those with one copy appeared to thin at an intermediate rate. Price and colleagues explored the effect of combining additional genomic copies of wild type rhodopsin with the human RHO^{P23H} transgene and found that the greater the expression of wild type protein the slower the observed rate of disease progression up to the equivalent of three normal copies⁷⁵. Rod OS ultrastructure was also found to be markedly improved in the presence of additional wild type protein⁷⁵. Relative rescue of ONL degeneration has been demonstrated following delivery of wild type mouse Rho cDNA by subretinal injection of AAV in the RHO^{P23H} transgenic mouse on a $Rho^{+/+}$ background¹⁹¹. When this approach was however applied to a RHO^{P23H} transgenic strain on a $Rho^{+/-}$ background (which arguably better models the human disease state), no such treatment effect was observed¹²¹. As part of their CRISPR-based study, Tsai and colleagues found that wild-type gene supplementation in isolation improved ERG signal versus sham injection in the $Rho^{P23H/P23H}$ homozygous knock-in mouse but not in the $Rho^{P23H/+}$ or $Rho^{D190N/+}$ heterozygous knock-in lines⁵⁶. Additionally, no benefit has been observed following subretinal delivery of AAV expressing wild type human RHO in mice expressing the human RHO^{P347S} transgene on a $Rho^{+/-}$ background¹²⁰. Thus, the efficacy of the wild type gene augmentation approach seems

to be dependent not only upon the specific mutation in the rhodopsin gene but also upon the genetic background of the model being investigated.

Whilst supplementation with normal protein appears to be of benefit in certain mouse models of rhodopsin-related RP, toxicity has been demonstrated when overexpression has been induced in wild type rods^{75,76,191}. Tan and colleagues generated mouse lines expressing increased levels of normal rhodopsin and reported outer retinal degeneration even when expression levels were just 23% greater than that of the wild type line⁷⁶. Mao and colleagues further demonstrated that AAV-induced rhodopsin augmentation in wild type mice results in a similar toxic effect¹⁹¹. Theories as to the mechanism of rhodopsin overexpression related rod toxicity include misfolding and aggregation of the excess protein, a mismatch between levels of opsin and 11-cis-retinal chromophore availability, increased susceptibility to light-induced damage, a saturation of rod translational machinery, and overcrowding due to increased OS volume^{75,76,191}. P23H rhodopsin mouse models may exhibit greater resistance to the deleterious effects of rhodopsin overexpression observed in wild type animals by virtue of their baseline rhodopsin expression levels being lower^{105,192}.

1.4.3.2. RNA replacement

Gene supplementation alone is unlikely to be of benefit for rhodopsin mutations that cause cell death by toxic gain of function mechanisms. Several research groups have thus focussed on RNA replacement strategies whereby all genomically encoded rhodopsin is knocked down using an AAV-delivered short hairpin RNA (shRNA), and replacement is provided by a 'hardened' copy of the gene which is co-delivered either within the same or within a different vector. Hardened rhodopsin cDNA is generated by switching the codons that constitute the shRNA target sequence to synonymous codons that encode the same amino acid. In this way, the replacement transcript is rendered resistant to the shRNA which relies on complementary base

pair matching with the target sequence to exert its silencing effect. O'Reilly and colleagues developed an approach in which a shRNA was delivered together with a resistant human *RHO* transgene within a single AAV¹⁹³. Following subretinal injection in new-born *RHO*^{P23H} transgenic mice on a *Rho*^{+/-} background, only minimal evidence of rescue was observed which the authors attributed to the low level of replacement transcript expression and the rapid rate of degeneration in this model¹⁹³. Members of this research group subsequently divided the shRNA and *RHO* replacement arms of the treatment into separate AAV constructs which were individually optimised^{194,195}. Using the resulting dual vector approach, they were later able to demonstrate both structural and functional rescue in a *RHO*^{P347S} transgenic mouse on a *Rho*^{+/-} background, a model that exhibits a much slower rate of retinal degeneration¹²⁰. Using a similar dual vector strategy, Greenwald and colleagues reported rescue of a rapidly degenerating *RHO*^{P347S} transgenic mouse on a rhodopsin null background¹⁹⁶. They found however that no additional benefit was conferred by the combination of a rhodopsin-targeting micro RNA (miRNA)-expressing vector together with a hardened rhodopsin-expressing AAV beyond that observed following delivery of the latter in isolation¹⁹⁶. Gorbatyuk and colleagues independently identified candidate target sequences within the human rhodopsin mRNA transcript using small interfering RNAs (siRNAs) *in vitro*¹⁹⁷. The most successful of these was incorporated into an AAV as a shRNA under the control of the H1 RNA polymerase III promoter, together with a codon-modified resistant mouse rhodopsin transgene driven by the mouse opsin proximal promoter. Using this single vector, the authors were able to achieve long-term rescue of degeneration in a slow-progressing *RHO*^{P23H} transgenic mouse on a *Rho*^{+/-} background¹²¹. More recently, a single vector comprising a shRNA under the control of the H1 promoter together with a resistant human *RHO* transgene driven by the human rhodopsin promoter has been used to rescue retinal degeneration in the naturally occurring *RHO*^{T4R/+} canine model of RHO-ADRP¹⁹⁸.

DNA nanoparticles, which unlike AAV are not limited in packaging capacity, have been employed as an alternative vehicle of gene delivery as part of an RNAi-based knockdown/replacement gene therapy strategy¹⁹⁹. The authors demonstrated a modest slowing of the rate of disease progression in the rapidly degenerating murine *Rho*^{P23H/P23H} homozygous knock-in model¹⁰⁵ when they co-delivered a short genomic form of the human rhodopsin DNA sequence and mouse Rho-targeting shRNAs within separate nanoparticles¹⁹⁹. Transgenic DNA appears to have a shorter duration of expression when delivered in nanoparticles rather than viruses which may limit the suitability of this technology for downstream clinical application^{200,201}.

Ribozymes are RNA polynucleotide sequences with catalytic activity that may be designed to degrade mRNA transcripts in a highly specific fashion based on Watson-Crick base-pair complementarity. Hammerhead ribozymes have been developed *in vitro* to target rhodopsin transcripts and have shown efficacy in reducing the expression of mutant rhodopsin in the P23H transgenic rat when delivered within rAAVs^{202–204}. A rhodopsin-targeted hammerhead ribozyme was subsequently delivered together with a codon-modified resistance transcript within a rAAV2/5 in the *RHO*^{T4R/+} dog model. Although effective rhodopsin suppression was demonstrated *in vitro* as well as in both wild type and mutant dogs, severe inflammatory complications were observed at AAV titres required to achieve this and the strategy was abandoned by the authors¹⁹⁸.

1.4.3.3. *DNA editing and transcriptional silencing*

Zinc finger nucleases (ZFNs) are synthetic proteins that have been engineered to target and cleave specific DNA sequences. DNA repair then follows by way of either non-homologous end joining or homology-directed repair where the sister chromosome or exogenously delivered polynucleotides may act as a template. Using engineered ZFNs targeting rhodopsin, Greenwald and colleagues were able to correct the P23H mutation *in vitro* using an ectopically delivered

DNA fragment²⁰⁵. Mussolino et al. developed zinc finger based transcriptional repressors and demonstrated mutation-independent silencing of human rhodopsin gene expression in the P347S transgenic mouse model of rhodopsin-related ADPR²⁰⁶. Whilst ZFN-based approaches appear promising, they do not appear to be completely specific with toxicity having been demonstrated secondary to off-target cleavage elsewhere in the genome²⁰⁷.

Clustered regulatory interspersed short palindromic repeats (CRISPR)/Cas9 is a newly described technology that has rapidly become widely adopted for the purpose of genome editing. CRISPR are DNA sequences found in prokaryotes that form part of a bacterial adaptive immune system. The CRISPR-associated protein Cas9 is an endonuclease that is capable of inducing double-stranded breaks in DNA at specific locations directed by guide RNAs through complementary base pairing²⁰⁸. Such breaks may then be repaired by non-homologous end joining with insertions or deletions that result in gene silencing. Alternatively, an oligonucleotide template may be provided to correct a mutation by homology-directed repair. In the retina, CRISPR has been used to selectively silence several human transgene targets. Bakondi and colleagues used electroporation to deliver Cas9 and guide RNA from a subretinally injected plasmid in the *RHO*^{S334ter} transgenic rat model²⁰⁹. The resulting induction of loss of function mutations in the human transgene resulted in a relative preservation of photoreceptors and an improvement in measured visual function. In a similar fashion, Latella et al. reported successful ablation of human *RHO*^{P23H} gene expression in a transgenic mouse model²¹⁰. Specific silencing of the P23H allele with relative photoreceptor preservation has also been demonstrated in the *Rho*^{P23H/+} knock-in mouse using plasmid delivered CRISPR/Cas9 technology²¹¹. These proof-of-concept studies all involved specific targeting of a mutant allele and are therefore mutation-dependent. CRISPR/Cas9 could however be applied as part of a mutational independent strategy for rhodopsin-related ADPR if a codon-modified resistant version of the rhodopsin gene were to be co-delivered with the Cas9 CDS and rhodopsin-targeting guide RNA. This

would however far exceed the packaging capacity of a single AAV vector. One novel approach to this problem involved delivery of Cas9 under a ubiquitous promoter within one AAV and human rhodopsin cDNA together with mouse rhodopsin-targeting guide RNAs in another⁵⁶. After sub-retinal delivery of a mixture of these two vectors, rhodopsin knockdown would be expected to occur only in those cells that had been successfully co-transduced with both AAVs. Using this strategy, the authors of this study achieved successful gene editing and partial structural and functional rescue in the murine *Rho*^{P23H/+}, *Rho*^{P23H/P23H}, and *Rho*^{D190N} knock-in models of ADRP.

1.5. Other Approaches to the Treatment of RP

1.5.1. Neuroprotection

Neurotrophic factors are thought to play a significant role in the mechanisms of photoreceptor cell death in RP⁵⁴. A slowing of cone degeneration in the *Rho*^{-/-} mouse retina has been demonstrated *ex vivo* following supplementation with both ciliary neurotrophic factor (CNTF) and glial-cell derived neurotrophic factor (GDNF)²¹², and AAV-mediated delivery of CNTF by subretinal injection appears to confer lifelong neuroprotection of cones in this model²¹³. These neurotrophic factors also led to a preservation of both the ONL and cone photoreceptors when delivered by AAV in the transgenic RHO^{S334ter} rat model of ADRP^{214,215}. Although neuroprotective, CNTF appears to have a detrimental impact on photoreceptor function as measured by ERG²¹⁶. Intraocular delivery of both RdCVF and basic fibroblast growth factor (bFGF) proteins similarly resulted in relative preservation of photoreceptors in rat models of RP^{50,217}. Delivery of neurotrophic factors represents a treatment strategy for RP that is potentially both gene and mutation independent, although the potential for off-target effects on overall retinal function must be considered.

1.5.2. Pharmacotherapy

Class II rhodopsin mutations such as P23H are characterised by misfolding of the protein and retention in the ER⁸⁹. Small molecules that act as chaperones may be of therapeutic benefit in such cases by acting to stabilize the mutant protein and/or suppress apoptosis thus prolonging photoreceptor survival. Retinoids and retinoid analogues appear to promote mutant rhodopsin folding and ER exit *in vitro*⁸⁹. Further, vitamin A supplementation has been shown to slow the rate of degeneration in the (Class II) transgenic *RHO*^{T17M} mouse model but not in the (Class I) transgenic *RHO*^{P347S} mouse model¹²². In a randomised controlled trial, Berson and colleagues demonstrated a slowing in the rate of retinal degeneration in patients with RP taking 15000 IU/day oral vitamin A over a four to six year period²¹⁸. This trial was not however conducted in genotyped patients and certain forms of retinal degeneration such as Stargardt disease related to *ABCA4* mutations may in fact be exacerbated by vitamin A²¹⁹. High levels of vitamin A may also lead to hepatotoxicity and are known to be teratogenic in pregnancy. Non-retinoid molecular chaperones including curcumin²²⁰, tauroursodeoxycholic acid (TUDCA)⁵⁸, and BiP/Grp78²²¹ have also proved beneficial in slowing outer retinal degeneration in transgenic P23H rat models.

1.5.3. Restoring vision after photoreceptor death

The treatment strategies for RP outlined above all aim to slow or arrest degeneration of rods and cones, and would therefore be of little value in end stage disease when very few photoreceptors remain. Although RP can lead to eventual complete loss of the ONL, the inner retina may retain the capability of transmitting signals to the brain in spite of the significant tissue remodelling that occurs^{222,223}. Three broad experimental strategies have been developed in this regard: retinal implants, optogenetics, and cell transplantation.

Electronic implants are devices that detect light and stimulate the remaining retina in order to restore some level of vision to blind patients. Two different approaches have been developed which involve placing the implant in either the sub- or epiretinal space. A commercial epiretinal implant developed by Second Sight and known as Argus II has gained both FDA approval and is CE marked in the European Union. This implant has a 60-electrode array that is affixed over the macular using a tack²²⁴. A polymer wire from this array exits the eye through a pars plana sclerotomy and connects to an electronics case outside of the eye. Images are captured by a camera fixed to a pair of glasses worn by the patient and the signal is passed to the electronics case of the implant via an induction coil²²⁴. A subretinal implant known as the Alpha-IMS device has been developed by the German company Retinal Implant AG. This consists of a 3x3mm 1500-pixel photodiode array that detects light, amplifies the signal, and directly stimulates the overlying retina²²⁵. This is linked by a cable that passes in the subdermal space to a coil behind the ear where it may be powered by external induction in a similar manner to a cochlear implant. This device has the advantage of being entirely self-contained without relying on an external camera as well as having a greater pixel density. Further, because it stimulates the overlying retina directly, the subretinal implant preserves the retinotopic map making mislocalization of visual stimuli less likely. Surgical implantation of the subretinal implant is however lengthier and more technically demanding compared to that for the Argus II. Both implants are capable of providing a rudimentary level of vision and are associated with improved performance in visually guided tasks²²⁶. The promising results from trials of retinal prostheses prove that ganglion cells in end-stage RP are capable of transmitting useful visual signals from the eye to the brain. This in itself is an important proof of principle for other regenerative therapies in the retina²²².

Optogenetics as a treatment strategy for end-stage RP aims to render the surviving cells of the inner retina sensitive to light through the introduction of intrinsically light-sensitive peptides

such as rhodopsin or melanopsin. Restoration of some degree of visual function has been demonstrated using this approach in mouse models of end-stage retinal degeneration^{227,228}.

Much research has also focussed on reconstitution of the degenerate ONL in models of advanced disease through cellular transplantation. Transplantation studies have shown that photoreceptor precursors derived from non-degenerate mice are capable of forming functional connections with bipolar cells when injected into the subretinal space of degenerate mice with no surviving ONL, and that these cells are capable of driving the pupillary light reflex²²⁹. In a recent study, sheets of neuroretinal tissue derived from human embryonic stem cells were transplanted into the sub-retinal space of non-human primates and were noted to form synaptic connections with the host retina²³⁰.

1.6. Thesis Overview

In this thesis I aim to develop treatments for RP caused by dominantly inherited mutations in the rhodopsin gene. Mutation in rhodopsin is one of the most common causes of RP and, at present, no treatment is available for such patients. Given the large degree of mutational heterogeneity observed in the population⁸⁹, targeting therapies at specific mutations would be both impractical and commercially unviable. Such treatments would be unlikely to reach clinical trial let alone to gain regulatory approval. Identifying treatment approaches for rhodopsin-related ADRP that may confer benefit in a mutation-independent fashion is challenging given the potential variety of non-mutually exclusive mechanisms that may be involved in its pathophysiology^{72,89}. These mechanisms may be broadly divided into those that cause rod cell death by dominant negative effects and those that do so through a toxic gain of function, although this distinction is not always easy to define⁸⁹. In the former case, supplementation with wild-type rhodopsin may be sufficient to slow degeneration, whilst in the latter, concomitant knockdown of the toxic allele would be required²³¹. For this to be mutational-independent,

knockdown would need to be targeted at both mutant and wild type alleles and this would need to be combined with a replacement transgene that has been rendered resistant to the gene silencing effector as part of a ‘knockdown/replacement’ gene therapy strategy.

The *Rho*^{P23H/+} mouse knock-in line was selected as a model system in this thesis for several reasons. Firstly, P23H is the most common single point mutation in the USA. Secondly, it is the mutation that has been most widely studied and is arguably the best understood. Thirdly, transcription and expression levels more likely faithfully mimic those of the human heterozygous condition which cannot be said for equivalent transgenic models. This strain also has the added advantage of ready commercial availability from the Jackson laboratories, USA.

The P23H mutation falls between Classes II and IV of the classification system of Mendes et al.^{72,89} and the associated degeneration appears to be exacerbated by light^{110,112}. The precise mechanism by which this mutation causes rod cell death remains controversial and may be dominant negative, toxic gain of function or both⁸⁹. Based on these characteristics, three treatment strategies are explored in this thesis:

1. **Long-pass light filters.** Complete exclusion of light appears to slow degeneration in P23H animal models^{106,110,111}. Whilst this would clearly be inappropriate as a clinical treatment, exclusion of shorter wavelength light to which rods are maximally sensitive using red ocular filters might be similarly effective. Little prior work has been done to explore this idea. The effect of long-pass light filters on the disease course in the *Rho*^{P23H/+} knock-in mouse model will be investigated in Chapter 3.
2. **Supplementation with wild type rhodopsin.** Wild type rhodopsin forms dimers in the OS disc membrane. P23H rhodopsin shows an enhanced ability to oligomerize and may

thus exert a dominant negative effect on the wild type protein causing OS destabilization and rod death²³¹. Supplementation with wild type rhodopsin may slow this process by competing with the mutant protein, and success with this strategy has previously been demonstrated in a *RHO*^{P23H} transgenic mouse on a *Rho*^{+/+} background¹⁹¹. In Chapter 4, I explore whether simple AAV-mediated gene augmentation may prove effective in the more clinically relevant *Rho*^{P23H/+} knock-in mouse model of ADRP.

3. **Rhodopsin RNA replacement.** If the mutant opsin in the *Rho*^{P23H/+} knock-in mouse model exerts its dominant effect via a toxic gain of function mechanism, then gene augmentation alone would likely be ineffective as a treatment regime and simultaneous knockdown of the damaging protein would be required as part of a knockdown/replacement approach. Given that both rhodopsin over- and underexpression may exacerbate the degenerative phenotype, the balance between the two arms of the therapy would need to be carefully balanced. In Chapter 5, I develop a novel molecular technique for rhodopsin mRNA knockdown using artificial mirtrons. These RNA interference (RNAi) effectors are a subclass of naturally occurring micro RNAs (miRNAs) that are spliced out of pre-mRNA transcripts as short introns. In Chapter 6, rhodopsin-targeting mirtrons are combined with the most effective rhodopsin supplementation vector developed in Chapter 4 and the effect of the resulting rAAVs on retinal structure and function in the *Rho*^{P23H/+} knock-in mouse determined.

2. Materials & Methods

2.1. Molecular Biology

All molecular biology experiments were conducted under controlled laboratory conditions using calibrated instruments. Plasmids referred to in subsequent chapters were designed *in silico* using commercially available software (Gencious v11.0.2, Biomatters Ltd., Auckland, NZ).

2.1.1. Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to amplify DNA from custom primers (Sigma-Aldrich, UK) using a thermocycler (Multigene, Labnet International Inc.). Primers were designed where possible according to the following criteria (Thermo Fisher Scientific):

1. Length 18-30 nucleotides
2. Melting temperature (T_m) 65-75°C with paired primer T_m within 5°C of each other
3. GC content 40-60%
4. At least two nucleotides before/after an enzyme restriction site to facilitate efficient cutting

A list of primers is provided in the Appendix and are referenced by number throughout this thesis. KOD Hot Starter Mastermix, an optimised high-fidelity 2x mixture containing DNA polymerase, ultra-pure deoxynucleotides, and reaction buffer (Novagen, EMD Chemicals, Merck, UK) was used for all cloning work in accordance with manufacturer's instructions. Briefly, reactions were carried out in a total volume of 20µl containing 10µl KOD Mastermix, 10-20ng template DNA, forward and reverse primers at a final concentration of 0.5µM each,

with the remaining volume made up with molecular biology grade water. PCR conditions are outlined in Table 2.1.

Step	Amplicon<0.5kb	Amplicon 0.5-1kb	Amplicon>1kb
1. Polymerase activation	95°C for 2 mins	95°C for 2 mins	95°C for 2 mins
2. Denature	95°C for 20 s	95°C for 20 s	95°C for 20 s
3. Annealing	60°C for 10 s	60°C for 10 s	60°C for 10 s
4. Extension	70°C for 10s	70°C for 15s	70°C for 20s/kb

Table 2.1. Standard PCR conditions for amplification of DNA for cloning applications. The number of cycles of steps 2-4 was optimised for each reaction and varied between 25 and 35.

2.1.2. Restriction Digests and Agarose Gel Electrophoresis

DNA amplicons generated by PCR, and plasmid vectors where applicable, were subjected to restriction endonuclease digestion to allow subsequent DNA ligation. Restriction digests were performed in accordance with manufacturer's instructions (New England Biolabs, UK). All such reactions were carried out in a total volume of 50µl containing 1µl of enzyme (20 units, where one unit is defined as the quantity of enzyme required to digest 1µg DNA under recommended conditions), and 5µl 10x buffer solution. Mixtures were incubated in a water bath set to the optimal working temperature for the enzyme in question for a period of one hour. Enzymes were subsequently heat inactivated where possible, and 10µl 6x Gel Loading Dye (New England Biolabs, UK) added. The total volume of this solution was then loaded into a well of an agarose gel containing 0.5µg/ml ethidium bromide. Gels were manufactured using agarose at a concentration of 1% or 2% in tris/acetate/ETDA (TAE) buffer (Thermo Fisher, UK). One percent agarose gels together with a 1kb DNA ladder (UltraRanger, Norgen, UK) were used for isolation of amplicons >1kb in length, whilst 2% agarose gels with a 100bp DNA

ladder (PCRRanger, Norgen, UK) were used for DNA amplicons <1kb in length. Gels were completely submerged in a tank containing TAE buffer across which a voltage of 100-120V was applied for a period of 1-2 hours. Following electrophoresis, gels were visualised and photographed using an ultraviolet (UV) transilluminator (U:Genious, Syngene, UK). In cases where DNA was required for downstream application, bands were excised from the gel and DNA extracted using the QIAquick gel extraction kit (Qiagen, UK) in accordance with manufacturer's instructions. In all cases, DNA was eluted in 30µl of elution buffer. A Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) was used to determine DNA concentration with ratios of absorbance 260:280 nm and 260:230 nm recorded as measures of sample purity.

2.1.3. DNA Ligation & Bacterial Transformation

DNA was ligated using 1µl T4 DNA ligase and 1µl T4 ligase 10x buffer (New England Biolabs) in a total reaction volume of 10µl at room temperature. In all instances, 20-40ng vector DNA was included with a 3:1 insert: vector molar ratio. Cohesive- and blunt-ended ligations were incubated for periods of 40 minutes and two hours respectively. In all cases, a ligation control reaction was performed in parallel using vector DNA alone. At the end of the incubation period, T4 ligase enzyme was inactivated by heating samples to 65°C on a heat block for ten minutes.

Ligation products were used to transform XL-10 Ultracompetent *E. coli* cells (Stratagene, USA). Prior to use, cells were thawed from -80°C on ice. For each transformation reaction, 2µl of 1.43 M β-mercaptoethanol was added to 50µl of XL-10 cell suspension which was then stirred every two minutes for ten minutes. Subsequently, 5µl of ligation mixture was added and samples incubated on ice for a further 30 minutes. At the end of this period, the tubes were heat-pulsed in a 42°C water bath for 30 seconds, and then incubated on ice for two minutes. Nine-hundred

mls of pre-warmed Super Optimal Broth (SOC) culture media (Thermo Fisher, UK) was added to each sample and the total volume transferred to a 15ml polypropylene Falcon tube to be incubated in a 37°C shaker at 250 rpm for one hour. Following incubation, 120µl of each bacterial suspension was spread over a Luria's Broth (LB)-Agar plate with ampicillin 100µg/ml using a sterile spatula and the plates incubated overnight at 37°C. All plasmids described in this thesis included an ampicillin resistance gene so that these plates selected for successfully transformed bacteria.

2.1.4. Colony Selection, Plasmid Isolation & Amplification

Efficiency of ligation between vector and insert was estimated by comparing the number of colonies present on the experimental plate with that on the vector-only control plate. Individual colonies were selected from the ligation plate using sterile 200µl pipette tips, each of which was transferred in an aseptic fashion into a 15ml polypropylene Falcon tube containing 4mls of autoclaved LB to which ampicillin had been added to a final concentration of 100µg/ml. Samples were incubated overnight in a 37°C shaker at 250 rpm. Five-hundred µl of turbid overnight culture was set aside for each sample and stored at 4°C. Plasmid was isolated from the remaining volume by alkaline-SDS lysis using a commercial kit according to manufacturer's instructions (GeneElute Plasmid Miniprep Kit, Sigma-Aldrich), and eluted in 100µl elution buffer. Plasmid minipreps were screened for correct ligation products using a restriction digest assay, or by commercial Sanger sequencing (Source Bioscience, UK), or both. Having identified a miniprep sample with the correct plasmid sequence, the corresponding 500µl of retained turbid culture media was used to create a bacterial stock though the addition of 500µl autoclaved 50% glycerol solution. All glycerol stocks were stored at -80°C. For downstream cellular or *in vivo* application, plasmids were amplified from bacterial glycerol stocks using commercial

endotoxin-free kits in accordance with manufacturer's instructions (EndoFree Plasmid Maxi or Mega Kit, Qiagen, UK).

2.1.5. RNA Extraction & Reverse Transcription

Prior to RNA extraction, all surfaces and instruments were treated with decontamination solution to remove environmental RNases (RNaseZAP wipes, Invitrogen, UK). RNA was extracted from cell pellets using the RNeasy Plus Mini kit (Qiagen, UK), which includes a DNA removal step. Cellular lysis was achieved using the supplied RLT Plus buffer supplemented with 10µl β-mercaptoethanol per ml. Cell pellets were resuspended in this solution and then drawn up and down through a 25G needle six to eight times. RNA was then isolated in accordance with manufacturer's instructions and yields determined by spectrophotometry. For RNA extraction from neural retina, animals were sacrificed and retinal tissue rapidly dissected in sterile PBS. Tissue was then frozen on dry ice and stored at -80°C. The miRNeasy Mini kit (Qiagen, UK) was used for RNA extraction from retinal tissue. In the first step of this process, frozen retinas were thawed on ice and tissue disrupted in 700µl trizol by drawing it up and down through a 25G needle six to eight times. RNA was then isolated in accordance with manufacturer's instructions. Reverse transcription using the SuperScript III kit (Invitrogen, UK) was immediately performed using 1-2µg RNA with the remaining sample being stored at -80°C.

2.1.6. Real Time Quantitative PCR

Real time quantitative PCR (RT-qPCR) was used to determine levels of gene expression from complementary DNA (cDNA) samples using gene-specific primer pairs. All reactions were performed in triplicate using newly opened sterile filter pipette tips and opaque 96 well plates. Two different qPCR assays were used: SYBR Green and TaqMan (both Thermo Fisher

Scientific, UK). Primers for SYBR green assays were designed according to criteria outlined in Section 2.1.1, but additionally, were selected so as to span exon-exon junctions in order to reduce the likelihood of detection of potentially contaminating genomic DNA. SYBR Green assays were performed in a total reaction volume of 10 μ l consisting of 1 μ l cDNA template, 1 μ l of both forward and reverse primers (each at a concentration of 10 μ M), 2 μ l molecular grade water, and 5 μ l SYBR Green x2 Mastermix. TaqMan assays were performed in a total volume of 20 μ l consisting of 2 μ l cDNA template, 1 μ l TaqMan assay mix (which includes a pair of unlabelled PCR primers and a specific TaqMan probe with a FAM dye label at the 5' end and minor groove binder/non-fluorescent quencher at the 3' end), 10 μ l TaqMan x2 Universal Mastermix and 7 μ l molecular grade water. RT-qPCR was performed using the CFX ConnectTM real time PCR detection system (Bio-Rad, UK). Prior to experimental use, reaction conditions were optimised for efficiency by constructing a standard curve using ten-fold DNA dilutions of linear target DNA. Reaction efficiencies between 90-105% were deemed acceptable for experimental application. PCR conditions for SYBR Green assays consisted of an initial polymerase activation period at 95°C for three minutes. This was followed by 40x two-step cycles of denaturation at 95°C for ten seconds and annealing/extension at the previously optimised temperature (typically 55-62°C) for 30 seconds. This was followed by a melt curve analysis to determine purity of the amplified product. TaqMan thermal cycling conditions consisted of an initial polymerase activation period at 95°C for ten minutes, followed by 40x PCR cycles of denaturation at 95°C for ten seconds and annealing/extension at 60°C for one minute. For transgene expression assays, qPCR reactions were run for detection of both the gene of interest and of a housekeeping gene for internal normalization. No template control (NTC) samples run using molecular-grade water in place of cDNA were included in every plate as negative controls. Gene expression levels were calculated from cycle threshold (C_t) values as measured during the exponential phase of the PCR reaction. Gene expression relative to

housekeeper was calculated using the transformation $2^{-\Delta C_t}$, where ΔC_t represents the difference between the gene and housekeeper geometric mean C_t values from three technical replicates:

$$\Delta C_t = \text{mean } C_t (\text{transgene}) - \text{mean } C_t (\text{housekeeper})$$

Gene expression in an experimental sample normalised to that of a control group was calculated using the transformation $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ represents the difference in ΔC_t values:

$$\Delta\Delta C_t = \Delta C_t (\text{experimental sample}) - \text{mean } \Delta C_t (\text{control samples})$$

2.1.7. Protein Extraction & Quantification

Protein was extracted from cell pellets and neuroretina through lysis in radioimmunoprecipitation (RIPA) buffer (Sigma Aldrich, UK) supplemented with protease inhibitor (PI, Roche Diagnostics, UK). Cell pellets were initially resuspended in a volume of 100 μ l RIPA/PI, then sonicated at 20% power for ten seconds. Retinal samples were briefly homogenised in 80-100 μ l RIPA/PI, then similarly sonicated. Samples were subsequently agitated using a tube rotator set at 20rpm at 4°C for 30 minutes before being centrifuged at 13,000rpm at 4°C for ten minutes. The supernatant protein lysate was then transferred to a new microcentrifuge tube and placed on ice. Total protein was quantified from lysates using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK) in accordance with manufacturer's instructions. This colourimetric assay quantifies protein of appropriately diluted samples against a standard curve of bovine serum albumin (BSA) of known concentration. All reactions were performed in duplicate, and colourimetric absorbance readings taken at 562nm using the iMarkTM microplate reader (Bio-Rad, UK).

2.1.8. Western Blot

Western blots were used for detection and quantification of rhodopsin protein from lysate samples. Briefly, samples were denatured before being loaded into wells of a pre-cast 10% Criterion™ Tris-Glycine extended (TGX™) gel (Bio-Rad, UK) for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein lysate samples were diluted in RIPA buffer/PI together with Laemmli loading buffer such that the 25µg of protein was prepared for each in a volume appropriate for the well size. Denaturation was achieved through incubation at room temperature for 30 minutes in Laemmli buffer. SDS-PAGE of denatured protein samples was performed in a gel tank in Tris/Glycine/SDS running buffer (National Diagnostics, USA) alongside 5µl of BLUEye prestained protein ladder (Geneflow, UK). The loaded gel was subjected to electrophoresis at 100V for a period of one hour and 45 minutes, at the end of which the gel was carefully removed from its styrene copolymer cassette for blotting. Protein from SDS-PAGE gels was blotted onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo transfer system (Bio-Rad, UK) in accordance with manufacturer's instructions. Blotted PVDF membranes were incubated in Odyssey blocking buffer (TBS, LI-COR, NE, USA) for 45 minutes at room temperature under agitation. Membranes were then incubated in primary antibodies (mouse anti-rhodopsin [1D4] monoclonal ab5417 and rabbit anti-β actin polyclonal ab8227, both Abcam, UK) diluted 1:1000 in PBS with 0.1% Tween-20 (PBST) supplemented with 10% blocking buffer under agitation for a period of two hours. This was followed by three washes in PBST under agitation for seven minutes each. Membranes were then incubated in fluorescence-tagged secondary antibodies (IRDye 800CW donkey anti-mouse and IRDye 680RD donkey anti-rabbit, both LI-COR, NE, USA) diluted 1:10,000 in PBST for 45 minutes under agitation. The membranes were subsequently washed a further three times as above before being removed and allowed to dry at room temperature. The processed PVDFs were imaged using the Odyssey Fc imaging system (LI-COR Inc., USA), with 700nm and 800nm detection channels and an exposure time of two

minutes. In cases where protein was to be quantified, expression relative to β -actin was calculated from acquired images by band densitometry using Image Studio Lite software (version 5.2.5, LI-COR Inc., USA).

2.2. Cell Culture & *In Vitro* Transfection

2.2.1. Cell Culture

Cell culture was performed in a designated tissue culture laboratory within positive pressure ventilation hoods using aseptic technique. All cells were initially acquired from master cell banks (Public Health England) and stored in liquid nitrogen. After resuscitation, cells were passaged at least three times prior to experimental application. All cell cultures were maintained in a designated humidified tissue culture incubator at 37°C with 5% CO₂. Human Embryonic Kidney 293 (HEK293) cells (#85120602, Culture Collections, Public Health England) were cultured in complete Dulbecco's Modified Eagle Medium (complete DMEM) consisting of DMEM supplemented with 10% filtered fetal bovine serum (FBS), L-glutamine (2mM), penicillin (100U/ml), streptomycin (100µg/ml), 1% non-essential amino acids, and phenol red pH indicator (Gibco, Thermo Fisher Scientific, UK). HEK293 cells were maintained within sterile T75 culture flasks and were passaged twice per week when 70-90% confluent. For passage, old culture media was removed and the cell monolayer gently washed with 5mls pre-warmed sterile phosphate buffered saline (PBS). Two mls TrypLE™ Express solution (Gibco, Thermo Fisher Scientific, UK) was then applied and the flask incubated at 37°C for five minutes. A further eight mls pre-warmed complete DMEM was then added and the cells completely resuspended by pipetting up and down. One to two mls of this cell suspension was then added to fresh pre-warmed DMEM in a new sterile T75 flask for ongoing culture. The remaining cells were either discarded or used for transfection experiments.

2.2.2. In Vitro Transfection

The remaining cell suspension acquired from a confluent T75 flask was centrifuged at 300g for five minutes at 20°C. The supernatant was discarded and the cells resuspended in 10mls fresh pre-warmed DMEM ± phenol red. A 10µl sample of this cell suspension was diluted with 40µl complete DMEM and 50µl 10% trypan blue for cell counting. Ten µl of diluted sample was loaded into a haemocytometer and viable cells counted in order to estimate cell density of the original suspension. Cells were then seeded into wells of 12-, 24-, 48-, or 96-well sterile culture plates using 1ml, 0.5ml, 0.25ml or 100µl respectively of suspension diluted to a density of 10⁵ cells/ml. Seeded plates were incubated overnight at 37°C before transfection. Transfection was performed using *TransIT*® LT-1 reagent (Mirus Bio, USA) in accordance with manufacturer's instructions. Prior to use, the reagent was brought to room temperature and briefly vortexed. Transfection medium was prepared by adding plasmid DNA to a volume of Opti-MEM™ serum-free culture media (Thermo Fisher Scientific, UK) appropriate to the well size. *TransIT*® LT-1 reagent was then added in a ratio of 3µl per 1µg DNA. This was mixed by gentle pipetting and incubated at room temperature for 30 minutes. The transfection media was then added directly into the DMEM within culture wells in a dropwise fashion, whilst the plate was gently rocked to ensure even distribution of the complexed DNA. The cell culture plates were then returned to the 37°C incubator.

2.3. AAV Manufacture

All AAVs referred to in this thesis were manufactured in a designated tissue culture laboratory using aseptic technique. Viruses were produced by co-transfection of HEK293T cells with three plasmids:

1. Transgene plasmid (pTransgene), containing the intended AAV transgenic genome flanked by inverted terminal repeat sequences (ITRs).
2. Rep/Cap plasmid (pRepCap), containing sequences required for viral replication and capsid synthesis.
3. Helper plasmid (pAdΔF6), containing genes from adenovirus essential for AAV replication.

Large scale plasmid purification was performed in all cases using the Qiagen Endotoxin-free Plasmid Mega Kit (Qiagen, UK) in accordance with manufacturer's instructions. Transfected cells containing AAV were lysed and the vector isolated and purified. The steps involved in this process are outlined in detail below.

2.3.1. HEK293T Cell Culture

HEK293T cells (#12022001, Culture Collections, Public Health England) were cultured in complete DMEM with phenol red (Gibco, UK) supplemented with 10% filtered FBS, L-glutamine (2nM), penicillin (100U/ml) and streptomycin (100μg/ml). Cells harvested from three confluent T75 flasks were used to seed two Corning Hyperflasks (Merck, UK) for each virus to be manufactured. Hyperflasks were topped up with complete DMEM until full (approximately 560mls total volume per flask), and bubbles carefully removed. Seeded hyperflasks were then incubated at 37°C 5% CO₂ for 72 hours.

2.3.2. Transfection

Two hyperflasks at ~80% confluence were used for the production of each AAV batch. For each hyperflask, a total of 500μg DNA was prepared for transfection. This consisted of pTransgene, pRepCap and pAdΔF6 in a 1:1:1 molar ratio. The three plasmids were well mixed

and the solution made up to 10mls with NaCl 150mM in a 50ml Falcon tube. In a separate 50ml Falcon tube, 1.125ml polyethylenimine (PEI) 10mM was mixed with 8.875ml NaCl 150mM. The DNA was then complexed with the diluted PEI by adding the total volume of the latter to the former in a dropwise fashion. The complexed DNA was incubated at room temperature for 20 minutes before being added in its entirety to pre-warmed transfection media consisting of DMEM with phenol red supplemented with 2% filtered FBS, L-glutamine (2mM), penicillin (100U/ml) and streptomycin (100µg/ml). The old media were carefully drained from cultured hyperflasks and the transfection media containing complexed DNA carefully added. The hyperflasks were topped up with fresh transfection media until full, bubbles were removed and the hyperflasks again incubated at 37°C 5% CO₂ for a further 72 hours.

2.3.3. Cell Harvesting & Lysis

After 72 hours of incubation, 200mls of old media were removed from each hyperflask and discarded. Hyperflasks were then shaken vigorously until all cells had been loosened into suspension (as confirmed by light microscopy). The cell suspension was poured into 500ml autoclaved bottles and centrifuged at 1200g, 20°C for 20 minutes to generate a loose cell pellet. The majority of the supernatant was carefully removed by pipette and cells resuspended in the last ~30mls media. This cell suspension was transferred into a 50ml Falcon tube and again centrifuged at 1200g, 20°C for 20 minutes to generate a more secure pellet which allowed complete removal of the remaining media. Fifteen mls of autoclaved lysis buffer (1M tris(hydroxymethyl)aminomethane with 150mM NaCl) was added to each cell pellet and the lysate carefully pipetted up and down. To this, 500µl lysis buffer/PI solution (prepared using one PI tablet in 1ml lysis buffer) was added and the lysate mixed further. The tube was then placed into the -80°C freezer for a period of at least one hour.

2.3.4. Isolation of AAV Particles

The frozen lysate was thawed in a 37°C water bath over 15 minutes with the sample being shaken vigorously halfway through the incubation time. At the end of this period, the thawed lysate was returned to the -80°C freezer and again left for at least one hour. Four such freeze-thaw cycles were completed, after which benzonase enzyme (Merck, UK) was added to the thawed lysate to a final concentration of 50U/ml. The lysates were then incubated at 37°C for 45 minutes with vigorous shaking every 15 minutes. Subsequently, the lysates were centrifuged at 3700g at room temperature for 20 minutes.

Iodixanol gradients (Sigma-Aldrich, UK) were assembled using fractions of different concentrations prepared as per Table 2.2.

Fraction	Iodixanol	5M NaCl	5x PBS-MK	H ₂ O	Phenol Red
15%	4ml	3.2ml	3.2ml	5.6ml	-
25%	5ml	-	2.4ml	4.6ml	20µl
40%	6.7ml	-	2ml	1.3ml	-
60%	10ml	-	-	-	20µl

Table 2.2. Constituent fractions used to construct iodixanol gradients for AAV isolation. Figures displayed are for the preparation of two iodixanol gradients. Iodixanol: OptiPrep™ density gradient medium (Sigma-Aldrich, UK); 5x PBS-MK: PBS with 5mM MgCl₂ plus 12.5mM KCl; H₂O: sterile molecular biology grade water (Sigma-Aldrich, UK); Phenol Red: 0.5% solution (Sigma-Aldrich, UK).

All iodixanol gradients were prepared using UV treated 5ml long-neck Pasteur pipettes. Three gradients were prepared for each AAV. A volume of 7.2mls of the 15% fraction was first pipetted directly into the base of a 32.4ml OptiSeal™ ultracentrifuge tube (Beckman-Coulter Life Sciences, USA). The 25% fraction (4.8ml) was then carefully pipetted beneath this by

passing the neck of the pipette through the 15% fraction to the base of the ultracentrifuge tube. In a similar fashion, 4mls of the 40% fraction were delivered beneath the 25% fraction, and finally, 4mls of the 60% fraction were delivered beneath the 40% fraction. The supernatant from the centrifuged lysates was then carefully pipetted on top of the iodixanol gradients (Figure 2.1). All microcentrifuge tubes were filled to the top with any remaining volume being made up with lysis buffer. Tubes were then sealed with caps and centrifuged at 59,000rpm in an Optima ultracentrifuge (Beckman-Coulter Life Sciences, USA) for one hour and 30 minutes at 20°C. An 18G needle attached to a 5ml syringe was then used to pierce each OptiSeal™ tube at the base of the 40% phase (where AAV particles are concentrated following centrifugation), and 1.5-2mls of this fraction were extracted from each. The collected fraction was stored overnight at 4°C.

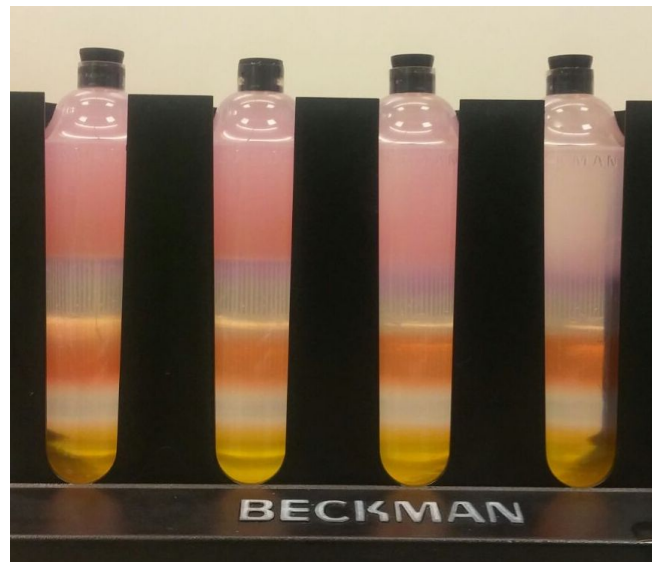


Figure 2.1. Iodixanol gradients within 32.4ml OptiSeal™ ultracentrifuge tubes. Layers from top to bottom: HEK293T cell lysate (pink), 15% iodixanol fraction (colourless), 25% iodixanol fraction (orange), 40% iodixanol fraction (colourless), 60% iodixanol fraction (yellow).

2.3.5. Purification & Concentration of AAV

Five mls of sterile PBS was added to an Amicon® Ultra 100K filter tube (Millipore, UK) which was then spun at 3000g for 15 minutes at 20°C in a table top centrifuge (Megafuge™, Thermo Fisher Scientific, UK). The flow-through liquid was subsequently discarded. Sterile PBS was added to the collected iodixanol fraction up to a volume of 14mls and the solution mixed well by pipetting up and down. The total volume was then transferred into the prepared Amicon filter and the sealed tube centrifuged at 3000g at 20°C until the volume remaining above the filter was less than 500µl (typically this took between 30 minutes and one hour). To this, a further 15mls sterile PBS was added and the solution mixed well by pipetting up and down. The sealed tube was then centrifuged as above until the volume remaining was again less than 500µl. Twice more, the collected fraction was washed with 15mls PBS and centrifuged as described above. After the final wash, the Amicon tube was centrifuged until the volume remaining above the filter was less than 250µl. This purified and concentrated AAV suspension was pipetted over the filter membrane 20 times on each side using a 200µl pipette and then divided into 10µl aliquots for storage at -80°C. After collection of the concentrated AAV suspension, 500µl PBS was pipetted into the empty filter compartment and rinsed over the membrane on each side 20 times. This 'wash' suspension was then divided into 50µl aliquots and stored at -80°C.

2.3.6. AAV Titration

Capsid yield and purity of the viral preparation was determined by SDS-PAGE. After staining the gel for protein, three distinct bands corresponding to proteins of 87, 72 and 62 kDa in size should be visible. These correspond to the three proteins that make up the capsid of an AAV virion: VP1, VP2 & VP3. Additional bands on the gel would indicate contamination of the viral preparation with cellular or transgenic protein contaminants. Although this assay gives an indication of total capsid yield from an AAV preparation, it cannot differentiate full from empty

capsids i.e. those viral particles containing a DNA genome from those that do not. This is best determined by electron microscopy. For SDS-PAGE of the concentrated AAV preparation, a 10 μ l aliquot was mixed with 10 μ l molecular grade water and 20 μ l 2x Laemmli buffer (Sigma Aldrich, UK). For the wash preparation, a 20 μ l sample was mixed with 20 μ l 2x Laemmli buffer. A negative control consisting of 20 μ l molecular grade water with 20 μ l 2x Laemmli buffer was included in all cases as a negative control. All samples were heated to 95°C for 15 minutes before being loaded into wells of a pre-cast 10% Criterion™ Tris-Glycine extended (TGX™) gel (Bio-Rad, UK). Electrophoresis was performed in Tris/Glycine/SDS running buffer (National Diagnostics, USA) at 100V for 1 hour and 45 minutes together with 5 μ l of BLUeye prestained protein ladder (Geneflow, UK). At the end of this period, the gel was carefully removed from its cassette and washed in water at room temperature with gentle agitation for five minutes. This wash step was repeated twice more with fresh water after which the gel was incubated in EZBlue™ protein stain (Sigma Aldrich, UK) with agitation for 45 minutes. The gel was then subjected to three further washes prior to photography.

Viral genome titre expressed as genome copies per ml of preparation (gc/ml) was determined by RT-qPCR. The first step of this assay involved digestion of samples with DNase enzyme to remove any contaminating DNA that may be present outside the viral capsids. Samples were prepared in a total volume of 10 μ l consisting of 1 μ l viral concentrate or wash, with 1 μ l 10x DNase buffer, 1 μ l DNase enzyme (both New England Biolabs, USA), and 7 μ l molecular biology grade water. Samples were incubated at 37°C for 30 minutes and then heated to 95°C for 10 minutes. The latter step was performed in order to inactivate the DNase enzyme and at the same time denature viral capsids to liberate their DNA genomes. Samples were subsequently diluted a further 1:10 and 1:100 for RT-qPCR. Standard curves were constructed using a six step 10x dilution series of linearised rhodopsin transgene. All reactions were performed in

triplicate and the geometric mean C_t used for analysis. RT-qPCR was performed as described in Section 2.1.6. Viral genome copy number was determined for AAV samples by interpolation from within the standard curve.

2.4. *In Vivo* Procedures

All animal procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986, UK and with the Association for Research in Vision & Ophthalmology (ARVO) statements on the care and use of animals in ophthalmic research under UK Home Office Personal License I4A76E11 (HO) and Project License 30/3363. Mice were housed at the Biomedical Sciences Division, University of Oxford with a 12-hour light/dark cycle and food and water available *ad libitum*.

2.4.1. Anaesthesia & Pupil Dilatation

Mice were anaesthetised by intraperitoneal injection of ketamine (Vetalar, Boehringer Ingelheim, Germany, 80mg/kg body weight) and xylazine (Rompun, Bayer, Germany, 10mg/kg body weight). Anaesthesia was reversed at the end of procedures by intraperitoneal injection of atipamezole (Antisedan, Zoetis, USA, 2mg/kg body weight). Pupil dilatation was achieved where required using guttae tropicamide 1% and phenylephrine hydrochloride 2.5% applied in sequence (both Minims, Bausch & Lomb, UK).

2.4.2. Subretinal Injection

Mice underwent subretinal injection at post-natal week (PNW) three at the time of weaning. Animals were anaesthetised and the pupils dilated as described above. Prior to injection, guttae proxymetacaine (Minims, Bausch & Lomb, UK) was applied to provide additional topical anaesthesia. The animal was positioned on a customised foam operating mat beneath a foot

pedal controlled microsurgical operating microscope. A paracentesis was performed by brief puncture of the mid-peripheral corneal with a 33G needle. This induced the efflux of a small bleb of aqueous after which the wound self-sealed. This purpose of this step was to lower intraocular pressure to minimize the risk of trans-scleral reflux during or after subsequent subretinal injection. Carbomer gel (Viscotears Liquid Gel, Alcon, UK) was then applied liberally to the corneal surface and a 5mm glass coverslip rested on top. This allowed direct visualization of the posterior pole through the operating microscope. A 35G bevelled NanoFil needle was mounted on a Nanofil 10 μ l syringe (both World Precision Instruments, UK) and 1.5 μ l of AAV suspension or PBS was drawn up in preparation for injection. The superior rectus muscle was grasped with notched forceps to stabilize the eye and the needle was carefully introduced through the adjacent sclera and into the subretinal space under direct visualisation (Figure 2.2). Once the entirety of the needle's bevel was visible beneath the retina, the superior rectus muscle was released and the syringe plunger depressed creating a bullous superior hemi-retinal detachment (Figure 2.2B). The needle was then carefully withdrawn, the glass cover slip removed, and guttae chloramphenicol (Minims, Bausch & Lomb, UK) instilled. Any complications such as epiretinal, subretinal or vitreous haemorrhage, extraocular reflux of injected substance, or inadvertent retinotomy were recorded at the time of surgery.

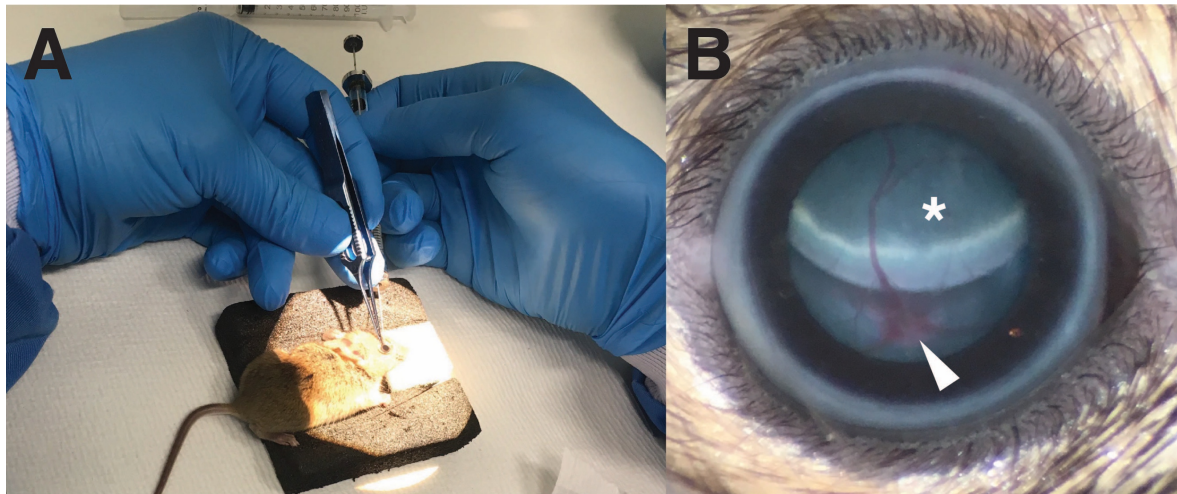


Figure 2.2. Subretinal injection. **(A)** Positioning of animal and instruments for subretinal injection. Following anterior chamber paracentesis, a glass cover slip was placed over a liberal quantity of Viscotears liquid gel covering the corneal surface. Whilst grasping the superior rectus muscle with notched forceps, the tip of a 35g NanoFil needle was passed trans-sclerally into the subretinal space under direct visualization. **(B)** Surgeon's view through the operating microscope following subretinal injection. The superior bullous subretinal detachment induced by the injection is marked by a white asterisk. The white arrow shows the position of the optic nerve head.

2.4.3. Retinal Imaging

Retinal imaging was performed using the Spectralis® ophthalmic imaging platform (Heidelberg Engineering, Germany) with a 55° camera lens. *En face* images were acquired using the inbuilt confocal scanning laser ophthalmoscope (cSLO), whilst spectral domain optical coherence tomography (SD-OCT) was used for cross-sectional imaging of the retina. Animals were anaesthetised and pupils dilated as described in Section 2.4.1. Guttæ hypromellose 0.3% (Blumont Healthcare Ltd., UK) was instilled bilaterally and polymethyl methacrylate contact lenses of specification 3.2mm diameter, 1.7mm back optic zone radius, 0.4mm central thickness and plano refractive power (Cantor & Nissel Ltd., Brackley, UK), were applied to both eyes and centred over the dilated pupils. The contact lenses served to create a uniform refractive surface

for imaging, as well as acting to prevent corneal dehydration, which would otherwise result in the formation of reversible cataract²³². Mice were then moved onto a custom platform affixed to the chinrest of the Spectralis machine, and the height adjusted such that the mouse's eyes were in line with the marker designed to indicate the optimal human patient's eye position. All images were acquired in 'High Resolution' mode (8bits/pixel and 1536 x 1536 pixel resolution) using automatic real-time (ART) functionality. In ART mode, multiple images are acquired and averaged to create a composite with increased resolution. The eye is actively tracked during this process based on high contrast landmarks, thus compensating for small movements of the eye such as those that occur as a result of respiration. In all cases, a reference image was first taken of the external aspect of the eye using the near infrared (NIR) reflectance mode (which utilizes an 815nm wavelength diode laser), before the laser arm was moved forwards and the image focussed on the retina with the optic nerve positioned at the centre of the field of view. The dioptric focus was adjusted to the confocal plane corresponding to that of highest NIR reflectance which is thought to be that of the RPE²³³, and the sensitivity set to give the brightest image possible whilst avoiding areas of overexposure. An NIR reflectance image was captured as an average of five or more stills taken in ART mode. Fluorescence cSLO images were subsequently acquired using the inbuilt blue autofluorescence (BAF) and indocyanine green angiography (ICGA) modules of the Spectralis platform. The former modality consisted of a 486nm blue diode excitation laser coupled with a 500nm barrier filter. Emission was recorded between 500nm and 700nm. The latter modality consisted of a diode laser of 768nm wavelength and barrier filter at 830nm, with detection of emission at longer wavelengths. Fluorescence fundal images were captured at the same focal plane as that used for NIR reflectance image acquisition. BAF images of retinas expressing green fluorescent protein (GFP) were acquired at sensitivity values (a parameter that can be adjusted between values of 31 and 107) of 50, 60 and 70 in ART mode (minimum five frames) with normalisation switched off to allow for

subsequent quantification of the fluorescence signal. ICGA images were acquired at maximum sensitivity (a value of 107).

For SD-OCT, the mouse remained in the same position on the viewing platform with the contact lens in situ. The imaging protocol consisted of eight equally spaced radially orientated scans centred on the optic disc. Each image was captured using a 55° lens in ART mode from an average of 25 individual B-scans. Photoreceptor layer (PRL) thickness was measured from these images at mid-peripheral locations using callipers built in to the Spectralis software between the high contrast OPL and RPE bands at a fixed distance of 22.5° from the optic nerve head. Mean PRL thickness for a given eye was calculated using eight measurements from superior, superonasal, nasal, inferonasal, inferior, inferotemporal, temporal and superotemporal retinal loci.

2.4.4. Electroretinography

Prior to electroretinography (ERG), mice were dark-adapted overnight (minimum 12 hours) within a purpose-built light-tight cabinet. The procedure was conducted in a designated windowless room with only dim red illumination used during setup where required. Mice were anaesthetised and pupils dilated as described in Section 2.4.1. The animal was then transferred onto a heated platform set to 38°C within a dark cabinet and ground and reference electrodes placed subcutaneously on the flank and between the eyes respectively. Guttae hypromellose 1% (Alcon, UK) was instilled bilaterally. Custom contact lenses produced from achromatic ACLAR (Honeywell International, USA) were used to hold silver thread Dawson, Trick & Litzkow (DTL) Plus electrodes (Diagnosys Ltd., Cambridge, UK) in place on each cornea. Having completed setup, the platform was manoeuvred into the centre of a Ganzfeld stimulator (Diagnosys, UK) and the housing cabinet door closed and secured. The animal was monitored

throughout the procedure by way of a video camera located within the cabinet. Once in position, the mouse was left to acclimatize for a period of 10 minutes prior to commencing the ERG protocol using Espion software (Diagnosys, UK). Dark-adapted responses to uniform white flashes of increasing light intensity were recorded, followed by a dark-adapted flicker protocol as per Table 2.3. Mice were subsequently exposed to full-field 30cd/m² white background light for a period of ten minutes, an intensity greater than that expected to achieve complete rod saturation. Animals were then subjected to further flash and flicker stimuli superimposed upon this background (Table 2.3). At the end of the procedure, anaesthesia was reversed as previously described and guttae chloramphenicol applied bilaterally.

Step	No. of sweeps	Inter-sweep delay (s)	Sweep length (s)	Inter-step delay (s)	Description (cd.s/m ²)	Log
0				600	Dark adaptation	
1	16	0	3	10	Single 0.5Hz 1x10 ⁻⁶ cd.s/m ²	-6
2	16	0	3	30	Single 0.5Hz 1x10 ⁻⁵ cd.s/m ²	-5
3	9	1	3	30	Single 0.5Hz 0.0001 cd.s/m ²	-4
4	9	1	3	60	Single 0.5Hz 0.001 cd.s/m ²	-3
5	9	5	3	60	Single 0.5Hz 0.01 cd.s/m ²	-2
6	4	13	3	120	Single 0.5Hz 0.1 cd.s/m ²	-1
7	4	29	3	120	Single 0.5Hz 1 cd.s/m ²	0
8	4	61	3	180	Single 0.5Hz 10 cd.s/m ²	1
9	1	0	3	60	Single 0.5Hz 25 cd.s/m ²	1.4
10	25	0	1.5	0	Continuous 6Hz 0.01 cd.s/m ²	-2
11	25	0	1.5	0	Continuous 6Hz 0.1 cd.s/m ²	-1
12	25	0	1.5	0	Continuous 6Hz 1 cd.s/m ²	0
13	25	0	1.5	0	Continuous 6Hz 3 cd.s/m ²	0.5
14	25	0	1.5	0	Continuous 6Hz 10 cd.s/m ²	1
15	25	0	1.5	0	Continuous 20Hz 10 cd.s/m ²	1
16	25	0	1.5	600	Continuous 30Hz 10 cd.s/m ²	1
					Light adaptation	
17	25	0	1	0	Single 1Hz 0.3 cd.s/m ²	-0.5
18	25	0	1	0	Single 1Hz 1 cd.s/m ²	0
19	25	0	1	0	Single 1Hz 3 cd.s/m ²	0.5
20	25	0	1	0	Single 1Hz 10 cd.s/m ²	1
21	25	0	1	0	Single 1Hz 25 cd.s/m ²	1.4
22	25	0	1	0	Continuous 20Hz 10 cd.s/m ²	1
23	25	0	1		Continuous 30Hz 10 cd.s/m ²	1

Table 2.3. ERG recording protocol. Mice were dark-adapted in a light-tight cabinet for a minimum of 12 hours prior to recording. After set-up, mice were left to dark adapt for a further ten minutes prior to initiating recordings. A ten-minute light adaptation time was included between steps 16 and 17.

2.5. Cell and Tissue Processing

2.5.1. Immunocytochemistry

Immunocytochemistry (ICC) was performed on HEK293 cells cultured in chamber slides (Nunc Lab-Tek II chamber slide system™, Thermo Fisher Scientific, UK) 48hrs after transfection (see Section 2.2.2). The ICC protocol followed is outlined below:

1. Media was removed from the cell monolayer and two PBS washes were applied, each consisting of the gentle addition and removal of 120µl of 0.01M PBS.
2. Cells were fixed in 120µl 4% formaldehyde (Thermo Fisher Scientific, UK) for 15 minutes at room temperature.
3. Two further PBS washes were performed as above.
4. Cells were permeabilised by incubation in 120µl 0.2% Triton X-100 in 0.01M PBS for ten minutes.
5. One PBS wash was applied as above.
6. Cells were incubated in 120µl 10% normal donkey serum (NDS) with 0.1% Triton X-100 in 0.01M PBS for 30 minutes.
7. One PBS wash was applied as above.
8. The cell monolayers were then incubated in 100µl primary antibody diluted 1:1000 in 1% NDS with 0.1% Triton X-100 in 0.01M PBS for two hours at room temperature.
9. Four PBS washes were applied as above.
10. The cell monolayers were then incubated in 100µl secondary antibody (conjugated with fluorescent dye) diluted 1:200 in 1% NDS with 0.1% Triton X-100 in 0.01M PBS for 30 minutes at room temperature in the dark.
11. Three PBS washes were applied as above.

12. Cells were then counterstained through incubation in 120µl Hoechst 33342 (Life Technologies, USA) diluted 1:1000 in 0.01M PBS for ten minutes.
13. One final PBS wash was applied as above.

Details of the specific antibodies used are outlined in the methods sections of the relevant experimental chapters. At the end of the ICC protocol above, chamber walls were removed from the glass slide base in accordance with manufacturer's instructions. ProLong[®] Diamond antifade mounting media (Thermo Fisher Scientific, UK) was then applied to the slides on top of which a glass coverslip was carefully placed. Slides were left to dry at room temperature overnight and then stored at 4°C ahead of microscopy.

2.5.2. Immunohistochemistry

Eyes were extracted from mice as soon after confirmation of death as possible. In cases where radial orientation of the final section was important, the eye was removed together with its superior rectus muscle which acted as a downstream marker for the dorsal aspect of the globe. The eye was transferred into sterile PBS and other extraocular muscles and conjunctival tags removed. The cornea was then punctured with a 30G needle after which the cornea, iris and lens were carefully removed using microsurgical instruments. The resulting eye cup was fixed in 4% formaldehyde for 30 minutes and then transferred sequentially into 10%, 20% and finally 30% sucrose solutions, each for a period of at least 20 minutes. Eye cups were left in 30% sucrose overnight at 4°C, washed briefly in PBS, dried, and then immersed in optimal cutting temperature compound (OCTC) within a mould in the desired orientation. Specimens were then frozen on dry ice and stored at -80°C prior to cryosectioning.

Eyes to be sectioned were removed from the -80°C freezer and transported on dry ice to a cryostat (Leica Biosystems, UK) that had been pre-chilled to between -20 and -22°C. Eyes were sectioned to a thickness of 18µm onto polylysine coated glass slides, left to dry at room temperature overnight, and then stored at -20°C.

For immunohistochemistry (IHC), slides were thawed at room temperature, condensation removed with a tissue and a perimeter border created with a hydrophobic barrier pen (Thermo Fisher Scientific, UK). On each slide, a small area containing at least two tissue sections was separately demarcated with hydrophobic barrier pen. Tissue sections in this region were incubated with PBS in place of primary antibody solution during the subsequent IHC protocol to act as negative control samples. The following steps were followed for IHC:

1. Slides were washed by submersion in 0.01M PBS for a period of five minutes. This process was repeated with fresh PBS on two further occasions.
2. Tissue sections were permeabilised by incubation of slides in 0.2% Triton X-100 in 0.01M PBS for 20 minutes.
3. Three further PBS washes were performed as above.
4. Tissue sections were then incubated in 10% NDS with 0.1% Triton X-100 in 0.01M PBS for one hour.
5. Three further PBS washes were performed as above.
6. Primary antibody diluted 1:1000 in 1% NDS with 0.1% Triton X-100 in 0.01M PBS was then applied to the main section of slides. The smaller control area of each slide was covered in 0.01M PBS only. Slides were incubated overnight at 4°C.

7. Slides were washed by submersion in 0.05% Tween-20 in 0.01M PBS for five minutes. This process was repeated in fresh Tween-20/PBS solution before a final five-minute wash in 0.01M PBS was performed.
8. Secondary antibody (conjugated to a fluorescent dye) diluted 1:500 in 0.01M PBS with 0.1% Triton X-100 was then applied to the slides which were incubated in the dark at room temperature for two hours.
9. Slides were washed twice in 0.05% Tween-20 in 0.01M PBS as described above.
10. Tissue sections were counterstained through incubation in Hoechst 33342 (Life Technologies, USA) diluted 1:1000 in 0.01M PBS for 30 minutes.
11. A final wash step was performed by submersion of slides in 0.01M PBS for five minutes.
12. Slides were removed from the PBS and excess liquid dried from the edges of the slide using tissues.
13. Small drops of ProLong[®] Diamond antifade mounting medium (Thermo Fisher Scientific, UK) were applied to the sections onto which glass cover slips were carefully placed.

Details of specific antibodies used may be found in the methods sections of relevant experimental chapters. At the end of the staining protocol, slides were left to cure overnight in the dark at room temperature after which they were stored at 4°C.

2.5.3. Microscopy

Fluorescence micrographs of processed cell and tissue slides as well as for cells in culture were acquired using the Invitrogen EVOS FL Auto 2 Cell Imaging System (Thermo Fisher Scientific, UK). For each image, the focus for transmission and fluorescent channels was individually

determined. The gain level for each channel was adjusted to a level which avoided image saturation and was kept the same for comparable slides or wells.

Confocal microscopy was performed using the LSM-710 inverted confocal microscope system (Zeiss, Germany). Areas of interest were first identified using epifluorescence illumination before XY confocal image capture with an optical section thickness of approximately 500nm. Blue, green and red channel fluorescence images were acquired using 350nm UV, 488nm argon, and 543nm HeNe excitation lasers respectively with appropriate filters. Images were processed using ImageJ software (Version 1.50c4, NIH, USA).

2.6. Statistical Analysis

All graphing and statistical analysis was performed using Prism software (Version 7.0a, GraphPad Inc., USA). Quantitative measures and errors are presented as mean $\pm$ standard error of the mean (SEM) unless otherwise stated. Where data sets were large enough, the Shapiro-Wilk test for normality was performed and, if established, parametric tests applied. When two matched or unmatched groups were to be compared, paired or unpaired two-tailed t-tests were used respectively, with Welch's correction applied in cases where the standard deviation between the groups could not be assumed to be equal. In circumstances where three or more groups were to be compared, or the effects of multiple factors were to be determined within two or more groups, one- or two-way analysis of variance (ANOVA) tests were performed respectively. If significant statistical interaction was confirmed between factors, appropriate post-hoc tests were applied which accounted for multiple comparisons. In all cases, p values less than 0.05 were considered statistically significant.

3. Filtration of Short Wavelength Light for the Treatment of Dominant RP

3.1. Introduction

Several forms of retinitis pigmentosa (RP), particularly those associated with dominant mutations around the N-terminus of the rhodopsin gene, demonstrate a more severe degeneration in the inferior than the superior retina (so called ‘sector RP’)^{16,28}. It has been proposed that the mechanism for this pattern is the modifying effect of incident light to which the inferior retina is more intensely exposed^{29,89,234}. In support of this hypothesis, rearing animal models of ADRP, including the *RHO*^{P23H} transgenic mouse¹¹⁰, rat¹¹¹ and *Xenopus laevis*¹⁰⁶, as well as the *RHO*^{T4R} dog²³⁵, in complete darkness has been shown to slow the degenerative process, and such animals appear to have an increased sensitivity to light-induced retinal damage^{110,112}. Further, cases of patients with rhodopsin-related RP who have degenerative patterns reflective of occupational light exposure have also been reported^{28,236}. Together, these studies would suggest that substantial reduction in light exposure might slow retinal degeneration in patients with sector RP. Complete occlusion is likely to be the most effective, but this has obvious drawbacks as a treatment strategy for use in patients. Given that RP is primarily a rod cell degeneration, and that rods themselves are only minimally receptive to light of wavelength greater than 600nm²³⁷ (Figure 3.1), it follows that filtration of short wavelength light may similarly slow the degenerative process whilst allowing patients to retain useful vision. Only one study has systematically examined the effect of coloured lighting and optical filters in an animal model of RP¹¹³. The authors used rhodopsin levels as a marker of retinal degeneration in the bovine transgenic *Rho*^{P23H} *Xenopus laevis* model and found evidence supporting a beneficial effect when short wavelength light is reduced or excluded.

In this chapter, I explore the effect of filtration of short wavelength light on the rate of outer retinal degeneration in the *Rho*^{P23H/+} knock-in mouse model of dominant RP which shows a superior-inferior gradient of outer retinal degeneration¹⁰⁵, and compare this with that of the *Rpgrr*^{-/-} mouse model of X-linked (XL) recessive RP which shows no such gradient²³⁸.

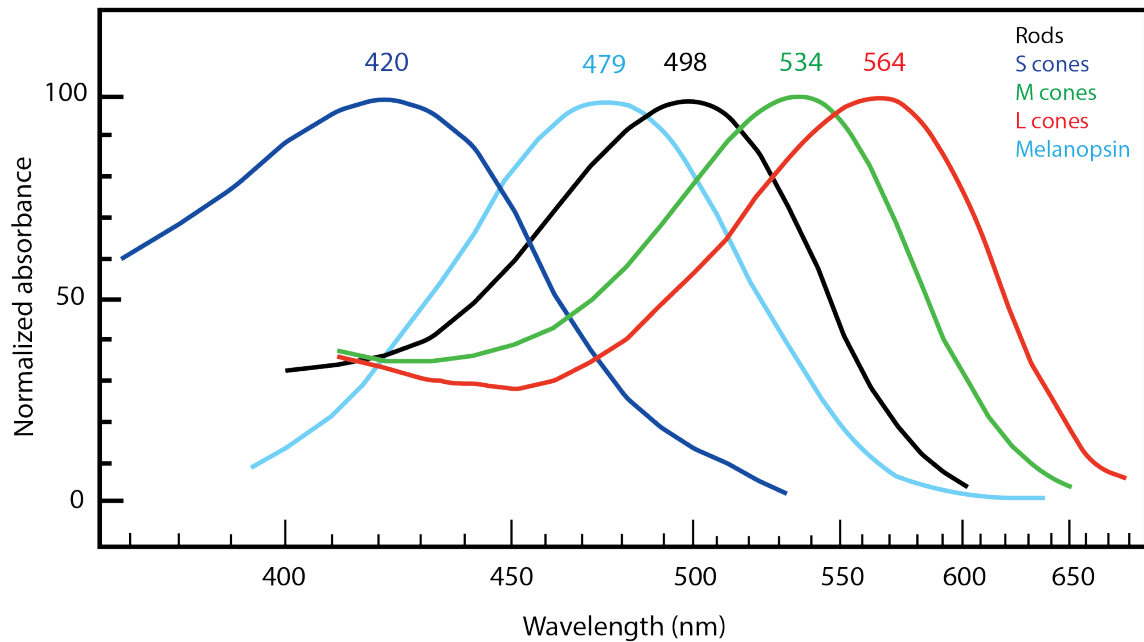


Figure 3.1. Mean absorbance spectra of rods, cones and melanopsin. The wavelength of maximal absorbance (λ_{max}) in nanometres for each photoreceptor/pigment type is displayed above its corresponding curve. Note that rods absorb relatively little light of wavelength $>600\text{nm}$. Figure adapted from Bowmaker & Dartnall, 1980²³⁷.

3.2. Aims

The principal aims of the experiments outlined in this chapter were as follows:

1. To determine the effect of rearing behind red optical light filters on the rate of photoreceptor degeneration in the *Rho*^{P23H/+} mouse model of ADRP.

2. To establish whether any such effect is present when animals raised in normal ambient light conditions are switched into filtered housing after visual maturation.
3. To determine the effect of red filters on the rate of degeneration in a mouse model of RP that does not show a superior-inferior difference in the rate of retinal degeneration (*Rpgr*^{-/-}, which is a cause of X-linked recessive RP).

3.3. Materials & Methods

3.3.1. Animals and housing conditions

Mice heterozygous for the *Rho*^{P23H} mutation (henceforth referred to as *Rho*^{P23H/+}) were generated by crossing B6.129S6(Cg)-*Rho*^{tm1.1Kpal}/J homozygotes¹⁰⁵ obtained from the Jackson Laboratory, ME with C57BL/6J wild type mice, obtained from Envigo, UK. *Rho*^{P23H/+} mice which additionally carried a green fluorescent protein (GFP) transgene under the control of the neural retina leucine zipper (Nrl) promoter (henceforth referred to as *Nrl-GFP*+/+, *Rho*^{P23H/+}) were generated by crossing *Rho*^{P23H/P23H} mice with those homozygous for the Tg(Nrl-EGFP)1Asw/J allele (gift from Anand Swaroop, National Eye Institute, Bethesda, MD). Mice carrying a knock-out mutation of the XL retinitis pigmentosa GTPase regulator (*Rpgr*) gene (henceforth referred to as *Rpgr*^{-/-})²³⁸ were obtained as a gift from Professor Tiansen Li (NIH, USA). Animals were housed in either clear or red-tinted (tint: RD2A067T) plastic individually ventilated cages (IVCs; Figure 3.2) of 2.5mm thickness in a dedicated facility as previously described. Paired clear and red cages housing littermates in any one experiment were positioned in an alternating fashion on the same shelf of the storage rack (second and third from top rows). Equal numbers of male and female animals were included for red and clear-housed groups in all experiments. IVCs were manufactured by Tecniplast, Buguggiate, Italy. All red IVC changes were performed in a dark room under dim red illumination in order to avoid exposing mice to ambient light.

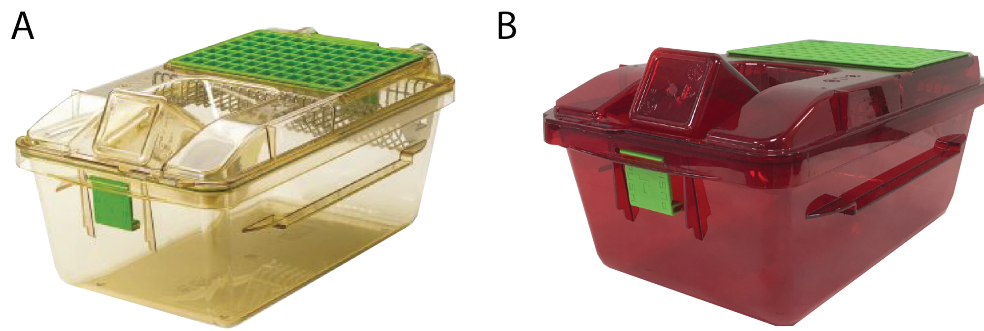


Figure 3.2. Individually ventilated cages (IVCs). (A) Standard clear IVC. (B) Red-tinted IVC (Tecniplast, Buguggiate, Italy). Both cage types include a white filter located on the posterior upper surface beneath a green plastic retainer.

3.3.2. Spectroradiometry

Readings were taken using a calibrated spectroradiometer (USB2000; Ocean Optics, Dunedin, FL). Transmission spectra for clear and red-tinted IVCs were generated by placing the cage lid between a bright halogen light source and the spectroradiometer detector and then dividing this value by the signal from the unimpeded light. Recordings below 450nm were not plotted as halogen light sources include very little low wavelength light (Figure 3.4A). The poor signal: noise ratio in this part of the visible light spectrum would thus result in an unreliable measurement. In situ recordings were acquired by fixing the probe at approximate mouse eye level, pointing forwards inside cages located within their housing rack. In all cases, measurements were taken at 0.33nm intervals and data were plotted using a 5nm boxcar average.

3.3.3. Assessment of retinal structure and function

Retinal structure was assessed by SD-OCT as described in Section 2.4.3. In all cases, the mean measurement from both eyes was used. For animals housed in red-tinted IVCs, this was

performed under dim red illumination. Measurement of photoreceptor layer thickness was performed in a masked fashion. Retinal function was quantified by dark- and light-adapted ERG under terminal anaesthesia six months post-weaning as described in Section 2.4.4.

3.3.4. Flatmount Immunohistochemistry

All steps were performed at room temperature. Following completion of ERG, mice were culled whilst still under anaesthesia and eyes extracted. The neural retina of the right eye of each animal was carefully dissected out in PBS solution and fixed in 4% paraformaldehyde (PFA) for 30 minutes before being transferred back into PBS. Retinas were permeabilised in 1% Triton X-100-PBS for 20 minutes then washed three times, each wash step consisting of incubation in PBS for ten minutes in 24 well plates on an orbital shaker set to approximately 45rpm. Retinas were then blocked by incubation in 10% normal donkey serum (NDS), 1% Triton X-100-PBS for three hours. Tissue was then transferred into new wells of a 24 well plate for overnight incubation in anti-cone arrestin primary antibody (rabbit anti-mouse cone arrestin polyclonal antibody AB15282, Millipore, UK) diluted 1:1000 in 1% Triton-X 100-PBS supplemented with 2.5% NDS. Three wash steps were then performed, each consisting of incubation in 0.2% Triton X-100-PBS on an orbital shaker set to a speed of approximately 45rpm for 30 minutes. Retinas were then transferred into secondary antibody solution (donkey anti-rabbit IgG-568, Alexa-Fluor ab175470, Abcam, UK) diluted 1:250 in 1% Triton X-100-PBS with 2.5% NDS, and incubated for three hours. Three further washes in 0.2% Triton X-100-PBS were performed as described above, after which retinas were incubated in Hoechst 33342 nuclear stain (Life Technologies, USA) diluted 1:1000 in PBS for 30 minutes. A final 30-minute wash in double distilled water was then performed, after which retinas were transferred onto polylysine coated glass slides, photoreceptor side down. Radial relieving incisions were made from the outer margins of each retinal cup towards the optic nerve head to allow the tissue to flatten. Prolong Diamond mounting media (Thermo Fisher Scientific, UK) was then applied to the upper

surface and cover slips placed carefully on top. Slides were left to cure overnight in the dark at room temperature ahead of confocal microscopy which was performed as described in Section 2.5.3.

3.3.5. Rpe65 polymorphism genotyping

Ear notch tissue was incubated in 100µl 50nM NaOH at 95°C for one hour, briefly agitated using a vortex mixer, and centrifuged at 13,000rpm for two minutes. Samples were then neutralised with 10µl tris-HCl and again agitated using a vortex mixer and centrifuged at 13,000rpm for two minutes. PCR reactions were set up using 1µl of the resulting supernatant as template DNA, together with 20µl ImmoMix (x2) PCR reaction mix (Bioline, UK) and 1µl of both forward and reverse Rpe65 primers at a concentration of 10µM, with the reaction volume being made up to 40µl with molecular biology grade water. The sequences of Rpe65 genotyping primers were as follows (Primers 1 & 2):

Rpe65 FW: 5'-ACCAGAAATTGGAGGGAAAC

Rpe65 RC: 5'-CCCTTCCATTCAGAGCTTCA

PCR reaction conditions consisted of a polymerase activation phase of ten minutes at 95°C followed by 30 cycles of 95°C for 20s, 58°C for 15s and 72°C for 15s, with a final extension period of five minutes at 72°C. DNA was subsequently purified using the QIAquick PCR purification kit (Qiagen, UK) in accordance with manufacturer's instructions and eluted in a total volume of 44µl molecular biology grade water. To this, 1µl MwoI enzyme and 5µl CutSmart buffer (both New England Biolabs, UK) were added and the samples incubated in a water bath at 65°C for one hour. The resulting DNA fragments were then separated in a 2%

agarose/ethidium bromide gel by electrophoresis and photographed under UV illumination as previously described.

3.4. Results

3.4.1. A superior-inferior difference in the rate of retinal degeneration is seen in *Rho*^{P23H/+} but not in *Rpgr*^{-/-} mice

Rho^{P23H/+} mice reared in standard clear cages were found by SD-OCT to have a thicker PRL superiorly than in the nasal, temporal and inferior retinal quadrants ($p=0.0001$ in all cases; Figure 3.3). At post-natal day (PND) 21, this difference was small but statistically significant. With advancing age, the difference in PRL thickness between superior and temporal, nasal and inferior quadrants increased. This would suggest that the superior retina degenerates more slowly in this model (Figure 3.3C). This superior-inferior difference was also confirmed histologically in the *Nrl-GFP/+*, *Rho*^{P23H/+} mouse in which green fluorescent protein (GFP) is expressed in rods under the control of the neural retina leucine zipper (*Nrl*) promoter (Figure 3.3B). By contrast, the PRL thickness of the superior retina was not significantly different to that of the inferior retina in either wild type mice ($p=0.15$; data not shown), or in the *Rpgr*^{-/-} mouse model of X-linked RP as measured by SD-OCT ($p=0.88$; Figure 3.3D).

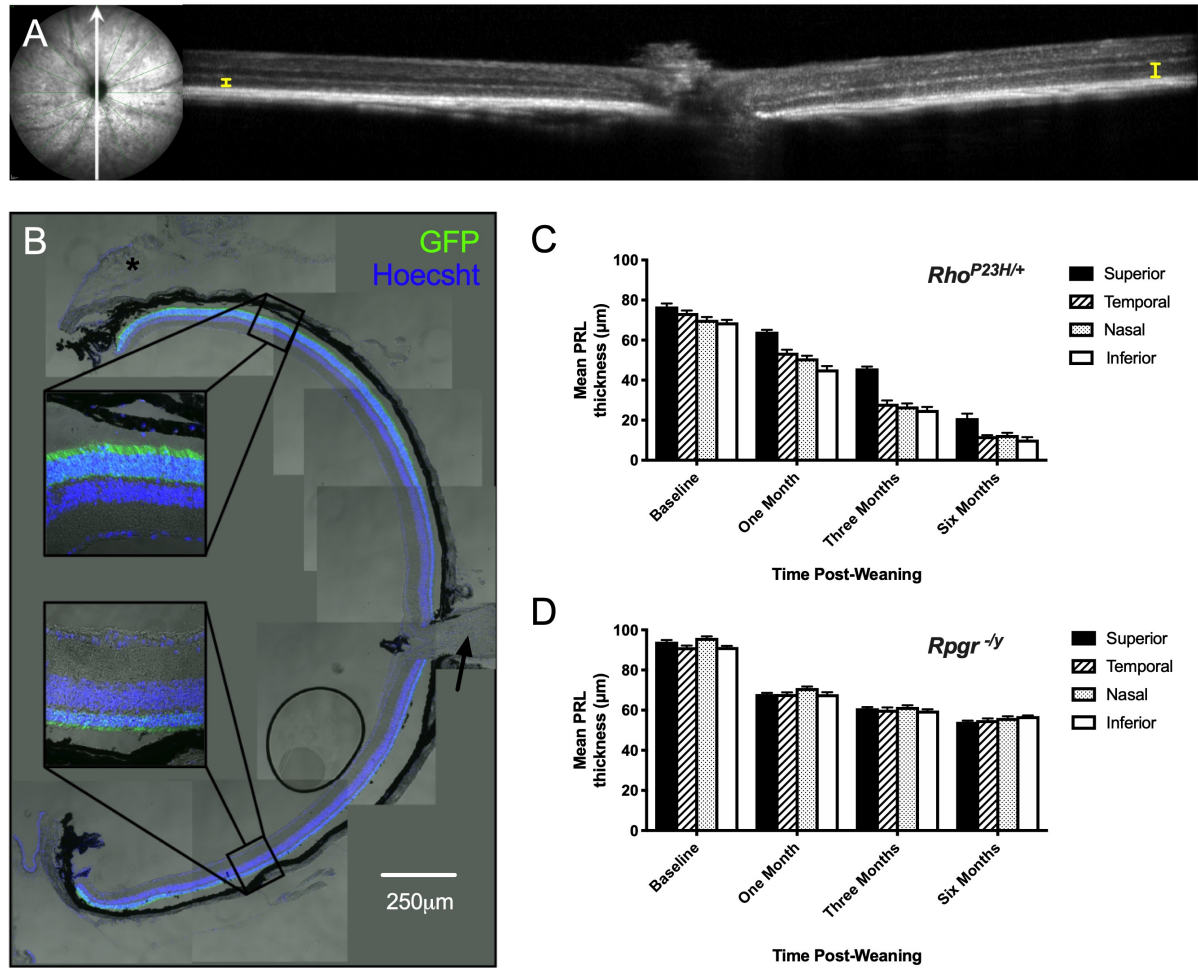


Figure 3.3. The inferior retina degenerates more rapidly than the superior retina in the *Rho*^{P23H/+} but not in the *Rpggr*^{-/-} mouse model of RP. Vertical SD-OCT retinal image from a *Rho*^{P23H/+} mouse aged three months. The white arrow through the accompanying en face infrared cSLO fundus image indicates the orientation of the section. Yellow callipers at the inferior and superior aspects of the section indicate the PRL at these locations. **(B)** Vertical cryosection through an *Nrl:GFP/+*, *Rho*^{P23H/+} mouse eye aged three months. The superior rectus muscle is marked with a black asterisk whilst the optic nerve is indicated by a black arrow. Rod photoreceptors express GFP in this model highlighting the ONL and IS in green. **(C, D)** Mean photoreceptor layer (PRL) thickness $\pm$ SEM by retinal quadrant as a function of time post-weaning for *Rho*^{P23H/+} (C; n=9) and *Rpggr*^{-/-} (D; n=9) mice. The superior PRL thickness was significantly greater than that of all other retinal quadrants in the *Rho*^{P23H/+} model and this difference increased over time (repeated measures two-way ANOVA with factors of retinal quadrant and time: $F(3, 24) = 336.1$, $p < 0.0001$ for effect of retinal quadrant; $F(9, 72) = 13.08$, $p < 0.0001$ for interaction time x

retinal quadrant; $p=0.0001$ for superior versus temporal, nasal and inferior retinal quadrants, Sidak's multiple comparison test). No superior-inferior difference in PRL thickness was detected in *Rpgr*^{-/-} mice ($p=0.88$, Sidak's multiple comparison test).

3.4.2. Red cages filter short wavelength light

Figure 3.4 shows the transmission spectra of clear and red-tinted IVCs recorded using a bright halogen light source. Absolute light measurements for the two cage types recorded in situ under fluorescent lighting are shown in Figure 3.4C. Note that the red tint results in the near complete attenuation of all peaks below 600nm with a significant reduction of the peak at 614nm. Thus, red IVCs used here appear to act as long-pass light filters significantly attenuating light transmission for wavelengths below ~600nm. Total absolute light levels recorded in situ were 659 lux for clear IVCs and 92 lux for red IVCs. The predicted effect of the tint on activation of the four mouse photopigments (rhodopsin, melanopsin, and short/long-wavelength cone opsins) was calculated using the formulae described by Lucas et al., 2014 in their irradiance toolbox²³⁹, and is shown in Figure 3.4D.

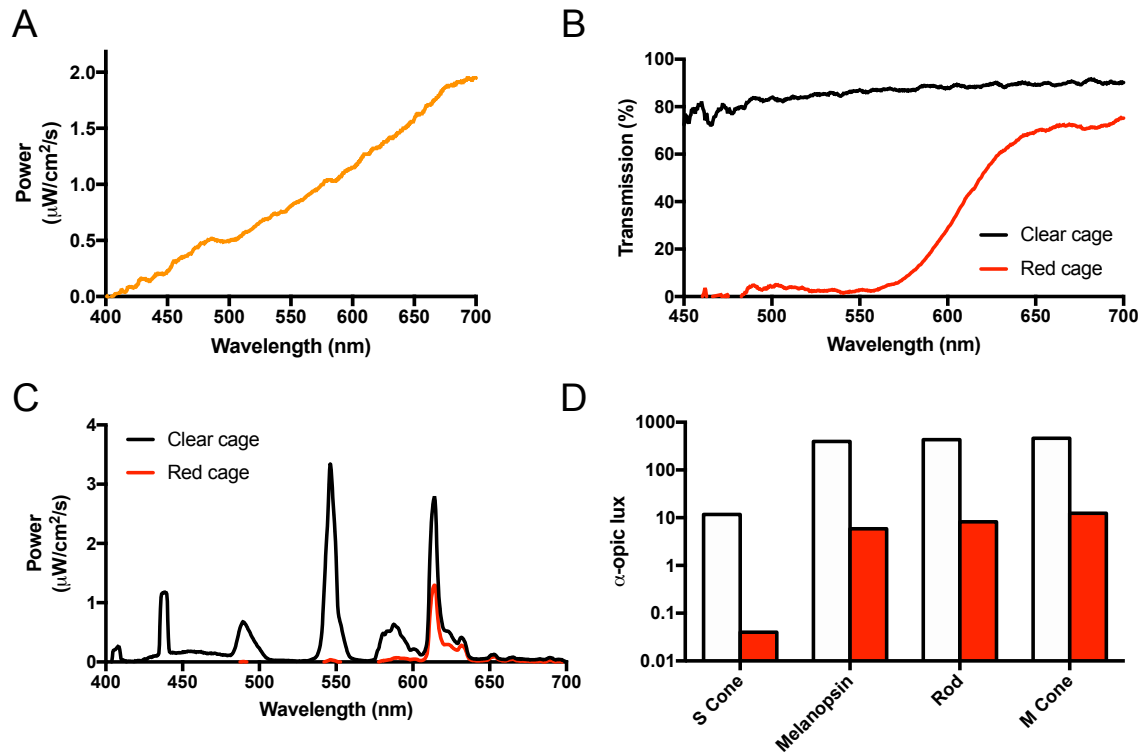


Figure 3.4. Spectroradiometer recordings from clear and red-tinted IVCs. (A) Spectral distribution of the halogen light source used to quantify IVC transmission. (B) Transmission spectra recorded for clear and red-tinted IVCs using a halogen light source. (C) Spectral distribution for both cage types recorded from within cages located in situ in the animal housing facility under fluorescent lighting. (D) Predicted activation of mouse photoreceptor classes for animals within clear (open bars) and red-tinted (red bars) IVCs.

3.4.3. Rearing within red IVCs slows retinal degeneration in the $Rho^{P23H/+}$ model

$Rho^{P23H/+}$ pups born within red IVCs were divided at post-natal week (PNW) three into clear or red-tinted IVCs. Photoreceptor layer thickness was subsequently measured by SD-OCT one, three and six months later, and retinal function determined by ERG six months post-weaning. Mean photoreceptor layer (PRL) thickness as measured by SD-OCT was significantly greater for animals housed in red-tinted IVCs than in those housed in standard conditions at all time points examined (repeated-measures two-way ANOVA with factors of cage type and time post-

randomisation: $F(1, 20) = 68.03$, $p < 0.0001$ for effect of cage type; $F(3, 60) = 29.61$, $p < 0.0001$ for interaction cage type x time; Figure 3.5). This difference was noted in all four retinal quadrants at all time points (Figure 3.5B-D).

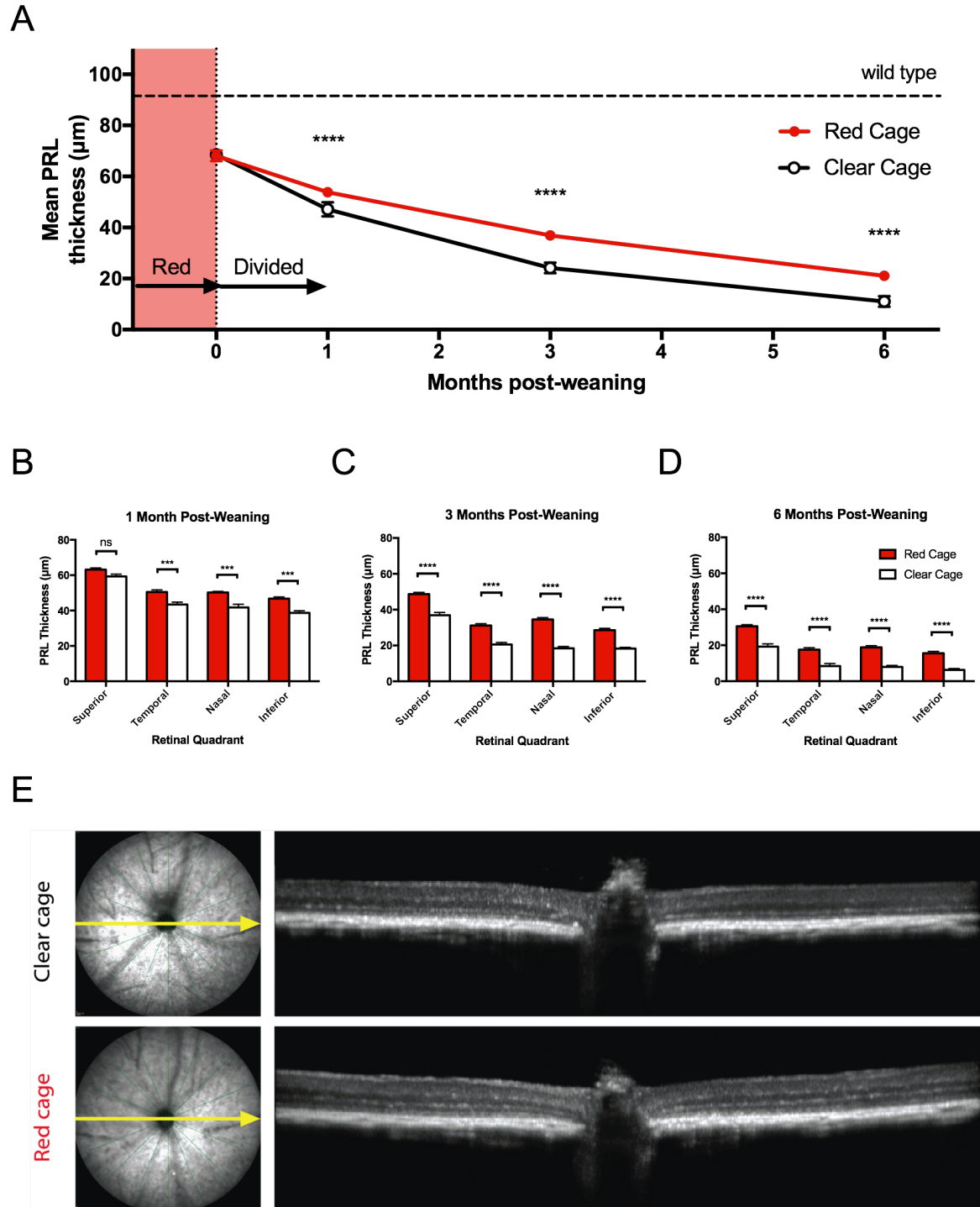


Figure 3.5. Rearing in red IVCs slows the rate of outer retinal degeneration in the *Rho*^{P23H/+} mouse as measured by SD-OCT. Animals born in red IVCs were divided between clear and red IVCs at the point of weaning on PND21 **(A)** Mean photoreceptor layer (PRL) thickness recorded by SD-OCT. Error bars represent the 95% confidence interval about the mean. For points where no error bar is displayed, the confidence interval is smaller than the size of the symbol. The dashed horizontal line

represents the mean PRL thickness measured from four C57BL/6J wild type mice at six months post-weaning. **** $p < 0.0001$, Sidak's multiple comparison test. **(B-D)** Mean PRL thickness by retinal quadrant at one-, three- and six-months post-weaning. *** $p < 0.001$, **** $p < 0.0001$, Sidak's multiple comparison test. **(E)** Representative NIR reflectance images and horizontal SD-OCT for littermates housed in clear and red IVCs. The particular cross section displayed is indicated by the yellow arrow in the corresponding en face NIR reflectance image.

The inferior: superior ratio of PRL thickness was also significantly greater for the red-housed group throughout the experiment, indicating a relative attenuation of the sectoral phenotype in these animals ($p = 0.001$; Figure 3.6)

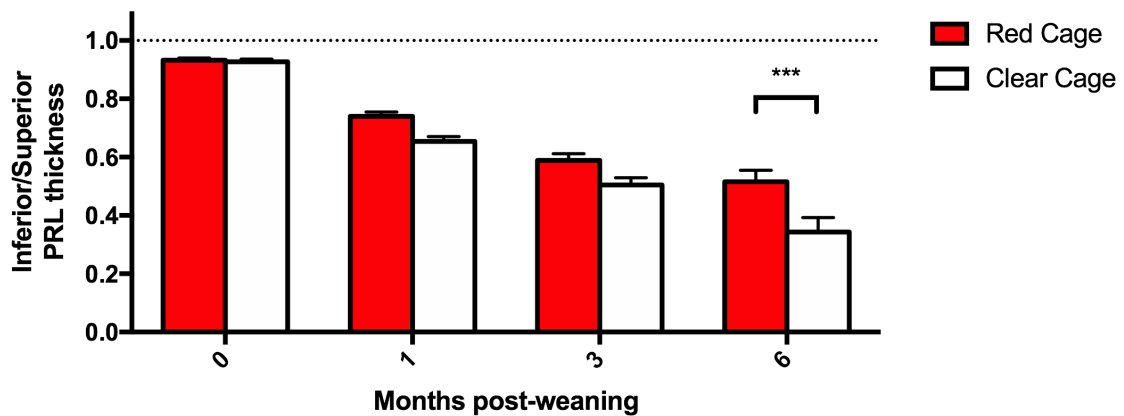


Figure 3.6. The inferior: superior ratio of PRL thickness is greater for *Rho^{P23H/+}* mice housed in red compared to clear IVCs suggesting an attenuation of the sector phenotype. Animals were born in red cages and divided between the two cage types at weaning. Points are plotted as mean $\pm$ SEM. The horizontal dotted line represents the PRL ratio of one that would be expected if no sector phenotype were to be present. Repeated measures two-way ANOVA with factors of cage type and time post-randomisation: $F(1, 20) = 14.47, p = 0.001$ for effect of cage type; $F(3, 60) = 3.75, p = 0.015$ for interaction cage type x time; *** $p < 0.001$, Sidak's multiple comparison test.

ERG performed six months post-weaning showed a greater preservation of both dark and light-adapted responses in the red IVC group than in the clear IVC group (Figure 3.7)

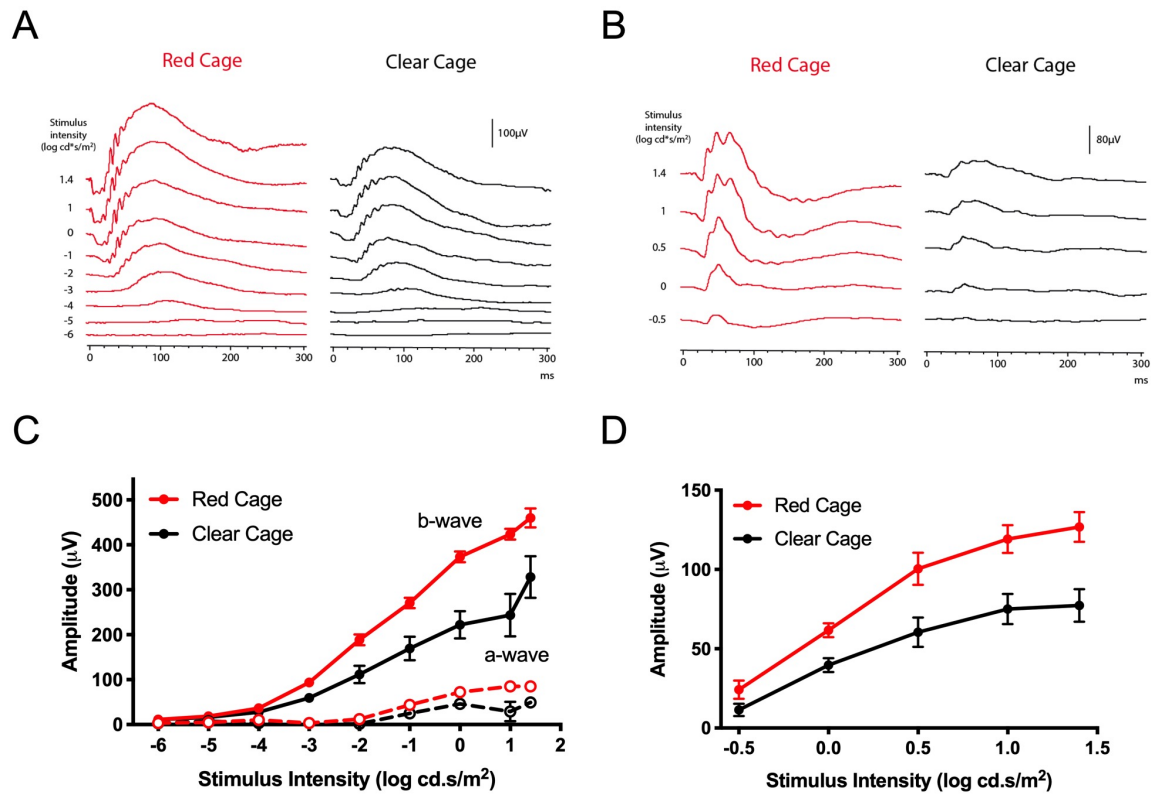


Figure 3.7. ERG recordings demonstrate a relative preservation of retinal function in red-housed animals when compared to their clear-housed littermates. (A) Representative dark-adapted traces. **(B)** Representative light-adapted traces. **(C)** Dark-adapted ERG irradiance-response curves (mean response $\pm$ SEM); a- and b-wave responses are represented by dashed and solid lines respectively. Repeated measures two-way ANOVA for effect of housing: $F(1, 18) = 18.13$, $p=0.0005$ for a-wave; $F(1, 18) = 12.95$, $p=0.002$ for b-wave. **(D)** Light-adapted ERG irradiance response curves (mean response $\pm$ SEM). Repeated measures two-way ANOVA for effect of housing: $F(1, 18) = 12.13$, $p=0.003$.

3.4.4. Red filters lead to a relative preservation of cone morphology in the $Rho^{P23H/+}$ model

Following ERG, mice that had been housed in red or clear IVCs were culled and eyes extracted. The retinas were immunostained for cone arrestin and flat-mounted for *en face* confocal fluorescence microscopy. Cones from animals housed in clear cages had disrupted pedicles, shortened axons, as well as fewer and more disorganised outer segments (Figure 3.8). Their red IVC housed littermates displayed cone anatomy that was closer in appearance to that observed in age-matched wild type animals. Long-term housing in red IVCs thus resulted in a relative preservation of cone morphology.

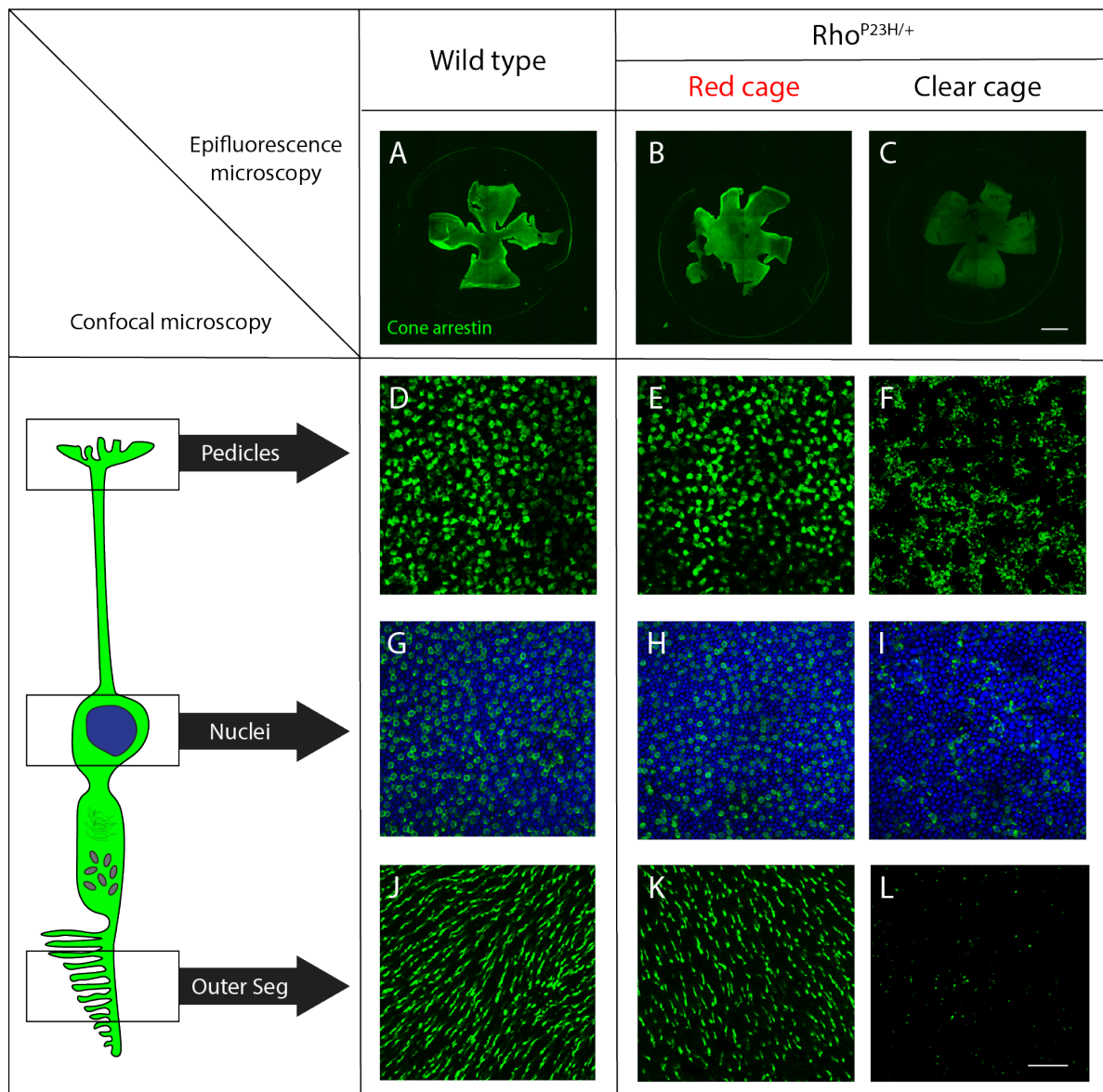


Figure 3.8. Rearing within red IVCs results in a relative preservation of cone morphology. Retinas from animals aged six months post-weaning were harvested, stained for cone arrestin (shown in green) and flat-mounted for microscopy. **(A-C)** A brighter signal was seen on low power epifluorescence microscopy for $Rho^{P23H/+}$ animals housed in red cages (B) than for their littermates housed in standard clear cages (C), with the staining pattern in the red-housed group appearing more similar to that observed in age-matched wild type controls (A). Scale bar: 2mm. **(D-L)** En face confocal fluorescence microscopy. Photoreceptor nuclei are stained with Hoechst 33342 nuclear stain (blue). Cone arrestin staining of retinas from clear-housed $Rho^{P23H/+}$ mice revealed disrupted pedicles (F), fewer nuclei (I) and

loss of outer segments (L). These anatomical features for their red-housed littermates (E, H, K) were again more similar to those observed in age-matched wild-type controls (D, G, J). Scale bar: 10 μ m.

3.4.5. Rearing within red IVCs has no effect on the rate of retinal degeneration in the *Rpgr*^{-/-} model

In a similar fashion, *Rpgr*^{-/-} pups born within red IVCs were divided at PNW3 into clear or red-tinted IVCs and photoreceptor layer thickness measured by SD-OCT after one, three and six months. In contrast to the *Rho*^{P23H/+} model, no significant difference in PRL thickness was observed between red and clear-housed groups at any time point (p=0.90; Figure 3.9).

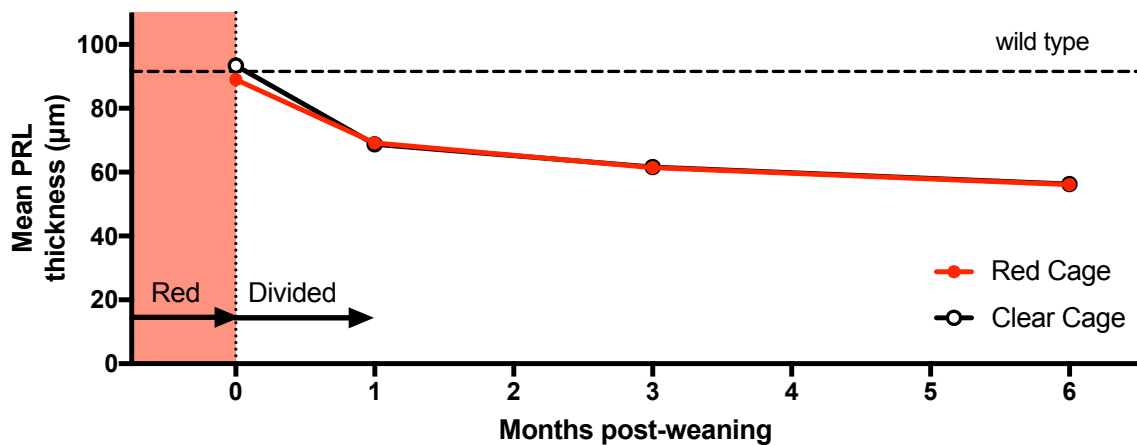
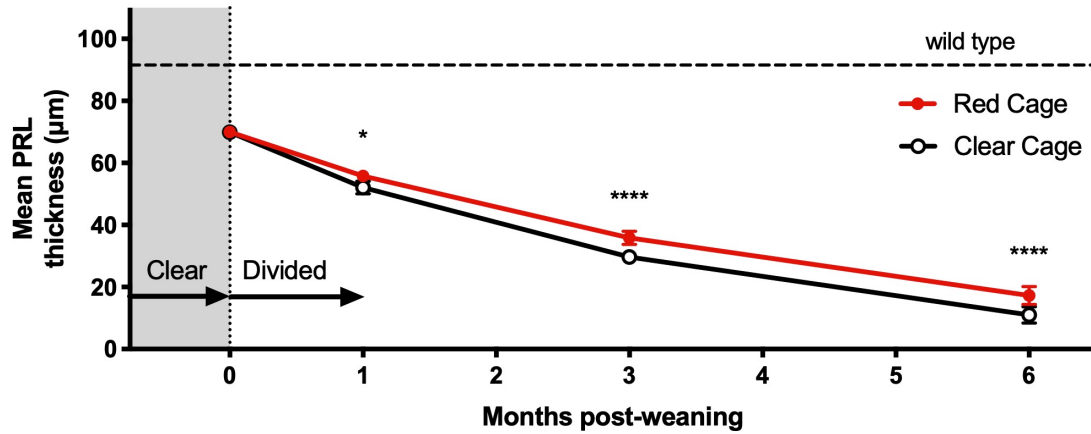


Figure 3.9. Rearing in red IVCs has no effect on the rate of outer retinal degeneration in the *Rpgr*^{-/-} mouse as measured by SD-OCT. Animals born in red IVCs were divided between clear and red IVCs at the point of weaning on PND21. Error bars representing the 95% confidence interval about the mean are smaller than the size of every symbol for both groups and so are not displayed. Repeated measures two-way ANOVA with factors of cage type and time post-weaning: $F(1, 16) = 0.016$, $p=0.90$ for effect of cage type. The dashed horizontal line represents the mean PRL thickness measured from four C57BL/6J wild type mice at six months post-weaning.

3.4.6. Effect of switching adult $Rho^{P23H/+}$ mice reared under standard conditions into red IVCs

To determine whether slowing of retinal degeneration in the $Rho^{P23H/+}$ mouse could be achieved when red filters are applied at more advanced stages of disease, further cohorts reared under standard conditions were divided between the two cage types at weaning (i.e. PNW3; n=8 per group) and one month post-weaning (at which point PRL thickness was already reduced to around 60% of that of C57BL/6J wild type animals; n=10 in red group, n=9 in clear group). A small but significant increase in PRL thickness was observed in the group of animals switched from clear into red IVCs at weaning compared to those remaining within standard conditions (p=0.0002; Figure 3.10A). No difference in PRL thickness was however detected between the two groups divided at PNW7 (p=0.71; Figure 3.10B). These results would suggest that early intervention may be required to see a treatment effect associated with filtration of short wavelength light in the $Rho^{P23H/+}$ mouse model.

A



B

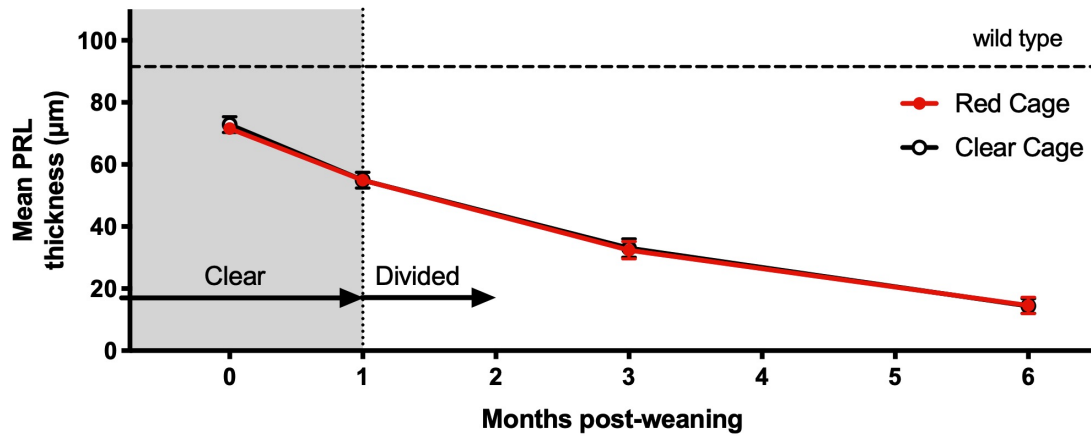


Figure 3.10. Moving *Rho*^{P23H/+} mice reared in standard conditions into red IVCs after increasing periods of time is associated with a reduction in the associated treatment effect. *Rho*^{P23H/+} mice were born and initially reared within standard clear cages and were divided between clear and red IVCs at the times indicated by vertical dotted lines. Error bars represent the 95% confidence interval about the mean. The dashed horizontal line represents the mean PRL thickness measured from four C57BL/6J wild type mice at six months post-weaning. **(A)** Cohort divided between cage types at weaning (PNW3); n=8 per group. Repeated measures two-way ANOVA with factors of cage type and time post-randomisation: $F(1, 14) = 23.81$, $p=0.0002$ for effect of cage type; $F(3, 41) = 8.87$, $p=0.0001$ for interaction cage type x time; * $p<0.05$, **** $p<0.0001$, Sidak's multiple comparison test. **(B)** Cohort divided between cage types at one month post-weaning (PNW7); n=10 in red group, n=9 in clear group.

Repeated measures two-way ANOVA with factors of cage type and time post-randomisation: $F(1, 17) = 0.14$, $p=0.71$ for effect of cage type.

3.4.7. *Rpe65* L450M polymorphism status of *Rho*^{P23H/+} mice

The L450M polymorphism within the *Rpe65* gene is known to influence the susceptibility of the mouse retina to light-induced damage²⁴⁰ with strains with the leucine variant appearing more vulnerable^{241,242}. *Rho*^{P23H/+} mice were genotyped according to the restriction fragment length polymorphism assay described by Kim et al.²⁴² to determine whether presence of the leucine variant (rather than, or in addition to, the P23H rhodopsin mutation) could be responsible for the observed effect of light on the retinal degenerative phenotype. All *Rho*^{P23H/+} mice genotyped by this method were found to be homozygous for the methionine variant of *Rpe65* (Figure 3.11). L450M polymorphism of *Rpe65* cannot therefore explain the sector phenotype observed in *Rho*^{P23H/+} mice.

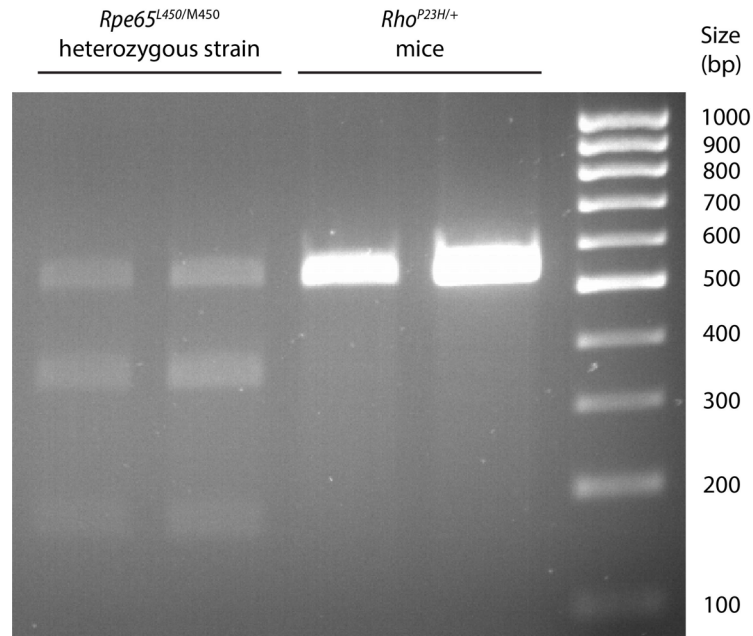


Figure 3.11. *Rho*^{P23H/+} mice are homozygous for the M450 variant of *Rpe65*. Restriction length polymorphism assay. The M450 polymorphism eliminates the MwoI restriction site within the 545bp *Rpe65* PCR amplicon. Presence of bands of length 180 and 360bp indicates the presence of an L450 allele. Three bands of 180, 360 and 545bp were thus detected for *Rpe65*^{L450/M450} heterozygous mice. Single bands of 545bp were apparent for *Rho*^{P23H/+} mice indicating *Rpe65*^{M450/M450} homozygosity in this strain.

3.5. Discussion

In this chapter I have explored the use of red-coloured long-pass light filters as a potential treatment option for sectoral RP. The findings presented above support the hypothesis that light, particularly of shorter wavelength, is a modifying factor in the natural history of degeneration in the *Rho*^{P23H/+} mouse model. Long-term housing within red-tinted IVCs resulted in a significant slowing of the rate of degeneration in the *Rho*^{P23H/+} retina which has a superior-inferior difference in PRL thickness, but not in the *Rpgt*^{-/-} model of RP that demonstrates no such difference. Furthermore, a more substantial difference in PRL thickness between superior and inferior retina was observed for mice housed in clear cages than for their littermates in red-

tinted housing. The sectoral phenotype may thus indicate the subset of RP patients that stand to benefit from long-pass light filters, although this would need to be confirmed in further RP models and in clinical trials.

The sector pattern of retinal degeneration has been described in dominant RP caused by a variety of missense mutations in the rhodopsin gene that appear to cluster within intradiscal (p.N15S²⁴³, p.T17M²⁴⁴, p.P23H²⁸, p.G106N²⁴⁵ and p.G182S²⁴⁶) and transmembrane (p.M39R²⁹, p.N55K²⁹, p.T58R/M^{245,247}, p.N78I²⁴⁸, p.W126L²⁴⁹) domains of the protein. These mutations appear to be those associated with protein misfolding, ER retention and instability (Class II rhodopsin mutations according to the classification of Mendes et al., 2005⁷²), and those with altered post-translational modifications and reduced stability (Class IV mutations). By contrast, amino acid substitutions at the C-terminus such as p.P347L and p.V345M which result in rhodopsin mistrafficking (Class I mutations) cause a classical concentric disease phenotype^{35,250}. A sector distribution has also been reported in relation to RP associated with mutations in other genes such as *CDH23*²⁵¹, *USH1C*²⁵², and *RPGR*³⁰. It has further been postulated that the retinas of a subset of patients presenting with a typical concentric distribution of degeneration may have shown a sectoral phenotype had they been examined at an earlier preclinical stage of the disease. The true prevalence of the sector phenotype may therefore be underestimated amongst the population of patients with RP²⁹.

The potential role for light in the pathogenesis of RP was first proposed as early as 1901 by Jules Gonin²⁵³. Berson later hypothesised that extended periods of near complete exclusion of light may be therapeutic and suggested that the visual lifetime of patients could be effectively doubled by occluding one eye to keep this retina “in reserve” whilst the fellow retina is used but degenerates²⁵⁴. This was not however borne out in a subsequent small trial in one patient with dominant and one patient with recessive RP where an occlusive contact lens was applied

unilaterally for six to eight hours per day over a period of five years²⁵⁵. Miyake et al. reported the case of a patient with RP who sustained anterior segment trauma which resulted in complete occlusion of the pupil²⁵⁶. This effectively excluded light transmission to the posterior segment over a 40-year period. After the pupillary membrane was opened surgically, no difference in the degenerative state of the retina was observed between the eyes. These cases were however reported prior to the era of molecular diagnostics, so it is unclear which mutations may have been included in the studies. Schwartz et al. mathematically modelled the distribution of blue and UV light falling on the human retina and concluded that given the observed pattern, this could not be the cause of the concentric centripetal field constriction characteristic of the majority of RP cases²⁵⁷. The authors did however recognize that sector RP may at least partially be explained by the distribution of shorter wavelength light that falls on the retina and proposed that appropriate light filters might provide therapeutic benefit.

Light has been shown to accelerate retinal degeneration in a number of animal models of RP, many of which may be at least partially rescued by dark-rearing²³⁴. Class II and Class IV rhodopsin missense mutations appear to confer a particular susceptibility to light damage. Retinal degeneration observed in the naturally occurring T4R dog is highly sensitive to light²³⁵, and accelerated light damage has been described in the T17M transgenic mouse²⁵⁸ and in multiple P23H animal models^{106,110,112}. The precise mechanism or mechanisms by which light may accelerate degeneration in a subset of patients with rhodopsin-related RP remains to be decisively elucidated. Class II mutant opsins harbouring N-terminus amino acid substitutions have been found to misfold and accumulate within the endoplasmic reticulum (ER) in both cell culture experiments⁹⁷⁻⁹⁹ and in the rods of animal models including the P23H transgenic *Xenopus laevis*^{67,100} and mouse^{101,102}. This is thought to trigger the unfolded protein response (UPR) and lead to apoptotic cell death in the P23H retina^{89,103,104,259}. Tam & Moritz established that dark-rearing rescued retinal degeneration in a *Xenopus laevis* model expressing bovine *Rho*^{P23H}, and that

this was associated with an increase in trafficking of mutant opsin to the OS. The authors further showed that this effect was dependent on an intact chromophore binding site¹⁰⁶. This would suggest a critical role for 11-cis-retinal in the process of ER export of the mutant opsin. Since light causes photoisomerization of the chromophore, they postulated that the higher availability of 11-cis-retinal under dark conditions may facilitate more efficient export of Rho^{P23H}, leading to a lesser degree of ER stress, UPR activation and cell death. In support of this hypothesis, dietary restriction of vitamin A, the precursor of 11-cis-retinal, induced an acceleration of retinal degeneration in the bovine *Rho*^{P23H} transgenic *X. laevis* model¹¹³, and genetic disruption of chromophore synthesis led to a more severe phenotype in the *Rho*^{P23H/+} knock-in mouse¹⁰⁵.

Direct light-induced destabilization of mutant rhodopsin within the OS may also contribute to the exacerbation of disease progression induced by light. Although most P23H rhodopsin is retained in the ER and degraded, a small amount reaches the OS^{105–107} where the mutant protein appears to aggregate. This destabilizes disc membranes causing them to form vesiculotubular structures which can be seen by electron microscopy^{105,108}, and these changes appear to be accelerated by light¹⁰⁹ and deficiency of 11-cis-retinal¹⁰⁵. Further, both a slower rate of degeneration and improved OS morphology have been observed in a transgenic mouse model expressing human RHO^{P23H} when combined with knockout of the guanine nucleotide binding protein G(t) Gnat1 α subunit of transducin²⁶⁰. These data would suggest that light-activation of mutant rhodopsin itself contributes to the degenerative process in the P23H retina, with the implication that dark-rearing may exert its beneficial effect, at least in part, via a total reduction in rhodopsin photoactivation along its biosynthetic pathway and within the OS. Ramon et al. investigated the behaviour of M39R and N55K mutant rhodopsins (both of which are associated clinically with sector RP) in response to light²⁹. In contrast to other described mutations, these proteins did not show a tendency to misfold or mistraffic but instead displayed

abnormal activation kinetics. This would again suggest a direct effect of light on the mutant opsin as the underlying cause of the sectoral pattern of retinal degeneration in these patients.

The red IVCs used here act as long-pass light filters and substantially impede transmission of light with wavelength below 550-600nm (Figure 3.4). Given that the peak spectral sensitivities of free 11-cis-retinal and chromophore-bound rhodopsin are approximately 370-400nm and 495-510nm respectively¹¹³, it follows that such filters should at least be partially effective, regardless of whether the mechanism of light-induced damage is mutant rhodopsin activation, chromophore depletion or a combination of the two.

It is interesting to note that no treatment effect was observed when red filters were applied to animals later in the course of the disease (at PNW7). It is possible that ER accumulation of mutant opsin may be so great at the point of intervention that the additional availability of 11-cis-retinal is insufficient to affect the fate of individual photoreceptors. Indeed, upregulation of proteins associated with the UPR has been detected as early as PND 30 in mice heterozygous for the P23H mutation¹⁰³. It is however difficult to establish the extent to which these findings can be applied to patients with P23H rhodopsin mutations in whom, unlike the *Rho*^{P23H/+} mouse model, disease progression is typically slow²⁶¹.

In this study, housing of mice within red IVCs was associated with a significant reduction in the altitudinal gradient of retinal degeneration observed although this gradient was not completely abolished. A greater treatment effect may be observed if long-pass filters with higher cut-off wavelengths were to be employed as has been previously demonstrated in the tadpole model¹¹³. This would however come at the expense of a further reduction in colour discrimination.

Short wavelength light-filtering IVCs were associated with a relative preservation of cone morphology as determined by immunofluorescence confocal microscopy, and of cone function as quantified by light-adapted ERG. Cones die in RP secondary to rod death, and it is this change which ultimately results in the symptomatic constriction of the visual field in photopic light conditions and eventual loss of central acuity^{46,55}. Degeneration of rods in RP reduces outer retinal oxygen consumption and leads to high levels of oxygen in the surviving tissue⁵⁴. This is thought to trigger cone death through a mechanism of oxidative stress⁵⁵. Reduced levels of rod-derived cone viability factor, an inactive thioredoxin secreted by rods which protects cones from oxidative stress⁵⁹, may also contribute. The relative conservation of cone structure and function observed here likely reflects a delay in the onset of secondary cone death that resulted from a slowing of rod degeneration for animals housed in red cages. Long-term use of red optical filters may thus allow patients with sector RP to retain useful photopic vision for longer.

This treatment could potentially be applied to patients in the form of red-tinted spectacles or contact lenses, although it is likely that the filters would need to be used for a substantial proportion of an individual's waking time over many years to see a significant treatment effect. Use in high luminance light conditions, for example when outdoors, would be of particular importance. Reasonable tolerability of red-tinted long-pass lenses has been reported amongst RP patients with many users reporting acceptable cosmetic appearance, increased ocular comfort and improved visual performance²⁶². No improvement in grating visual acuity has however been demonstrated for patients with RP when using such filters²⁶³.

There are a number of predictable disadvantages associated with the short- and long-term use of red light filters. Firstly, colour discrimination would undoubtedly be affected owing to a substantial reduction in the stimulation of S-cones, and to a lesser extent M-cones (Figure 3.4). This would be expected to produce a tritan-like colour vision abnormality. Some patients may

also dislike seeing the world with a red tint. Studies in healthy subjects have however demonstrated that plasticity of colour perception occurs in response to the extended use of coloured optical filters, suggesting that these perceptual changes may diminish over time²⁶⁴. Secondly, it is conceivable that nyctalopia, already a cardinal symptom in RP, may be exacerbated by the reduction in rod activation consequent upon filtration of short wavelength light. The extent to which this occurs would have to be determined experimentally. This may not however be so problematic, as in such light conditions, the benefit conferred by red-tinted spectacles would be marginal, and they could simply be removed by the patient if found to be troublesome. Thirdly, red filters would be expected to lead to a substantial reduction in activation of retinal melanopsin which has a peak spectral sensitivity of approximately 479nm²⁶⁵. Intrinsically photosensitive retinal ganglion cells express this photopigment and, through their projection to the suprachiasmatic nucleus of the hypothalamus, play a key role in the entrainment of normal sleep-wake cycles and circadian rhythms²⁶⁶. Indeed, sleep disturbance has been demonstrated in patients with advanced nuclear sclerotic type cataracts which act as yellow-tinted optical filters and thus absorb light in the blue part of the spectrum^{267–269}. Cataract surgery has been shown to improve these symptoms²⁷⁰. Interestingly, improvements in sleep quality and latency have been noted following cataract surgery regardless of whether a blue-filtering yellow-tinted or a UV-filtering untinted intraocular lens has been implanted²⁷⁰. One possible explanation for this result is that the melanopsin system may adapt to chronic changes in light spectral composition. Evidence for this comes from a study by Gimenez and colleagues which showed that the acute reduction in the melatonin-suppressing effect of light observed after the application of soft orange-tinted contact lenses, normalised after approximately two weeks of continuous use in healthy subjects²⁷¹. Red filters may not therefore have a substantial impact on sleep and circadian function when applied continuously, although patients would have to be closely monitored for related symptoms in any future clinical trial.

3.6. Conclusions

Long-pass light filters effectively slow the rate of outer retinal degeneration when applied long-term, and from an early stage, in the *Rho*^{P23H/+} mouse model of AD sector RP. No such treatment effect was observed in the *Rpgr*^{-/-} mouse model which shows no altitudinal gradient of retinal degeneration. It would be interesting to investigate the effect of such filters in further animal models of rhodopsin-related RP with both sector and non-sector phenotypes. Red light filters, in the form of tinted spectacles or contact lenses, may represent a low cost and low risk intervention that may slow the rate of photoreceptor loss, and thus prolong the lifetime of useful vision, in patients with sector RP. Prior to clinical trial, it would be important to establish the short- and long-term effects of red filters on visual acuity, contrast sensitivity, colour perception, and their overall tolerability in healthy volunteers and in RP patients.

4. Rhodopsin Supplementation for the Treatment of Dominant RP

4.1. Introduction

Point mutations that induce cell death by a dominant negative mechanism may be amenable to treatment by way of gene augmentation²³¹. Much debate exists as to whether the widely studied P23H mutation in rhodopsin (which falls into Class II according to the classification of Mendes et al.⁷²) causes cell death in this way or alternatively through direct toxicity. In support of a dominant negative mechanism, the human *RHO*^{P23H} transgene appears to induce a more rapid degeneration on an endogenous *Rho*^{+/-} background than on a null *Rho*^{-/-} background¹⁰¹, and additional genomic copies of wild type rhodopsin attenuate the degenerative phenotype⁷⁵. Other studies have however pointed to a toxic gain of function mechanism. In cell culture systems, P23H rhodopsin appears to misfold and accumulate in the ER which may trigger cell death via activation of the unfolded protein response (UPR)^{98,99,103}. Further studies have indicated that a proportion of mutant protein reaches the OS *in vivo* where it may induce cell death following destabilisation of discs^{106,108,109}.

Rhodopsin augmentation is an appealing gene therapy strategy for the treatment of RHO-related ADRP as it has the potential for application in multiple different dominant negative mutations. Since the rods of patients with rhodopsin-related RP already express normal rhodopsin protein, there is also very little risk of the transgenic protein triggering a detrimental immune response.

Mao and colleagues demonstrated that delivery of additional copies of wild type rhodopsin by subretinal injection of a recombinant AAV2/5 could attenuate the degeneration in a *RHO*^{P23H}

transgenic mouse on an endogenous *Rho*^{+/+} background¹⁹¹. This strategy however proved ineffective in an equivalent transgenic mouse on a *Rho*^{+/-} background¹²¹. The background genotype of the animal model used may thus dramatically influence the success or otherwise of the gene augmentation strategy. A knock-in mouse model has recently been developed which carries the P23H mutation at the endogenous rhodopsin locus on chromosome 6¹⁰⁵. Since the heterozygous *Rho*^{P23H/+} knock-in mouse mimics the genotype of affected patients, this is arguably a more appropriate model in which to test potential therapeutics. The effect of AAV-mediated *RHO* gene augmentation in this model has not been previously investigated and remains an important question, as demonstration of rescue may pave the way for human clinical trials for this and other Class II rhodopsin mutations.

4.2. Aims

The principal aims of the experiments outlined in this chapter were as follows:

1. Development and characterisation of *Nrl:GFP* mouse models. Neural retina-specific leucine zipper (*Nrl*) is a transcription factor expressed uniquely in cells of the rod lineage. In this chapter I develop mouse models of RP that also express an enhanced green fluorescent protein (eGFP) transgene under the control of the *Nrl*/promoter. Such mice have rods that fluoresce green and undergo degeneration. The potential advantage of such models is that rods may be directly visualised *in vivo* by blue autofluorescent (BAF) confocal scanning laser ophthalmoscopy (cSLO), and the retinal distribution of any rescue effect or toxicity induced by an intervention readily determined.
2. Development of an optimised human rhodopsin-expressing AAV. Rhodopsin is highly expressed in rod photoreceptors. An efficient rAAV is likely to be required in order to compete with such a high level of genomic expression. An efficient vector is further

desirable as it allows a lower vector dose to be administered to achieve any given therapeutic effect, thus minimizing the risk of capsid-related toxicity or immunogenicity. In the second part of this chapter, four different AAV2/8^{Y733F} rhodopsin expression vectors are designed, manufactured and compared for efficacy *ex vivo* and *in vivo* in the rhodopsin knock-out mouse.

3. To determine the effect of AAV-mediated rhodopsin supplementation on the rate of degeneration in the $Rho^{P23H/+}$ retina. For this, the most effective of the four rhodopsin-expressing AAVs was delivered at one of two doses into the subretinal space of the $Nrl.GFP/+$, $Rho^{P23H/+}$ mouse, and the effect on retinal structure and function determined by SD-OCT, cSLO and ERG.

4.3. Materials & Methods

4.3.1. Animal models & ocular imaging

The $Nrl.GFP/+$, $Rho^{-/-}$ mouse line was generated by an intercross between the rhodopsin knockout mouse as described by Humphries et al.⁷³, and a transgenic mouse line expressing enhanced green fluorescent protein (eGFP) under the control of the neural retina-specific leucine zipper (*Nrl*) promoter (Tg(*Nrl*-EGFP)), obtained as a kind gift from Professor A. Swaroop, Bethesda, MD, USA. The resulting mice have rods labelled in green that express no rhodopsin, do not elaborate OS, and undergo a rapid degeneration. Mice homozygous for the Rho^{P23H} knock-in mutation (B6.129S6(Cg)- $Rho^{tm1.1Kpal}/J$) were obtained from the Jackson laboratories, USA. $Nrl.GFP/+$, $Rho^{P23H/+}$ mice were bred by crossing $Rho^{P23H/P23H}$ knock-in with homozygous $Nrl.GFP$ transgenic mice. Longitudinal imaging was performed by cSLO as described in Section 2.4.3. Blue autofluorescence (BAF) images acquired at a variety of detector

sensitivity settings were imported into ImageJ software for analysis. A region of interest band centred on the optic nerve head with an inner radius of 600 pixels and a width of 255 pixels was used to measure mean grey value (mGV; Figure 4.1)²³². This quantifies the average signal intensity of the sampled region and was used as a global measure of rod survival in degenerate *Nrl.GFP* models. For each animal, the average mGV recorded from the two eyes was used for further analysis.

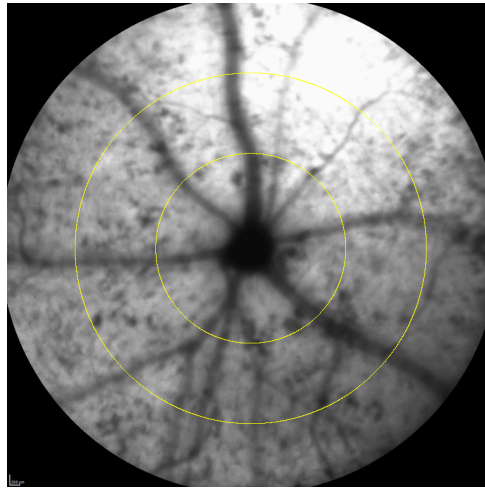


Figure 4.1. Measurement of mean grey value (mGV) from blue autofluorescence (BAF) cSLO fundus images. All images were centred on the optic disc. ImageJ software was used to create a sampling band (shown in yellow) with radius 600 pixels and width 255 pixels centred on the optic disc from which mGV was calculated. The mGV thus quantifies the average signal within the area between the two yellow rings.

4.3.2. Cloning of rhodopsin expression plasmids

Four rhodopsin-expressing AAV2/8^{Y733F} vectors were designed, manufactured and compared for potency (Figure 4.2). The AAV genome in all cases included the human rhodopsin CDS downstream of an 806bp human rhodopsin promoter and Kozak consensus sequence (GCCACC) at positions -1 to -6 relative to the translational start codon. A bovine growth

hormone polyadenylation (polyA) signal sequence was present downstream of the *RHO* CDS and the transgene cassette was flanked by the 3' and 5' ITR sequences derived from AAV2 in all vectors. The four AAVs are described below:

1. **AAV-ssRHO** (AAV2/8^{Y733F}.RHOp.RHO): a single-stranded AAV genome containing the human rhodopsin CDS under the control of the human rhodopsin promoter (Figure 4.2A).
2. **AAV-WPRE** (AAV2/8^{Y733F}.RHOp.RHO.WPRE): as for AAV-ssRHO but with the addition of a 3' WPRE sequence between the *RHO* stop codon and polyA signal (Figure 4.2B).
3. **AAV-Ex/Int** (AAV2/8^{Y733F}.RHOp.Ex/Int.RHO.WPRE): as for AAV-WPRE but with the addition of the first exon and intron of the chicken beta-actin gene together with the splice acceptor from the rabbit beta-globin gene (sequences derived from the CAG promoter) located between the RHOp sequence and Kozak consensus (Figure 4.2C). It is well established that inclusion of introns may lead to a significant enhancement in transgene expression^{272,273}.
4. **AAV-scRHO** (scAAV2/8^{Y733F}.RHOp.RHO): a self-complementary (sc) version of AAV-ssRHO. Self-complementary vectors lead to increased vector efficiency as they bypass intracellular second-strand synthesis, a step thought to be rate-limiting in the process of AAV transduction²⁷⁴. One major drawback of self-complementary vectors is that they have a packaging capacity that is effectively half that of their single-stranded counterparts. This vector is very close to capacity and so self-complementarity has to

be an alternative to the additional elements included in vectors 2 and 3 above (Figure 4.2D).

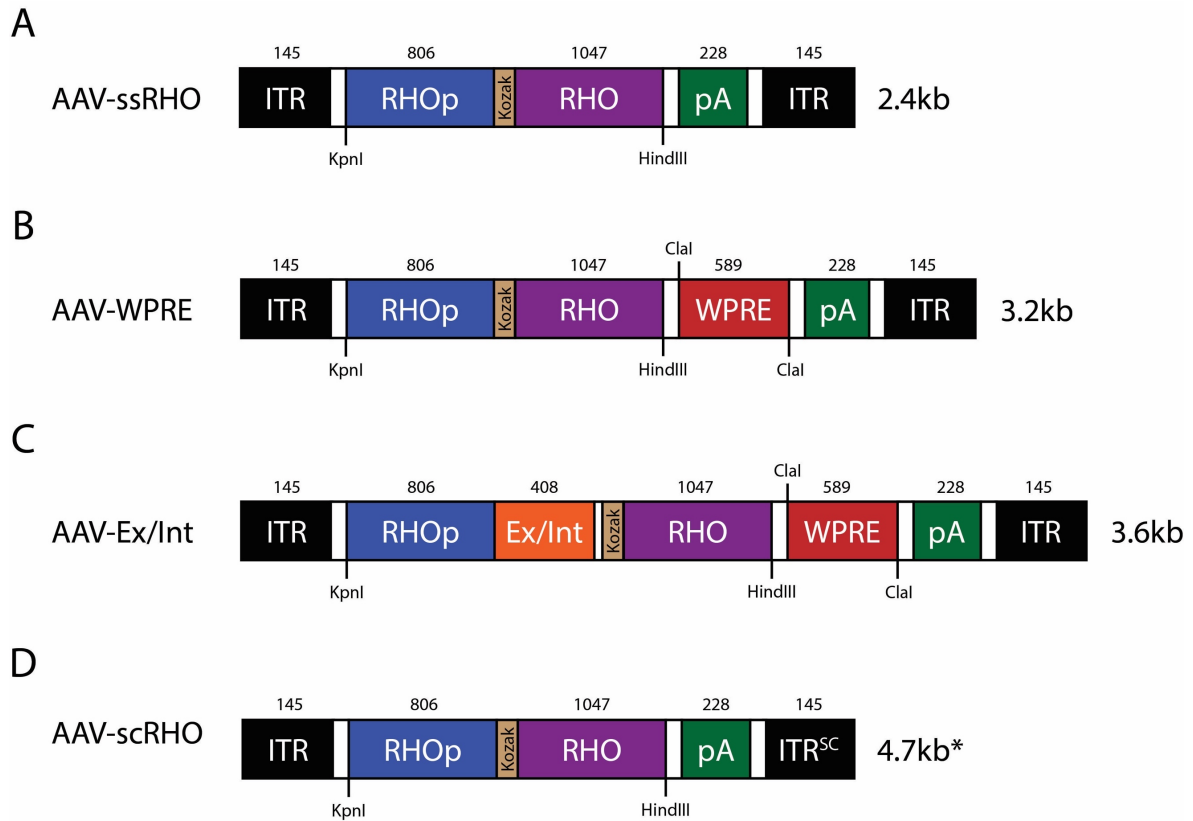


Figure 4.2. Schematics of rhodopsin-expressing rAAV genomes. Size in bp is indicated above each element. The locations of relevant restriction sites are indicated. The size of the entire genome is indicated to the right of each schematic. **(A)** AAV-ssRHO: a single-stranded rhodopsin vector. **(B)** AAV-WPRE: a single-stranded rhodopsin vector with a 3' WPRE sequence. **(C)** AAV-Ex/Int: a single-stranded rhodopsin vector with a 5' splice sequence derived from the CAG promoter and 3' WPRE sequence. **(D)** AAV-scRHO: a self-complementary rhodopsin vector. *Note that this figure reflects the size of the whole packaged genome which includes reverse complement sequences of the rhodopsin transgene, rhodopsin promoter and 5' ITR. ITR: inverted terminal repeat; RHOp: an 806bp length human rhodopsin promoter; Kozak: the Kozak consensus translational start sequence; RHO: the human rhodopsin CDS; WPRE: woodchuck hepatitis virus post-transcriptional regulatory element; pA: bovine growth hormone polyadenylation sequence; Ex/Int: the first exon and intron of the chicken beta-actin

gene together with the splice acceptor from the rabbit beta-globin gene, derived from the CAG promoter; ITR^{SC}: mutant 3' ITR to produce a self-complementary vector.

A self-complementary AAV2 rhodopsin expression plasmid was provided as a kind gift from Dr Daniyar Dauletbekov. The RHOp.RHO sequence was amplified from this using PCR primers that introduced 5' KpnI and 3' HindIII restriction sites (Primers 3 & 4):

KpnI RHOp FW: 5'-AGGTACCCAGATCTTCCCCAC

RHO HindIII RC: 5'-ATAAGCTTTTAGGCCGGGGCCA

An AAV2 CAG.eGFP.WPRE.pA plasmid in which the CAG.eGFP sequence was flanked by 3' KpnI and 5' HindIII restriction sites was provided as a kind gift from Dr Maria Patrício. The AAV-WPRE transgene plasmid was generated by ligating the KpnI/HindIII-digested RHOp.Kozak.RHO amplicon with the backbone generated from KpnI/HindIII digestion of the AAV2 pCAG.eGFP.WPRE.pA as described in Section 2.1 (Figure 4.3).

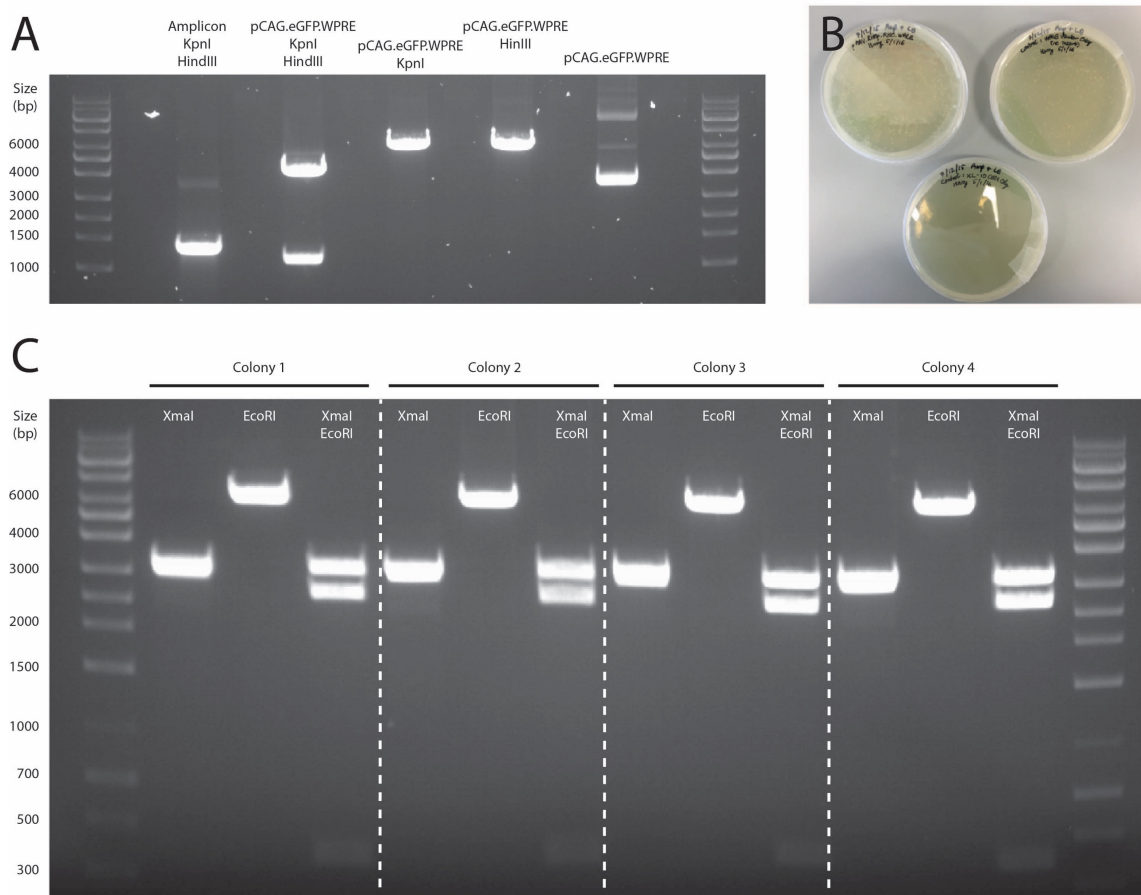


Figure 4.3. Cloning of the AAV-WPRE transgene plasmid. **(A)** Agarose gel of restriction digest products. The 1861bp KpnI/HindIII-digested RHOp.RHO amplicon (first column) was ligated with the 4378bp CAG.WPRE backbone (upper band, second column) and used to transform XL-10 bacteria. **(B)** A large number of colonies grew on the plate inoculated with bacteria transformed with ligation product (top left), whilst few and no colonies were present on the vector-only transformed (top right) and untransformed (bottom) bacterial plates respectively. **(C)** Presence of the transgene was confirmed in miniprep DNA derived from bacterial colonies from the ligation plate shown in (B) by restriction digest using XmaI and EcoRI enzymes. XmaI cut through both ITRs producing two similarly sized fragments of 3kb and 3.2kb, whilst EcoRI cut within the transgene to linearize the 6.2kb plasmid. The combination of both enzymes produced three fragments of 4.3kb, 2.6kb and 0.4kb as expected for all colonies tested.

The WPRE sequence within the AAV-WPRE plasmid was bounded by ClaI restriction sites (Figure 4.2). The AAV-ssRHO transgene plasmid was thus manufactured by restriction digest of the AAV-WPRE plasmid with ClaI and subsequent religation of the larger DNA fragment (Figure 4.4).

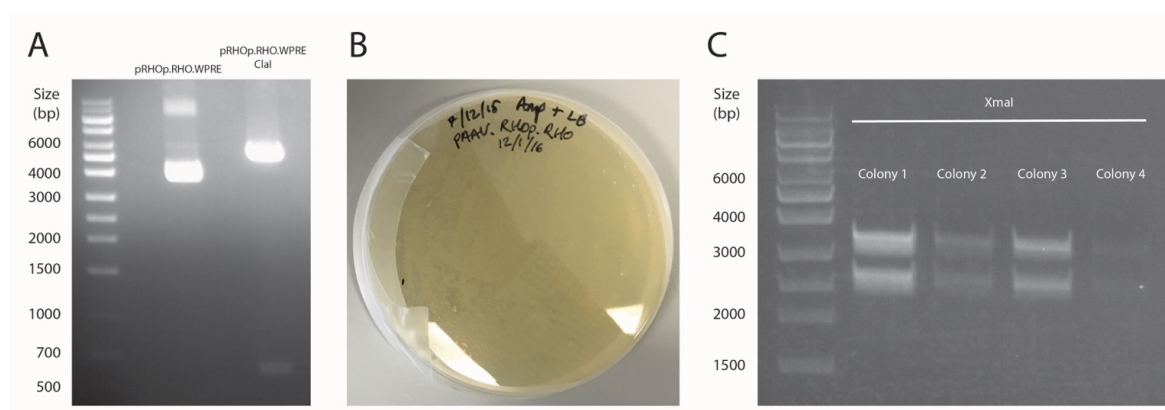


Figure 4.4. Cloning of the AAV-RHO transgene plasmid. (A) Agarose gel of restriction digest products. ClaI digestion allowed removal of the 595bp WPRE DNA fragment from the RHOp.RHO.WPRE transgene plasmid. DNA extracted from the larger 5.6kb fragment was ligated to from plasmid DNA which was used to transfect XL-10 bacteria. (B) Copious growth was achieved after these bacteria were inoculated onto an ampicillin LB-agar plate. (C) Confirmation of the presence of plasmid DNA within bacterial cultures amplified from selected colonies was achieved by XmaI digestion which produced fragments of the expected size (3.2kb and 2.4kb) in all cases.

The AAV-Ex/Int plasmid was cloned by inserting the 408bp chicken beta-actin/rabbit beta-globin sequence amplified from the CAG promoter between the RHOp and Kozak consensus of the AAV-WPRE plasmid by way of an overlap extension PCR strategy (Figure 4.5)²⁷⁵. Successful manufacture of the four transgene plasmids was confirmed in all cases by commercial Sanger sequencing (Source Bioscience, UK).

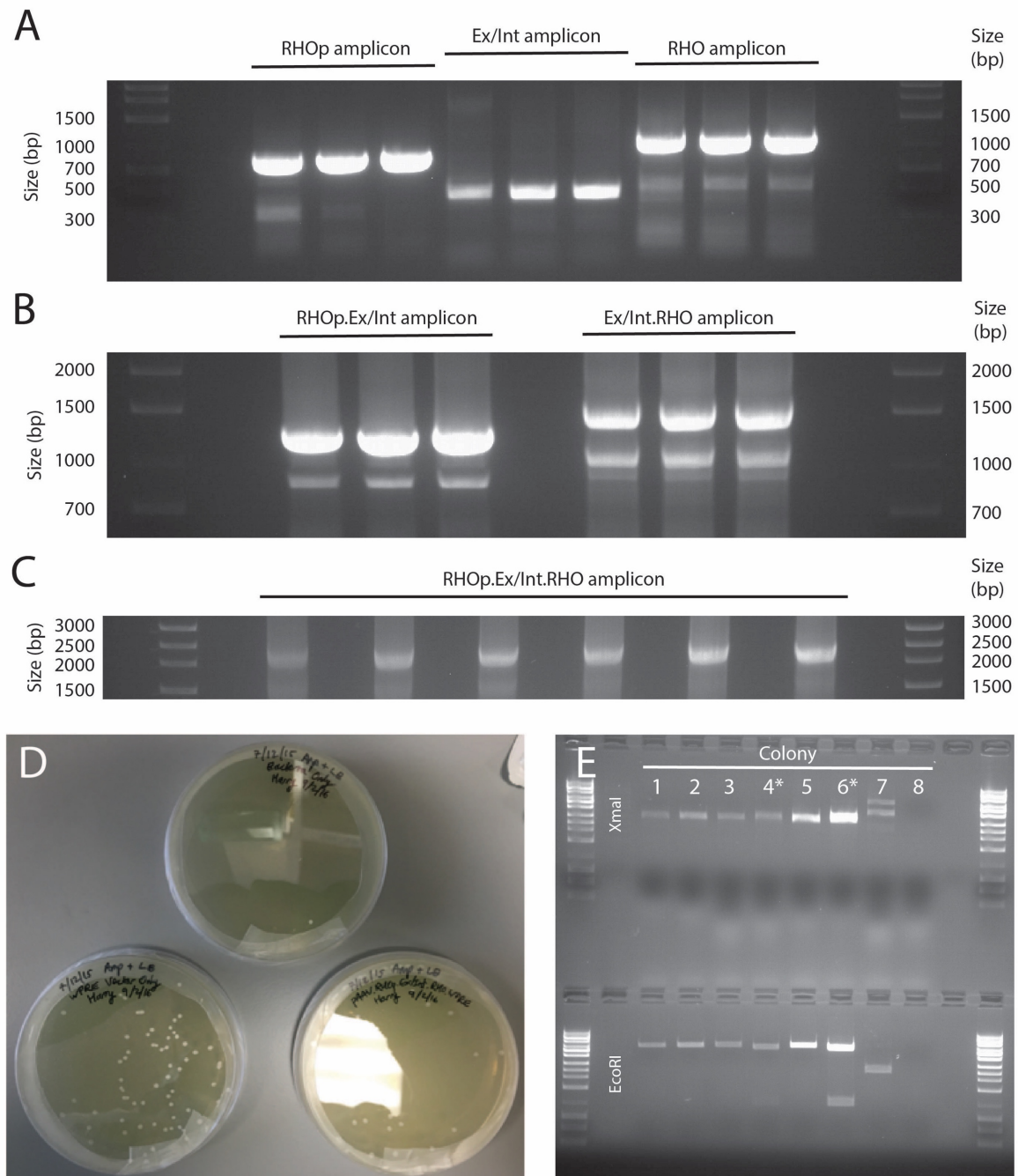


Figure 4.5. Cloning of the AAV-Ex/Int transgene plasmid. (A) RHOp (0.8kb, left) and RHO (1.0kb, right) DNA sequences were amplified from the AAV-RHO plasmid using Primers 3 & 5, and Primers 4 & 8 respectively, whilst the chicken beta-actin/rabbit beta-globin exon-intron-exon sequence (Ex/Int; 0.4kb; centre) was amplified from pCAG.GFP.WPRE template using Primers 6 & 7. (B) Approximate equimolar proportions of RHOp and Ex/Int amplicons were used as template with

Primers 3 & 7 in an overlap extension PCR to generate a composite RHO_{Op}.Ex/Int amplicon (1.2kb; left). In a similar fashion, an Ex/Int.RHO composite amplicon (1.4kb, right) was generated using a mixture of Ex/Int and RHO amplicon templates with Primers 4 & 6. **(C)** In the final step of the overlap extension PCR, the two composite amplicons generated in (B) were mixed in an approximate 1:1 molar ratio and used as template to generate a 2.2kb RHO_{Op}.Ex/Int.RHO insert using Primers 3 & 4 which introduced KpnI and HindIII restriction sites respectively. This amplicon was subcloned into the AAV2-WPRE backbone by KpnI/HindIII digestion and ligation as previously described for the AAV-RHO transgene plasmid. **(D)** Transformation of XL-10 bacteria with the resulting ligation product generated very few colonies (bottom right). Indeed, a greater number of colonies grew on the plate inoculated with bacteria transformed with the vector only control ligation mixture which suggested a high degree of vector self-ligation without incorporation of the insert. **(E)** Plasmid DNA was amplified and extracted from eight colonies selected from the ligation product plate and restriction digests performed using XmaI (top) to confirm presence of the ITR sequences, and EcoRI (bottom) to confirm presence of the insert. The RHO_{Op}.Ex/Int.RHO insert introduced a second EcoRI restriction site meaning that digestion of the AAV-Ex/Int plasmid should result in the presence of two DNA fragments of 5.7kb and 1kb in size. This was confirmed for plasmid DNA derived from colonies 4 and 6 (marked by *). Bacteria from colony 6 were subsequently cultured to generate the AAV-Ex/Int transgene plasmid.

4.3.3. AAV production

AAV-ssRHO, WPRE, Ex/Int and scRHO vectors were manufactured by co-transfection of HEK293T cells with the respective transgene plasmids together with Rep2Cap8^{Y733F} and Helper plasmids as described in Section 2.3. Viral genome titre for concentrated and wash preparations of the four vectors was established by SYBR Green RT-qPCR using primers directed against the bovine growth hormone polyA sequence and capsid purity determined by SDS-PAGE as described in Section 2.3.6. Concentrate vector titres of the order of 10¹³ gc/ml were achieved for AAV-ssRHO, AAV-WPRE and AAV-Ex/Int whilst a lower titre of 1.66 x 10¹² gc/ml was

recorded for AAV-scRHO (Table 4.1). SDS-PAGE analysis suggested a high level of purity for all four vector preparations (Figure 4.6).

	Concentrate Titre (gc/ml)	Wash Titre (gc/ml)
AAV-ssRHO	3.71×10^{13}	9.87×10^{11}
AAV-WPRE	1.54×10^{13}	6.84×10^{11}
AAV-Ex/Int	2.06×10^{13}	1.04×10^{11}
AAV-scRHO	1.66×10^{12}	1.81×10^{10}

Table 4.1. Titres for RHO-expressing AAVs. Titres were determined by SYBR Green RT-qPCR and interpolated from a standard curve constructed using a 10x dilution series of linear RHO.pA DNA. Reaction efficiency was 100%.

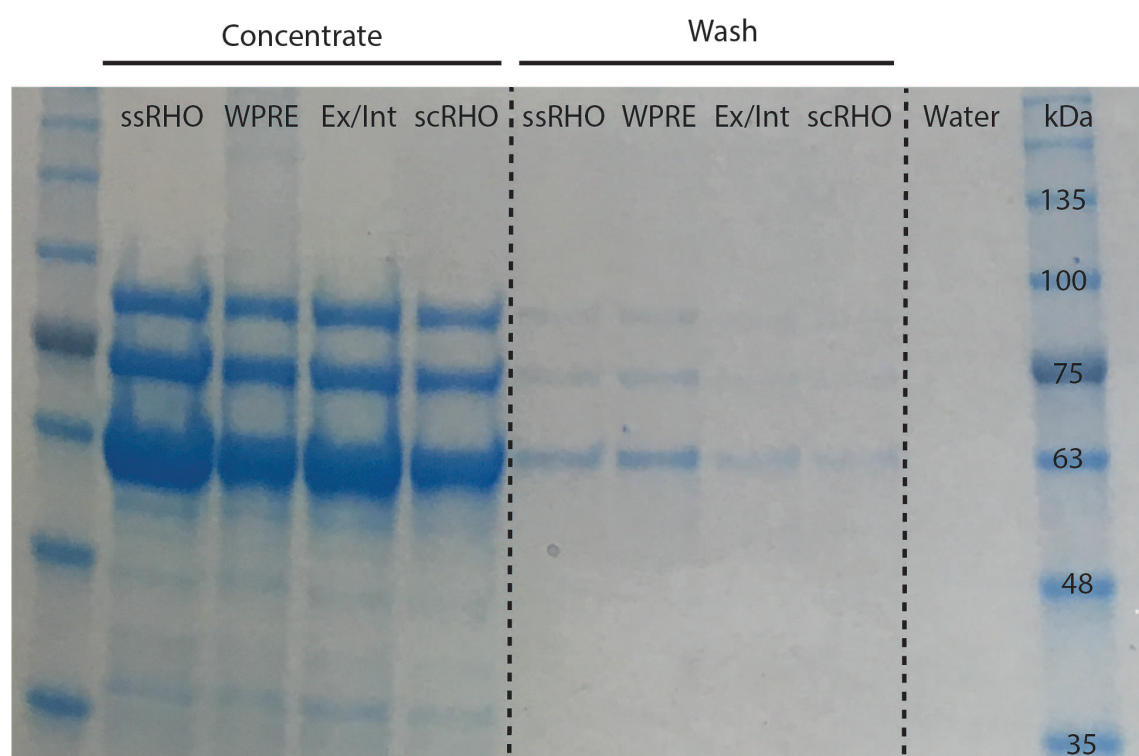


Figure 4.6. SDS-PAGE of recombinant AAV capsid proteins. Following electrophoresis, total protein was stained with EZBlue dye (Sigma Aldrich, UK). Bands corresponding to VP1 (87kDa), VP2 (72kDa) and VP3 (62kDa) proteins in the expected ratio of 1:1:10 were detected in concentrate and

wash samples for all four vectors. No significant additional bands were present indicating a high level of sample purity.

4.3.4. Culture of dissociated retinal cells

Nrl.GFP/+, *Rho^{-/-}* pups were sacrificed at PND three and their retinas extracted and transferred directly into Earle's balanced salt solution (EBSS). Retinal cells were dissociated using a papain-based dissociation system (Worthington Biochemical, USA) according to a modified version of the manufacturer's protocol. Briefly, the dissected retinal cups were placed into 250µl of papain solution made up of 20 units/ml papain and 0.005% DNase in EBSS and incubated in a water bath set to 37°C for ten minutes. The tubes were agitated by gentle flicking every two minutes over the course of this time, after which the suspension was triturated further by pipetting up and down twice through a 1ml tip. The suspension was centrifuged at 300g for five minutes and the supernatant discarded. The remaining pellet was resuspended in 525µl of resuspension solution (450µl EBSS, 50µl albumin-ovomucoid inhibitor, and 25µl DNase solution). The resulting cell suspension was carefully layered on top of 500µl albumin-ovomucoid inhibitor within a 2ml centrifuge tube which was then sealed and centrifuged at 70g for six minutes. The resulting supernatant was discarded and the remaining cell pellet resuspended in up to 1ml complete neurobasal A medium (neurobasal A with B27 and N2 supplements, 0.8mM L-glutamine, 100 units/ml penicillin, and 100µg/ml streptomycin). Cells were counted using a haemocytometer, seeded into 24-well plates at a density of 2×10^5 cells/well in 1ml complete neurobasal A, and incubated at 34°C with 5% CO₂. After 24 hours, cells were transduced with AAV at a multiplicity of infection (MOI) of 2×10^4 . Half of the volume of media was removed on alternate days thereafter and replaced with fresh complete neurobasal A. After ten days in culture, cells were harvested for RNA extraction or processed for ICC as described in Section 2.5.1. The primary antibody used for ICC was rabbit polyclonal anti-rhodopsin antibody ab3424

(Abcam, UK), whilst donkey anti-rabbit IgG-568, Alexa-Fluor ab175470 (Abcam, UK) was used as secondary antibody.

4.3.5. RT-qPCR

In order to differentiate between endogenous mouse and AAV-derived human rhodopsin transcripts, TaqMan gene expression assays (Thermo Fisher Scientific, UK) directed against each rhodopsin species were optimised prior to experimental application (supplier codes Hs00892431 and Mm01184405). Each probe was tested in a RT-qPCR (see Section 2.1.6) against a ten-fold dilution series of linear rhodopsin DNA of its corresponding species and reaction efficiencies of 100% were confirmed (Figure 4.7A, B). The human probe was further tested against a 10-fold dilution series of linear mouse *Rho* DNA and *vice versa* to ascertain the degree to which the probes may be considered specific for their intended species. The human TaqMan probe detected mouse *Rho* DNA around 20 cycles after it detected an equivalent copy number of the human gene (Figure 4.7C). The human probe was thus over 1,000,000 times less sensitive to the mouse sequence than the human sequence. The mouse probe on the other hand detected human *RHO* DNA approximately ten cycles after that for an equivalent copy number of the mouse gene (Figure 4.7D). This probe was thus over 1,000 times less sensitive to the human sequence than the mouse sequence. In a mixed population of cDNA, as might be expected after AAV-RHO gene therapy in the *Rho*^{P23H/+} retina, use of both probes would therefore be expected to detect the expression levels of both endogenous mouse and transgenic human with an error attributable to cross-reactivity of less than 0.1%. Further optimisation RT-qPCRs in which the probes were used against mixed populations of mouse and human rhodopsin DNA of known concentrations revealed that each probe was indeed able to accurately determine the starting concentration of its corresponding species. As the mouse TaqMan probe spans the fourth and fifth exons of the rhodopsin gene, cDNA derived from both wild type and P23H endogenous alleles should be detected by the assay.

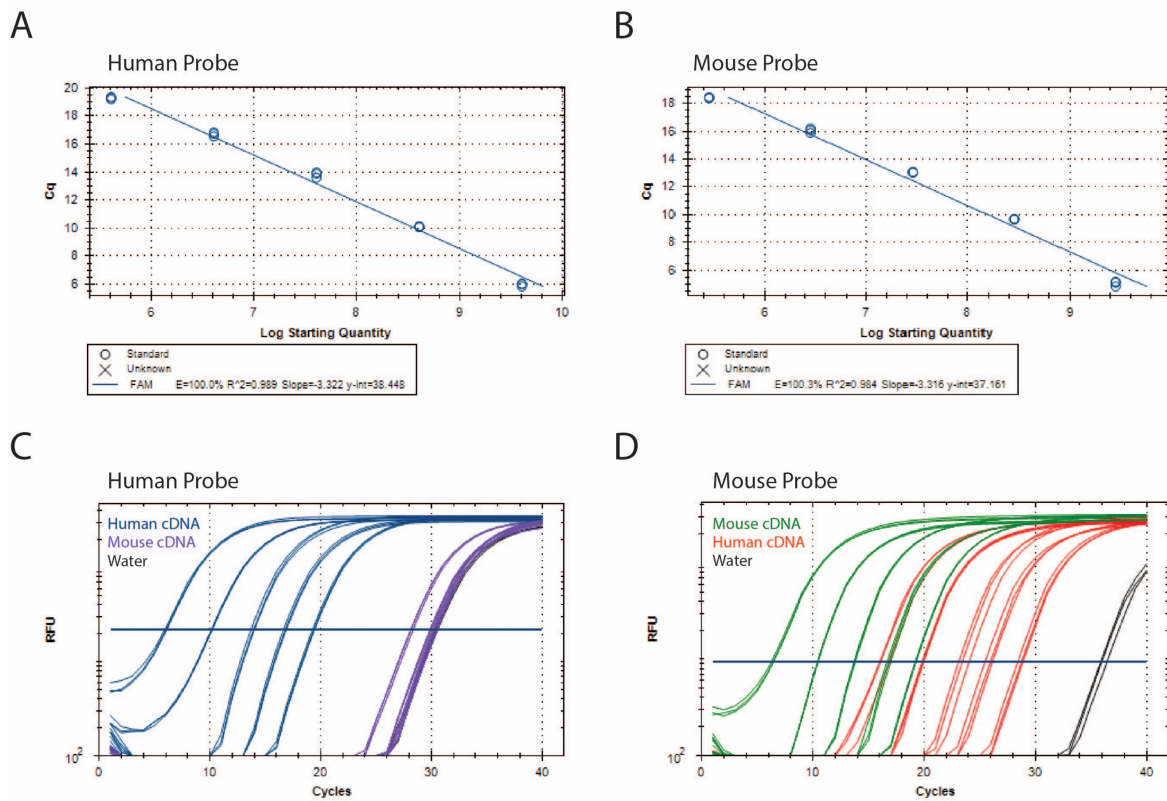


Figure 4.7. Optimisation of RT-qPCR using TaqMan probes designed to detect human and mouse rhodopsin cDNA. (A & B) Standard curves constructed using each TaqMan probe with a dilution series of template DNA of its corresponding species. **(A)** Human probe with human DNA template in a ten-fold dilution series from 2.03×10^9 copies to 2.03×10^5 copies. Reaction efficiency was 100.0%. **(B)** Mouse probe with mouse DNA template in a ten-fold dilution series from 1.42×10^9 copies to 1.42×10^5 copies. Reaction efficiency was 100.3%. **(C & D)** Reaction curves plotting signal in relative fluorescent units (RFU) on a log scale against cycle number. **(C)** Human probe. Blue curves represent the reactions using the human DNA ten-fold dilution series from 2.03×10^9 copies to 2.03×10^5 copies that were used to calculate reaction efficiency. Purple curves plot the reactions using a mouse DNA ten-fold dilution series from 1.42×10^9 copies to 1.42×10^5 copies. **(D)** Mouse probe. Green curves represent the reactions using the mouse DNA ten-fold dilution series from 1.42×10^9 copies to 1.42×10^5 copies that were used to calculate reaction efficiency. Red curves plot the reactions using a human DNA ten-fold dilution series from 2.03×10^9 copies to 2.03×10^5 copies.

4.3.6. Subretinal injection

Subretinal injection of mice was performed as described in Section 2.4.2. All subretinal injections were delivered superiorly to mice aged PNW3. A total volume of 1.5µl of vector suspension in PBS vehicle (or PBS alone in the case of sham injection) was delivered into the subretinal space and in all cases this was supplemented with 0.0001% Pluronic F-68 non-ionic surfactant (Thermo Fisher Scientific, UK) to reduce adherence and retention of AAV capsids within the injection system²⁷⁶.

4.3.7. Immunohistochemistry

Retinal tissue was processed and IHC was performed as described in Section 2.5.2. The primary antibody used for IHC was rabbit polyclonal anti-rhodopsin antibody ab3424 (Abcam, UK), whilst donkey anti-rabbit IgG-568, Alexa-Fluor ab175470 (Abcam, UK) was used as secondary antibody.

4.4. Results

4.4.1. Natural history of fluorescence decay in the *Nrl.GFP/+*, *Rho*^{-/-} retina

To validate the use of confocal scanning laser ophthalmoscopy (cSLO) as a tool to directly observe and track the progressive loss of rods in degenerate *Nrl.GFP* models, longitudinal imaging along with histology was performed first in the *Nrl.GFP/+*, *Rho*^{-/-} mouse (Figure 4.8). This technique demonstrated a uniform distribution of fluorescence decay (and thus photoreceptor loss) over time (Figure 4.8A). At PNW3 the mean grey value (mGV) measured from blue autofluorescence (BAF) images was reduced to 66.1% of that recorded from age-matched *Nrl.GFP/+*, *Rho*^{+/+} retinas at a detector sensitivity of 70. Over the following nine weeks, mGV decreased further in the *Nrl.GFP/+*, *Rho*^{-/-} model, whilst that recorded from *Nrl.GFP/+*, *Rho*^{+/+} eyes remained stable at PNW15 (Figure 4.8C; $F(3, 54)=7.79$, $p=0.0002$ for

effect of time; $F(1, 18)=264.4$, $p<0.0001$ for effect of model, repeated measures two-way ANOVA). Fluorescence microscopy of histological sections over this time period demonstrated progressive thinning of the green fluorescent ONL in the *Nrl.GFP/+*, *Rho*^{-/-}, but not in the *Nrl.GFP/+*, *Rho*^{+/+} retina (Figure 4.8B).

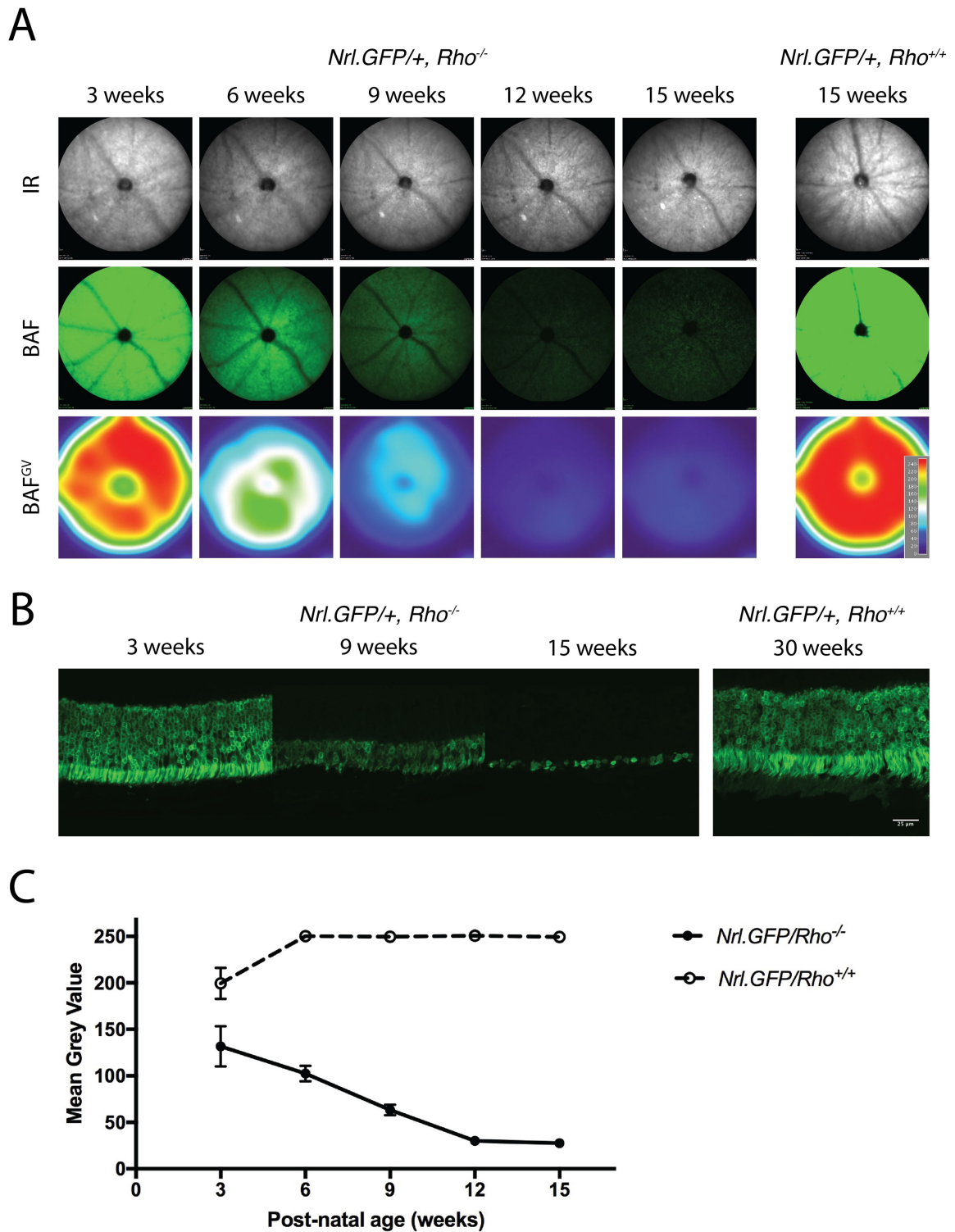


Figure 4.8. Natural history of fluorescence decay in the *Nrl.GFP/+*, *Rho*^{-/-} mouse retina. (A) Longitudinal confocal SLO imaging of a representative animal between postnatal weeks 3 and 15. Upper panels show near infrared (IR) images. Middle panels show blue autofluorescence (BAF) micrographs captured in the same focal plane as their corresponding IR images, acquired at a detector sensitivity of

70. Lower panels show corresponding surface plots in which grey value for each pixel within BAF images has been colour coded according to the inset scale (BAF^{GV}). **(B)** Fluorescence microscopy of retinal cryosections derived from *Nrl.GFP/+*, *Rho*^{-/-} mice at PNW 3, 9 and 15. **(C)** Mean grey value recorded from BAF images at a sensitivity of 70 as a function of time in *Nrl.GFP/+*, *Rho*^{-/-} (n=12) and *Nrl.GFP/+*, *Rho*^{+/+} (n=8) eyes.

4.4.2. Natural history of fluorescence decay in the *Nrl.GFP/+*, *Rho*^{P23H/+} retina

The same imaging protocol was next applied to *Nrl.GFP/+*, *Rho*^{P23H/+} mice. At PNW3, mGV was 83.6% of that recorded in *Nrl.GFP/+*, *Rho*^{+/+} mice at a detector sensitivity of 70. The mGV steadily decreased over time in the *Nrl.GFP/+*, *Rho*^{P23H/+} model (F(5, 90)=18.0, p<0.0001 for effect of time, repeated measures two-way ANOVA), but at a rate slower than that observed in the knockout eye (Figure 4.9). As previously described in the *Rho*^{P23H/+} knock-in mouse²⁷⁷, degeneration in the *Nrl.GFP/+*, *Rho*^{P23H/+} retina was markedly faster in the inferior than in the superior retina (Figure 4.9B). This difference was detectable *in vivo* by cSLO BAF imaging (Figure 4.9A). Histological examination of these retinas revealed shortened IS and rudimentary or absent OS when compared with wild type (Figure 4.9B).

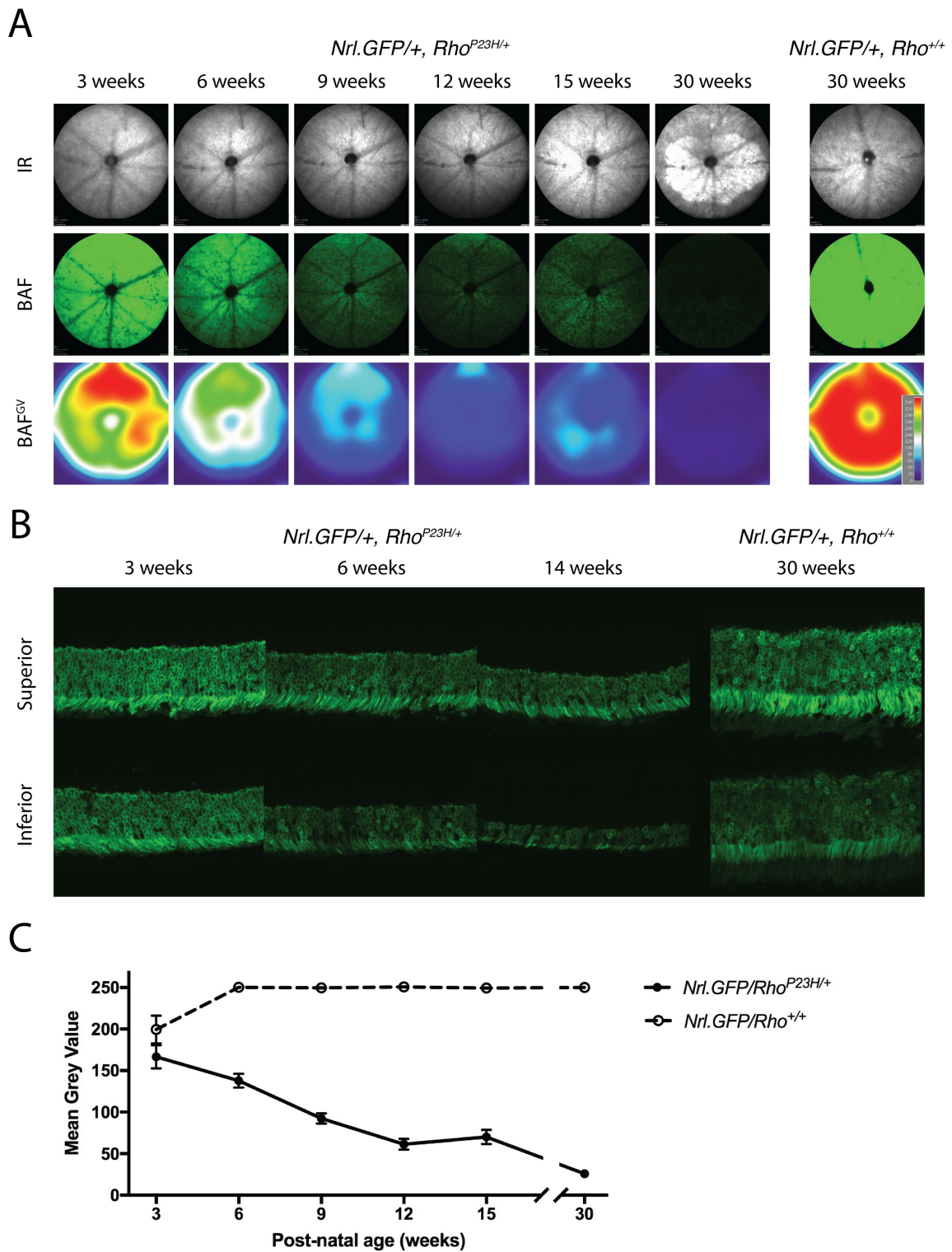


Figure 4.9. Natural history of fluorescence decay in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse retina.

(A) Longitudinal confocal SLO imaging of a representative animal between postnatal weeks 3 and 15. Upper panels show near infrared (IR) images. Middle panels show blue autofluorescence (BAF) micrographs captured in the same focal plane as their corresponding IR images, acquired at a detector

sensitivity of 70. Lower panels show corresponding surface plots in which grey value for each pixel within BAF images has been colour coded according to the inset scale (BAF^{GV}). **(B)** Fluorescence microscopy of retinal cryosections derived from *Nrl.GFP/+*, *Rho^{P23H/+}* mice at PNW 3, 6 and 14. Images were acquired of the midperipheral retina from vertically orientated cryosections in the plane of the optic disc at equal distances superior and inferior of the nerve head margin. Note the more rapid thinning of the green fluorescent photoreceptor layer in the inferior than the superior retina. **(C)** Mean grey value recorded from BAF images at a sensitivity of 70 as a function of time in *Nrl.GFP/+*, *Rho^{P23H/+}* (n=12) and *Nrl.GFP/+*, *Rho^{+/+}* (n=8) eyes.

4.4.3. SD-OCT phenotype of the *Nrl.GFP/+*, *Rho^{P23H/+}* retina

The low signal band corresponding to the ONL on SD-OCT sections was thinner in *Nrl.GFP/+*, *Rho^{P23H/+}* retinas compared with that observed in *Nrl.GFP/+*, *Rho^{+/+}* mice and this difference became more marked with increasing age (Figure 4.10A-C). Progression of photoreceptor layer (PRL, measured between OPL and RPE high contrast lines) degeneration recorded from SD-OCT images could be modelled as an exponential decay function with formula:

$$\text{PRL}(t) = 92.68e^{-0.175t} + 8.12$$

where t = time in PNW (goodness to fit $r^2 = 0.930$; Figure 4.10B).

In keeping with the histological data presented in Section 4.4.2, a more rapid rate of degeneration was apparent in the inferior than the superior retina in *Nrl.GFP/+*, *Rho^{P23H/+}* mice (Figure 4.10C). The ellipsoid zone (EZ) band is thought to represent the outer portions of photoreceptor inner segments which are packed with mitochondria⁶. This band was prominent in *Nrl.GFP/+*, *Rho^{+/+}* animals, but was difficult to distinguish from background signal in the *Nrl.GFP/+*, *Rho^{P23H/+}* model (Figure 4.10D).

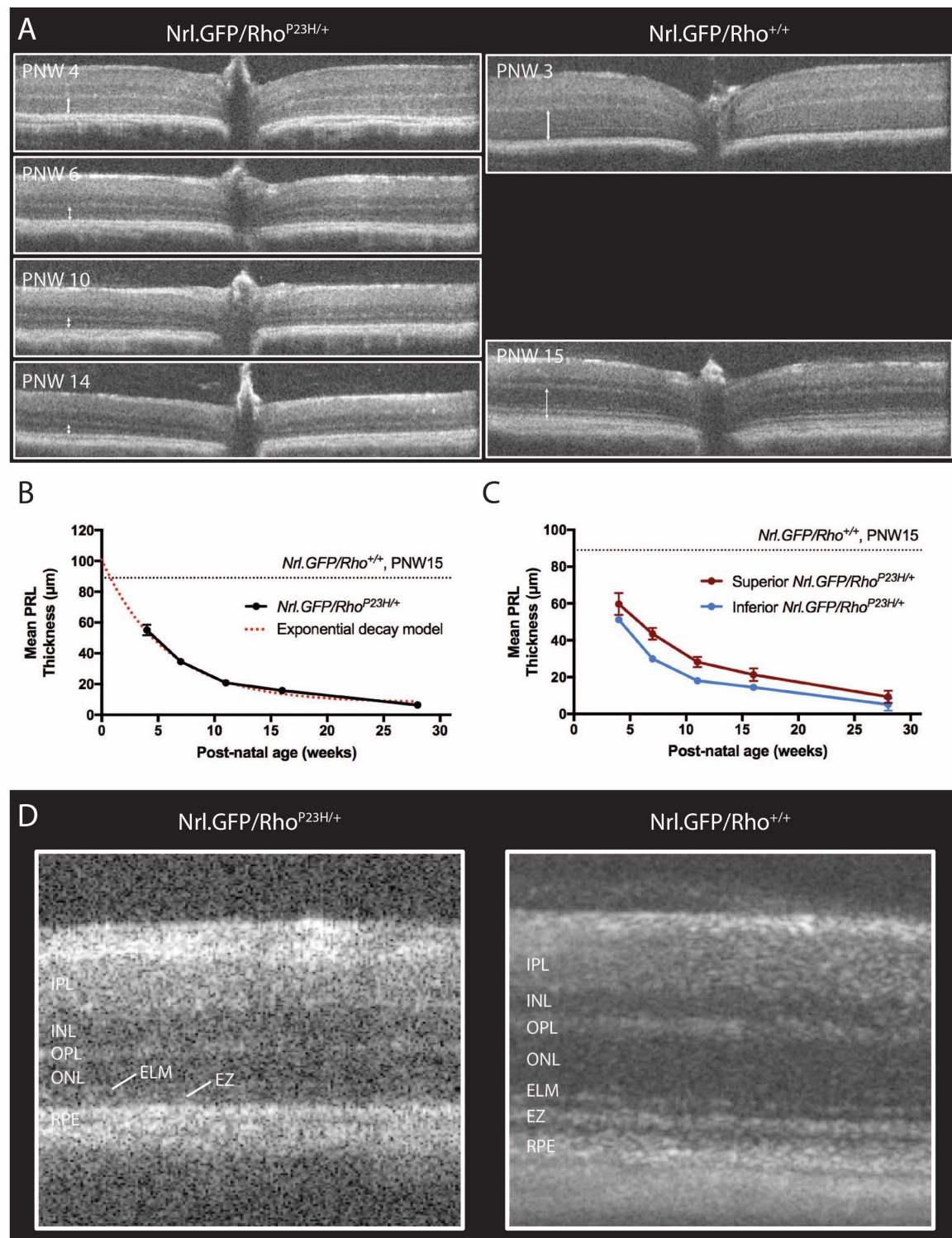


Figure 4.10. SD-OCT natural history of retinal degeneration in the *Nrl.GFP/+*, *Rho^{P23H/+}* mouse. (A) Representative SD-OCT sections taken through the horizontal meridian of *Nrl.GFP/+*, *Rho^{P23H/+}* retinas of mice aged PNW 4, 6, 10 and 14 and *Nrl.GFP/+*, *Rho^{+/+}* mice aged PNW 3 and 15.

The thickness of the surviving photoreceptor layer (PRL, measured as the vertical distance between the high contrast OPL and RPE bands) is indicated in each example by a white arrow. A steady decline in PRL thickness was observed in *Nrl.GFP/+*, *Rho^{P23H/+}* (left), but not in *Nrl.GFP/+*, *Rho^{+/+}* (right) animals. **(B)** Mean PRL thickness as a function of time in the *Nrl.GFP/+*, *Rho^{P23H/+}* retina (n = at least six for each data point). Error bars represent the 95% confidence interval about the mean. The horizontal dotted line represents mean PRL thickness measured from *Nrl.GFP/+*, *Rho^{+/+}* eyes (n=6), whilst the red dotted curve plots the exponential decay best-fit model defined as $PRL(t) = 92.68e^{-0.175t} + 8.12$ ($r^2 = 0.930$). **(C)** PRL thickness of the superior and inferior retina as a function of time in the *Nrl.GFP/+*, *Rho^{P23H/+}* retina (n = at least 6 for each data point). Error bars represent the 95% confidence interval about the mean. The horizontal dotted line represents mean PRL thickness measured from *Nrl.GFP/+*, *Rho^{+/+}* eyes (n=6). The PRL was thinner in the inferior retina than superior retina at any given age (repeated measures two-way ANOVA with time and retinal location as factors: $F(1, 32)=15.2$, $p<0.0001$ for effect of retinal location). **(D)** Magnified view of SD-OCT sections from equivalent retinal loci of PNW6 *Nrl.GFP/+*, *Rho^{P23H/+}* (left) and *Nrl.GFP/+*, *Rho^{+/+}* (right) retinas. IPL: Inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; ELM: external limiting membrane; EZ: ellipsoid zone; RPE: retinal pigment epithelium.

4.4.4. Comparison of RHO-AAVs in dissociated *Nrl.GFP/+*, *Rho^{-/-}* retinal cells

Cultured dissociated retinal cells from PND3 *Nrl.GFP/+*, *Rho^{-/-}* mice were transduced with the four previously manufactured *RHO* rAAVs: AAV-ssRHO, AAV-WPRE, AAV-Ex/Int and AAV-scRHO. After staining for rhodopsin using a secondary antibody tagged with a 568nm fluorophore, red fluorescent signal could be detected in a proportion of cells in all groups (Figure 4.11). All cells with a red fluorescent signal also showed a strong green signal suggesting that expression of the transgene was confined to cells of the rod photoreceptor lineage in which the *Nrl* promoter is active (Figure 4.11).

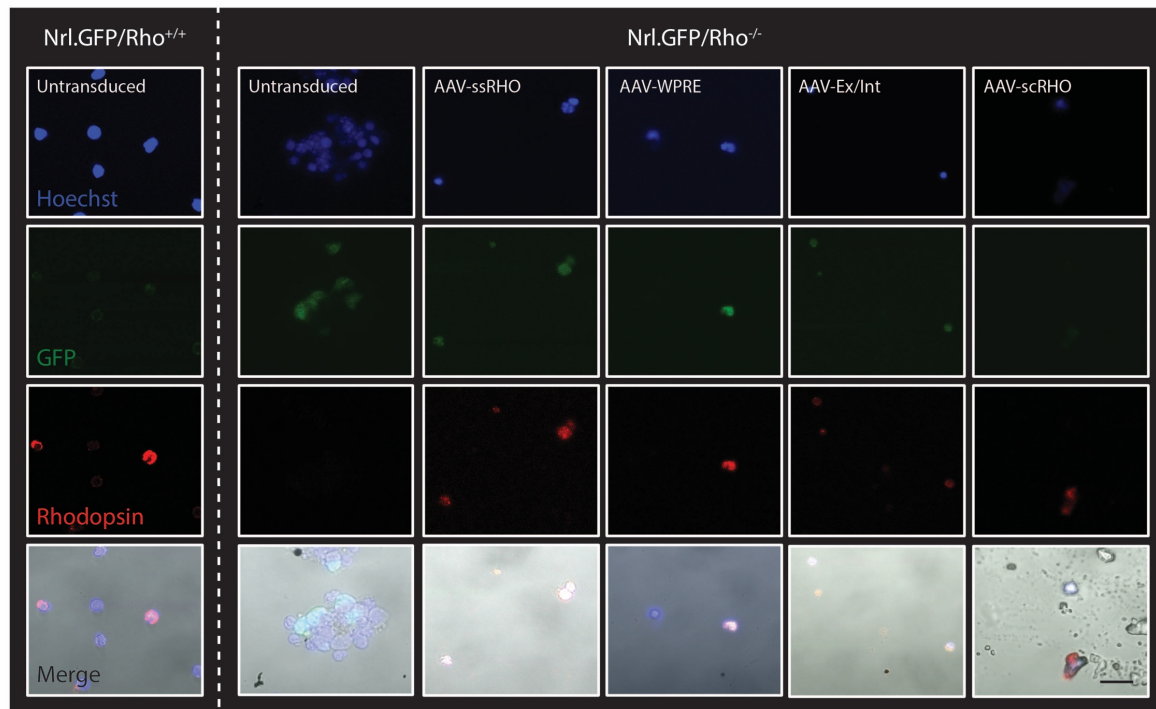


Figure 4.11. Transduction with RHO-AAVs induces expression of rhodopsin in dissociated cells derived from *Nrl.GFP/+*, *Rho*^{-/-} retinas. ICC was performed in which untransduced and transduced dissociated retinal cells were stained for rhodopsin. Left-hand panels demonstrate the staining pattern of cultured PND3 *Nrl.GFP/+*, *Rho*^{+/+} retinal cells in which the subset of cells expressing GFP stained for rhodopsin. Untransduced *Nrl.GFP/+*, *Rho*^{-/-} retinal cells on the other hand did not express rhodopsin protein. In transduced *Nrl.GFP/+*, *Rho*^{-/-} samples, a proportion of GFP-positive cells stained positively for rhodopsin indicating expression of the transgene in cells of the rod lineage for each of the four vectors. Scale bar = 20μm.

Transgene expression in dissociated *Nrl.GFP/+*, *Rho*^{-/-} retinal cells transduced with AAV-scRHO was found to be approximately four times greater than that of cells transduced with the other three single-stranded vectors (Figure 4.12; $F(3, 8)=10.87$, $p=0.003$, ordinary one-way ANOVA). This difference may be attributed to the bypassing of second strand synthesis by self-complementary vectors, a process thought to be rate-limiting in the AAV transduction pathway^{149,187}.

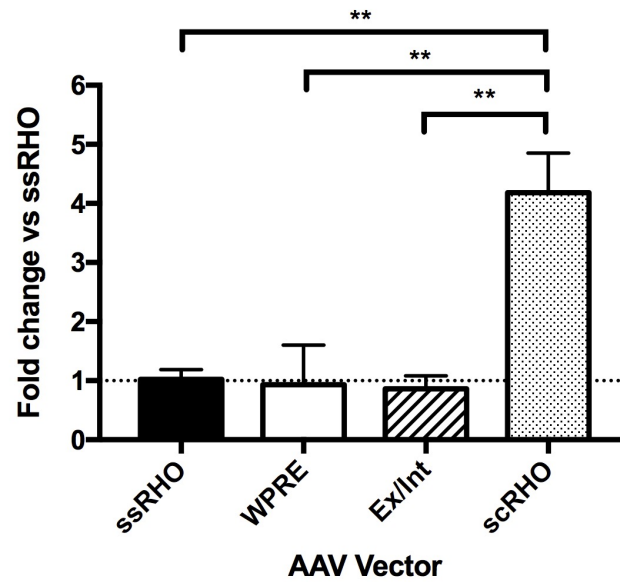


Figure 4.12. Human rhodopsin transgene expression in dissociated cultured *Nrl.GFP/+*, *Rho*^{-/-} cells following transduction with RHO-AAVs. RT-qPCR for transgenic human *RHO* using Primers 11 & 12 demonstrated increased expression levels for AAV-scRHO compared with the other vectors. n=3 per group, **p<0.01, Tukey's multiple comparisons test.

4.4.5. Comparison of RHO-AAVs in *Nrl.GFP/+*, *Rho*^{-/-} mice *in vivo*

Each of the four AAVs was injected subretinally at a dose of 2×10^9 gc in a total volume of 1.5 μ l in PNW3 *Nrl.GFP/+*, *Rho*^{-/-} mice. Four weeks later, animals were subjected to SD-OCT imaging. Mice were then sacrificed and retinas harvested for IHC (n=6 eyes per vector) and western blot (n=6 eyes per vector) for human transgene detection. Figure 4.13 shows retinal cryosections from such mice stained for rhodopsin. All four viruses were capable of driving human rhodopsin protein expression in the murine retina. Transduced rods elaborated OS packed with rhodopsin that were absent in untransduced eyes. Rhodopsin expression was confined to this layer mimicking the wild type state and confirming promoter specificity. Rhodopsin expression appeared greatest in AAV-Ex/Int and AAV-scRHO injected eyes.

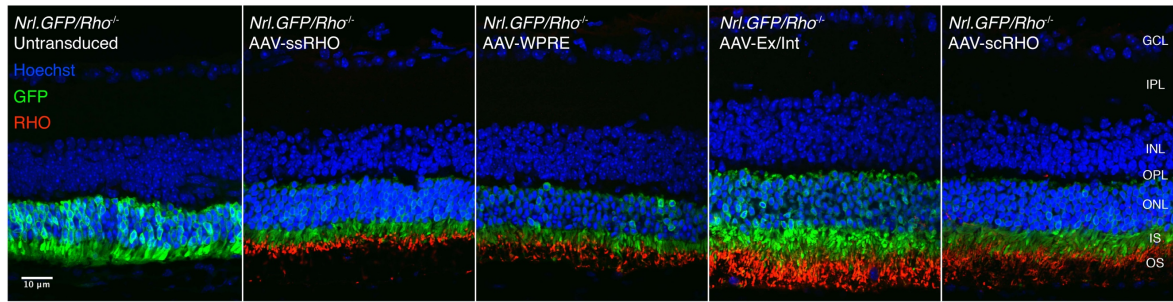


Figure 4.13. Transgenic human rhodopsin expression in the *Nrl.GFP/+*, *Rho*^{-/-} retina following subretinal injection of RHO-AAV vectors. IHC of retinal cryosections processed from *Nrl.GFP/+*, *Rho*^{-/-} eyes harvested four weeks after subretinal injection of AAV-RHO vectors. Representative images are shown following immunostaining for rhodopsin (red). The outer segment (OS) layer was absent in untransduced retina (left-hand panel), whilst all four AAVs were capable of inducing OS growth (remaining panels). Rhodopsin expression within OS appeared greatest for retinas transduced with AAV-Ex/Int and AAV-scRHO.

Mean PRL thickness as determined from SD-OCT image analysis was greatest for *Nrl.GFP/+*, *Rho*^{-/-} eyes injected with AAV-Ex/Int and AAV-scRHO (Figure 4.14). Similarly, these two vectors induced the highest levels of rhodopsin protein expression in *Nrl.GFP/+*, *Rho*^{-/-} eyes as measured by western blot performed using protein lysates derived from transduced neural retinas (Figure 4.15). The performance of the AAV-Ex/Int vector in dissociated retinal cells *ex vivo* (Figure 4.12) was not therefore predictive of that determined *in vivo* which might limit the utility of the dissociated cell assay for validation of such vectors. Taken together, these data would suggest that the addition of the WPRE and chicken beta-actin/rabbit beta-globin exon-intron-exon sequences can allow similar levels of rod-specific rhodopsin transgene expression to be achieved using a single-stranded construct as that associated with a self-complementary vector, but with a significantly smaller AAV genome.

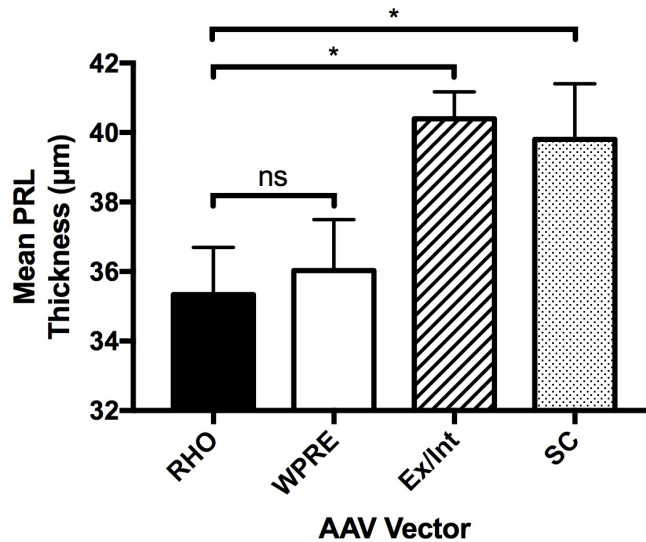


Figure 4.14. Mean PRL thickness measured by SD-OCT following subretinal injection of RHO-AAV vectors in *Nrl.GFP/+*, *Rho*^{-/-} mice. n=9 eyes per group. *p<0.05, Holm-Sidak multiple comparisons test.

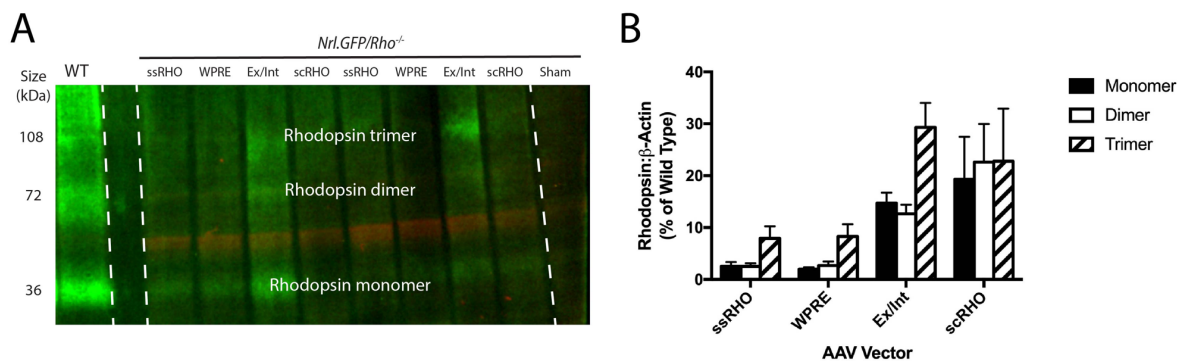


Figure 4.15. Comparison of rhodopsin protein expression in the *Nrl.GFP/+*, *Rho*^{-/-} mouse following subretinal injection with RHO-AAVs. Retinas were harvested four weeks after subretinal injection with 2x10⁹ gc AAV-ssRHO, AAV-WPRE, AAV-Ex/Int, and AAV-scRHO (n=6 each) or PBS (sham). (A) Western blot using whole neural retinal protein lysates stained using fluorescent secondary antibodies for rhodopsin (green) and β-actin (red). WT: age-matched wild type retinal lysate diluted 1:10. (B) Western blot rhodopsin protein expression levels normalised to β-actin loading control in transduced retinas expressed as a percentage of that measured from untransduced wild type eyes.

Relative expression levels of monomeric, dimeric and trimeric forms of the protein are shown for each group (n=6). Two-way ANOVA with vector and multimeric structure as factors: $F(3, 20)=8.3$, $p=0.0009$ for effect of vector, no statistically significant interaction between factors. Tukey's multiple comparison test: ssRHO vs WPRE $p>0.9999$; ssRHO vs Ex/Int $p=0.0212$; ssRHO vs scRHO $p=0.0057$; WPRE vs Ex/Int $p=0.0211$; WPRE vs scRHO $p=0.0057$; Ex/Int vs scRHO $p=0.9343$.

4.4.6. RHO rAAVs induce rhodopsin overexpression in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse

To determine the level of rhodopsin overexpression achievable following subretinal delivery of RHO-expressing rAAVs in the *Nrl.GFP/+*, *Rho*^{P23H/+} retina, the two most efficient vectors- AAV-Ex/Int and AAV-scRHO- were delivered at a dose of 2×10^9 gc unilaterally into the subretinal space of PNW3 *Nrl.GFP/+*, *Rho*^{P23H/+} mice. Four weeks later, retinas were harvested and RHO protein levels compared between injected and uninjected eyes by western blot (Figure 4.16). A higher level of RHO was detected in injected versus uninjected retinas for both AAV-Ex/Int and AAV-scRHO, although this difference did not reach statistical significance for the latter vector (two-way ANOVA with eye and multimeric structure as factors: $F(1, 5)=10.3$, $p=0.023$ and $F(1, 5)=2.1$, $p=0.21$ for AAV-Ex/Int and AAV-scRHO respectively for effect of eye). The greatest difference between the two eyes in both cases was for monomeric forms of the protein (Figure 4.16). Taking all forms of rhodopsin into account, mean level of overexpression was 51.4% for AAV-Ex/Int and 52.6% for AAV-scRHO. It is interesting to note that the ratio of multimeric forms of rhodopsin observed by western blot differed depending upon whether the protein was of transgenic (Figure 4.15) or genomic (Figure 4.16) origin.

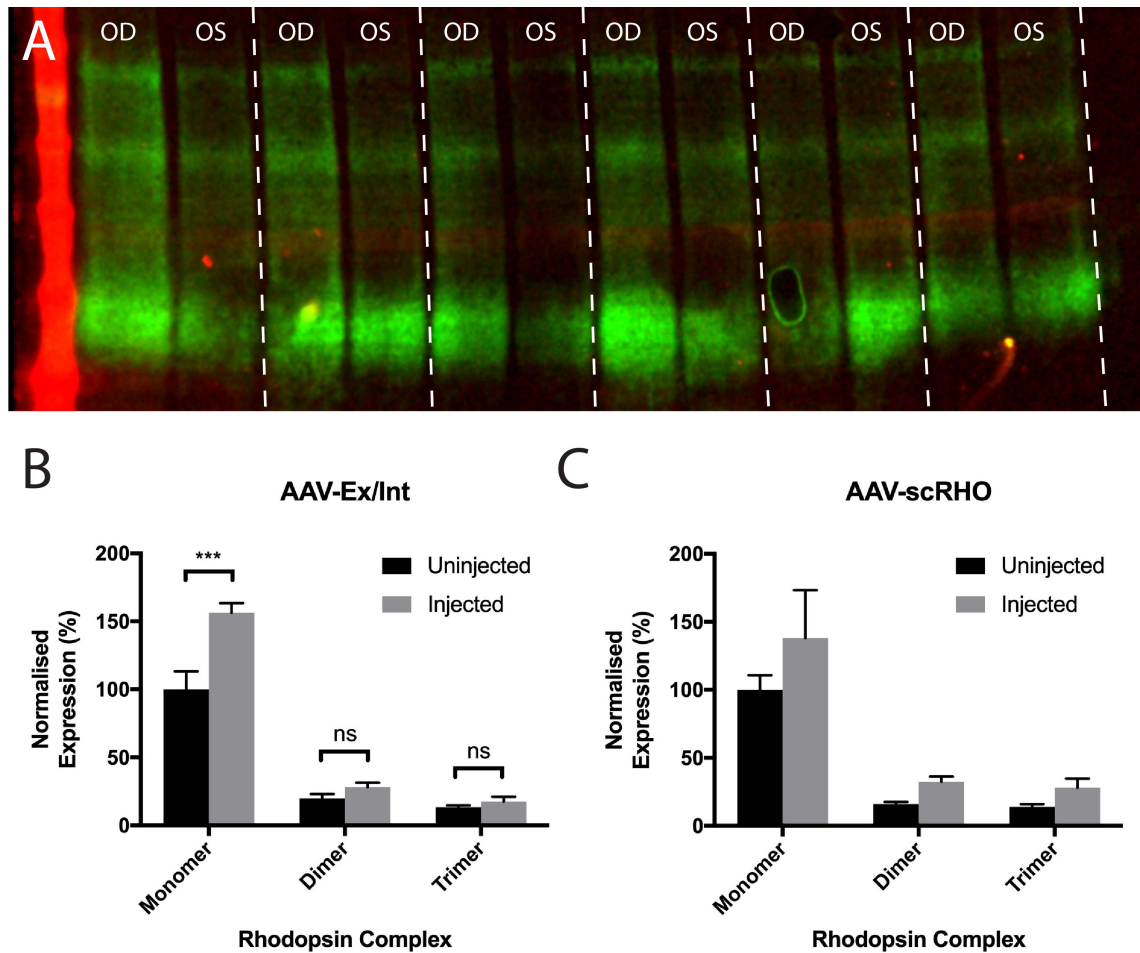


Figure 4.16. RHO rAAVs induce rhodopsin overexpression in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse.

Mice received unilateral injections of AAV-Ex/Int (n=6) or AAV-scRHO (n=6) into the right eye (OD) whilst the left eye (OS) was uninjected in all cases. **(A)** Western blot stained for rhodopsin (green) and β -actin (red) for the Ex/Int-injected cohort. OD: oculus dexter (right eye); OS: oculus sinister (left eye). **(B)** Densitometric analysis of western blot images for the AAV-Ex/Int-injected cohort. ***p=0.0003, Sidak's multiple comparison test. **(C)** Densitometric analysis of western blot images for the AAV-scRHO-injected cohort. Differences between right and left eyes failed to reach statistical significance, repeated measures two-way ANOVA.

Gene expression analysis by RT-qPCR was next performed to determine the extent to which levels of AAV-derived human rhodopsin expression compared with those of endogenous mouse rhodopsin. For this, the right eyes of *Nrl.GFP/+*, *Rho*^{P23H/+} mice were injected with

AAV-Ex/Int at either low dose (2×10^8 gc) or high dose (2×10^9 gc) whilst left eyes received isovolumetric sham injections of PBS. Retinas were harvested four weeks later from which cDNA was derived for RT-qPCR analysis (Figure 4.17). At low dose, AAV-Ex/Int achieved a transgenic human *RHO* expression level that was 0.64 that of endogenous mouse *Rho* in the sham-injected eye (Figure 4.17A; $p=0.6603$, two-way ANOVA, Sidak's multiple comparison test). At high dose, the expression of the transgene was 2.5x that of the endogenous mouse *Rho* in the fellow-eye (Figure 4.17B; $p=0.0055$, two-way ANOVA, Sidak's multiple comparison test).

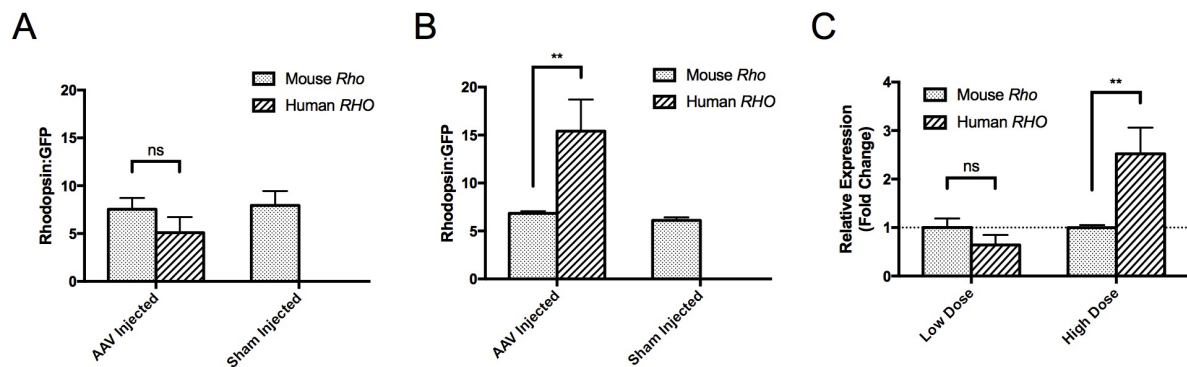


Figure 4.17. Gene expression analysis following subretinal injection of AAV-Ex/Int in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse. Right eyes were injected with vector whilst left eyes received sham injections of PBS. To account for differences in photoreceptor number between eyes, rhodopsin gene expression was normalised to *eGFP* which was used as a housekeeping gene (all TaqMan assays, Thermo Fisher Scientific, UK). **(A)** Low dose cohort (2×10^8 gc; n=5). **(B)** High dose cohort (2×10^9 gc; n=5). **(C)** Rhodopsin: GFP expression levels from low and high dose cohorts expressed as fold change relative to that measured for endogenous mouse rhodopsin in the sham-injected eye. The dotted line at $y=1$ thus represents the level of expression expected from two endogenous genomic copies of rhodopsin.

** $p < 0.01$, two-way ANOVA, Sidak's multiple comparison test.

4.4.7. Effect of rhodopsin supplementation on retinal structure in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse

To establish whether AAV-mediated RHO supplementation is sufficient to slow the rate of retinal degeneration in the P23H knock-in mouse model, unilateral injections of AAV-Ex/Int at one of two doses, 2×10^8 gc (low dose) or 2×10^9 gc (high dose), were performed in *Nrl.GFP/+*, *Rho*^{P23H/+} mice. A third cohort received isovolumetric unilateral injections of PBS and served as a control group to determine the effect of surgery alone. The impact of these interventions on retinal structure was determined by cSLO and SD-OCT at one, two and three months post-injection, whilst the effect on retinal function was ascertained by ERG at one and three months post-injection.

Mean PRL thickness was significantly reduced in injected versus uninjected eyes of animals in the sham-injected cohort (a relative reduction of 8.8%, 11.2% and 9.9% at one-, two- and three-months respectively; Figure 4.18C). The surgical procedure itself thus induced a small but significant thinning of the PRL. No significant overall difference was however detected between the mean PRL thickness of treated and untreated eyes in either the low or high dose cohorts (Figure 4.18A & B). To enable comparison of the three interventions directly, the numerical difference in mean PRL thickness between injected and uninjected eyes was calculated for each animal for the three cohorts. In this analysis, positive values would suggest a slowing of degeneration in the treated compared to the untreated eye whilst negative values would indicate an exacerbation of degeneration relative to the fellow eye. The graph in Figure 4.18D plots mean PRL thickness difference for the three groups as a function of time. No significant difference between the groups was found and there was no statistical interaction between the factors of experimental group and time.

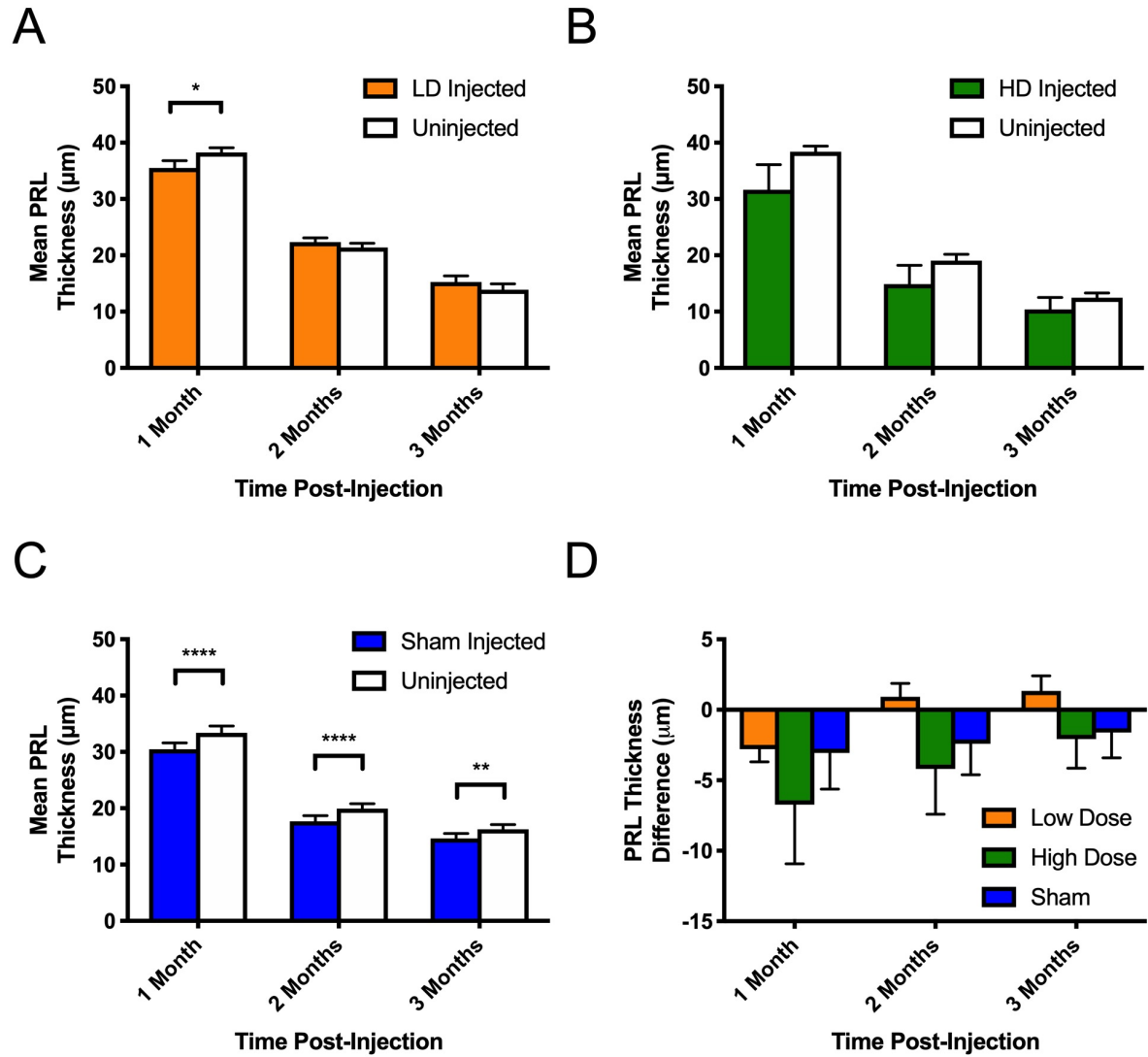


Figure 4.18. Effect of subretinal injection of AAV-Ex/Int on mean PRL thickness in the *Nrl.GFP/+*, *Rho^{P23H/+}* mouse. In all cases, right eyes received single superior subretinal injections of either AAV-Ex/Int at low dose (LD: 2×10^8 gc, $n=7$), high dose (HD: 2×10^9 gc, $n=7$), or PBS (sham, $n=13$). Left eyes were uninjected as internal controls. Values for each eye represent the mean photoreceptor layer (PRL) thickness measured at eight points distributed around the mid-peripheral retina as described in Section 2.4.3. Repeated measures two-way ANOVA tests with eye and time as factors were used throughout. **(A)** Low dose cohort, $F(1, 6)=0.08$, $p=0.79$ for overall effect of injection. Statistical interaction eye x time $F(2, 12)=6.1$, $p=0.015$. **(B)** High dose cohort, $F(1, 12)=1.7$, $p=0.2198$ for overall effect of injection. Statistical interaction injection x time $F(2, 24)=2.2$, $p=0.13$ **(C)** Sham cohort, $F(1, 12)=24.8$, $p=0.0003$ for overall effect of injection. Statistical interaction injection x time

$F(2, 24)=2.3$, $p=0.12$ **(D)** Mean PRL thickness difference between injected right and uninjected left eyes as a function of time for the low dose, high dose and sham cohorts. * $p<0.05$; ** $p<0.01$; *** $p<0.0001$, Sidak's multiple comparison tests.

Given that all injections in this study were performed in the superior retina and that a dorso-ventral gradient of degeneration exists in the *Rho*^{P23H/+} model, analysis of mean PRL thickness in isolation may mask regional differences. PRL thickness as a function of retinal location was therefore assessed for the three cohorts. In all groups, a thinning of the PRL was apparent in the superior regions of the retina corresponding to the area of injection (Figure 4.19). This loss of rods was also evident on blue autofluorescence cSLO imaging (Figure 4.20).

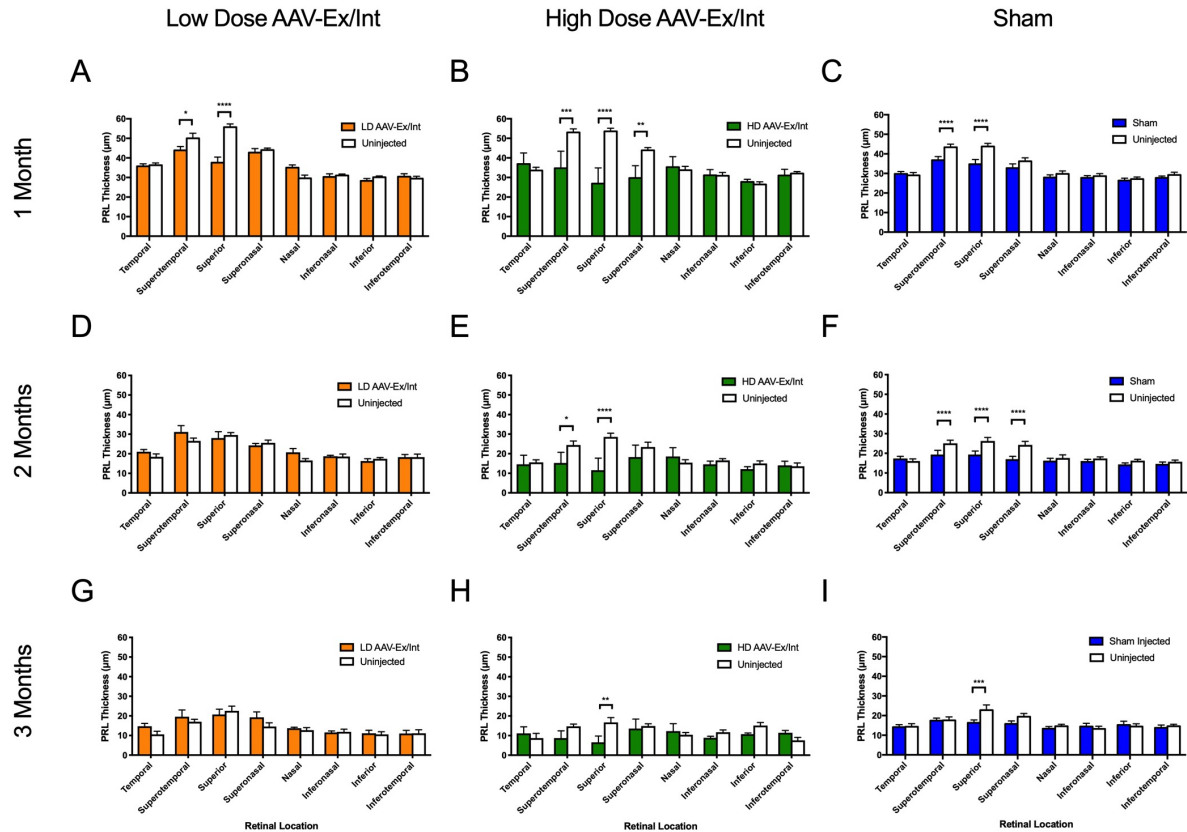


Figure 4.19. Effect of subretinal injection of AAV-Ex/Int on mean PRL thickness by retinal location in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse. All measurements were taken at a fixed eccentricity

of approximately 22.5° from the optic nerve head in the stated direction. **(A, D, G)** Low dose (2×10^8 gc) AAV-Ex/Int-injected cohort at one (A), two (D), and three (G) months post-injection. **(B, E, H)** High dose (2×10^9 gc) AAV-Ex/Int-injected cohort at one (B), two (E), and three (H) months post-injection. **(C, F, I)** Sham (PBS)-injected cohort at one (C), two (F), and three (I) months post-injection. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, all other comparisons not significant, Sidak's multiple comparison test.

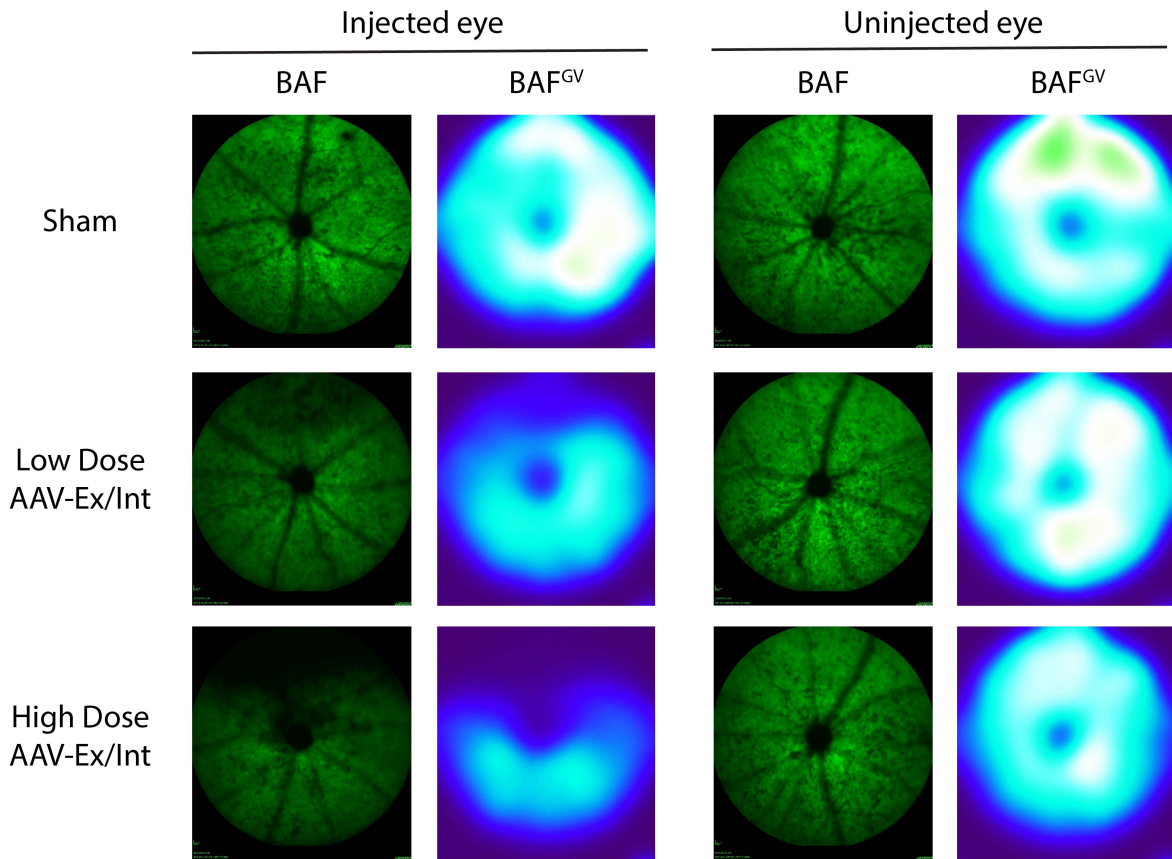


Figure 4.20. En face fluorescence imaging of *Nrl.GFP/+*, *Rho^{P23H/+}* retinas following subretinal injection of PBS (sham) or AAV-Ex/Int. Representative BAF images and BAF mGV surface plots taken four weeks following treatment. Paired right (injected) and left (uninjected) eyes are shown in each case.

To establish whether the inclusion of AAV in the subretinal injection had an additional effect above and beyond that of surgery alone, the mean PRL thickness difference between injected

and uninjected eyes was compared between groups as a function of retinal location (Figure 4.21). This analysis suggested that a greater degree of PRL thinning was induced by high dose vector than sham injection in the superior regions of the retina which would imply a degree of induced toxicity.

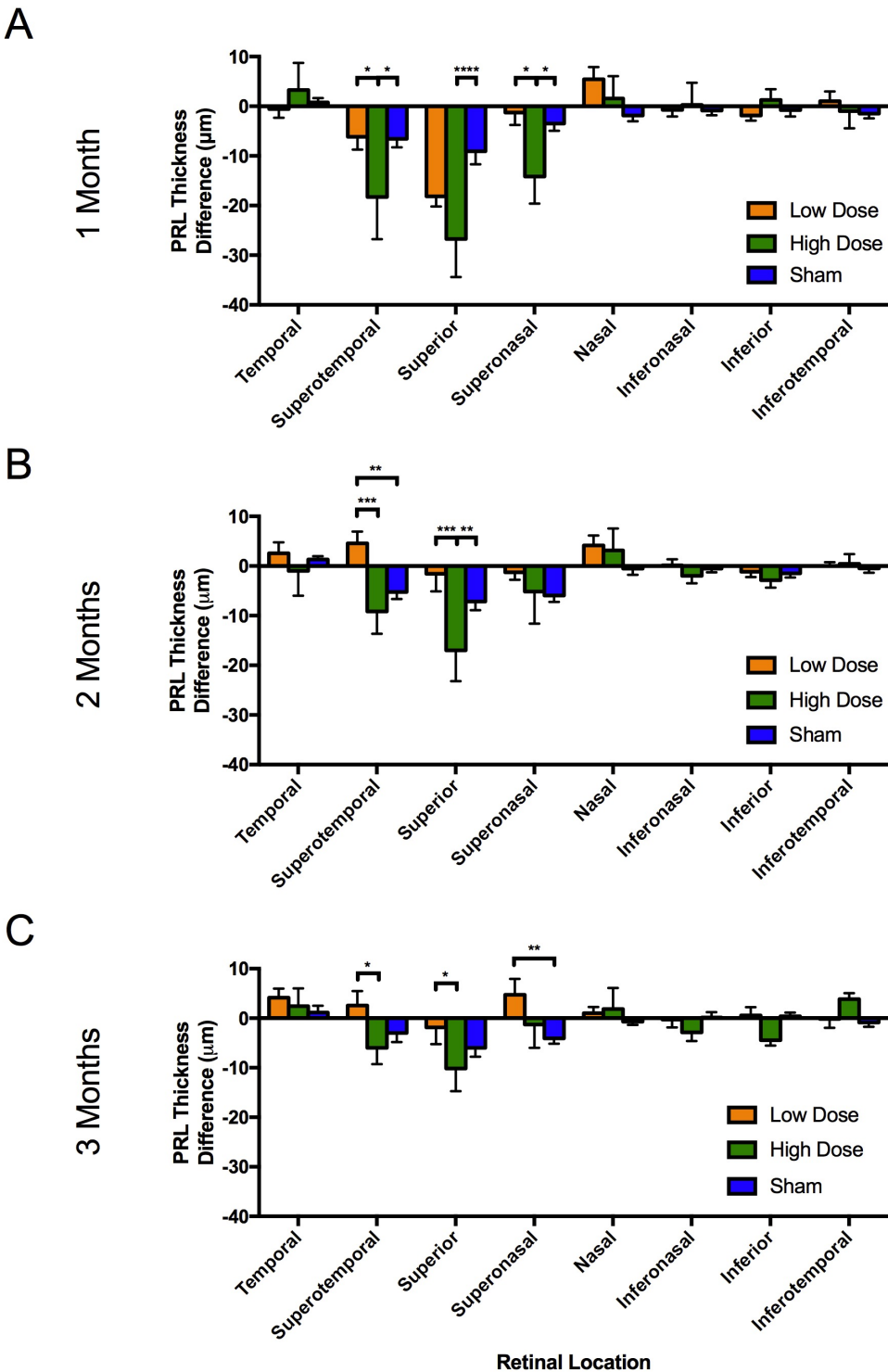


Figure 4.21. Difference in PRL thickness between AAV-Ex/Int-injected and uninjected fellow eyes as a function of retinal location. Values greater than zero suggest a benefit, whilst those below zero suggest damage as a result of subretinal injection. **(A)** One month post-injection. **(B)** Two months post-injection. **(C)** Three-months post-injection. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, all other comparisons not significant, repeated measures two-way ANOVA, Sidak's multiple comparison test.

4.4.8. Effect of rhodopsin supplementation on retinal function in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse

Full-field dark- and light-adapted ERG was performed for low dose, high dose and sham cohorts one and three months post-injection (Figure 4.22 and Figure 4.23). A significant reduction in dark-adapted responses of injected compared with fellow uninjected eyes was apparent for the high dose and sham cohorts at one month (repeated measures two-way ANOVA with stimulus intensity and eye as factors: $F(1, 6)=14.4$, $p=0.009$ and $F(1, 6)=7.8$, $p=0.0311$ for a- and b-waves in the high dose cohort, and $F(1, 11)=21.1$, $p=0.0008$ and $F(1, 11)=6.4$, $p=0.028$ for a- and b-waves in the sham cohort), but these differences failed to reach statistical significance at three months. No difference in dark-adapted response was detected between treated and untreated eyes in the low dose group at either time-point (Figure 4.22A, B).

For the light-adapted response, no significant difference in overall response between injected and uninjected eyes was present at either one- or three-months post-treatment for any of the three experimental cohorts (Figure 4.23). A statistical interaction between the factors of stimulus intensity and eye was however confirmed by repeated measures two-way ANOVA testing for the high dose cohort at one-month ($F(4, 24)=3.6$, $p=0.019$) and significant differences between right and left eyes were detected at higher stimulus intensities in post hoc

analyses (Sidak's multiple comparison test; Figure 4.23C). This would suggest a detrimental effect of high dose AAV-Ex/Int injection on overall cone function.

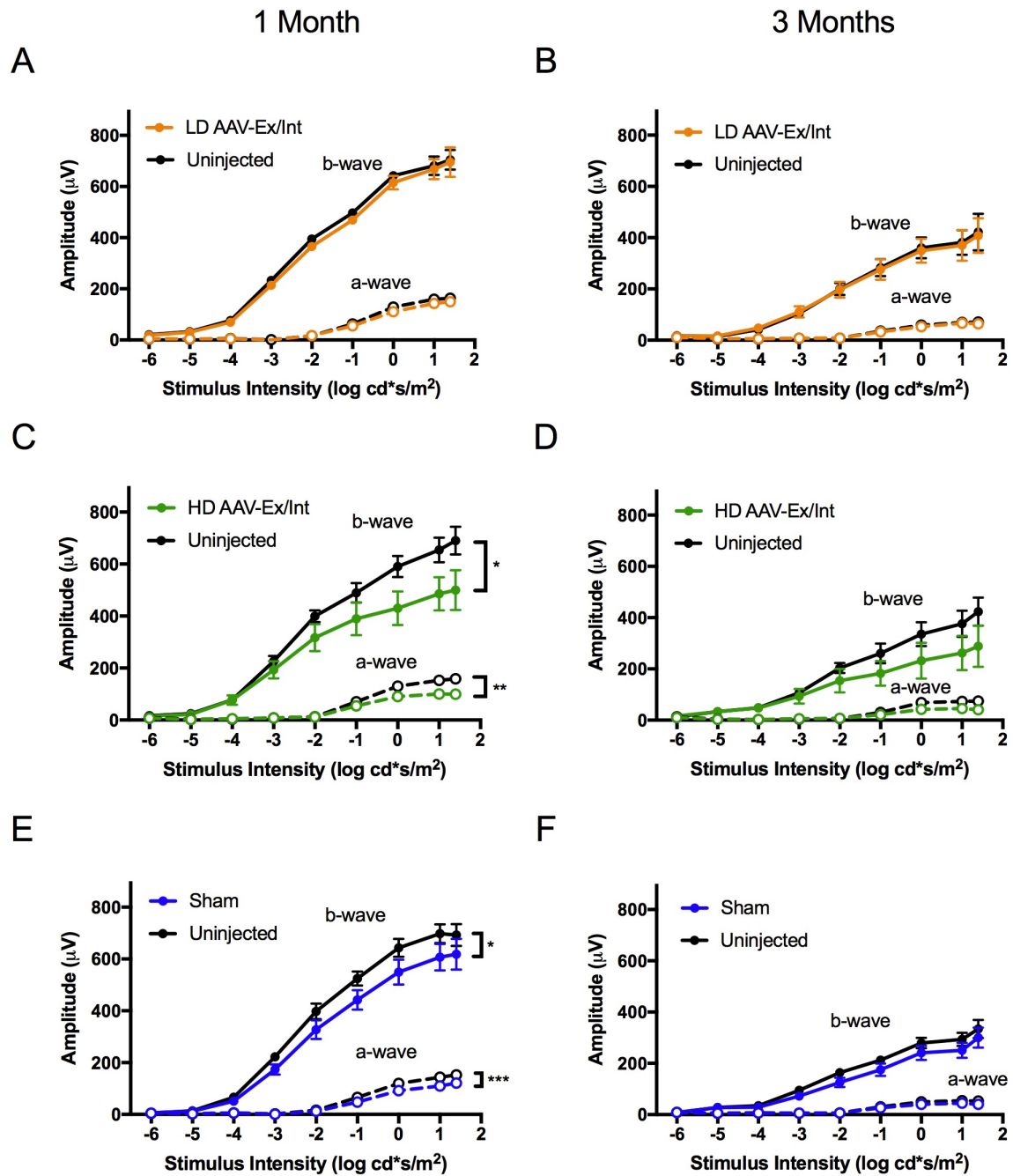


Figure 4.22. Full field dark-adapted ERG irradiance-response curves for low and high dose AAV-Ex/Int injected and sham-injected cohorts one and three months post-injection. All data points represent mean signal $\pm$ SEM. (A & B) Low dose AAV-Ex/Int-injected cohort, $n=7$. (C & D)

High dose AAV-Ex/Int-injected cohort, n=7. **(E & F)** Sham (PBS)-injected cohort, n=13. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, all other comparisons not significant, repeated measures two-way ANOVA for effect of injection.

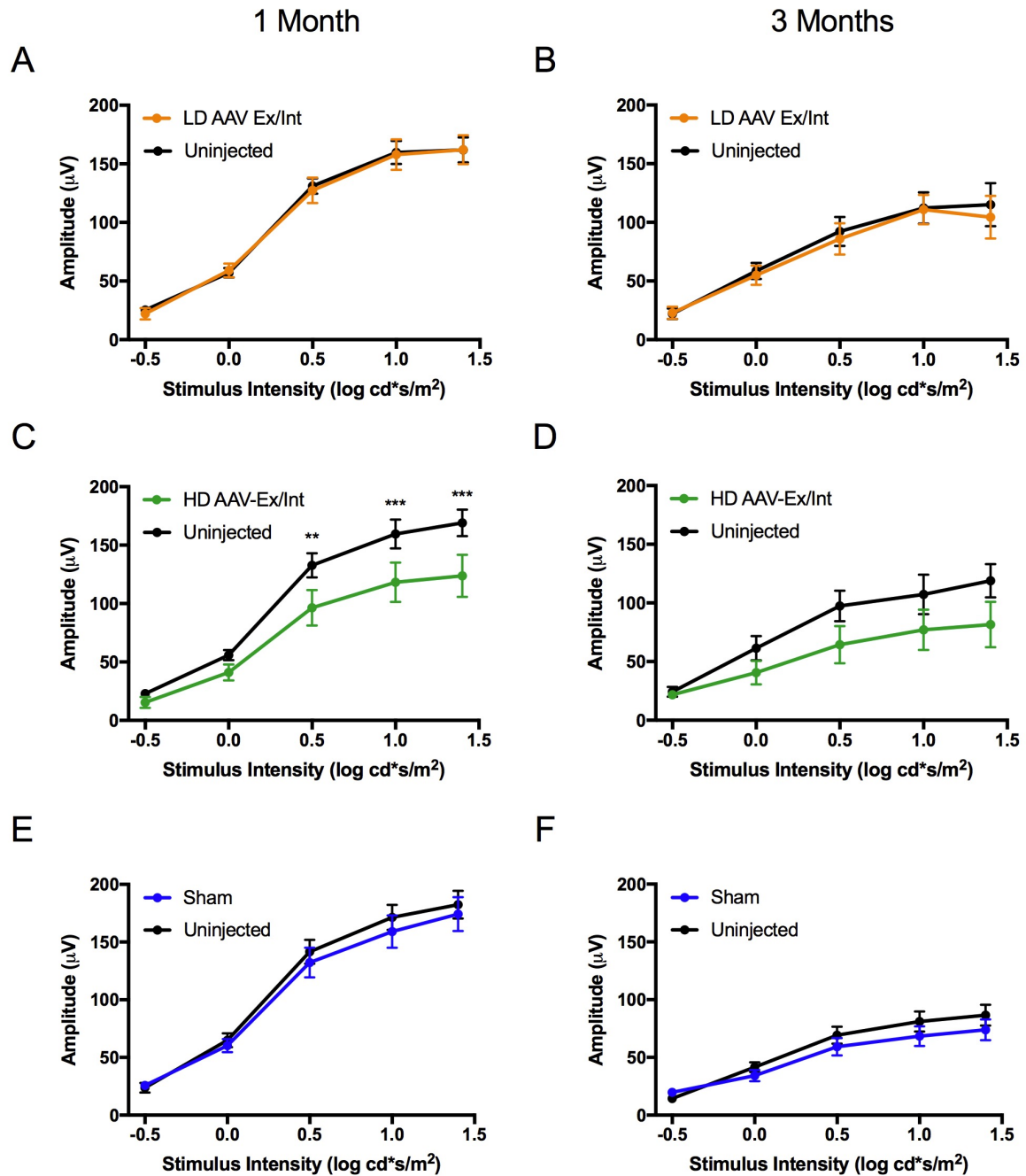


Figure 4.23. Full field light-adapted ERG irradiance-response curves for low and high dose AAV-Ex/Int-injected and sham-injected cohorts. All data points represent mean signal $\pm$ SEM. (A & B) Low dose AAV-Ex/Int-injected cohort, n=7. (C & D) High dose AAV-Ex/Int-injected cohort, n=7. (E & F) Sham (PBS)-injected cohort, n=13. **p<0.01; ***p<0.001, Sidak's multiple comparison test.

To allow a comparison to be made between groups, further irradiance response curves (IRCs) were plotted using the difference in recorded amplitude between injected and uninjected eyes for the three cohorts. Positive values represent improved signal from injected eyes relative to their uninjected counterparts, whilst the reverse applies for negative values. No statistically significant differences were detected between the groups for dark- or light-adapted responses at one- or three-months post-injection (Figure 4.24). However, a statistical interaction between stimulus intensity and cohort was detected for dark-adapted a- and b-waves one-month post-injection ($F(16, 200)=2.4$, $p=0.0025$ and $F(16, 184)=2.5$, $p=0.0016$ respectively) and post-hoc multiple comparison tests (Sidak) showed significant differences between the response of high dose and low dose/sham groups for the brightest flash intensities (e.g. a-wave 1.4cd.s/m^2 : HD vs Sham $p=0.0013$; HD vs LD $p<0.0001$; and b-wave 1.4cd.s/m^2 : HD vs Sham $p=0.0171$; HD vs LD $p=0.0005$).

In summary, no form of injection appeared to improve ERG response compared with the fellow untreated eye (all curves lie below the zero line in Figure 4.24), and at high dose, the AAV-Ex/Int vector appeared more damaging than an equivalent low dose or sham injection.

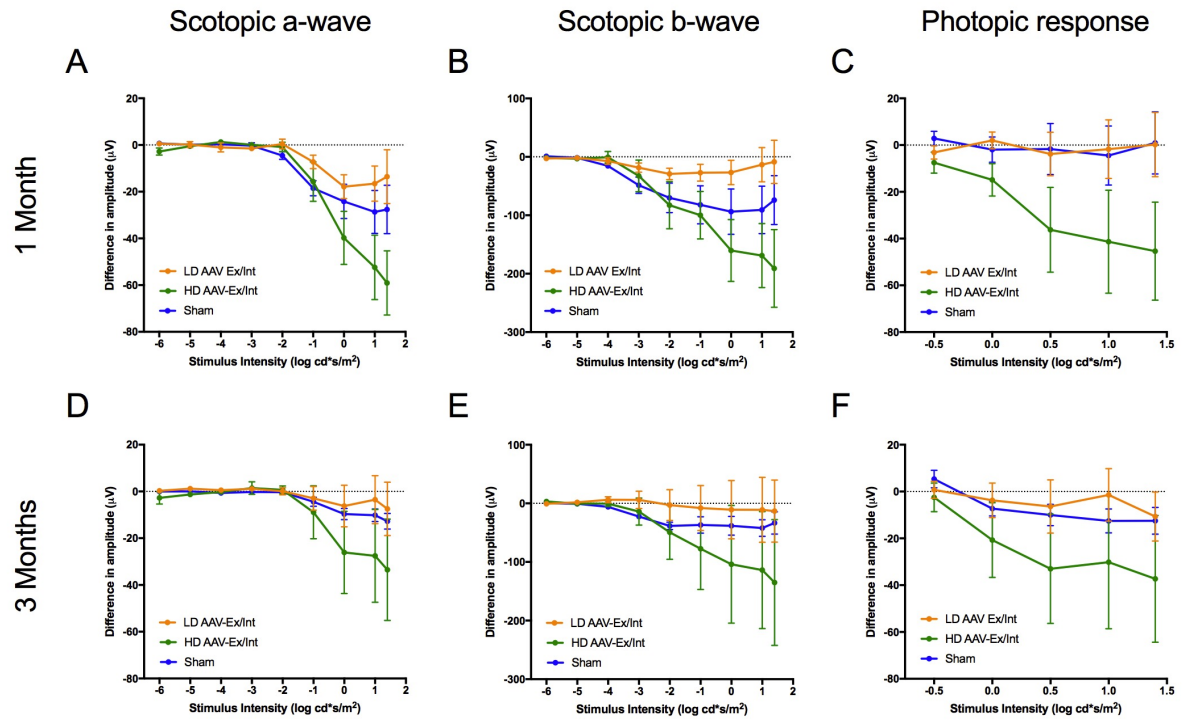


Figure 4.24. Difference in ERG amplitudes between treated and untreated eyes for low and high dose AAV-Ex/Int-injected and sham-injected cohorts. All data points represent mean signal difference $\pm$ SEM. Values greater than zero (dotted line) suggest a benefit, whilst those below zero suggest damage as a result of subretinal injection. No significant difference between the groups was detected for dark-adapted a- or b-wave or light-adapted responses at either one or three months post-injection.

4.5. Discussion

4.5.1. Development of *Nrl.GFP* mouse models

The pre-clinical development of new treatments for RP is heavily reliant upon the availability of suitable animal models that closely mimic the human phenotype²⁷⁸. To maximise the utility of such model systems, an ability to track temporal patterns of outer retinal degeneration with and without intervention is critical. Traditionally, anatomical changes in the retina over time and the effect on this of experimental treatments have been determined by histological means. Whilst this remains an important technique in preclinical studies, tissue sectioning for the

purpose of tracking retinal degeneration has a number of notable limitations: the introduction of artefactual changes consequent upon tissue processing²⁷⁹; low sensitivity for detecting subtle and regional changes; an inability to visualise a single specimen in more than one plane; and the inability to measure changes longitudinally in a single animal. Moreover, deriving quantitative measures from histological sections is both labour intensive and open to interpretation. Contemporary imaging technologies such as SD-OCT which are now used extensively in both clinical and laboratory practice, have revolutionised the assessment of animal models of retinal disease and circumvent many of the problems associated with traditional histology as presented above²³³. In spite of these advances, it has still proved challenging to objectively map broad patterns of outer retinal degeneration *in vivo* which would be of great benefit in the longitudinal assessment of interventions in animal models of RP. Manual segmentation of OCT images is time-consuming and subject to inter-observer variability, and the development of reliable automated systems is a current area of active investigation²⁸⁰. Further, segmentation algorithms built into current OCT software systems fail to accurately and consistently delineate the outer nuclear layer in rodents, particularly in degenerative states or following intervention where retinal architecture may be grossly abnormal. In this chapter, I have developed and validated two novel *Nrl.GFP* transgenic mouse models of rhodopsin-related RP: *Nrl.GFP/+*, *Rho*^{-/-} and *Nrl.GFP/+*, *Rho*^{P23H/+}. These mice have rods that are labelled with eGFP permitting their direct visualization *in vivo* using *en face* BAF cSLO imaging. This provides a simple and convenient means of tracking the loss of rods longitudinally in any given animal over time, and allows broad spatial patterns of degeneration to be readily determined. The *Nrl.GFP/+*, *Rho*^{P23H/+} mouse was thus used as the model system for ADRP in which to test vectors in this chapter and in Chapter 6.

The method does however suffer from a number of notable limitations. Firstly, the widespread expression of eGFP in models of RP has the potential to mask the signal from other retinal

fluorophores that may accumulate in such animals. Indeed, hyperfluorescent spots which are thought to represent debris from degenerating photoreceptors have been noted in several rodent models of RP, including *Rho*^{-/-}, when imaging by cSLO in BAF mode²⁸¹. These lesions were not discernible from the background eGFP signal in either the *Nrl.GFP/+*, *Rho*^{-/-} or *Nrl.GFP/+*, *Rho*^{P23H/+} retina. Secondly, the BAF signal recorded from these models may be influenced by a number of other factors aside from ONL thickness including pupil size, media opacity, contact lens centration and variation in confocal plane²³². The accuracy of mGV measurements may further be adversely affected if areas of signal saturation occur within an image. This may necessitate the acquisition of multiple BAF images at a range of detector sensitivity settings. These factors may limit the accuracy of mGV as a global representative measure of surviving ONL, and measurements derived from SD-OCT are likely to represent more consistent and clinically relevant metrics. The strength of BAF cSLO imaging in degenerate *Nrl.GFP* models may thus lie in the generation of the images themselves, which represent qualitative *en face* maps of regional rod survival that do not rely on processing or analysis following image acquisition. Finally, it is possible that the expression of eGFP within rods may have an impact on the natural history of photoreceptor loss in degenerate models, which would of course detract from their clinical relevance. Although no degeneration was observed in the *Nrl.GFP/+*, *Rho*^{+/+} mouse, the possibility of such an interaction cannot be excluded.

4.5.2. Recombinant AAVs for delivery of human rhodopsin

Rhodopsin constitutes around 90% of the total protein found in the rod OS and has a high level of turnover²⁸². Correspondingly high levels of RHO expression from an AAV are thus likely to be required if the transgenic wild type protein is to overcome any dominant negative effect of the genomically-expressed mutant. An efficient RHO vector would also be required as part any 'knockdown/replacement' treatment paradigm. In this chapter, I have compared

four AAV2/8^{Y733F} vectors carrying wild type copies of the human rhodopsin CDS. An *in vitro* validation of these constructs was not possible since no immortalised cell line is known to have sufficiently strong rhodopsin promoter activity. The murine *Rho*^{-/-} retina thus represents the most appropriate current model in which to validate and compare rhodopsin-expressing constructs, and was thus used here and has been used by others for this purpose^{195,283}. These mice do not express rhodopsin, lack OS and their dark- and light-adapted ERG responses derive solely from cones²⁷⁸. Any rhodopsin expressed following gene therapy in this model must therefore be transgenic in origin. Of the four vectors developed and tested in this chapter, AAV-Ex/Int and AAV-scrRHO appeared to be the most efficient. The self-complementary vector achieved a higher level of mRNA expression in dissociated *Rho*^{-/-} cells when compared with that of the single-stranded vectors. This likely reflects the fact that AAV-scrRHO bypasses the rate-limiting step of second-strand synthesis during viral transduction. Although self-complementary AAVs are thought through this mechanism to result in a higher level of transgene expression with a more rapid onset¹⁸⁷, their clinical application may be limited due to the significant loss of packaging capacity, lower achievable titres during manufacture and potential increased immunogenicity²⁸⁴. AAV-Ex/Int, which includes two cis-acting elements intended to enhance gene expression (see Section 1.4.2.3), was able to induce similar levels of rhodopsin protein expression as AAV-scrRHO. As a single-stranded vector, AAV-Ex/Int does not suffer from the limitations listed above and has ample remaining packaging space for the subsequent inclusion of additional elements to mediate rhodopsin knockdown. In a previous study, Palfi and colleagues compared six RHO-AAV2/5 vector designs in the *Rho*^{-/-} model¹⁹⁵. They found that the highest levels of transgene expression were achieved when the vector included the WPRE sequence and a longer mouse rhodopsin promoter. The authors were additionally able to demonstrate a reconstitution of rod OS following subretinal RHO-AAV injection at PND0 which was accompanied by a modest rescue of the rod-derived ERG^{195,283}. In this chapter, I have shown that a similar reconstitution of OS with appropriate trafficking of

transgenic wild type human rhodopsin is achievable following injection at PNW3 i.e. after the point of terminal rod differentiation. This is an important observation as terminally-differentiated rods rather than photoreceptor precursors would be the target for treatment in any human clinical trial.

4.5.3. Effect of RHO supplementation in the *Nrl.GFP/+*, *Rho*^{P23H/+} model

Overexpression of wild type rhodopsin has been proposed as a possible treatment for the subset of rhodopsin mutations that act in a dominant negative fashion^{191,231}. I have tested this hypothesis by subretinal injection of the optimised AAV-Ex/Int vector at two different doses in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse and found no evidence of either structural or functional rescue. Indeed, at the higher dose tested (2x10⁹ gc), a degree of outer retinal toxicity was observed with superior PRL thinning and ERG attenuation relative to the uninjected eye being greater than that observed in sham-injected animals. These results contrast to those found in a similar study by Mao and colleagues in which wild type *RHO* cDNA was delivered by AAV in a transgenic *RHO*^{P23H} mouse on a *Rho*^{+/+} background where a significant treatment effect was observed. A number of notable differences exist between the Mao study and the work presented here which may, at least in part, explain this discrepancy. Firstly, the mouse used in this chapter is a knock-in as opposed to a transgenic species. Although this more faithfully models the human P23H mutation at a molecular level¹⁰⁵, the rate of degeneration in this mouse is much more rapid than that observed in the transgenic line used by Mao and colleagues, and contrasts with the relatively slow rate of progression which characterises *RHO*^{P23H} dominant disease in humans²⁶¹. It is interesting to note that in a subsequent study involving the same transgenic mutation, but on a mouse with *Rho*^{+/-} genetic background, Mao and colleagues reported no treatment effect from AAV-mediated wild type rhodopsin supplementation¹²¹.

Any patient who is to undergo gene therapy treatment for rhodopsin-related RP is likely to have long established disease at the point of intervention. For this reason, animals were injected in this chapter after weaning at PND21 and not at earlier time points when fewer rods would already have degenerated. By contrast, Mao et al. injected mice in their study at PND15. The combination of genetic background and rate of natural disease progression in their mouse model, combined with an earlier timing of intervention by these researchers, may explain why the gene supplementation strategy proved successful in their study, but failed to impart a measurable benefit here.

AAV-mediated supplementation has proven ineffective in treating rhodopsin mutations known to mediate cell death by toxic gain of function mechanisms such as the Class I P347S mutation¹²⁰. The fact that no rescue was apparent following overexpression of rhodopsin in the knock-in model supports the hypothesis that the genomic P23H mutation, when present in the heterozygous state, predominantly induces cell death by a toxic gain of function rather than a dominant negative mechanism.

A degree of toxicity was observed in the treated area of retina when the AAV-Ex/Int vector was injected at high dose. The possible causes of this are discussed in detail in Chapter 7.

4.6. Conclusions

In summary, simple gene augmentation appears insufficient to slow disease progression in the *Nrl.GFP/+*, *Rho*^{P23H/+} knock-in mouse model of ADRP. Knockdown of the toxic mutant protein is thus likely to be required. The ‘knockdown/replacement’ paradigm, in which both wild type and mutant transcripts are indiscriminately targeted for knockdown whilst resistant copies of the wild type gene are concomitantly supplied, can potentially achieve this in a mutation-independent manner. With a genome size of 3.6kb, the efficient AAV-Ex/Int vector

validated in this chapter has ample residual packaging capacity to incorporate additional DNA elements directed towards suppression of genomically-derived rhodopsin. In the following chapter, novel RNA interference (RNAi) effectors are developed for this purpose and the human *RHO* cDNA sequence is modified to confer RNAi resistance.

5. Mirtron Mediated Knockdown of Rhodopsin

5.1. Introduction

The results presented in the previous chapter would suggest that simple supplementation of wild type rhodopsin is insufficient in itself to slow the rate of degeneration in the *Nrl.GFP/+*, *Rho*^{P23H/+} knock-in mouse retina. More successful studies published by other groups have utilised a knockdown/replacement strategy where native rhodopsin suppression with RNA interference (RNAi) is combined with delivery of a codon-modified resistant *RHO* transgene. Millington-Ward et al. reported structural and functional improvement after PND5 *RHO*^{P347S} transgenic mice were injected with a mixture of two AAVs, one carrying a rhodopsin-targeting short hairpin RNA (shRNA) suppressor under the control of the H1 promoter, and the other a codon-modified *RHO* replacement gene^{120,194}. Mao and colleagues combined both knockdown and replacement arms of their therapy within a single AAV, and showed rescue in a *RHO*^{P23H} transgenic line with a slow degenerative phenotype following injection at PND15. A codon-modified version of mouse *Rho* was expressed in their vector under the control of the mouse opsin proximal (RNA polymerase II) promoter, whilst a rhodopsin-targeting shRNA was expressed under the control of the H1 (RNA polymerase III) promoter¹²¹. More recently, an efficient shRNA has been identified which targets both human and canine *RHO*¹⁹⁸. This was placed downstream of the H1 promoter, and combined with a codon modified human *RHO* cDNA sequence under the control of a 536bp human rhodopsin promoter within an AAV2/5 vector. Following subretinal injection in the *RHO*^{T4R/+} dog, the authors were able to demonstrate efficient suppression of endogenous rhodopsin RNA. They further demonstrated that the area of retina treated by the recombinant AAV showed resistance to the severe damage usually caused by acute intense light exposure in this model¹⁹⁸.

Whilst efficacy has been proven for both the single and the dual vector approaches, a number of potential limitations are associated with these strategies, which may have implications for downstream clinical application. Delivery of the two arms of the therapy with separate viruses means that the relative knockdown and replacement of rhodopsin in any given cell may be variable: an excess of supplementation over knockdown may lead to rhodopsin overexpression and cell death^{76,191}, whilst excessive suppression with insufficient replacement may act to accelerate the degeneration by conferring a rhodopsin-null phenotype⁷³. Moreover, the need to deliver two vectors effectively doubles the capsid dose to which retinal cells are exposed, and this has been reported to induce severe inflammatory complications *in vivo*¹⁹⁸. Control of the two arms of the therapy within the same vector, but under different promoters, may lead to similar problems should the relative strength of the promoters change over time, a phenomenon that has previously been observed following delivery of transgenic DNA under the transcriptional control of the CMV promoter^{285,286}. Severe cellular toxicity has also been observed due to saturation of the shRNA pathway when these hairpins are expressed under strong RNA polymerase III (Pol-III) promoters such as H1 or U6²⁸⁷. Subsequent studies have demonstrated a more favourable safety profile for RNA polymerase II (Pol-II)-driven effectors^{288,289}. This latter approach has the additional advantage of allowing improved cell-specific targeting. Inherent promiscuity of Pol-III promoters means that downstream shRNAs will be expressed in all cells transduced by the vector. By contrast, in the case of a cell-specific Pol-II promoter, expression will be confined to the target cell in question. This reduces the potential for off-target effects in other cell types such as RPE which are inescapably transduced following subretinal injection of vector.

Delivery of an RNAi effector together with its codon-modified replacement transgene under the combined control of a single cell-specific Pol-II promoter may therefore represent the

optimal strategy for knockdown and replacement of genes for the treatment of dominant disease. Such a strategy would ensure perfect spatiotemporal matching between the two arms of the therapy, whilst potentially avoiding fatal saturation of a vital cellular processing pathway and restricting expression of the RNAi effector to the intended cellular population. One such strategy involves the use of artificial mirtrons as mediators of RNAi. Mirtrons are pre-micro RNAs (pre-miRNAs) that are spliced out of mRNA transcripts as short introns. Although first described in *Drosophila*²⁹⁰, naturally occurring mirtrons have since been identified in a host of mammalian tissues²⁹¹, including the murine retina²⁹². The canonical miRNA pathway relies on processing by an intracellular microprocessing complex consisting of the endonuclease Drosha together with a cofactor known as Di George syndrome critical region 8- DGSCR8^{293,294}. Mirtrons are instead precisely defined and excised from a nascent mRNA transcript following recognition of their 5' splice donor and 3' splice acceptor sequences by the spliceosome²⁹¹. The resulting pre-miRNAs are transported from the nucleus into the cytosol of the cell by the nuclear membrane transporter protein exportin-5²⁹⁵, at which point the mirtron and canonical miRNA pathways converge²⁹¹. The cytoplasmic endonuclease Dicer removes the loop from the pre-miRNA hairpin to form a double-stranded RNA complex which is recognised by the RNA-induced silencing complex (RISC). One strand of the duplex (known as the guide strand) is selected by the RISC, whilst the other (the passenger strand) is discarded^{296,297}. The miRNA-RISC recognises and binds to specific target mRNA sequences by base complementarity, and may induce gene silencing through cleavage of the transcript, through translational repression, or both²⁹⁸.

Artificial mirtrons have been designed for the purpose of targeted gene knockdown in a number of recent proof of concept studies^{299–302}. Seow and colleagues demonstrated efficient *in vitro* knockdown of the myotonic dystrophy protein kinase (DMPK) gene responsible for autosomal dominant type 1 myotonic dystrophy using artificial mirtrons based on the primate miR-1226

structure³⁰⁰. Subsequently, artificial 3'-tailed mirtrons which mediate the suppression of vascular endothelial growth factor A (VEGF-A) expression *in vitro* have been described³⁰¹. The location of the intronic lariat branch point of these mirtrons outside of the stem-loop structure means that fewer sequence constraints exist for their design. In their 2017 publication, Curtis et al. investigated how artificial mirtrons based on the naturally-occurring murine miR-1224 design targeted against the ataxin 7 gene (responsible for spinocerebellar ataxia 7) might be co-delivered with a codon-modified resistant version of the gene under the control of a single Pol-II promoter³⁰². The authors found that placing the mirtron downstream of the replacement transgene ORF led to large reductions in splicing efficiency likely due to distance from the promoter to the first splice site. Similarly, placement of the mirtron within the 5' untranslated region (UTR) of the replacement transcript appeared to abrogate splicing. By locating the mirtron within the coding region of the transcript, the authors were able to achieve efficient splicing and gene silencing, albeit at the expense of transgene expression³⁰². Achieving a construct capable of efficient knockdown together with adequate gene replacement, an essential requirement for the application of artificial mirtrons for the treatment of dominant disease, thus remains a significant challenge.

In this chapter, artificial 3'-tailed mirtrons targeted against rhodopsin, as well as a resistant *RHO* CDS, are developed.

5.2. Aims

The principal aims of the experiments outlined in this chapter were as follows:

1. To design artificial mirtrons that are both accurately spliced, and capable of knocking down expression of both human and mouse rhodopsin RNA transcripts *in vitro*.

2. To investigate factors that contribute to the efficacy of mirtron-mediated RNAi as applied to the rhodopsin gene.
3. To determine specificity of rhodopsin-targeting mirtrons through testing against predicted potential off-targets.
4. To develop an efficient expression cassette in which one or more mirtrons may be co-delivered with a transgene under the control of a single Pol-II promoter.
5. To codon-modify the human *RHO* CDS in order to render the transcript resistant to mirtron-induced degradation.

5.3. Materials & Methods

5.3.1. Cloning

Double stranded mirtron inserts with 5' and 3' overhangs complementary to those of the BstBI restriction site were engineered by annealing complementary oligonucleotides. Forward oligonucleotides comprised the desired 76bp mirtron sequence with a 5'-P-CGAAG motif (where P represents a phosphate group) and corresponding omission of CGAAG from the 3' end. Reverse oligonucleotides comprised the reverse complement of the desired mirtron sequence with omission of the 5'-CTT motif, a 5' phosphate and inclusion of CTT at the 3' end. Oligonucleotides were manufactured by Sigma-Aldrich, UK, and reconstituted to a concentration of 10 μ M upon receipt. Ten μ l forward and 10 μ l reverse solution were thoroughly mixed and heated to a temperature of 95°C for five minutes using a PCR thermocycler (Multigene, Labnet International Inc.). Samples were then slowly cooled in a stepwise manner to room temperature over a period of six minutes to facilitate efficient annealing. The resulting

double stranded mirtron sequence (which by design already had BstBI adhesive ends) was ligated directly into the BstBI/shrimp alkaline phosphatase (both New England Biolabs, UK) treated CAG.eGFP.WPRE plasmid backbone. The *eGFP* CDS is shown below. The BstBI restriction site is highlighted in yellow, with the cut point marked by a vertical line:

```

ATGAGCAAGGGCGAGGAACTGTTCACTGGCGTGGTCCCAATTCTCGTGGAAGCTGGATGGCGATGTGAATGGG
CACAAATTTTCTGTGTCAGCGGAGAGGGTGAAGGTGATGCCACATACGGAAAGCTCACCCCTGAAATTCATCTGC
ACCACTGGAAAGCTCCCTGTGCCATGGCCAACACTGGTCACTACCCCTGACCTATGGCGTGCAGTGCTTTTCC
AGATACCCAGACCATATGAAGCAGCATGACTTTTTCAAGAGCGCCATGCCCGAGGGCTATGTGCAGGAGAGA
ACCATCTTTTCAAAGATGACGGGAACTACAAGACCCGCGCTGAAGTCAAGTT|CGAAGGTGACACCCTGGT
GAATAGAATCGAGCTGAAGGGCATTGACTTTAAGGAGGATGGAAACATTCTCGGCCACAAGCTGGAATACAA
CTATAACTCCCACAATGTGTACATCATGGCCGACAAGCAAAAGAATGGCATCAAGGTCAACTTCAAGATCAG
ACACAACATTGAGGATGGATCCGTGCAGCTGGCCGACCATTATCAACAGAACACTCCAATCGGCGACGGCCC
TGTGCTCCTCCCAGACAACCATTACCTGTCCACCCAGTCTGCCCTGTCTAAAGATCCCAACGAAAAGAGAGA
CCACATGGTCCTGCTGGAGTTTGTGACCGCTGCTGGGATCACACATGGCATGGACGAGCTGTACAAGTGA

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Correct orientation of the mirtron within the construct was confirmed by commercial Sanger sequencing with Primer 13 (Source Biosciences, UK).

To evaluate the efficacy of mirtrons, human and mouse rhodopsin target sequences were subcloned from plasmid DNA and murine retinal cDNA respectively into the multiple cloning site (MCS) of the PsiCHECK2 vector (Promega Inc., UK) in accordance with manufacturer's instructions. This plasmid is designed to provide a rapid approach for the evaluation of RNAi *in vitro*. The plasmid contains two luciferase genes- *Renilla* and firefly- both under the control of strong ubiquitous promoters. The *Renilla* luciferase has an MCS located between its translational stop codon and polyA sequence into which the target gene of interest is cloned (Figure 5.1A). Cleavage of the resulting mRNA transcript by an RNAi effector will result in its deadenylation and degradation. This would be expected to result in a reduction of the recorded *Renilla*

luciferase signal. The firefly luciferase signal acts as an internal control to which the *Renilla* luciferase signal is normalised (Dual Glo[®] assay- see Figure 5.1B and Section 5.3.3).

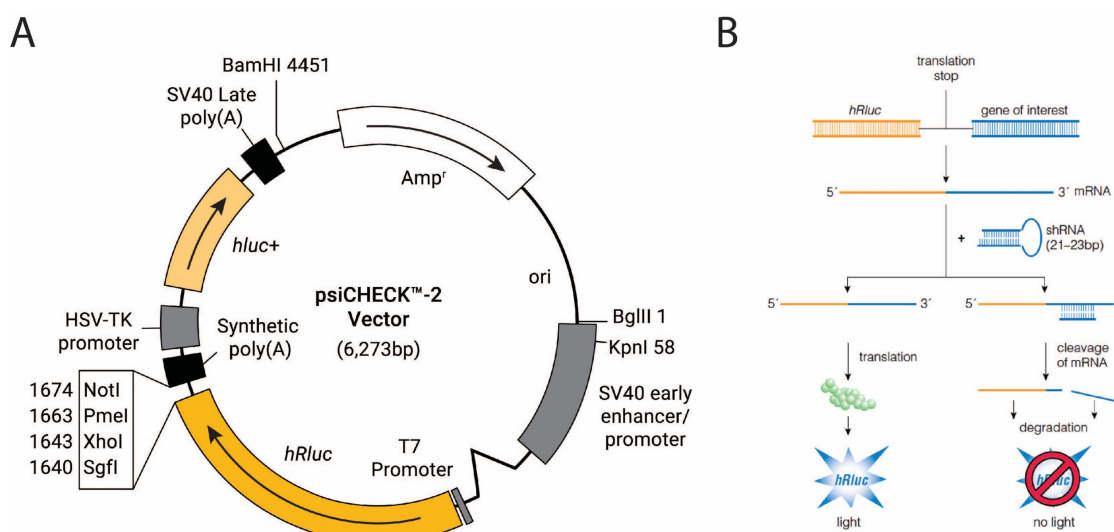


Figure 5.1. PsiCHECK2 vector and Dual Glo[®] assay. **(A)** PsiCHECK2 vector plasmid map. The *Renilla* luciferase (*hRluc*) and firefly luciferase (*hLuc+*) genes are located downstream of separate ubiquitous promoters. The gene of interest is cloned into the MCS (consisting of NotI, PmeI, XhoI and SgfI restriction sequences) which is located between the *Renilla* luciferase stop codon and its polyA sequence. **(B)** Overview of the Dual Glo[®] luciferase assay. RNAi effectors bind to target sequences in the 3'-UTR of the *Renilla* luciferase transcript causing cleavage and deadenylation. This will lead to a reduction in *Renilla* luciferase signal. Firefly luciferase signal will be unaffected and acts as an internal control. Effective RNAi will therefore result in a reduction in the luciferase ratio *Renilla*: firefly. Figures adapted from Promega Inc., UK.

To generate the PsiCHECK2-RHO vector, a human rhodopsin amplicon was amplified by PCR using CAG.RHO.WPRE plasmid template and the following primers (Primers 14 & 15):

XhoI RHO FW: 5'-ATCTCGAGATGAATGGCACAGA
RHO NotI RC: 5'-ATGCGGCCGCTTAGGCCGGGGCCA

The resulting 1065bp DNA fragment, identified by agarose gel electrophoresis, was extracted and digested with XhoI and NotI enzymes (both New England Biolabs, UK). The digestion product was then ligated into the XhoI/NotI-digested PsiCHECK2 vector, and the correct sequence of the resulting PsiCHECK2-RHO plasmid confirmed by commercial Sanger sequencing.

To generate the PsiCHECK2-mRho vector, a mouse rhodopsin amplicon was amplified by PCR using mouse retinal cDNA template and the following primers (Primers 16 & 17):

AsiSI mRho FW: 5'-ATGCGATCGCATGAACGGCACAGA

mRho NotI RC: 5'-ATGCGGCCGCTTAGGCTGGAGCCA

The resulting 1065bp DNA fragment identified by agarose gel electrophoresis was extracted and digested with AsiSI (an isoschizomer of SgfI) and NotI enzymes (both New England Biolabs, UK). The digestion product was then ligated into the AsiSI/NotI-digested PsiCHECK2 vector and the correct sequence of the resulting PsiCHECK2-mRho plasmid confirmed by commercial Sanger sequencing using Primer 18.

5.3.2. Fluorescence spectroscopy

Fluorescence spectroscopy assays were performed for quantification of splicing for mirtron constructs nested within the *eGFP* CDS (Section 5.4.2), and for assessment of the effect of 5'-UTR mirtrons on downstream transgene expression (Section 5.4.10). For the splicing assay, HEK293 cells were seeded into 12 well plates and transfected with 1µg pCAG.eGFP.WPRE or pCAG.eGFP-M1-7.WPRE. For gene expression assays, cells seeded in 12 well plates were transfected with 7.22×10^{10} copies of pCAG.eGFP.WPRE or Mirtron-UTR plasmid. In either

case, wells were photographed 48 hours later using standardised acquisition settings, after which cells were pelleted and resuspended in 100µl of fluorescence immunoprecipitation (FIPA) buffer (NaCl 150mM, Tris base 50mM, EDTA 2mM, 1% TritonX-100; adjusted to pH7.4 with HCl) supplemented with protease inhibitor (PI, Roche Diagnostics, one tablet per 10mls). Samples were then processed for protein extraction and quantification as described in Section 2.1.7. For each sample, 10µg of protein was loaded into wells of a black plastic 96 well plate (Greiner Bio-One, UK) in duplicate and the volume made up to 300µl with FIPA buffer/PI. Fluorescence (in arbitrary units) was measured using the Viktor³ 1420 Multilabel plate reader (Perkin Elmer, UK), with excitation at 485nm, and detection at 535nm and an exposure time of 1s per well. To account for background fluorescence, the mean reading taken from protein lysates derived from untransduced cells was subtracted from all transduced cell fluorescence readings. Signals obtained from protein lysates extracted from pCAG.eGFP-M1-7.WPRE, or Mirtron-UTR transfected cells were then normalised to that recorded from pCAG.eGFP.WPRE transfected cell lysates to obtain a measure of splicing efficiency, or normalised reporter gene expression respectively.

5.3.3. The Dual Glo[®] luciferase assay

For the Dual Glo[®] assay, HEK293 cells were seeded into 96 well culture plates. After 24 hours, cells were co-transfected with 65ng mirtron plasmid and 32.5ng PsiCHECK2-target plasmid. Forty-eight hours later, 30µl of media was removed from each well and the assay performed in accordance with manufacturer's instructions. Briefly, 70µl of *Renilla* luciferase substrate was added to each well and the plate left to incubate for 20 minutes. *Renilla* luminescence signal was then recorded (in arbitrary units) using the Viktor³ plate reader. Seventy µl of Stop & Glo reagent (which quenches *Renilla* luciferase activity and includes the substrate for Firefly luciferase) was then added to each well and the plate again left to incubate at room temperature

for 20 minutes. Firefly luminescence was then similarly read (in arbitrary units) using the Viktor³ plate reader. Mirtron-mediated mRNA knockdown was estimated by calculating the relative luciferase ratio *Renilla*/Firefly, which was then normalised to that recorded from cell samples transfected with the mirtronless CAG.GFP.WPRE plasmid.

5.3.4. Immunocytochemistry

Immunocytochemistry (ICC) was performed as outlined in Section 2.5.1. Antibodies used are detailed in Table 5.1 (all Abcam, UK).

Primary antibody target	Clonality/Epitope	Reference	Secondary antibody	Reference
Rhodopsin	Polyclonal	ab3424	Alexa-fluor donkey anti-rabbit IgG 568	ab175470
Rhodopsin	Monoclonal/ 1D4 (C-terminus)	ab5417	Alexa-fluor donkey anti-mouse IgG 568	ab175472
Rhodopsin	Monoclonal/ 4D2 (N-terminus)	ab98887	Alexa-fluor donkey anti-mouse IgG 568	ab175472
<i>Renilla</i> luciferase	Polyclonal	ab187338	Alexa-fluor donkey anti-rabbit IgG 488	ab150105

Table 5.1. Antibodies used for immunocytochemistry. All antibodies were manufactured by Abcam, UK. Primary antibodies were diluted 1:1000, whilst secondary antibodies were diluted 1:200.

5.4. Results

5.4.1. Design and *in silico* analysis of candidate mirtrons

The core structure of mirtrons used in this study is based on the 3'-tailed design described and validated by Kock et al. 2015³⁰¹. The advantage of artificial 3'-tailed over canonical mirtrons is

that they have fewer sequence constraints by virtue of the intronic branch point and polypyrimidine tract being located outside of the stem-loop structure which contains the guide strand (Figure 5.2). For all artificial mirtrons used in this study, the guide strand was located in the 5' arm of the hairpin stem, rather than the 3' arm where cellular processing may be less accurate³⁰². The guide strand was connected to a complementary 3' passenger arm by a TTCAAGAGA loop motif. Mismatches between guide and passenger strands of the stem were purposefully introduced at the 5' end to reduce local internal stability and facilitate correct selection of the guide strand (as opposed to the passenger strand) by the RISC (Figure 5.3A)^{297,303}.

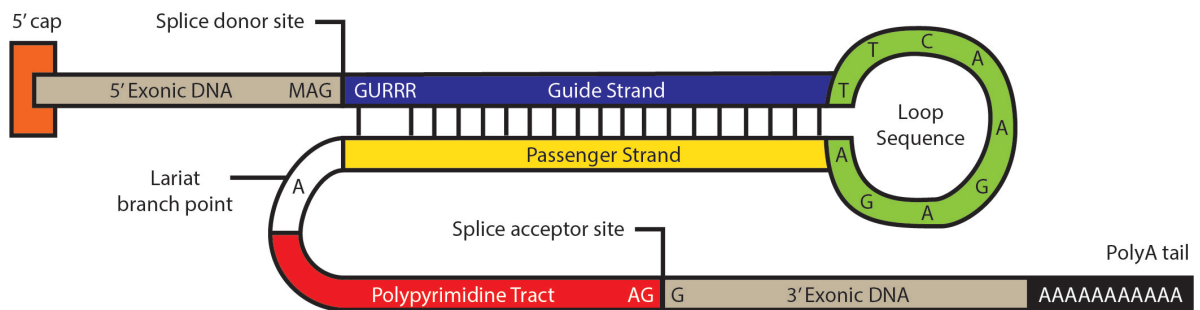


Figure 5.2. General structure of 3' tailed mirtrons. The schematic illustrates a 3' tailed mirtron as an intron within a pre-mRNA transcript. The mirtron itself consist of (from 5' to 3'): guide strand, loop sequence (TTCAAGAGA), passenger strand, lariat branch point (A), and polypyrimidine tract. The guide and passenger strands form a hairpin structure through Watson-Crick base pair complementarity, with mismatches being purposefully introduced at the base of the stem to facilitate guide strand selection by the RISC. M = A or C; R = A or G.

Potential targets for artificial mirtrons within the rhodopsin cDNA sequence were identified according to several criteria. Firstly, only areas of at least 21bp in length with perfect or near-perfect homology between the mouse and human rhodopsin mRNA transcript were considered. This is so that mirtrons designed for incorporation into a treatment for ADRP in

man could first be validated in a relevant mouse model. Secondly, since a 21bp guide sequence located within the 5' arm of a mirtron must commence with a splice donor motif, only potential target sequences that featured the reverse complement of such a motif at their 3' end were considered. Thirdly, mirtron candidates that contained an additional strong splice donor motif elsewhere within the 21bp guide sequence were not considered as such mirtrons would likely be preferentially spliced incorrectly.

Candidate mirtron sequences nested within the *eGFP* CDS were input into online splice prediction software (Spliceport: <http://spliceport.cbcb.umd.edu> and Human Splice Finder: <http://www.umd.be/HSF3/HSF.shtml>) to identify those that included strong donor motifs. Scores for the mirtrons that were subsequently manufactured and tested *in vitro* are shown in Table 5.2. The siRNA at Whitehead online RNAi prediction tool (<http://sirna.wi.mit.edu>) was also used to identify which candidate sequences were likely to make effective RNAi targets.

Mirtron Design	Spliceport		Human Splice Finder	
	Donor	Acceptor	Donor	Acceptor
1	0.26	1.60	75.21	86.7
2	0.9	2.38	76.61	86.7
3	1.83	2.13	95.95	86.7
4	0.41	2.06	83.94	86.7
5 ^H	0.95	1.84	78.54	86.7
5 ^M	1.12	1.93	78.54	86.7
6	0.79	2.16	83.79	86.7
7	2.15	2.19	95.71	86.7

Table 5.2. Splice donor and acceptor site strength prediction for artificial mirtron designs. The CDS for *eGFP* with artificial mirtrons nested at the BstbI cut site were input into Spliceport and Human Splice Finder online software. Higher scores in both programmes denote a greater likelihood of the intended splice donor and acceptor splice sites being recognised as such by cellular splice machinery.

With the above in mind, seven 3'-tailed mirtrons (designated M1-M7) were designed targeting different parts of the rhodopsin CDS (Figure 5.3). The 21bp guide sequences of M1-4 and M6 were perfect antisense matches to both the human and mouse transcripts. That of M7 was a complete antisense match for the human CDS, but contained a single mismatch with the mouse sequence. This mismatch would however be predicted to form a non-Watson & Crick G-U 'wobble' base pair at the RNA level, and so may nonetheless allow effective targeting by this mirtron³⁰⁴. The sequence targeted by M5 was identified by the siRNA at Whitehead online tool as a strong candidate for effective RNAi-induced cleavage. The mouse and human M5 targets however differed by two base pairs. Two versions of M5 were therefore created: M5^H matching the reverse complement of the human CDS, and M5^M matching that of the mouse sequence.

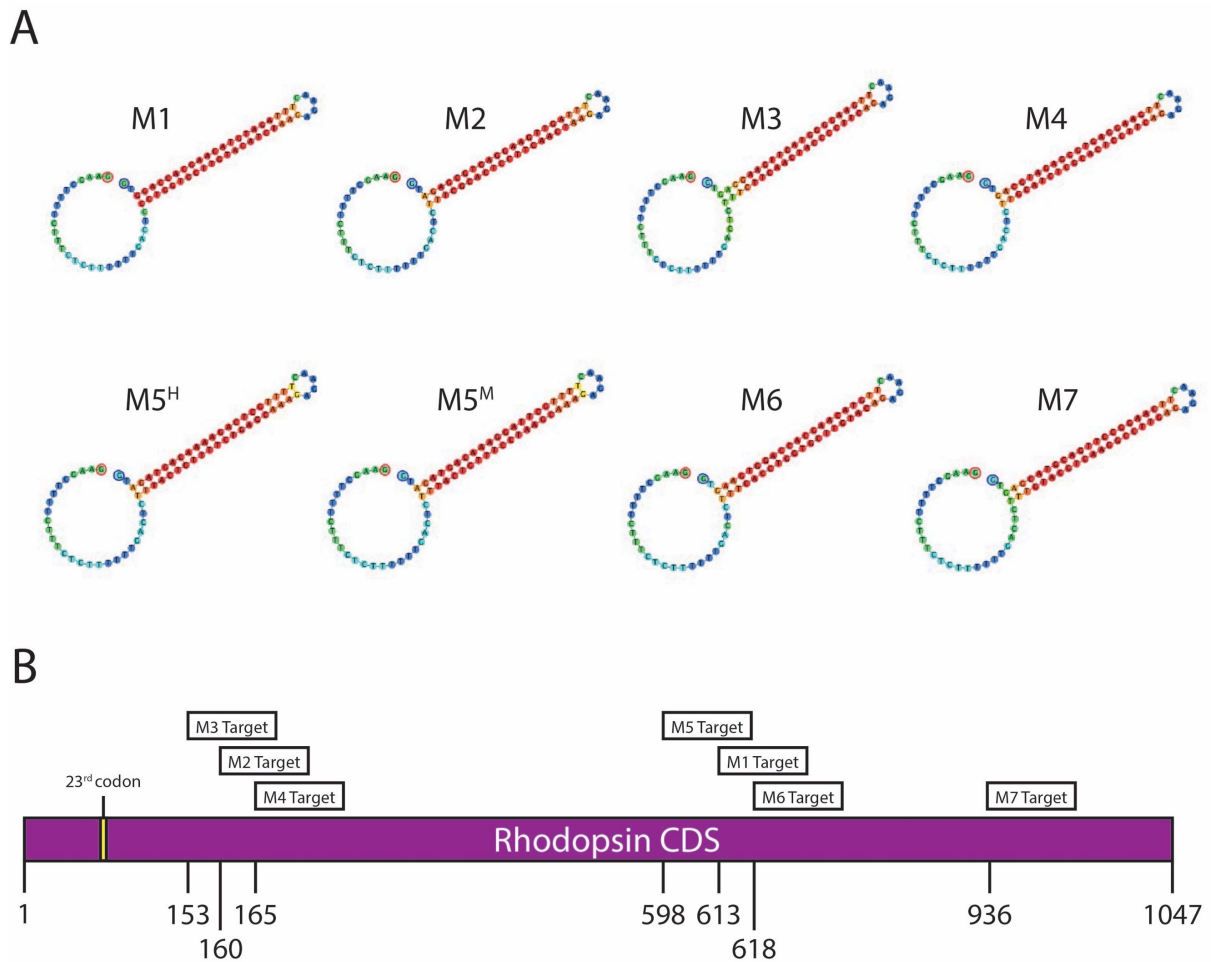


Figure 5.3. Mirtron designs. (A) RNA fold diagrams of the 76bp structures of mirtrons 1-7. RNA secondary structures are based on the probabilistic energy model described by Matthews et al., 2004³⁰⁵. Thermodynamic stability of predicted base pairing is indicated by colour with red representing the highest probability and blue the lowest. The 5' and 3' ends are indicated by blue and red circles respectively. Note that in all cases, the predicted internal stability at the base of the stem structure is lower than that of the distal end near the loop. This design feature was intended to favour selection of the guide (5') strand by the RISC²⁹⁷. Images generated using Gencious software (v11.0.2, Biomatters Ltd., Auckland, New Zealand) (B) Relative positions of the mirtron target sequences within the rhodopsin CDS. The 1047bp rhodopsin coding sequence is represented by a purple box. The relative locations of the 21bp target sequences for mirtrons 1 to 7 are indicated above the RHO CDS whilst the position of the first nucleotide of each target is shown beneath it. The position of the 23rd codon is

indicated by a yellow box and was not targeted by any of the seven mirtron designs. Schematic not to scale.

The guide strand and target sequences together with their respective target positions within the rhodopsin CDS are shown in Table 5.3.

Mirtron	Guide Strand Sequence	Target Sequence	Target Position
1	GTGGACCACGAACATGTAGAT	ATCTACATGTTCTGGTCCAC	613-633
2	GTAGAGCGTGAGGAAGTTGAT	ATCAACTTCCTCACGCTCTAC	160-180
3	GTGAGGAAGTTGATGGGGAAG	CTCCCCATCAACTTCCTCAC	153-173
4	GTGACGTAGAGCGTGAGGAAG	CTTCCTCACGCTCTACGTCAC	165-185
5 ^H	GTAGATGACAAAAGACTCGTT	AACGAGTCTTTTGTCTATCTAC	598-618
5 ^M	GTAGATGACAAAGGATTCGTT	AACGAATCCTTTGTCTATCTAC	598-618
6	GTGAAGTGGACCACGAACATG	CATGTTCTGGTCCACTTCAC	618-638
7	GTGAGCATGCAGTCCGGAAC	GTTCCGGAAGTGCATGCTCAC	936-956

Table 5.3. Mirtron guide and target sequences. The 21bp guide strands and corresponding target sequences within rhodopsin for mirtrons 1-7 are displayed. The position of each target sequence within the rhodopsin CDS in bp is shown in the right-hand column.

5.4.2. Mirtron splicing efficiency is dependent on splice donor sequence strength

To determine splicing efficiency of the seven candidate mirtron designs, each was individually cloned into the BstbI cut site of the coding sequence of *eGFP* as an artificial intron, and amplified in a plasmid under the control of the CAG promoter with a 3' WPRE sequence (designated M1-GFP to M7-GFP plasmids; Figure 5.4B). Since each mirtron was 76bp in length, failure to splice would be expected to induce a frame shift and result in abolition of eGFP protein expression following transfection. eGFP-derived fluorescent signal should therefore reflect splicing efficiency of the nested mirtron.

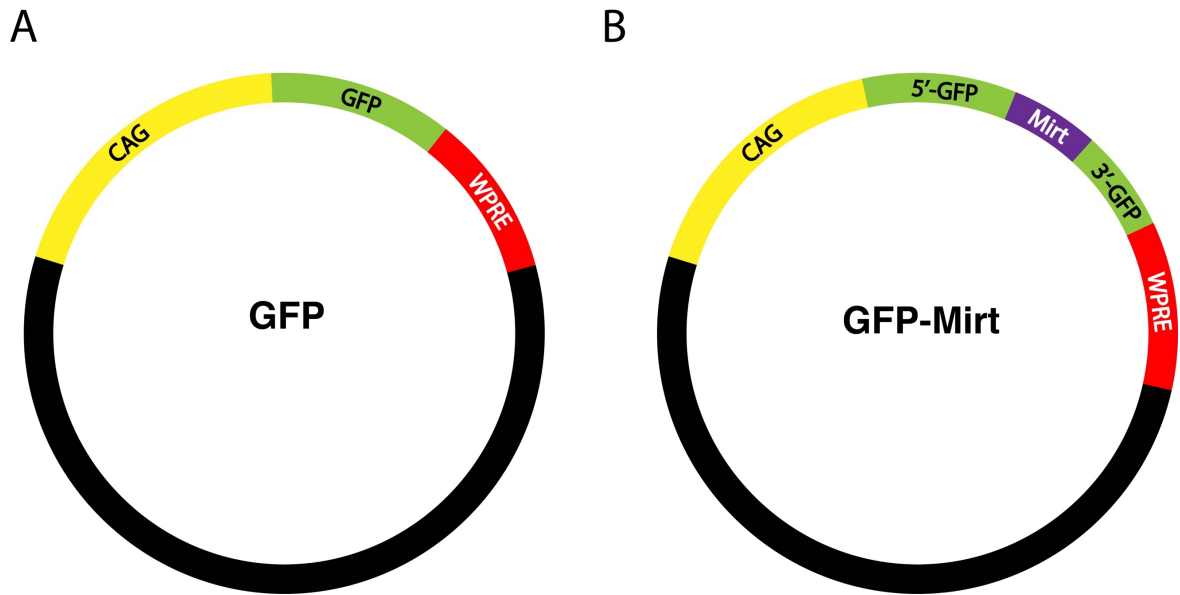


Figure 5.4. GFP and Mirtron-GFP plasmid maps. (A) Structure of the CAG.eGFP.WPRE (GFP) plasmid. **(B)** General structure of Mirtron-GFP plasmids (M1-GFP to M7-GFP). The 76bp mirtron is nested within the *eGFP* CDS dividing it into 5' and 3' fragments. Failure to splice would be predicted to cause a frame shift and nonsense-mediated decay of the transcript.

Figure 5.5A shows fluorescence micrographs of HEK293 cells taken 48 hours after transfection with equal quantities of Mirtron-GFP plasmids together with images of positive (GFP plasmid-transfected) and negative (untransfected) control samples. eGFP expression was detected for all plasmids but the strength of this signal varied significantly between the seven mirtron designs. Green fluorescence was subsequently quantified from protein lysates derived from these samples by fluorescence spectroscopy (Figure 5.5B).

To investigate splice variants, RNA from Mirtron-GFP transfected cells was extracted and reverse transcribed into cDNA. A set of mirtron-spanning primers directed against the flanking *eGFP* sequence (Primers 19 & 20) was used to amplify the spliced region. The resulting PCR products were run through a 2% agarose gel by electrophoresis. Each resulting band

corresponded to a different splice product: a band of 225bp suggested cDNA template derived from correctly spliced mRNA, a band of 301bp suggested cDNA template derived from unspliced mRNA, whilst bands of intermediate length indicated the presence of incorrectly spliced transcripts (Figure 5.5D). The detection of such intermediate bands suggested the presence of unintended cryptic splice donor or acceptor sites within mirtron sequences. The relative frequencies of splice products for each mirtron construct could be inferred from their relative band densities (Figure 5.5C). Taken together, these data suggest that rhodopsin-targeting synthetic mirtrons may be spliced out of a transcript as artificial introns following *in vitro* transfection, although the efficiency and accuracy of this process is sequence dependent. The estimated efficiency of correct splicing ranged from less than 1% for M1 to near 100% for M5 and M7. The degree of correct splicing for each mirtron broadly reflected the strength of its donor consensus sequence as determined by previous *in silico* analysis (Table 5.2). This was clearly further influenced by the presence and strength of alternative cryptic splice donor or acceptor motifs within the mirtron stem-loop hairpin.

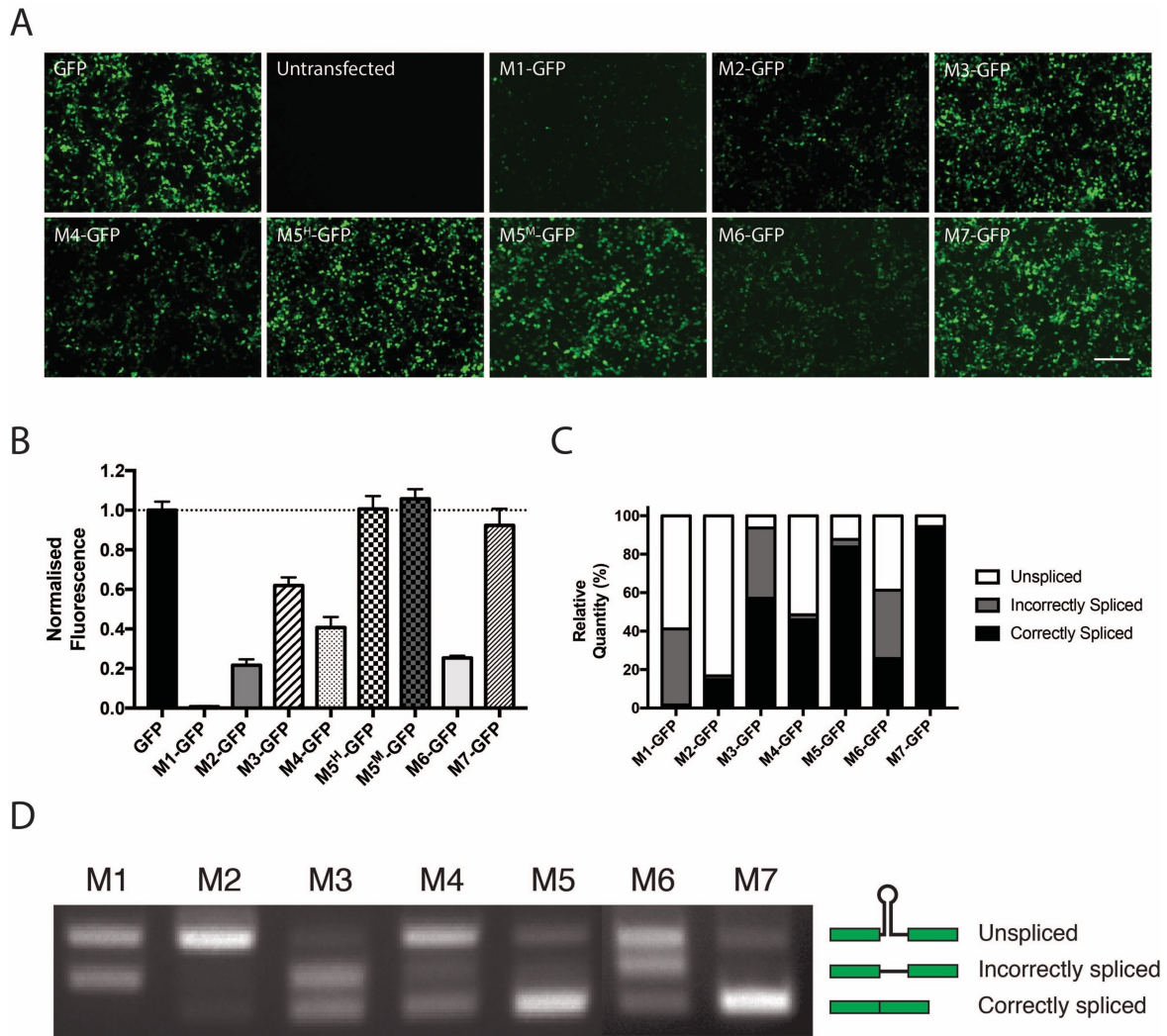


Figure 5.5. Splicing of mirtrons as artificial introns from within the *eGFP* CDS. HEK293 cells were transfected with CAG.eGFP.WPRE (GFP) or CAG.eGFP-M1-7.WPRE (M-GFP) plasmids. **(A)** Fluorescence micrographs of HEK293 cells taken 48 hours post-transfection using identical acquisition settings. Scale bar = 200µm. **(B)** Fluorescence levels of protein lysates from M-GFP transfected HEK293 samples normalised to that of the GFP transfected control group (n=3 each). **(C)** Relative densitometry of bands corresponding to PCR products derived from a mirtron-spanning primer pair. Note how the proportion of correctly spliced product for mirtrons as determined by this method (black bars) broadly reflect corresponding estimates of splicing efficiency derived from the fluorescence assay shown in (B). **(D)** Representative UV agarose gel image of splice PCR products. The schematic to the right indicates the likely origin of the various bands detected.

5.4.3. Artificial mirtrons can effectively knock down human and mouse rhodopsin mRNA

The human and mouse rhodopsin coding sequences were individually cloned into the MCS of the PsiCHECK2 (PC) vector (Promega, UK) in accordance with manufacturer's instructions (Section 5.3.1). The resulting plasmids, designated PC-RHO & PC-mRho, were cotransfected with Mirtron-GFP plasmids in HEK293 cells, and M1-7 knockdown efficiency against each rhodopsin species then determined using the Dual Glo[®] luciferase assay (Figure 5.6). Significant knockdown of both human and mouse rhodopsin was observed for M2 and M3, and for each version of M5 when tested against the PC vector corresponding to its targeted species (all $p < 0.0001$ versus control GFP sample). No knockdown was apparent when M5^H was tested against PC-mRho, or when M5^M was tested against PC-RHO, suggesting that mismatches between the mirtron guide sequence and its target may not be tolerated.

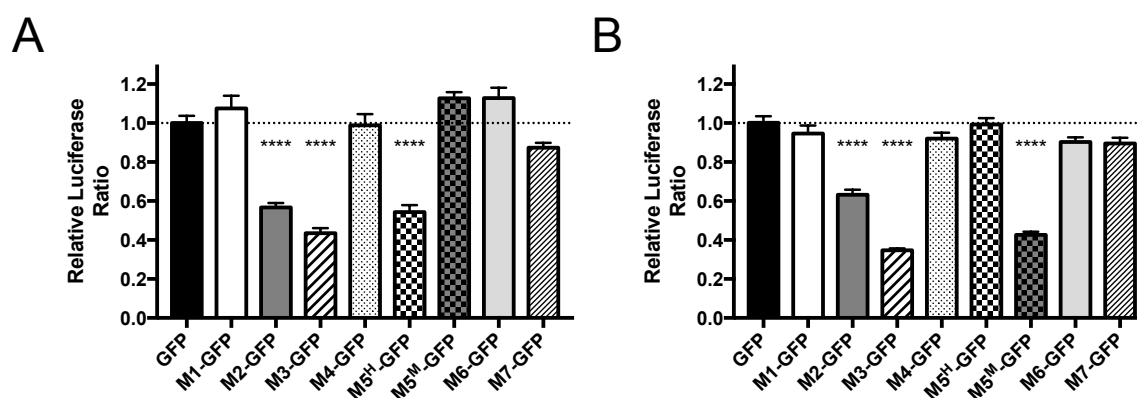


Figure 5.6. Determination of mirtron-mediated rhodopsin suppression by the Dual Glo[®] luciferase assay. Mean $\pm$ SEM is plotted in all cases. **(A)** Human rhodopsin target (n=12). **(B)** Mouse rhodopsin target (n=6). Note that each version of M5 was only effective at targeting the rhodopsin sequence of its corresponding species. Ordinary one-way ANOVA for effect of mirtron type on relative luciferase ratio for human target $F(8, 99)=41.9$, $p < 0.0001$; Ordinary one-way ANOVA for effect of mirtron type on relative luciferase ratio for mouse target $F(8, 45)=77.88$, $p < 0.0001$; **** $p < 0.0001$ versus the mirtronless CAG.eGFP.WPRE plasmid, Dunnett's multiple comparisons test.

5.4.4. Both splicing and a specific antisense sequence are required for mirtron efficacy

The requirement of correct splicing and a specific antisense sequence for mirtron-mediated rhodopsin knockdown was investigated by cloning two alternative versions of M2 into the BstBI restriction site of the *eGFP* CDS. The first was a so-called ‘unspliceable’ version (M2^U). M2 and M2^U were identical in all but the first nucleotide: the guanine at position 1 of the canonical splice donor site in M2 was substituted for an adenine nucleotide in M2^U. The M2^U guide strand was thus ATAGAGCGTGAGGAAGTTGAT. The second was a ‘scrambled’ version (M2^S) where the nucleotides of the guide strand not predicted to be crucially involved in splicing (positions 7-21) were randomly re-ordered. The guide strand for M2^S was GTAGAGATGTGGTTGAGGATT. Figure 5.7 shows that M2^U did not splice out of *eGFP* (indicated by a lack of green fluorescence) and was incapable of mediating rhodopsin knockdown. Although M2^S spliced out to a similar degree to M2, it too failed to induce rhodopsin knockdown. Thus, both splicing and a specific antisense sequence are required for mirtron-induced mRNA suppression.

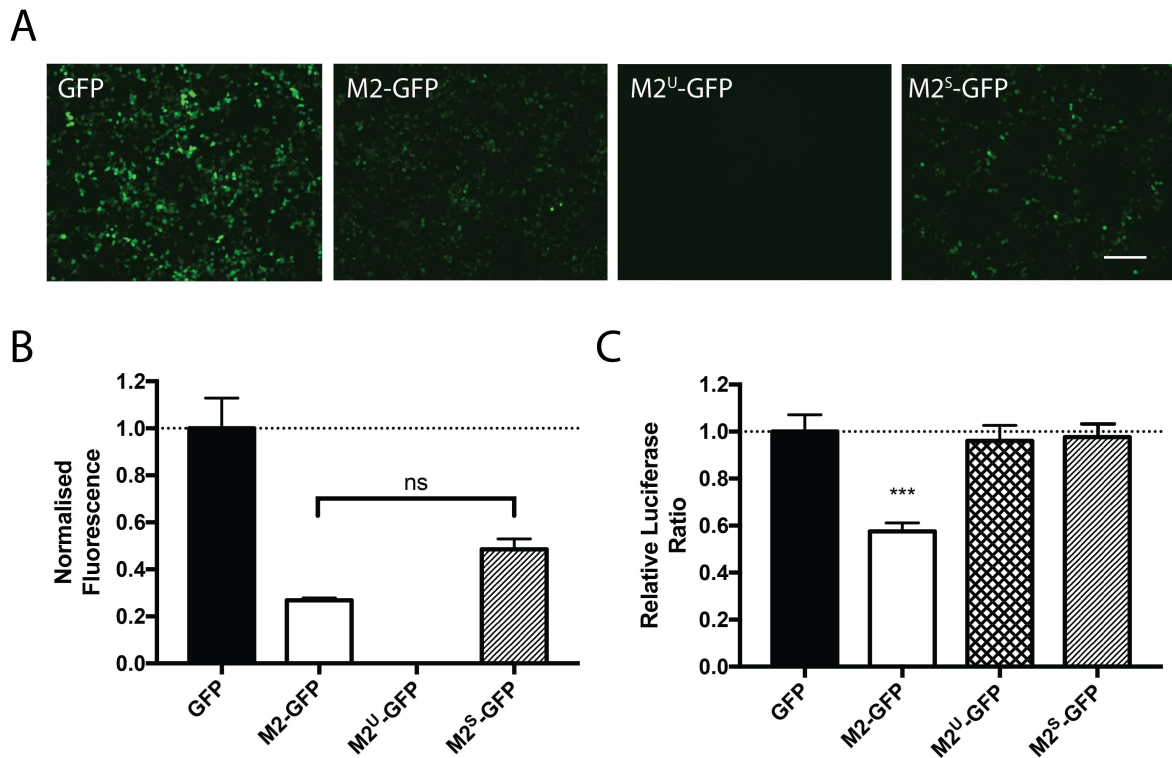


Figure 5.7. Mirtron-mediated rhodopsin suppression is dependent on splicing and a specific antisense sequence. (A) Fluorescence micrographs following transfection of HEK293 cells with the mirtronless *eGFP* plasmid (GFP), or M2-GFP, M2^U-GFP and M2^S-GFP plasmids. Scale bar = 200µm. **(B)** Fluorescence signal from mirtron-transfected HEK293 protein lysates normalised to that recorded from GFP plasmid transfected cell lysates (n=3). Dual Glo® luciferase assay. ***p=0.0001 M2-GFP versus GFP, Dunnett's multiple comparisons test.

5.4.5. mRNA target sequence context influences potency of mirtrons

To investigate the effect of target sequence context on mirtron potency, a 'short' version of the human rhodopsin target comprising the 21bp M3 target sequence with 25bp of upstream and 25bp downstream DNA was cloned into the MCS of the PsiCHECK2 vector (using Primers 21 & 22 with XhoI and NotI restriction enzymes). M3 efficacy against this construct was compared with that measured against the full-length sequence (Figure 5.8). Interestingly, M3 was significantly more effective against the RHO target with shorter flanking sequence. Context

of the target site can thus have a significant impact on the potency of mirtrons. This is likely to be attributable to local differences in mRNA secondary structure which may variously facilitate or restrict access of the RISC to the transcript target site. This phenomenon may underlie the differences in potency observed when mirtrons were tested against human and mouse targets (Figure 5.6).

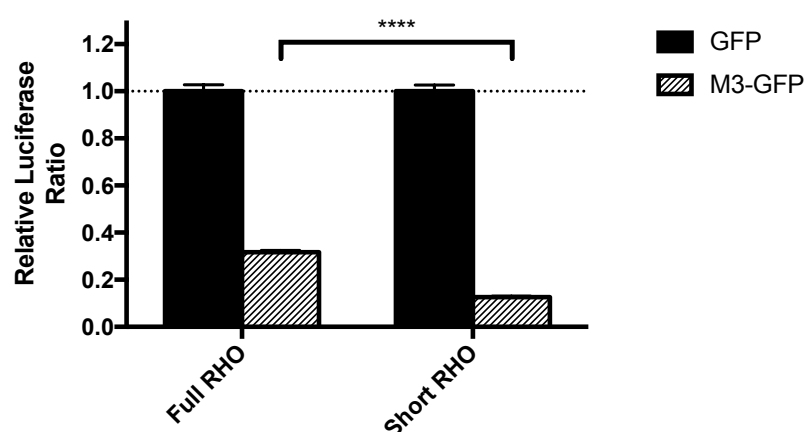


Figure 5.8. mRNA target context influences potency of mirtrons. ‘Full RHO’ corresponds to the PsiCHECK2 vector into which the full-length human rhodopsin coding sequence had been cloned. The ‘Short RHO’ PsiCHECK2 plasmid contained just the region of the human rhodopsin sequence corresponding to the 21bp M3 target sequence with 25bp of 5’ and 3’ flanking DNA. **** $p < 0.0001$, two-way ANOVA, Sidak’s multiple comparison test, $n = 6$.

5.4.6. A novel luciferase fusion assay for the determination of RNAi potency

The Dual Glo[®] luciferase assay quantifies efficacy of RNAi effectors which target the gene of interest cloned into the 3’ UTR of *Renilla* luciferase. It does not necessarily follow that mRNA cleavage will occur to the same extent *in vivo* in cases where the target site is located within an actively translated CDS, where ribosomes and their associated nascent peptides may restrict access of the RISC to the transcript. Indeed, it is thought that native miRNAs that target coding sequences may exert their effect more through translational inhibition rather than mRNA

cleavage, and such miRNAs seem to have a lesser influence on target protein levels³⁰⁶. To investigate the efficacy of mirtrons directed against a translated sequence, the human rhodopsin gene was cloned into the PsiCHECK2 vector using an overlap extension PCR technique using Primers 23 – 26 in order to create a RHO-*Renilla* luciferase fusion protein, with the luciferase being tagged to the cytosolic C-terminus of rhodopsin via an alanine-proline-valine-alanine-threonine (APVAT) link peptide (RHO-Luc.PsiCHECK2). The validity of this approach was first established by cloning a RHO-APVAT-GFP sequence (generated by overlap extension PCR using Primers 24, and 27 - 29) into the CAG.WPRE plasmid backbone. This was used to transfect HEK293 cells that were subsequently immunostained using the 4D2 anti-rhodopsin antibody (Abcam, UK) which targets the extracellular N-terminus of the protein. Confocal fluorescence microscopy revealed the RHO-GFP fusion protein to be visible as a double ring of fluorescence (Figure 5.9).

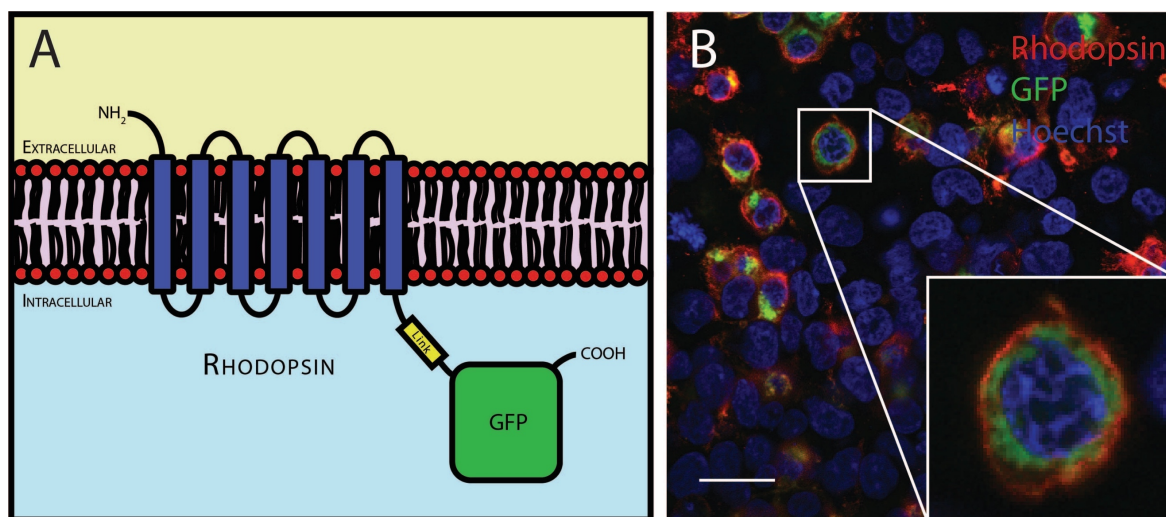


Figure 5.9. The RHO-GFP protein traffics to the plasma membrane. (A) Schematic representation of the RHO-GFP fusion protein structure. GFP is tagged to the intracellular C-terminus of rhodopsin via an APVAT link peptide. **(B)** Staining of HEK293 cells expressing the RHO-GFP protein for rhodopsin using the 4D2 N-terminus anti-rhodopsin primary antibody revealed a double ring of fluorescence signal (see inset). Scale bar = 20µm.

HEK293 cells were then transfected with the RHO-*Renilla* luciferase fusion PsiCHECK2 plasmid and stained for rhodopsin and *Renilla* luciferase. Fluorescence from the two antibodies co-localised. By contrast, staining of cells transfected with PsiCHECK2-RHO (where the human rhodopsin sequence had been cloned into the MCS of the *Renilla* luciferase 3' UTR) showed *Renilla* luciferase localization within the cytosol and no rhodopsin signal (Figure 5.10C). Having confirmed that mirtron target sequences within RHO-Luc.PsiCHECK2 are indeed translated into protein, the fusion plasmid was used to assess mirtrons 1-7 in further Dual Glo[®] luciferase assays. The results were comparable to that of the standard assay in all cases except for that of M2, which appeared ineffective when tested using the fusion assay (Figure 5.10D). This supports the hypothesis that mirtron-based RNAi efficacy may, in certain cases, differ when a translated CDS rather than a 3' UTR is targeted. The psiCHECK2 fusion vector approach presented here may have general applicability for *in vitro* testing of RNA interference effectors that are designed to target coding sequences. The results of this assay suggested that M3 and M5 are likely to be the most effective of the seven candidate mirtron designs for downstream *in vivo* application.

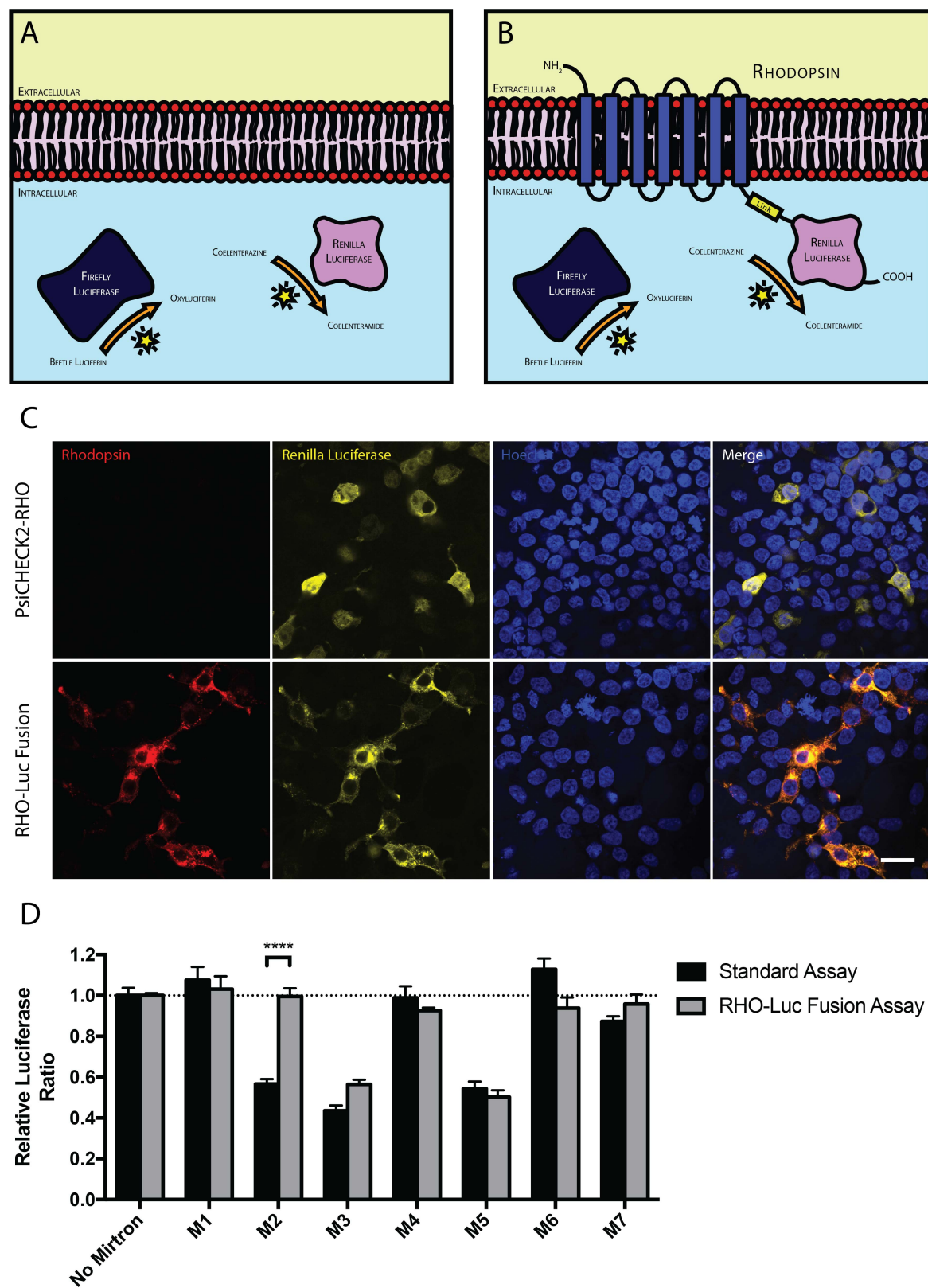


Figure 5.10. The RHO-Luc fusion assay for assessment of mirtron-based suppression when target sequences are located within the translated portion of the transcript. (A) Illustration of the

Dual Glo® assay following transfection with the PsiCHECK2-RHO plasmid. In this case the *RHO* CDS is located in the 3'-UTR of the *Renilla* luciferase mRNA and is thus not translated. Both firefly and *Renilla* luciferases are expressed in the cytosol and catalyse reactions that produce luminescence. **(B)** Illustration of the Dual Glo® assay following transfection with the RHO-Luc.PsiCHECK2 fusion plasmid. Here the *Renilla* luciferase is tagged to the C-terminus of rhodopsin via an APVAT link peptide and would therefore be expected to localize to the plasma membrane. **(C)** ICC of HEK293 cells transfected with the PsiCHECK2-RHO plasmid (upper panels) or the RHO-Luc.PsiCHECK2 plasmid (lower panels), and stained for rhodopsin and *Renilla* luciferase. No rhodopsin protein staining was detected following transfection with PsiCHECK2-RHO and *Renilla* luciferase localised to the cytosol. Following transfection with the RHO-Luc.PsiCHECK2 plasmid, colocalization of rhodopsin and *Renilla* luciferase signals was apparent confirming expression of the fusion protein. Scale bar = 20µm. **(D)** Comparison between standard and fusion Dual Glo® assays for assessment of efficacy of mirtrons 1-7 (M1-7). Note that M2 induced significant rhodopsin suppression as measured using the standard assay but was ineffective when tested using the RHO-Luc fusion assay. Two-way ANOVA with mirtron number and assay type as factors: $F(1, 27)=2.51$, $p=0.12$ for effect of assay, $F(7, 127)=7.43$, $p<0.0001$ for interaction mirtron number x assay type; **** $p<0.0001$, Sidak's multiple comparison test.

5.4.7. Predicted off-targets are not subject to mirtron-induced knockdown

If mirtrons are to be used for the treatment of patients with RP, it is important that off-target effects within transduced rods are minimal. To investigate this, the 21bp target sequences for the three most effective mirtrons (M2, M3 and M5) were subjected to a BLAST search (www.endeml.org) for homology within the mouse and human transcriptomes. All matches with an E-value (the calculated probability of the alignment between the input and subject sequences being due to chance) of less than ten were considered (Table 5.4). Potential targets for the passenger strand sequences of these mirtrons were also considered and are shown in Table 5.5. The longest guide strand off-target match was an 18bp complementary sequence (with one internal mismatch) within the GPT2 CDS (E-value=5.5). Several 14bp alignments

within the human transcriptome were also identified for M3 and 5H which included a match between the M3 guide sequence and the 3'-UTR of the SEC14L2 gene (E-value=5.5). This potential target (along with GPT2) was explored further, as expression within rod OS has been documented³⁰⁷. To quantify the extent to which these predicted sequences are true off-targets for M3, the target sequence for each (along with 25bp of upstream and downstream flanking DNA) was ligated into the MCS of the PsiCHECK2 vector. The subsequent Dual Glo[®] assay performed against M3 suggested that mRNA for GPT2 and SEC14L2 are not subjected to M3-mediated knockdown (Figure 5.11).

Mirtron	Human targets	Match (bp)	E-value	Mouse targets	Match (bp)	E-value
2	<i>RHO</i>	21	0.0004	<i>Rho</i>	21	0.0002
				<i>Tenm2</i>	14	3.7
3	<i>RHO</i>	21	0.0004	<i>Rho</i>	21	0.0002
	<i>GPT2</i>	18 [†]	5.5	<i>Ctsc</i>	14	3.7
	<i>IL34</i>	14	5.5	<i>Lgals4</i>	14	3.7
	<i>PTF1A</i>	14	5.5	<i>Tenm2</i>	14	3.7
	<i>SEC14L2*</i>	14	5.5			
	<i>GOLGA8B</i>	14	5.5			
	<i>AKNA</i>	14	5.5			
	<i>INPL1</i>	14	5.5			
5^H	<i>RHO</i>	21	0.0004	<i>Rho</i>	21	0.0002
	<i>ZBTB10</i>	14	5.5	<i>Ptprb</i>	15	0.94
	<i>RHPT1</i>	14	5.5	<i>Tgm7</i>	14	3.7
	<i>RHOT1</i>	14	5.5	<i>Mtm</i>	14	3.7
	<i>C11orf40</i>	14	5.5	<i>Mfsd8</i>	14	3.7
				<i>Ikbkap</i>	14	3.7
5^M	None	-	-	<i>Rho</i>	21	0.0003
				<i>Pnn</i>	14	3.9
				<i>Dync1h1</i>	14	3.9
				<i>Erg</i>	14	3.9

Table 5.4. Predicted off-targets for the guide strand of mirtrons 2, 3 and 5. The 21bp guide sequences were input into BLAST searches of the human and mouse cDNA databases (transcripts/splice variants). The length of the continuous match for each hit is shown in bp together with the corresponding E-value. *Gene expression previously described in photoreceptors. †One internal mismatch.

Mirtron	Human targets	Match (bp)	E-value	Mouse targets	Match (bp)	E-value
2	<i>SYNDIG1L</i>	15	1.4	<i>Swap70</i>	15	0.98
	<i>TENM2</i>	14	5.7			
3	<i>LAMA2</i>	15	1.4	<i>Olfir1384</i>	16	0.24
	<i>GOLGA8N</i>	14	5.5	<i>Gpr139</i>	16	0.24
	<i>CES2</i>	14	5.5	<i>Olfir281</i>	14	3.7
	<i>CWC22</i>	14	5.5	<i>Il34</i>	14	3.7
	<i>SERPIND1</i>	14	5.5			
	<i>PTPN9</i>	14	5.5			
5^H	<i>ADAMTSL1</i>	18	0.023	<i>Olfir715</i>	15	0.94
	<i>DRAXIN</i>	15	1.4	<i>Mcpb1</i>	15	0.94
	<i>ZNF326</i>	14	5.5	<i>Ndufaf4</i>	14	3.7
	<i>HERC1</i>	14	5.5			
	<i>JPH1</i>	14	5.5			
	<i>RXFP1</i>	14	5.5			
	<i>FGF18</i>	14	5.5			
5^M	<i>ASPA</i>	16	0.36	None	-	-
	<i>CHST9</i>	14	5.7			
	<i>FSHB</i>	14	5.7			
	<i>ZNF624</i>	14	5.7			
	<i>ZNF480</i>	14	5.7			
	<i>GCLC</i>	14	5.7			
	<i>GMNC</i>	14	5.7			

Table 5.5. Predicted off-targets for the passenger strand of mirtrons 2, 3 and 5. The 21bp passenger sequences were input into BLAST searches of the human and mouse cDNA databases

(transcripts/splice variants). The length of the continuous match for each hit is shown in bp together with the corresponding E-value.

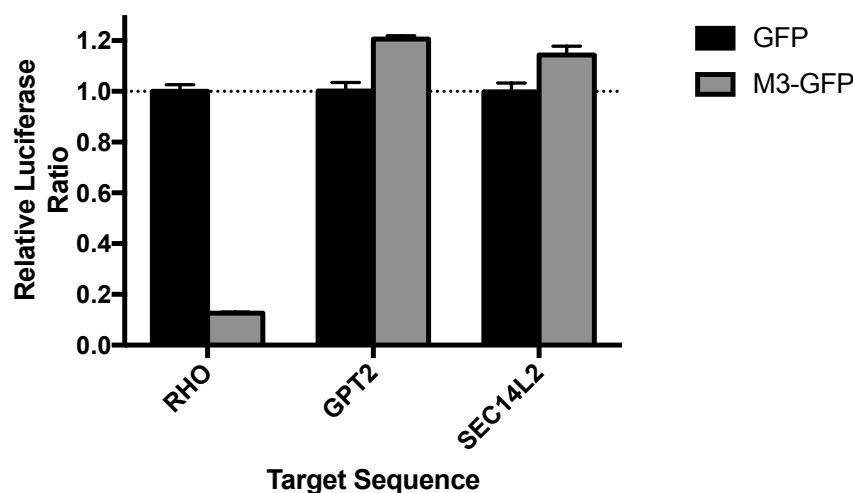


Figure 5.11. Mirtron 3 does not target the predicted off-target mRNA transcripts of the human *GPT2* and *SEC14L2* genes. Target sequences within *RHO*, *GPT2* and *SEC14L2* were cloned into the PsiCHECK2 vector and a Dual Glo® assay performed. No knockdown of gene expression was detected for either of the putative off-targets. Bars represent mean $\pm$ SEM, n=6.

5.4.8. Mirtrons are more potent when spliced from the 5'-UTR than the coding sequence of a transgenic mRNA

A recent study exploring the molecular biology of artificial mirtrons concluded that placement of the mirtron as an intron within the CDS of the replacement transcript leads to a significant reduction in protein expression from that gene³⁰². Placement of the mirtron in the 3'-UTR of the transgene would also be likely to result in reduced gene expression as splice sites downstream of a stop codon are thought to trigger nonsense-mediated decay³⁰⁸. It therefore seemed logical to locate mirtrons in the 5'-UTR of the replacement gene CDS. Curtis et al. however, reported that mirtrons in this location failed to splice out efficiently, which adversely affected potency, and attributed this to a paucity of exonic splice enhancers (ESEs, hexamer

DNA sequences that act to enhance splicing of pre-mRNA³⁰⁹) around the mirtron. To investigate this, mirtrons 2, 3 and 5 were cloned into the 5'-UTR of the CAG.GFP.WPRE plasmid. To encourage efficient splicing at this new location, flanking sequences around the original mirtron insertion site within *eGFP* were also included (i.e. around the BstBI restriction site where efficient splicing had already been confirmed- see Figure 5.5), as these were found to contain numerous ESEs (as identified using the online RESCUE-ESE tool³¹⁰, <http://genes.mit.edu/burgelab/rescue-ese/>). An *in silico* splice analysis was performed using online software (Spliceport, <http://spliceport.cbc.umd.edu>) to identify the optimal 5' and 3' flank length, whilst avoiding 'ATG' motifs which may act as cryptic translational start sites³¹¹. Table 5.6 shows the splice scores calculated using the Spliceport online tool for Mirtron 3 with flanks from the neighbouring *eGFP* CDS of different sizes when placed at the intended insertion site within the 5'-UTR: an EcoRV restriction site (which creates a blunt-ended cut) between the CAG promoter and Kozak consensus sequence.

Upstream flank size (bp)	Donor site score	Downstream flank size (bp)	Acceptor site score
0	-1.01	0	2.41
5	0.8	5	3.02
10	0.59	10	2.65
15	0.4	15	2.51
20	1.07	20	2.32
25	1.21	25	2.26
30	0.92	30	2.1
35	1.56	35	2.47
41	1.73	40	2.27
		45	2.06
		50	2.35
		53	2.55

Table 5.6. *In silico* optimisation of mirtron flank sequence length. Having determined that mirtrons splice effectively from the BstbI restriction site within the *eGFP* CDS, an *in silico* analysis was performed to determine the optimal likely upstream and downstream flanking sequence length that would ensure this splicing efficiency is maintained when cloned into the 5'-UTR. Scores >0 give a false positive rate for splice donor and acceptor sites of 1.41% and 1.54% respectively whilst scores >1 give a false positive rate of 0.09% and 0.12% (Spliceport online tool; <http://spliceport.cbcb.umd.edu>).

The maximum 5' flank size which avoided an 'ATG' motif was 41bp and this gave the highest donor consensus score. The 3' flank length had little effect on acceptor score which was found to be strong in all cases. Upstream flank size did not have a significant effect on acceptor score, and downstream flank size similarly had little effect on donor score. Thus, a 41bp upstream flank and 53bp downstream flank from the *eGFP* CDS around the BstBI cut site was used (amplified using Primers 30 & 31). The sequences of the upstream (5') and downstream (3') flanks are shown below in black. The positions of ESE hexamer motifs identified using the RESCUE-ESE online tool are shown above each in red:

Upstream flank:

```

      ACAAGA      TTCGAA
      TACAAG      GTCAAG
      ACTACA      TGAAGT
      AACTAC      CTGAAG
TGACGGGAACTACAAGACCCGCGCTGAAGTCAAGTTCGAAG

```

Downstream flank:

```

                                GAGGAT
                                GGAGGA
      TGAAGG      AAGGAG
      CTGAAG
GTGACACCCTGGTGAATAGAATCGAGCTGAAGGGCATTGACTTTAAGGAGGAT

```

The new CAG.Mirton-UTR.eGFP.WPRE constructs, designated M2-UTR, M3-UTR, M5^H-UTR and M5^M-UTR, were compared to the mirtronless CAG.eGFP.WPRE plasmid for

rhodopsin knockdown using the Dual Glo[®] luciferase assay, and for downstream gene expression by the fluorescence assay as described previously (Figure 5.12).

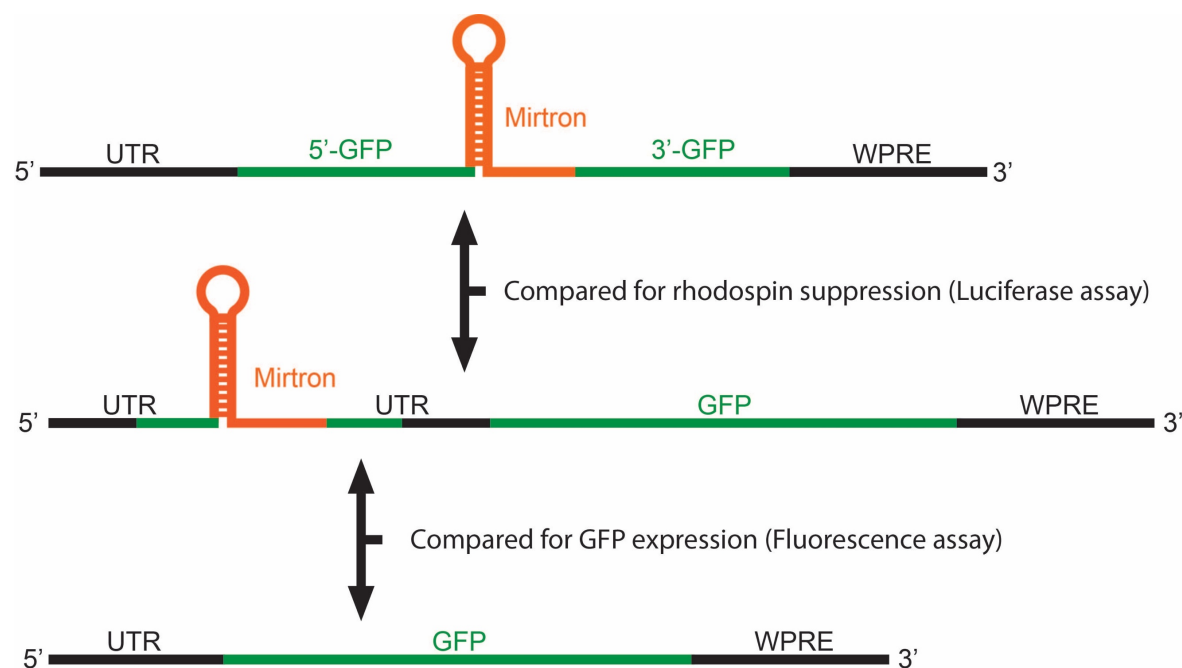


Figure 5.12. Experimental scheme for the comparison of nested and 5'-UTR mirtrons in HEK293 cells. Rhodopsin knockdown efficacy was compared for constructs in which the mirtron was nested as an intron within *eGFP* (top) and those in which the mirtron was located in the 5'-UTR of *eGFP* (middle). To determine the effect of placement of the mirtron in the 5'-UTR on downstream gene expression (i.e. promoter strength), *eGFP* fluorescence signal was compared for CAG.Mirtron-UTR.*eGFP*.WPRE (middle) and CAG.*eGFP*.WPRE (bottom) constructs using the fluorescence spectroscopy assay (Section 5.3.2).

All mirtrons were found to be more effective when located in the 5'-UTR than when nested within the *eGFP* coding sequence for both human and mouse rhodopsin targets (Figure 5.13). A PCR splice analysis using cDNA from transfected cells as template was conducted as described previously. Band densitometry performed on the subsequent gel image revealed that a greater proportion of transcripts were correctly spliced for 5'-UTR mirtrons when compared

with that of their nested counterparts (Figure 5.14). Confirmation that the lowest band did indeed represent correctly spliced mRNA was achieved by Sanger sequencing of extracted DNA amplicons (Figure 5.14C).

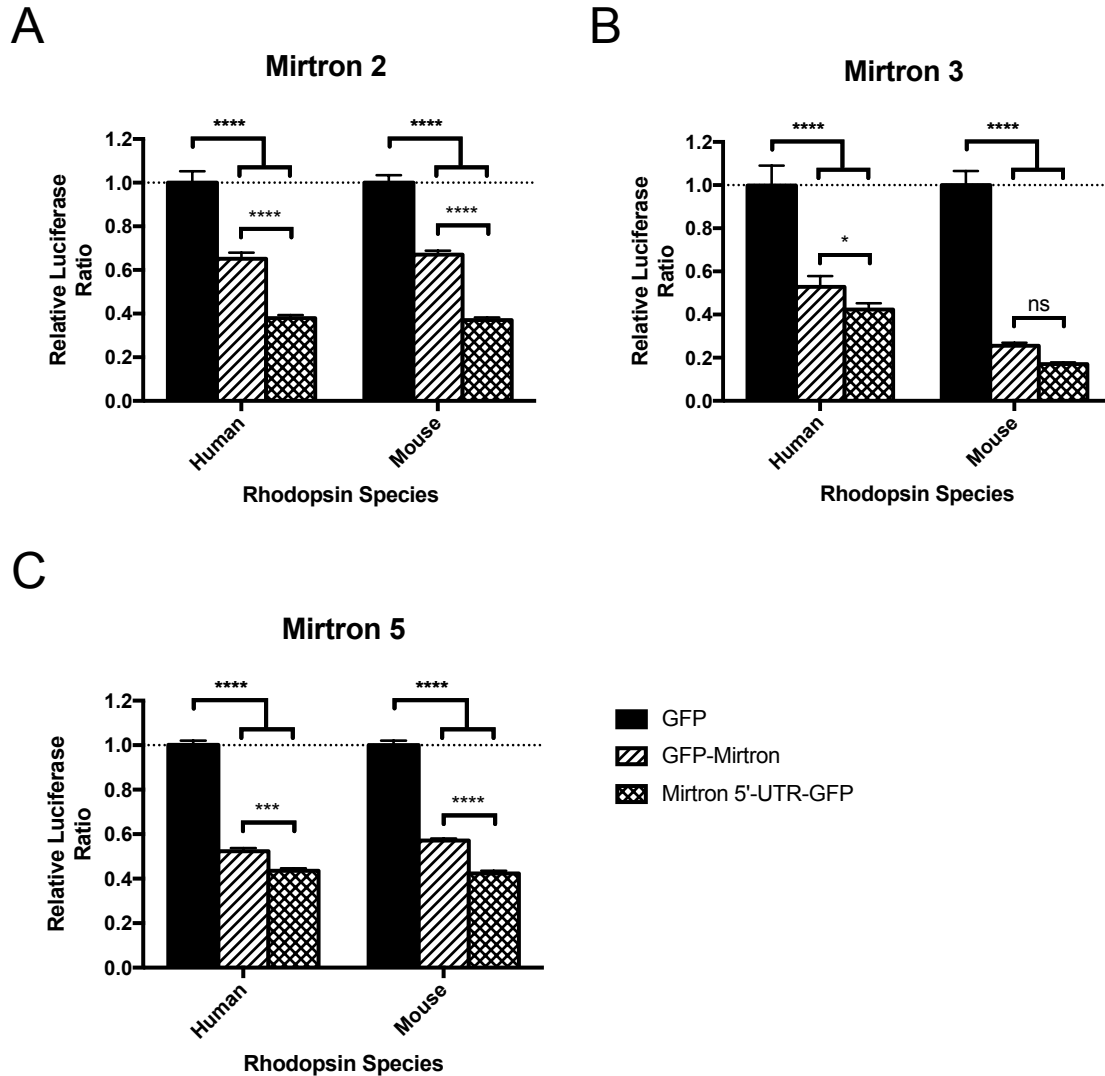


Figure 5.13. Mirtrons are more potent when located in the 5'-UTR than when nested within the CDS of the *eGFP* transgene. Knockdown of rhodopsin was compared using the Dual Glo® luciferase assay for 5'-UTR mirtrons (cross-hatched bars) and their nested equivalents (hatched bars) for mirtrons 2 (A), 3 (B), and 5 (C) in HEK293 cells. Data are shown for the version of mirtron 5 directed against its corresponding rhodopsin species. Mean $\pm$ SEM plotted in all cases, $n=6$. ns, not significant; * $p<0.05$; *** $p<0.001$; $p<0.0001$, two-way ANOVA, Sidak's multiple comparison test.

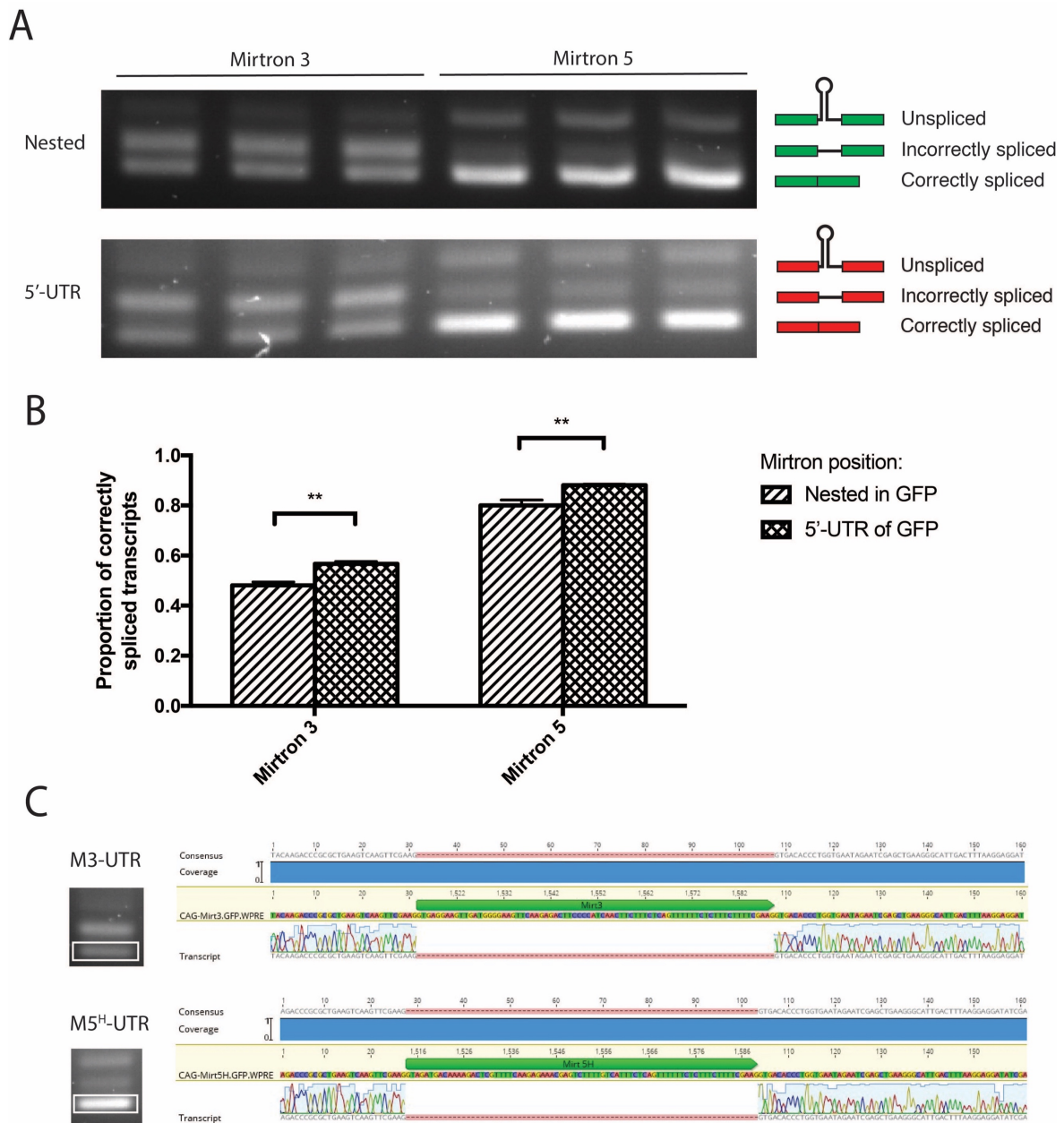


Figure 5.14. Mirtrons are efficiently and accurately spliced out from the 5'-UTR of *eGFP*. (A) PCR products generated using mirtron-spanning primers (Primers 32 & 33) and template cDNA derived from HEK293 cells transfected with either nested or 5'-UTR versions of mirtrons 3 and 5 ($n=3$ biological replicates for each). (B) Band densitometry analysis revealed a greater proportion of correctly spliced transcripts for mirtrons located in the 5'-UTR for both mirtrons 3 and 5. $**p<0.01$, two-way ANOVA, Sidak's multiple comparison test. (C) Sanger sequencing of the smallest of the three generated bands (highlighted in white boxes shown to the left of the figure) confirmed accurate splicing of mirtrons 3 and 5 from the 5'-UTR of transgenic mRNA. Alignment of amplicon electropherograms with their

corresponding CAG.Mirtron-UTR.GFP.WPRE reference sequences confirmed absence of the 76bp nucleotides corresponding to the mirtrons.

5.4.9. Delivery of multiple mirtrons in tandem results in increased rhodopsin knockdown

Having established that mirtrons remain effective when spliced out of the 5'-UTR of the supplemental transcript, the effect of multiple mirtrons in tandem within the 5'-UTR was investigated. The following plasmids were cloned (see Figure 5.16 A & C):

1. M3/3-UTR: Two copies of M3 in series, each with their own ESE flanking sequences, cloned into the 5'-UTR of the CAG.eGFP.WPRE plasmid.
2. M3/5^H-UTR: One copy of M3 and one copy of M5^H in series, each with their own ESE flanking sequences, cloned into the 5'-UTR of the CAG.eGFP.WPRE plasmid.
3. M3/5^M-UTR: One copy of M3 and one copy of M5^M in series, each with their own ESE flanking sequences, cloned into the 5'-UTR of the CAG.eGFP.WPRE plasmid.

HEK293 cells were transfected with these plasmids. Two days later, RNA was extracted and converted to cDNA. PCR splice analysis was performed using these samples together with mirtron-spanning primers (Primer 32 & 33), as previously described. Two prominent bands were detected in the resulting gel (Figure 5.15A). The smallest of the two (260 bp) corresponded in size to that predicted from independent splicing of the two mirtrons. The larger band of approximately 280 bp is likely to have resulted from incorrect splicing of one or both mirtrons. Sanger sequencing of DNA extracted from the smaller bands confirmed that tandem mirtrons

are independently and precisely spliced (Figure 5.15B). Sanger sequencing of the larger band produced a mixed readout, suggesting that the extracted DNA had derived from a combination of several mis-spliced transcripts.

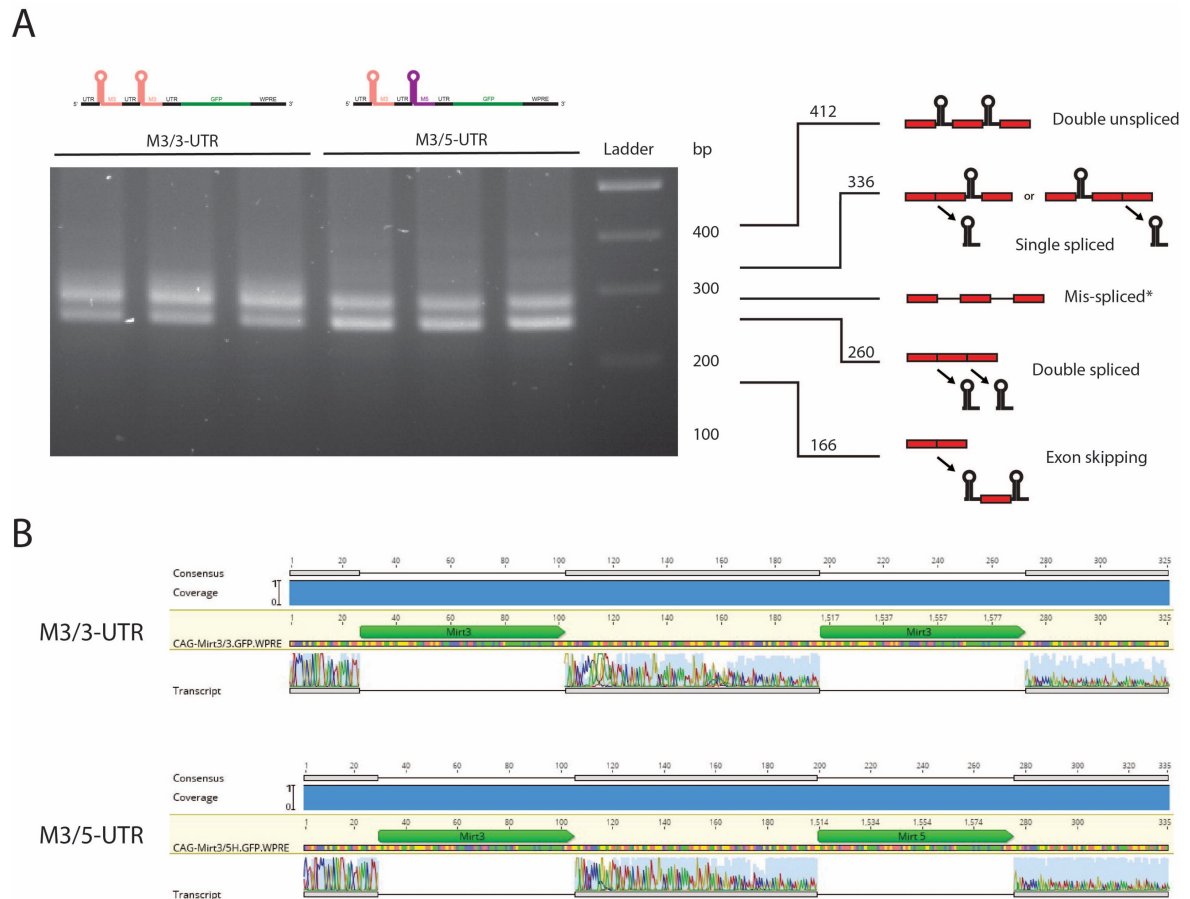


Figure 5.15. Mirtrons in tandem are individually and accurately spliced out of the 5'-UTR of *eGFP*. (A) PCR splice analysis of tandem mirtrons. To the right of the figure are schematics illustrating the predicted band sizes for the various possible alternative splice products. Note that no bands corresponding to double unspliced, single spliced or exon skipping splice products were apparent. *This band may represent a mixture of products which might include transcripts with correct splicing of the first mirtron and mis-splicing of the second, mis-splicing of the first and correct splicing of the second, or mis-splicing of both mirtrons. (B) Sanger sequencing of the smallest of the two generated bands confirmed accurate splicing of tandem mirtrons from the 5'-UTR of transgenic mRNA.

Comparison with single 5'-UTR mirtrons for knockdown of human and mouse rhodopsin using the Dual Glo[®] luciferase assay showed superior efficacy of the tandem construct in both instances (Figure 5.16). The fact that luciferase knockdown was greater following equimolar transfection with M3/3-UTR than with M3-UTR would suggest a dose-response relationship between intracellular mirtron expression and target gene suppression. That M3/5-UTR was more effective than either M3- or M5-UTR would suggest that mirtrons targeting different regions of a transcript can act in an additive fashion when simultaneously expressed within a cell. A direct comparison between M3/3-UTR and M3/5-UTR (testing each version against its corresponding rhodopsin species) indicated that the latter was the more effective when targeting human rhodopsin mRNA, whilst no difference between the constructs was apparent when targeting the mouse sequence (two-way ANOVA for overall effect of mirtron construct: $p=0.0004$. Statistical interaction between the factors of mirtron construct and rhodopsin species: $p=0.0094$. Sidak's multiple comparison test M3/3-UTR versus M3/5-UTR: $p<0.0001$ for human *RHO*, $p=0.6935$ for mouse *Rho*).

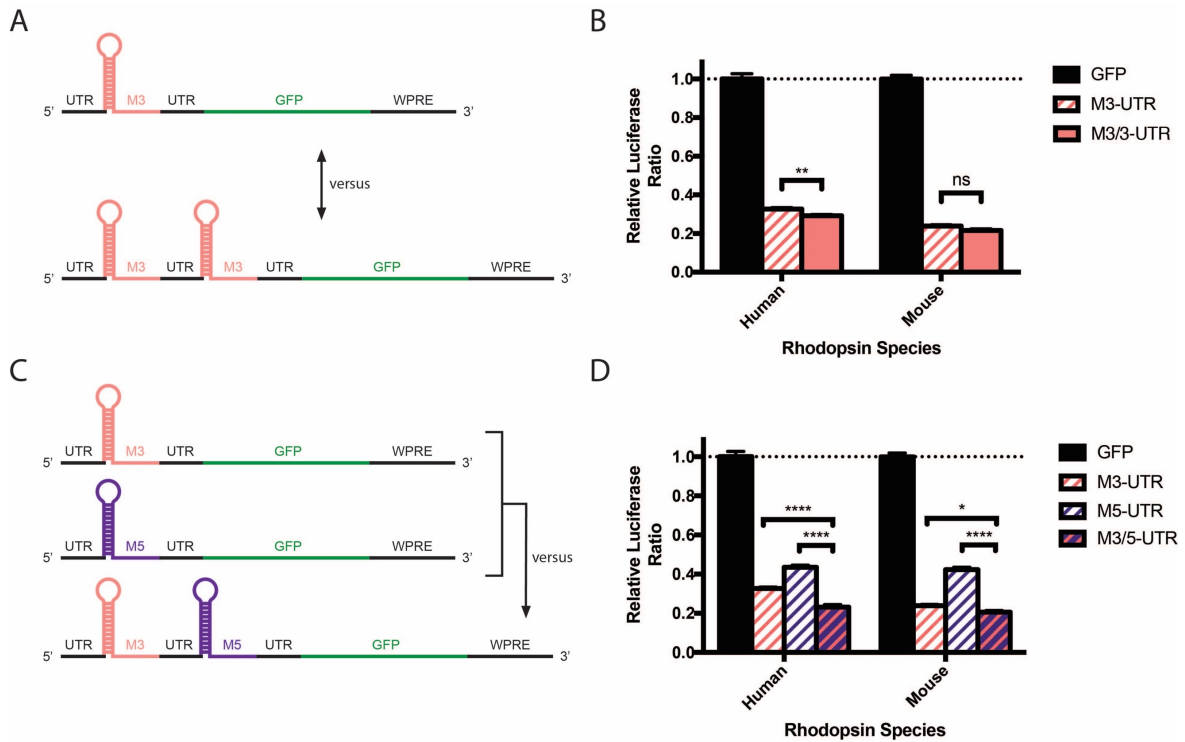


Figure 5.16. Gene suppression effects of mirtrons in tandem within the 5-UTR are additive. (A) Pre-mRNA schematics of M3-UTR and M3/3-UTR constructs. **(B)** These vectors were compared for rhodopsin knockdown in a Dual Glo® assay (n=12). M3/3-UTR induced a greater level of rhodopsin suppression than M3-UTR (two-way ANOVA with rhodopsin species and mirtron construct as factors: $F(1, 44)=15.8$, $p=0.0003$ for effect of construct; $**p<0.01$, Sidak's multiple comparison test). **(C)** Pre-mRNA schematics of M3-UTR, M5-UTR and M3/5-UTR constructs. **(D)** These vectors were compared for rhodopsin knockdown in a Dual Glo® assay ($n\geq 6$ in all cases; $*p<0.05$; $****p<0.0001$, Sidak's multiple comparison test). Only data for each version of M5-UTR and M3/5-UTR directed against its corresponding rhodopsin species are shown. M3/5^M-UTR did not provide additional knockdown of human *RHO* beyond that of M3-UTR. Similarly, no difference in mouse *Rho* suppression was detected between M3/5^H-UTR and M3-UTR.

All 5'-UTR-nested mirtrons tested remained effective when directed against the RHO-Luciferase fusion protein (Figure 5.17). Interestingly, the potency of M2-UTR was found to be

significantly lower, and that of M3/M3-UTR significantly higher, in this assay than when measured using the standard assay.

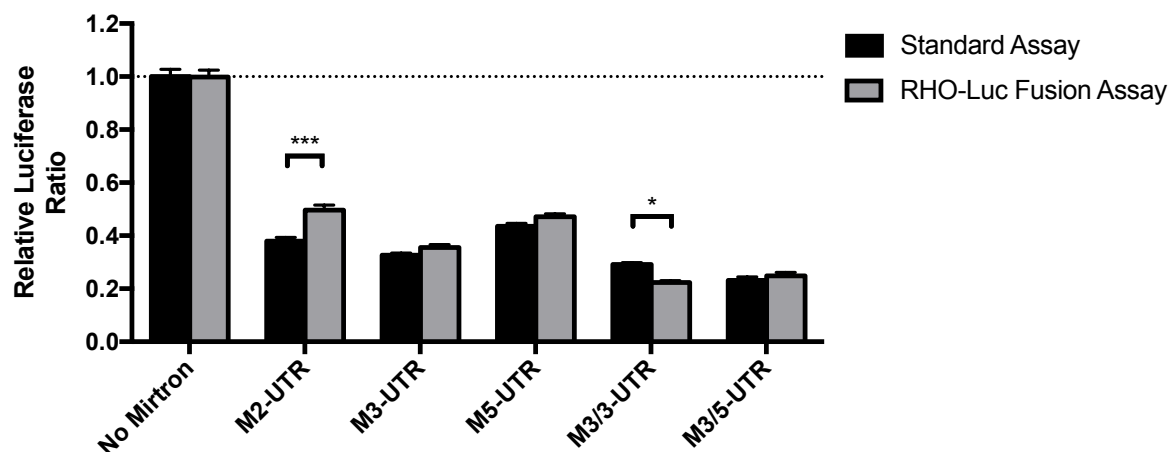


Figure 5.17. Comparison of 5'-UTR mirtron potency with the PsiCHECK2-RHO and RHO-Luc fusion vectors measured using the Dual Glo® luciferase assay. Two-way ANOVA with mirtron number and assay type as factors: $F(1, 84)=4.48$, $p=0.0373$ for effect of assay, $F(5, 84)=5.7$, $p=0.0001$ for interaction mirtron number x assay type ($n \geq 6$ in all cases); * $p < 0.05$; *** $p < 0.001$, Sidak's multiple comparison test.

5.4.10. Effect of 5'UTR mirtrons on downstream transgene expression

The effect of mirtrons and their flanking ESE sequences located within the 5'-UTR on downstream gene expression, was determined by fluorescence spectroscopy using lysates derived from transfected HEK293 cells. In this way, GFP expression following transfection with the mirtronless CAG.eGFP.WPRE plasmid was compared with that following equimolar transfection with M2-UTR, M3-UTR, M5^H-UTR, M3/3-UTR, and M3/5^H-UTR. Any negative effect of the additional sequences within the 5'UTR on downstream gene expression would be expected to result in an observed reduction in eGFP fluorescence by this assay. Figure 5.18 shows that eGFP expression was unaffected by the inclusion of M2, M3 and M5^H alone in the

5'UTR. For tandem mirtrons (M3/3-UTR and M3/5^H-UTR), a small but statistically significant reduction in eGFP expression was observed (23.2% and 24.2% respectively).

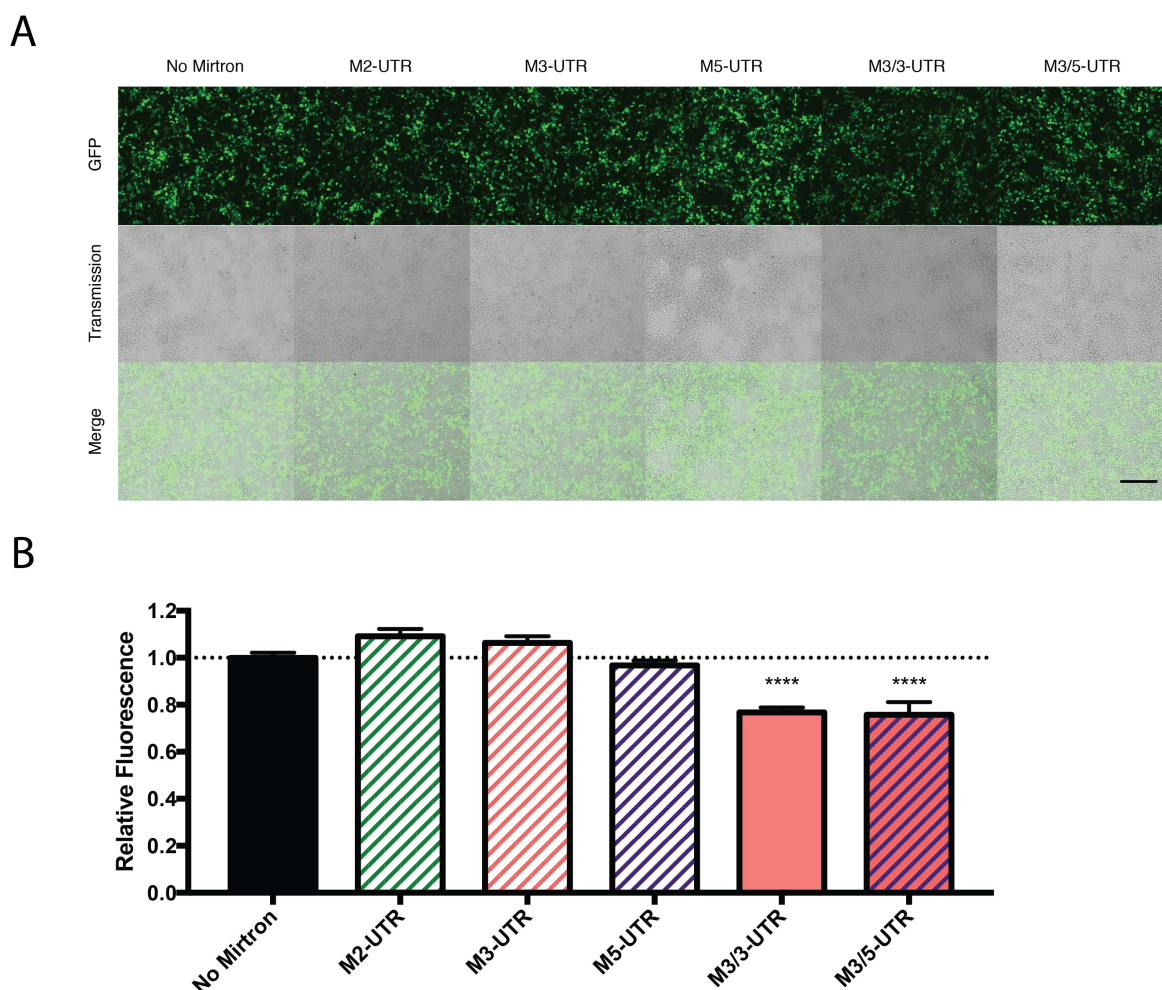


Figure 5.18. Effect of 5-UTR mirtrons on downstream transgene expression. (A) Fluorescence micrographs of HEK293 cells taken 48 hours after transfection with CAG.Mirtron-UTR.eGFP.WPRE expression plasmids. Scale bar = 200 μ m. **(B)** Fluorescence spectroscopy assay using transfected HEK293 cell lysates (n=6). Ordinary one-way ANOVA for effect of mirtron inclusion on relative fluorescence: $F(5, 36)=20.8$, $p<0.0001$; **** $p<0.0001$ versus the mirtronless CAG.eGFP.WPRE plasmid, Dunnett's multiple comparison test.

In summary, mirtrons 3 and 5 appeared the most effective designs, and when delivered in tandem within the 5'-UTR of the *eGFP* transgene, were capable of suppressing both human and mouse versions of rhodopsin *in vitro* by over 75%, whilst still permitting high levels of downstream transgene expression.

5.4.11. Codon modification of human rhodopsin confers resistance to mirtrons

For an effective knockdown/replacement vector, the CDS of human rhodopsin included in the AAV construct must be resistant to co-expressed mirtrons. To achieve this, codons that constitute the target sites for mirtrons 3 and 5 were exchanged, where possible, for alternative sequences that encode the same amino acids (synonymous codons). Human and mouse codon frequencies were considered, and rare codons for both species avoided (Table 5.7)³¹². The resulting mRNA sequence will not contain the mirtron 3 or 5 target sequences, and should not therefore be subject to degradation, but should nonetheless result in translation of normal human rhodopsin protein. To test this, codon-modified versions of human rhodopsin resistant to M3 (*RHO*^{M3R}), M5 (*RHO*^{M5R}) and both (*RHO*^{M3/5R}) were individually cloned into the MCS of the psiCHECK2 vector (using Primers 14, 15 and 34-37 in an overlap extension PCR technique). Resistance of these transcripts to degradation by their corresponding mirtrons was then established by the Dual Glo[®] assay (Figure 5.19). The exact codon modifications and amino acids they encode are shown below. Nucleotide replacements are highlighted in black. White text represents modification from both the human and mouse target sequences, whilst yellow text indicates modification from the human sequence only:

M3 target in <i>RHO</i> for both species (nucleotides 153-173):	CTTCCCCATCAACTTCCTCAC
Codon modified M3 target site (nucleotides 153-173):	ATTTCCTCAATTAAATTTCTGAC
Amino acid sequence (residues 51-57):	G--F--P--I--N--F--L--

M5 target in human *RHO* (nucleotides 598-618): AACGAGTCTTTTGTTCATCTAC

M5 target in mouse *Rho* (nucleotides 598-618): AACGAATCCTTTGTTCATCTAC

Codon modified M5 target site (nucleotides 598-618): AATGAATCCTTCGTGATTTAT

Amino acid sequence (residues 200-206): --N--E--S--F--V--I--Y

		Human				Mouse			
		Wild type		Modified		Wild type		Modified	
		Codon	Proportion	Codon	Proportion	Codon	Proportion	Codon	Proportion
M3 Target		GGC	0.34	GGA	0.25	GGC	0.33	GGA	0.26
		TTC	0.55	TTT	0.45	TTC	0.57	TTT	0.43
		CCC	0.33	CCA	0.27	CCC	0.31	CCA	0.28
		ATC	0.48	ATT	0.36	ATC	0.50	ATT	0.34
		AAC	0.54	AAT	0.46	AAC	0.57	AAT	0.43
		TTC	0.55	TTT	0.45	TTC	0.57	TTT	0.43
		CTC	0.20	CTG	0.41	CTC	0.20	CTG	0.39
M5 Target		AAC	0.54	AAT	0.46	AAC	0.57	AAT	0.43
		GAG	0.58	GAA	0.42	GAA	0.40	GAA	0.4
		TCT	0.18	TCC	0.22	TCC	0.22	TCC	0.22
		TTT	0.45	TTC	0.55	TTT	0.43	TTC	0.57
		GTC	0.24	GTG	0.47	GTC	0.25	GTG	0.46
		ATC	0.48	ATT	0.36	ATC	0.50	ATT	0.34
		TAC	0.57	TAT	0.43	TAC	0.58	TAT	0.43

Table 5.7. Codon frequencies within mirtron targets of wild type and codon modified rhodopsin.

Displayed figures represent the proportional frequency of usage of each of the associated codons in the coding DNA of the relevant species according to published data tables (GenScript; www.genscript.com/tools/codon-frequency-table).

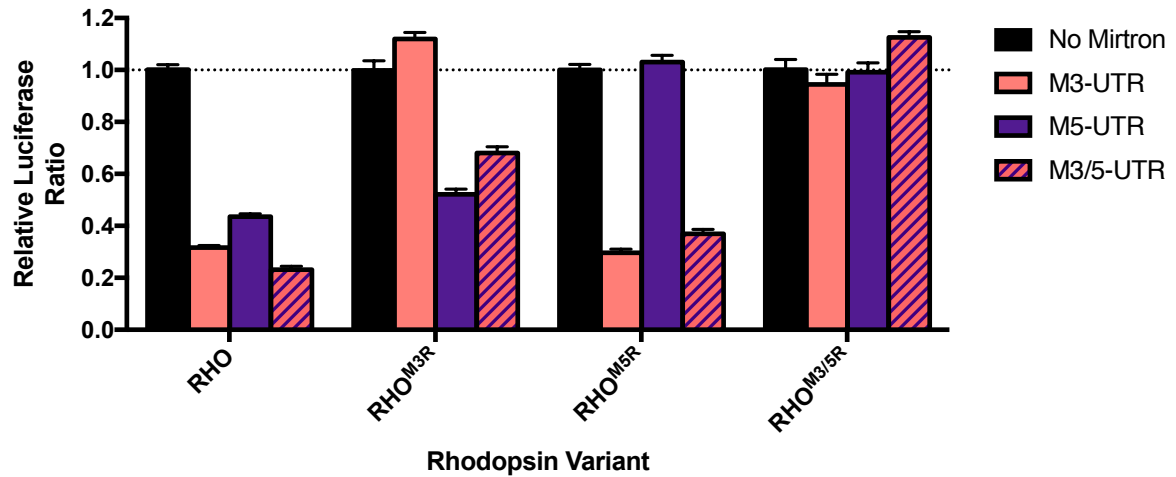


Figure 5.19. Codon modification of the *RHO* CDS confers resistance to rhodopsin-targeting mirtrons. HEK293 cells were cotransfected with the mirtronless CAG.eGFP.WPRE plasmid, M3-UTR, M5-UTR or M3/5-UTR and PsiCHECK2 vectors in which codon-modified versions of the human rhodopsin CDS had been cloned into the MCS. A subsequent Dual Glo[®] assay demonstrated that codon-modification of target regions conferred resistance to suppression by the corresponding mirtron(s). RHO^{M3R}: M3-resistant *RHO*; RHO^{M5R}: M5-resistant *RHO*; RHO^{M3/5R}: M3- and M5-resistant *RHO*.

5.4.12. Assessment of *RHO*^{M3/5R}-derived rhodopsin structure and trafficking

To confirm that the *RHO*^{M3/5R} CDS expresses normal rhodopsin protein, the sequence was subcloned into a plasmid under the control of the CAG promoter with a 3' WPRE (pCAG.RHO^{M3/5R}.WPRE). This plasmid was then used to transfect HEK293 cells which were subsequently stained for rhodopsin by immunocytochemistry using three different primary antibodies: two monoclonal antibodies raised in mouse, one directed against the N-terminus (4D2) and the other against the C-terminus (1D4) of rhodopsin, and a polyclonal antibody raised in rabbit. No difference in staining was observed in these cells compared to that in cells transfected with a CAG.RHO.WPRE plasmid (Figure 5.20).

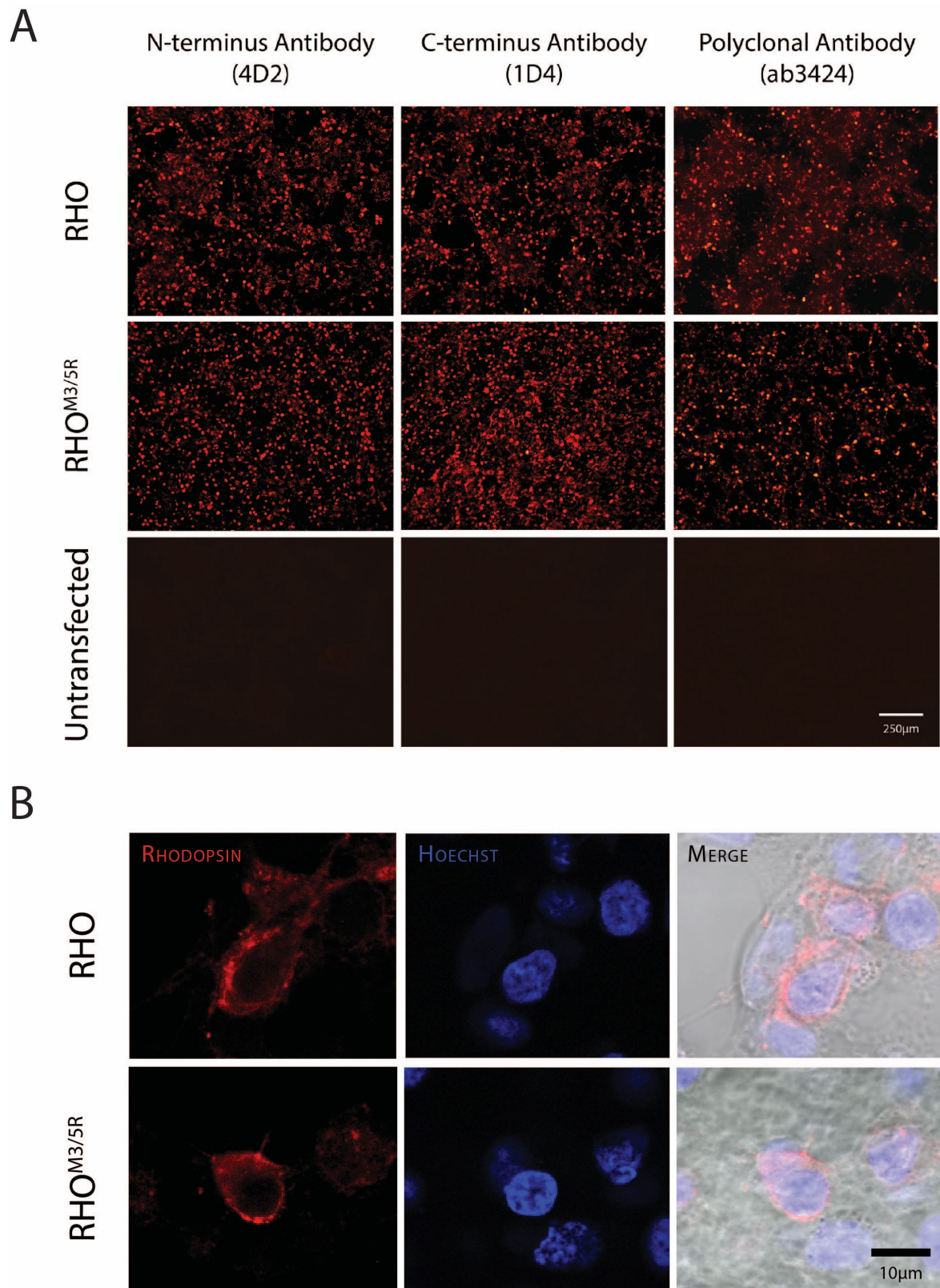


Figure 5.20. RHO^{M3/5R} protein appears indistinguishable from RHO when expressed in HEK293 cells. (A) Rhodopsin immunostaining of wild type RHO- and codon modified RHO^{M3/5R}-

transfected HEK293 cells using amino-terminus (4D2) and carboxyl-terminus (1D4) directed primary monoclonal antibodies and a polyclonal primary antibody (ab3424). **(B)** High magnification view confirming that RHO and RHO^{M3/5R} traffic in a similar fashion when expressed in HEK293 cells.

5.5. Discussion

In this chapter, I have developed artificial mirtrons that are capable of efficient knockdown of both human and mouse rhodopsin expression and may be co-delivered with a transgene under the control of a single Pol-II promoter. The use of *RHO*-directed mirtrons as part of a knockdown/replacement gene therapy strategy for dominant RP has a number of theoretical advantages over the dual vector^{120,196} or dual promoter^{121,198} approaches employed by other researchers. The single-promoter construct developed here should allow for consistent matching of the two arms of the therapy within any given cell, which may reduce the risk of toxicity related to any imbalance between rhodopsin suppression and supplementation. An improved safety profile^{288,289} and greater transcriptional accuracy³¹³ have also been demonstrated for RNAi hairpins expressed under the control of Pol-II promoters when compared with those of Pol-III promoters such as U6 or H1 which have been employed by other research groups as part of a single-vector strategy^{121,198}. Mirtrons represent one of several methods by which shRNAs may be expressed by Pol-II promoters. Samakoglu and colleagues placed a shRNA in either the 5'-UTR of a recombinant transgene or embedded within an endogenous intron at one of two locations and demonstrated effective gene suppression³¹⁴. This approach did however result in the induction of potentially detrimental interferon responses. Naturally-occurring intronic clusters of polycistronic miRNAs have also been modified for gene therapy application, and gene knockdown of the order of 75% has been achieved^{315,316}. The miRNAs generated by these constructs rely on Drosha/DGSCR8 processing. The point of microprocessor-induced cleavage at the 5' end of primary microRNA transcripts is however variable, meaning that a variety of guide strand sequences are likely to be produced in such

cases³¹⁷. By contrast, the mirtron pathway is Drosha-independent with the 5' end of the mirtron guide strand being precisely defined instead by the splice donor consensus sequence. Although this introduces further target sequence constraints for mirtron design, it may provide a purer and more precise population of RNAi effectors than alternative Drosha-dependent strategies with a lower theoretical risk of off-target effects²⁹¹. Indeed, a recent study demonstrated that 3'-tailed mirtrons produced a less heterogeneous pool of mature miRNA species than equivalent intronic miRNA mimics, and also showed superior knockdown efficacy³⁰¹.

Curtis and colleagues combined artificial mirtrons with a replacement transgene under the control of a single Pol-II promoter as part of a knockdown/replacement gene therapy construct for spinocerebellar ataxia 7³⁰². The authors found that mirtrons that spliced efficiently from within the CDS of *GFP* failed to do so in other contexts, and stipulated that the hairpin secondary structure of the mirtron may have caused this by restricting access for the spliceosome. They further postulated that a lack of ESEs at alternative mirtron locations may have contributed to this effect. In this chapter, rather than cloning the mirtron into its new location in isolation, 5' and 3' flanking regions of the *eGFP* reporter in which splicing and potency of mirtrons had initially been validated were included. This ensured that the ESE-rich context from which mirtrons were so effectively spliced was preserved. This strategy is of course only possible for mirtron placement in the UTR region of a transcript as additional nucleotides at either end of a mirtron located within the CDS would disrupt translation of the transgene after splicing. Placement within the 5'-UTR also allowed multiple mirtrons to be co-expressed alongside the replacement gene. These mirtrons were individually and accurately spliced from the parent transcript, and had additive suppressive effects against rhodopsin targets. Curtis et al. found that delivery of multiple mirtrons as separate introns within the CDS of a transgene reduced the level of splicing³⁰². This may have explained why they observed no significant increase in overall potency for double versus single mirtron constructs. Inclusion of

two or more independently functioning mirtrons in tandem within the 5'-UTR may be a useful general strategy for knockdown/replacement gene therapy application, as it potentially allows a construct to retain efficacy even when the dominant mutation carried by a particular patient happens to fall within the 21 bp target region of one of the mirtron guide strands. Indeed, several ADRP-related rhodopsin mutations have been described in the M3 target region which corresponds to amino acid residues 51–58 (F52Y³¹⁸, P53R³¹⁹, N55K²⁹, F56Y³²⁰, L57R³²¹, T58R³⁴, and T58M³²²), whilst none have been identified within the M5 target region at codons 200-206. A vector combining both mirtrons would thus allow all patients to be treated irrespective of underlying mutation. Tandem mirtrons could also be applied for the treatment of complex non-Mendelian disease, such as age-related macular degeneration, where they could potentially suppress the expression of multiple genes within a particular molecular pathway³²³.

A single mirtron placed within the 5'-UTR of the CAG.eGFP.WPRE plasmid had no effect on downstream transgene expression, whilst two mirtrons in tandem in this location led to a modest fall in recorded eGFP fluorescence (Figure 5.18). Although systematic investigation as to the cause of this was not conducted, possible explanations include the increase in 5'-UTR length consequent on addition of the second flanked mirtron which may suppress translation^{324,325}, or a direct effect of either mirtron DNA secondary structure on transcriptional efficiency or of unspliced/mis-spliced mirtron RNA secondary structure on translation³²⁶. The effect of three or more artificial mirtrons in the 5'-UTR on transgene expression was not investigated here, but would be an interesting question for further study.

In this chapter, all possible mirtron guide strand designs that targeted both the human and mouse transcripts were tested. These were found by aligning the reverse complement of the two sequences and identifying contiguous 21bp sequences that commenced with a 'GT' motif. Targeting an area of the UTR that is free from single nucleotide polymorphisms (SNPs) might

form an alternative approach to mirtron design that would obviate the need for codon alteration in the replacement transcript. For the purposes of this study however, no suitable areas of homology between the human and mouse sequences existed in either the 5'- or 3'-UTR of rhodopsin, with significant differences being apparent between the transcripts of the two species. For this reason, all mirtrons designed and tested here targeted the CDS. It is, however, conceivable that more potent mirtrons could be designed that specifically target the human *RHO* transcript, but it would not be possible to validate these in the knock-in murine model. Such mirtrons could be tested in a transgenic mouse model expressing a human opsin.

Splicing efficiency for mirtrons 1-7 varied dramatically, from less than 1% for M1 to close to 100% for M5, and broadly reflected that predicted by online splice prediction tools. These designs, with the exception of M4, fulfilled the qualifying criterion of Seow et al. that artificial mirtrons should commence with a GURRR motif to encourage splicing³⁰⁰. M4 had a C at position +5 but nevertheless demonstrated a reasonable degree of splicing (Figure 5.5). Conversely, M1 met this criterion but spliced poorly. The presence or absence of three purines at positions +3 to +5 did not therefore predict splicing efficiency in this study. It is of further interest to note that the 5' donor splice sites of the efficiently spliced mirtrons M5 and M7 differed between positions +4 and +6 from those that define the canonical splice consensus of GURAGU³²⁷ by 2 and 1 nucleotides respectively. This region of the intron is thought to be critical for donor splice site recognition by the small nuclear RNA U6 component of the spliceosome^{328,329}. Taken together, these data would suggest that the mammalian splicing machinery is able to identify a range of donor motifs that differ significantly from the GURAGU consensus in artificial mirtrons.

In keeping with prior studies³⁰⁰⁻³⁰², the splicing efficiency of mirtrons validated in this chapter did not correlate with potency. Further, delivery of two copies of M3 in tandem only provided

a marginal improvement in knockdown efficacy over that of a single copy (Figure 5.16B). Thus, target sequence choice would appear to have a greater impact than mirtron dose on the overall level of mirtron-mediated gene suppression.

A significant proportion of transcripts for all seven mirtron designs either did not splice or spliced incorrectly (Figure 5.5). This may be attributed to the presence of additional splice donor and/or acceptor motifs within the 76bp mirtron insert. The presence of alternative splice products provides a further compelling argument for locating artificial mirtrons within the 5'-UTR rather than within the CDS of a transgene. In the latter case, mis-splicing would be predicted to induce an insertion within the transgenic ORF. This may variously lead to production of mutant protein, or nonsense-mediated decay of the transcript with reduced gene expression, depending on whether the insertion was in- or out-of-frame. Conversely, mis-splicing of mirtrons located within the 5'-UTR would be expected to have minimal impact on the level of downstream gene expression, and importantly would not be expected to affect transgenic protein structure.

5.6. Conclusions

In summary, potent artificial mirtrons that target different parts of the human and mouse transcript have been developed, and a codon-modified resistant version of the human rhodopsin CDS has been cloned. An expression system has also been validated in which these effectors may be co-expressed with a transgene under the control of a single RNA polymerase II promoter without compromise of mirtron splicing, mirtron potency or downstream gene expression. This represents a significant milestone in the development of artificial mirtrons for knockdown/replacement gene therapy applications. In the next chapter, the most effective mirtrons (M3 & M5) are combined with the *RHO*^{M3/5R} CDS within the AAV-Ex/Int backbone (as developed in Chapter 4) to create a knockdown/replacement gene therapy vector for the

treatment of RHO-related ADRP. This combined vector is then tested in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse model to assess for evidence of structural and functional rescue of the degenerative phenotype.

6. Mirtron-Rhodopsin rAAVs for the Treatment of Dominant RP

6.1. Introduction

Artificial mirtrons are newly-developed molecular tools that particularly lend themselves to knockdown/replacement gene therapy applications, as they may be expressed from the same cell-specific RNA polymerase II promoter as the delivered transgene^{291,302}. Although mirtron-based knockdown/replacement constructs have been developed previously *in vitro*, these elements have not yet been combined within a gene therapy vector³⁰². Consequently, the viability of such a strategy for RNA replacement as applied to the treatment of dominant disease has yet to be determined in any model system *in vivo*.

In the previous chapter, artificial 3'-tailed mirtrons directed against regions of sequence homology between the human and mouse rhodopsin coding sequences were designed and validated within cell culture systems. Mirtron designs M2, M3 and M5 were capable of mediating effective suppression of rhodopsin gene expression for both species, and were found to be more potent effectors when located upstream of the transgene within its 5'-UTR. The combination of M3 and M5^H in tandem induced over 75% knockdown of both rhodopsin RNA species *in vitro*. Further, a codon-modified version of the human rhodopsin CDS was engineered that conferred resistance to the most effective mirtron designs, M3 and M5. In this chapter, M3 and M5^H are packaged within the 5'-UTR of the previously validated AAV2/8^{Y733F}-Ex/Int vector (see Chapter 4), in which the wild type human *RHO* CDS has additionally been replaced with the mirtron-resistant version: *RHO*^{M3/5R}. The resulting vector is then tested as a treatment for ADRP in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse model.

6.2. Aims

The principal aims of the experiments outlined in this chapter were as follows:

1. To develop recombinant AAV2/8^{Y733F} vectors in which one or more active mirtrons targeting human and mouse rhodopsin mRNA transcripts are packaged within the 5'-UTR of the mirtron-resistant *RHO*^{M3/5R} CDS, all under the control of the rod-specific RNA polymerase II human rhodopsin promoter.
2. To assess the intracellular trafficking and function of rhodopsin protein derived from transgenic *RHO*^{M3/5R} DNA following subretinal delivery of RHO-mirtron vector in the rhodopsin knockout mouse.
3. To determine the accuracy and efficiency of mirtron splicing that occurs in the retina following subretinal injection of RHO-mirtron vectors *in vivo*.
4. To quantify the degree of rhodopsin RNA replacement that occurs in the *Nrl.GFP/+*, *Rbo*^{P23H/+} retina following subretinal injection of RHO-mirtron vectors.
5. To determine the long-term effects of treatment with RHO-mirtron rAAVs on retinal structure and function in the *Nrl.GFP/+*, *Rbo*^{P23H/+} mouse model of ADRP.

6.3. Materials & Methods

6.3.1. Cloning

Genome maps for the mirtron-expressing AAVs manufactured and tested here, namely AAV-M3.M5^H.WPRE, AAV-M3.M5^H and AAV-M3.M5^M, are shown in Figure 6.1.

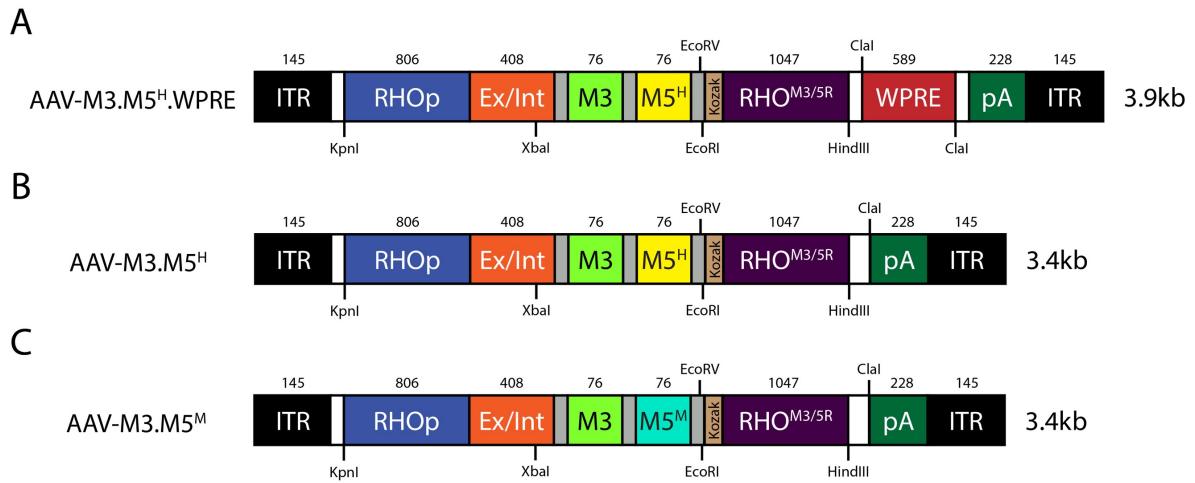


Figure 6.1. Genome maps of mirtron-AAVs. Size in bp is indicated above each element. The locations of relevant restriction sites are indicated. The size of the entire genome is shown to the right of each schematic. **(A)** AAV-M3.M5^H.WPRE. **(B)** AAV-M3.M5^H. **(C)** AAV-M3.M5^M. ITR: inverted terminal repeat; RHOp: an 806bp length human rhodopsin promoter; Ex/Int: the first exon and intron of the chicken beta-actin gene together with the splice acceptor from the rabbit beta-globin gene, derived from the CAG promoter; M3: mirtron 3; M5^H: mirtron 5 with guide strand complementary to human rhodopsin mRNA; M5^M: mirtron 5 with guide strand complementary to mouse rhodopsin mRNA; Kozak: the Kozak consensus translational start sequence; RHO^{M3/5R}: the codon modified M3 and M5-resistant human rhodopsin CDS; WPRE: woodchuck hepatitis virus post-transcriptional regulatory element; pA: bovine growth hormone polyadenylation sequence. Splice enhancer sequences flanking mirtrons are represented by shaded grey boxes.

The AAV-M3.M5^H.WPRE transgene plasmid (AAV2 pRHOp.Ex/Int.M3.M5^H.RHO^{M3/5R}.WPRE) was generated by way of a multi-step cloning strategy. First, the mirtron 3- and 5-resistant codon-modified human rhodopsin sequence (RHO^{M3/5R}) was amplified from the PsiCHECK2-RHO^{M3/5R} vector by PCR using primers that introduced a 5' EcoRI restriction site and Kozak consensus sequence, and a 3' HindIII restriction site (Primers 38 & 4):

EcoRI RHO FW: 5'-TCGAAT^TTCCGCCACCATGAATGGCAC
RHO HindIII RC: 5'-ATAAGCTT^TTAGGCCGGGGCCA

The resulting amplicon was digested with EcoRI and HindIII enzymes (New England Biolabs, UK) and ligated into the EcoRI/HindIII-digested M3/M5^H-UTR plasmid backbone (see Section 5.4.9). Following transformation of XL-10 bacteria and plasmid amplification and purification, the correct sequence of the resulting CAG.M3.M5^H.RHO^{M3/5R}.WPRE plasmid was confirmed by commercial Sanger sequencing (Source Bioscience, UK).

Next, the CAG.M3.M5^H.RHO^{M3/5R}.WPRE and RHOp.Ex/Int.RHO.WPRE (AAV-Ex/Int, see Chapter 4) plasmids were digested with XbaI (a restriction enzyme expected to cut uniquely within the chicken beta-actin sequence common to both plasmids) and HindIII (a restriction enzyme expected to cut uniquely between the rhodopsin stop codon and WPRE sequence in both plasmids). The digestion products were run through a 1% agarose gel by electrophoresis, and relevant fragments- the 1.5kb M3.M5^H.RHO^{M3/5R} insert and the 5.5kb RHOp.Ex/Int.WPRE backbone- were extracted and ligated. Following bacterial transformation, plasmid amplification and isolation, the correct sequence of the AAV2 RHOp.Ex/Int.M3.M5^H.RHO^{M3/5R}.WPRE (AAV-M3.M5^H.WPRE) transgene plasmid was confirmed by commercial Sanger sequencing (Source Bioscience, UK).

The WPRE sequence within the AAV-M3.M5^H.WPRE transgene plasmid was flanked at its 5' and 3' ends by ClaI restriction sites. The AAV-M3.M5^H transgene plasmid (AAV2 pRHOp.Ex/Int.M3.M5^H.RHO^{M3/5R}) was thus generated by ClaI digestion and religation of pAAV-M3.M5^H.WPRE as described previously (see Section 4.3.2).

The AAV-M3.M5^M transgene plasmid was cloned by digestion of the AAV-M3.M5^H transgene and M3/M5^M-UTR plasmids with XbaI and EcoRV restriction enzymes (note that both plasmids have a unique EcoRV restriction site located between the M5 and Kozak consensus sequences). The 455bp M3.M5^M DNA fragment generated from M3/M5^M-UTR digestion was then ligated with the 6.6kb backbone generated from AAV-M3.M5^H digestion. The correct sequence of the resulting AAV-M3.M5^M transgene plasmid (AAV2 pRHOp.Ex/Int.M3.M5^M.RHO^{M3/5R}) was subsequently confirmed by commercial Sanger sequencing (Source Bioscience, UK).

6.3.2. AAV manufacture and titration

Recombinant AAVs described in this chapter (AAV-M3.M5^H.WPRE, AAV-M3.M5^H and AAV-M3.M5^M) were each manufactured by co-transfection of HEK293T cells with the relevant transgene plasmid together with Rep2Cap8^{Y733F} and Helper plasmids, as described in Section 2.3. Viral genome titres for concentrated and wash preparations of the vectors were established using a human rhodopsin TaqMan RT-qPCR assay (Thermo Fisher Scientific, UK), and capsid purity determined by SDS-PAGE as described in Section 2.3.6. Viral copy number titre for the three vectors is shown in Table 6.1.

	Concentrate Titre (gc/ml)	Wash Titre (gc/ml)
AAV-M3.M5^H.WPRE	5.40 x 10 ¹²	5.49 x 10 ¹⁰
AAV-M3.M5^H	8.90 x 10 ¹²	7.6 x 10 ¹⁰
AAV-M3.M5^M	8.35 x 10 ¹²	7.42 x 10 ¹⁰

Table 6.1. Titres for RHO-Mirtron AAVs. Titres were determined by TaqMan-qPCR and interpolated from a standard curve constructed using linear RHOp.RHO.pA DNA. Reaction efficiency was between 99% and 100% in all cases.

Figure 6.2 shows the SDS-PAGE gels stained for total protein. Three distinct bands were generated in all cases corresponding in size to the three AAV capsid subunits VP1, VP2 and VP3. No significant additional bands were detected, indicating a high degree of vector purity.

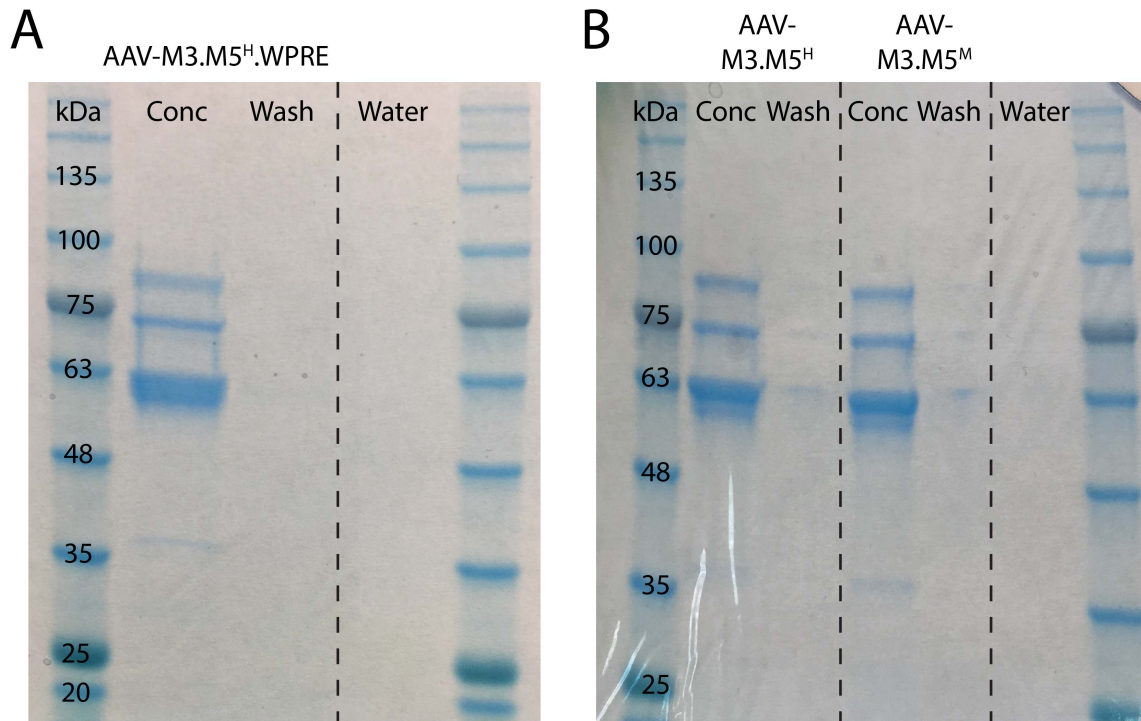


Figure 6.2. SDS-PAGE of RHO-Mirtron AAV capsid proteins. Following electrophoresis, total protein was stained with EZBlue dye (Sigma Aldrich, UK). Bands corresponding to VP1 (87kDa), VP2 (72kDa) and VP3 (62kDa) proteins in the expected ratio of 1:1:10 were detected in concentrate and wash samples for all four vectors. No significant additional bands were present.

6.3.3. Gene expression analysis

For gene expression analysis studies, PNW3 *Nrl.GFP/+*, *Rho*^{P23H/+} mice received single superior subretinal injections of AAV at low (2×10^8 gc) or high (2×10^9 gc) dose in the right eye, and isovolumetric sham injections of PBS in the left eye. Four weeks later, animals were sacrificed and retinas harvested. RNA extraction and cDNA production from injected *Nrl.GFP/+*, *Rho*^{P23H/+} retinal samples were performed as described in Section 2.1.5. All RT-qPCR gene

expression assays in this chapter used previously optimised TaqMan assays with probes against human *RHO*, mouse *Rho*, and *eGFP* (all Thermo Fisher Scientific, UK) as described in Sections 2.1.6 and 4.3.5. The *eGFP* signal was used as housekeeper so that rhodopsin expression was normalised for the number of surviving rod cells (which uniquely express the *eGFP* transgene).

6.3.4. Sample size and power calculations

Sample sizes for the AAV efficacy experiments were based on power calculations using PRL measurements recorded from untreated left eyes of the AAV-Ex/Int-injected cohorts described in Chapter 4 (n=14). Two-sample, two-sided calculations were performed using the following formula:

$$n = \frac{2\sigma^2(z_{1-0.5\alpha} + z_{1-\beta})^2}{(\mu_1 - \mu_2)^2}$$

where:

n = predicted sample size requirement

σ = standard deviation of mean μ_1

z = critical Z value for a given α or β

α = probability of type 1 error (0.05)

β = probability of type 2 error (0.2)

μ_1 = mean PRL thickness of uninjected eyes from previous sample

μ_2 = anticipated mean PRL thickness of injected eyes

Mean (μ_1) and standard deviation (σ) PRL thickness in these eyes at PNW7 (one month after fellow eye treatment) were 38.32 μ m and 2.32 μ m respectively, whilst at PNW15 (three months after fellow eye treatment), mean and standard deviation PRL thickness were 13.18 μ m and 2.50 μ m. Using a power of 80% ($\beta = 0.2$) with 5% probability of type 1 error ($\alpha = 0.05$), a predicted sample of six mice would be required to detect a 10% difference in PRL thickness

between treated and untreated eyes at one month post-injection, whilst a predicted sample size of 14 mice would be required to detect a 20% difference at three months post-injection.

6.4. Results

6.4.1. Rhodopsin expressed from the $RHO^{M3/M5R}$ transcript traffics to outer segments and is capable of driving the dark-adapted electroretinogram

To test for appropriate expression, localization and function of rhodopsin protein derived from the $RHO^{M3/5R}$ transgene, 2×10^9 gc of the AAV-M3.M5^H.WPRE vector in a total volume of 1.5 μ l were injected subretinally into the right eyes of $Nrl.GFP/+$, $Rho^{-/-}$ mice. Four weeks later, dark-adapted electroretinography (ERG) was performed, after which animals were sacrificed and retinal cryosections immunostained for rhodopsin. A dark-adapted irradiance-response curve (IRC) was constructed for injected right and uninjected left eyes, which confirmed rescue of rod-derived dark-adapted ERG responses, and thus functionality of the transgenic protein (Figure 6.3).

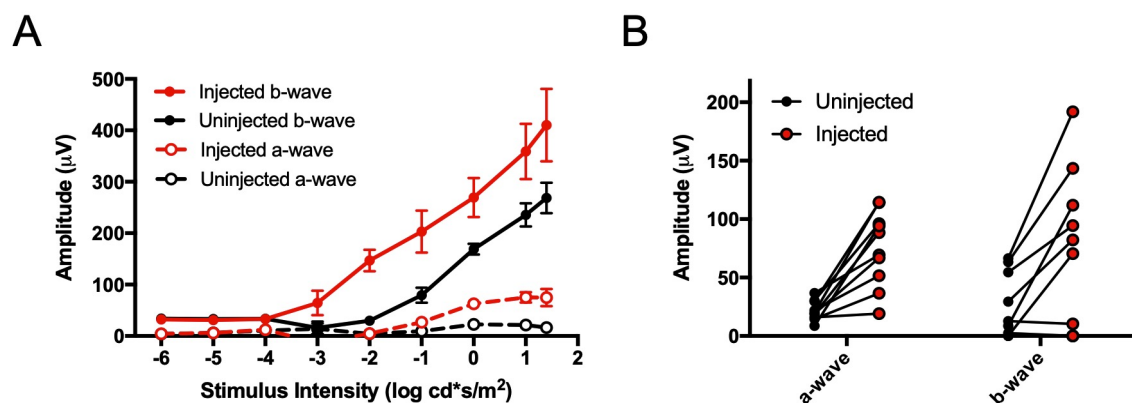


Figure 6.3. ERG confirms functionality of human transgenic rhodopsin protein expressed from the codon modified $RHO^{M3/5R}$ transcript following subretinal delivery of AAV-M3.M5^H.WPRE in rhodopsin knockout mice. (A) Dark-adapted irradiance-response curves (IRCs) for a- and b-wave

responses of injected (red curves) and uninjected (black curves) eyes (n=10). Injected versus uninjected responses: $p=0.0022$ for a-wave and $p=0.0043$ for b-wave, repeated measures two-way ANOVA. **(B)** Dark-adapted a- and b-wave responses of paired uninjected (left) and injected (right) eyes. The a-wave measurements displayed are those recorded at a flash intensity of 10 cd.s/m^2 and represent a mixed rod-cone response. The b-wave responses shown are those that followed dim flashes of 0.0001 cd.s/m^2 and were thus derived predominantly from rods.

IHC of retinal sections from injected eyes revealed presence of outer segments that were absent from those of uninjected fellow eyes (Figure 6.4A). These were packed with rhodopsin. No ectopic rhodopsin expression (i.e. outside of the rod OS) was identified. Dual immunostaining for rhodopsin and cone arrestin showed no overlap of the two signals suggesting a lack of transgene expression in cone photoreceptors and specificity of the promoter (Figure 6.4B). Thus, human rhodopsin protein translated under the control of a rod cell-specific promoter from an mRNA transcript resistant to both mirtrons 3 and 5 traffics to the outer segment of rods, and is capable of driving the scotopic light response.

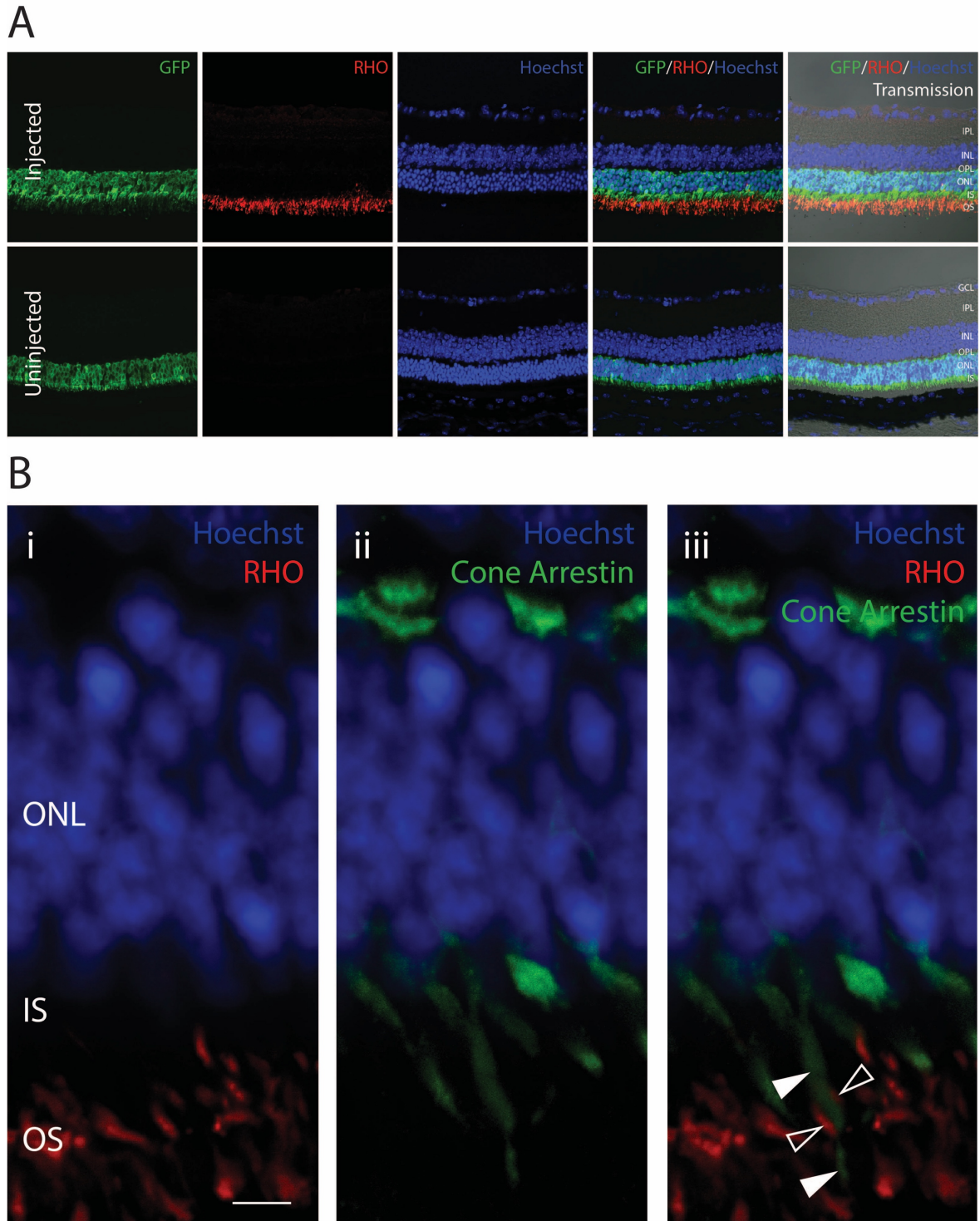


Figure 6.4. Transgenic human rhodopsin is expressed in rods of *Nrl.GFP/+*, *Rho*^{-/-} mice following subretinal injection of AAV-M3.M5^H.WPRE. **(A)** Retinal cryosections from the injected and uninjected eyes (upper and lower panels respectively) of an individual *Nrl.GFP/+*, *Rho*^{-/-} mouse stained for rhodopsin. Note that transgenic *RHO*^{M3/5}-derived protein expression is restricted to OS which are absent in uninjected eyes. Scale bar = 200μm. **(B)** High magnification section of an injected

retina double stained for rhodopsin (red) and cone arrestin (green). No overlap of the two stains was detected confirming that transgenic protein expression is restricted to rod photoreceptors. In the right-hand panel the IS/OS of a single cone (solid arrows) located between two rod OS expressing transgenic human rhodopsin (open arrows) is shown, suggesting rod-specific expression. Scale bar = 20µm.

6.4.2. Mirtrons are correctly and efficiently spliced from transgenic pre-mRNA *in vivo*

To confirm that mirtrons are liberated from transgenic mRNA *in vivo*, Nrl.GFP/+, Rho^{P23H/+} mice were injected subretinally with AAV-M3.M5^H.WPRE. Right eyes were injected with the vector at either low dose (2×10^8 gc) or high dose (2×10^9 gc), whilst left eyes received subretinal sham injections of PBS. Four weeks later, retinas were harvested from which cDNA was derived. PCR reactions were then performed using this cDNA as template together with primer pairs that spanned the mirtron-containing region of the transgenic 5'-UTR (Primers 32 & 39). When the resulting amplicons were run through an agarose gel by electrophoresis, the predominant bands detected corresponded in size to those expected from transcripts in which both mirtrons had been individually spliced out (Figure 6.5A). Densitometric analysis suggested that these transcripts accounted for over 90% of the total AAV-derived mRNA population in these samples (Figure 6.5B). A band of lower intensity and shorter DNA length was additionally detected in all lanes. The size of this fragment approximated to that which would be predicted to have arisen from mRNA in which the splice donor site of M3 had ligated to the splice acceptor site of M5 and the intervening exon skipped. Sanger sequencing of the predominant 409bp amplicon demonstrated accurate splicing of the M3 sequence (Figure 6.5C). The quality of the sequencing readout however dropped downstream of this, and it was not possible to confirm the accuracy of M5 splicing from these samples. Similarly, sequencing reactions performed using DNA derived from the less prominent 305bp bands failed to produce accurate readouts.

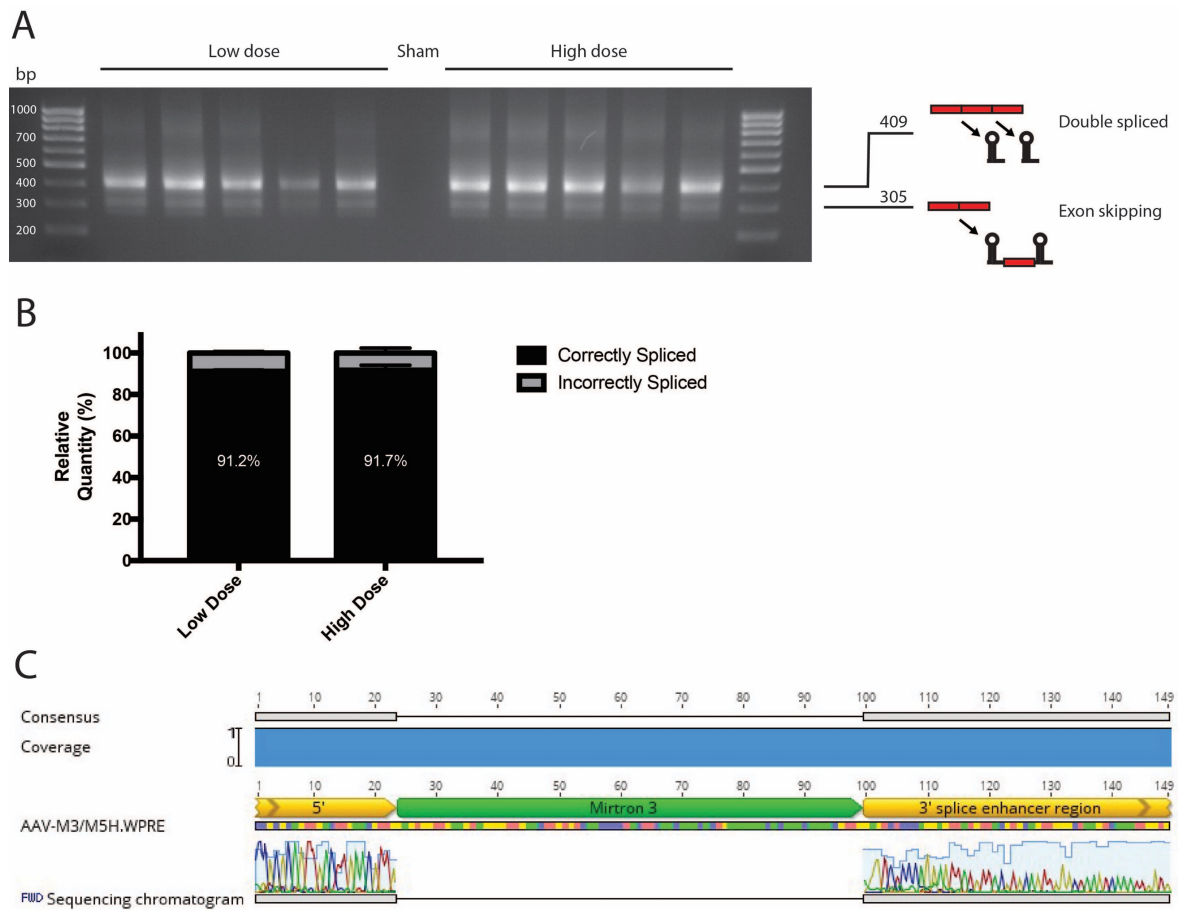


Figure 6.5. Artificial mirtrons delivered by a recombinant AAV vector are efficiently spliced out of transgenic pre-mRNA *in vivo*. **(A)** DNA gel electrophoresis of amplicons generated using mirtron-spanning primers with template cDNA derived from AAV-M3.M5^H.WPRE-injected retinas. Schematics which illustrate the possible origins of the bands detected, together with their predicted size in bp, are shown to the right of the figure. The predominant band was of the size expected for transcripts from which both mirtrons had been individually spliced. **(B)** Mean relative band density $\pm$ SEM for low and high dose samples ($n=5$). Displayed figures relate to the percentages of amplicons predicted to have arisen from correctly spliced transcripts. **(C)** Sanger sequencing of DNA extracted from the predominant (larger) band confirmed accurate splicing of the 76bp M3 sequence. Sequencing of the M5 region, and of the smaller of the two bands, was inaccurate.

6.4.3. Rhodopsin mRNA replacement induced by AAV-M3.M5^H.WPRE *in vivo*

An effective rhodopsin knockdown/replacement vector would ideally achieve maximal suppression of endogenous gene expression, and at the same time provide a level of gene supplementation that approximates to that of the wild type state. Whole retinal gene expression studies revealed knockdown of endogenous rhodopsin in eyes injected with high dose (2×10^9 gc) AAV-M3.M5^H.WPRE of $34.1 \pm 5.0\%$ compared with fellow sham-injected eyes ($p=0.0024$). No gene suppression was apparent in the low dose (2×10^8 gc)-injected cohort ($p=0.9939$; Figure 6.6A). The level of human $RHO^{M3/5R}$ transgene expression in low dose-injected retinas was similar to that of genomic mouse *Rho* detected in fellow sham-injected eyes. At high dose, $RHO^{M3/5R}$ expression in injected retinas was 4.6 ± 1.0 times greater than that of *Rho* in uninjected fellow eyes ($p=0.0011$; Figure 6.6B).

No knockdown of mouse rhodopsin was detected following injection of the mirtronless AAV-Ex/Int vector (developed in Chapter 4) at either low (2×10^8 gc) or high (2×10^9 gc) dose (Figure 6.6C), and human *RHO* expression in retinas injected with this vector did not correlate with levels of mouse *Rho* ($r^2=0.005923$, $p=0.844$; Figure 6.6E). By contrast, for AAV-M3.M5^H.WPRE, a strong and highly significant negative relationship existed between the rhodopsin gene expression levels of the two species variants ($r^2=0.731$, $p=0.0016$; Figure 6.6F): i.e. for the mirtron-containing vector, retinas with the highest levels of transgene expression showed the highest level of endogenous *Rho* suppression and *vice versa*, whilst for the mirtronless vector, mouse *Rho* levels were unrelated to transgenic *RHO* expression.

Taken together, these data support the ability of AAV-M3.M5^H.WPRE to function as a rhodopsin mRNA replacement vector. This represents the first demonstration to date of knockdown of gene expression by an artificial mirtron *in vivo*.

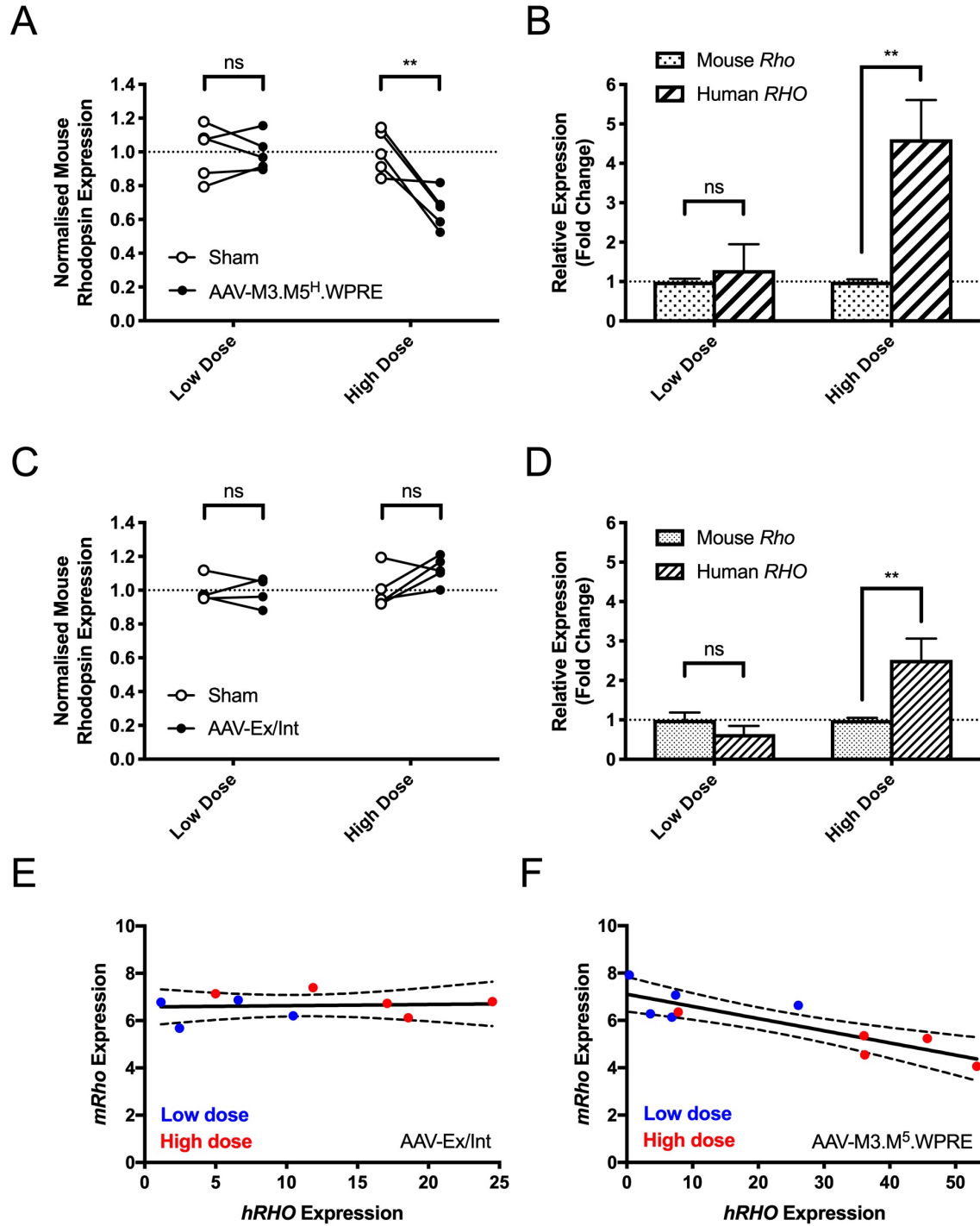


Figure 6.6. Gene expression analysis of knockdown and replacement arms of AAV-M3.M5^H.WPRE gene therapy following subretinal injection. *eGFP* was used as the housekeeper gene for all qPCR data. Thus gene expression levels refer to the ratio *Rhodopsin*: *eGFP*. **(A)** Suppression of endogenous mouse rhodopsin gene expression by AAV-M3.M5^H.WPRE. Paired right (AAV-injected) and left (sham PBS-injected) eyes are connected by joining lines. ***p*=0.0024, two-way ANOVA, Sidak's

multiple comparison test. **(B)** Human transgenic rhodopsin supplementation levels in AAV-M3.M5^H.WPRES injected retinas expressed as fold change relative to endogenous mouse rhodopsin in fellow eyes. **p=0.0011, two-way ANOVA, Sidak's multiple comparison test. **(C)** No suppression of the endogenous mouse rhodopsin gene was detected at either dose in eyes injected with the mirtronless AAV-Ex/Int vector. Paired right (AAV-injected) and left (sham PBS-injected) eyes are connected by joining lines. **(D)** Human transgenic rhodopsin supplementation levels in AAV-Ex/Int injected retinas expressed as fold change relative to endogenous mouse rhodopsin in fellow eyes. **p=0.0055, Sidak's multiple comparison test. **(E)** Scatter plot of human *RHO* versus mouse *Rho* gene expression relative to *eGFP* housekeeper following subretinal injection of AAV-Ex/Int as determined by qPCR ($r^2=0.005923$, $p=0.844$). **(F)** Scatter plot of human *RHO* versus mouse *Rho* gene expression relative to *eGFP* housekeeper following subretinal injection of AAV-M3.M5^H.WPRES as determined by qPCR ($r^2=0.731$, $p=0.0016$). Trend lines represent linear regression $\pm$ 95% confidence interval (dotted lines).

6.4.4. Structural effect of delivery of AAV-M3.M5^H.WPRES to the *Nrl.GFP/+*, *Rho*^{P23H/+} retina

AAV-M3.M5^H.WPRES was injected at one of two doses, 2×10^8 gc (low dose, n=17) or 2×10^9 gc (high dose, n=17), into the right eyes of *Nrl.GFP/+*, *Rho*^{P23H/+} mice, whilst left eyes remained uninjected and acted as internal controls. The effect on retinal structure and function was subsequently determined by SD-OCT/cSLO and ERG respectively. Subretinal injection of the vector at low dose was associated with a small but significant increase in mean PRL thickness relative to fellow eyes (repeated measures two-way ANOVA with eye and time as factors: $F(1,15)=7.0$, $p=0.0184$ for overall effect of injection; Figure 6.7A). At high dose however, no significant difference was detected ($F(1,16)=0$, $p=0.98$; Figure 6.7B). In the sham-injected cohort, a small detrimental effect was observed in injected eyes ($F(1,9)=24.6$, $p=0.0008$; Figure 6.7C). The difference in mean PRL thickness between injected and uninjected eyes for the low dose cohort was significantly greater than that of the sham group ($p=0.01$), but not significantly different from that of the high dose group ($p=0.10$, Sidak's multiple comparison test; Figure 6.7D).

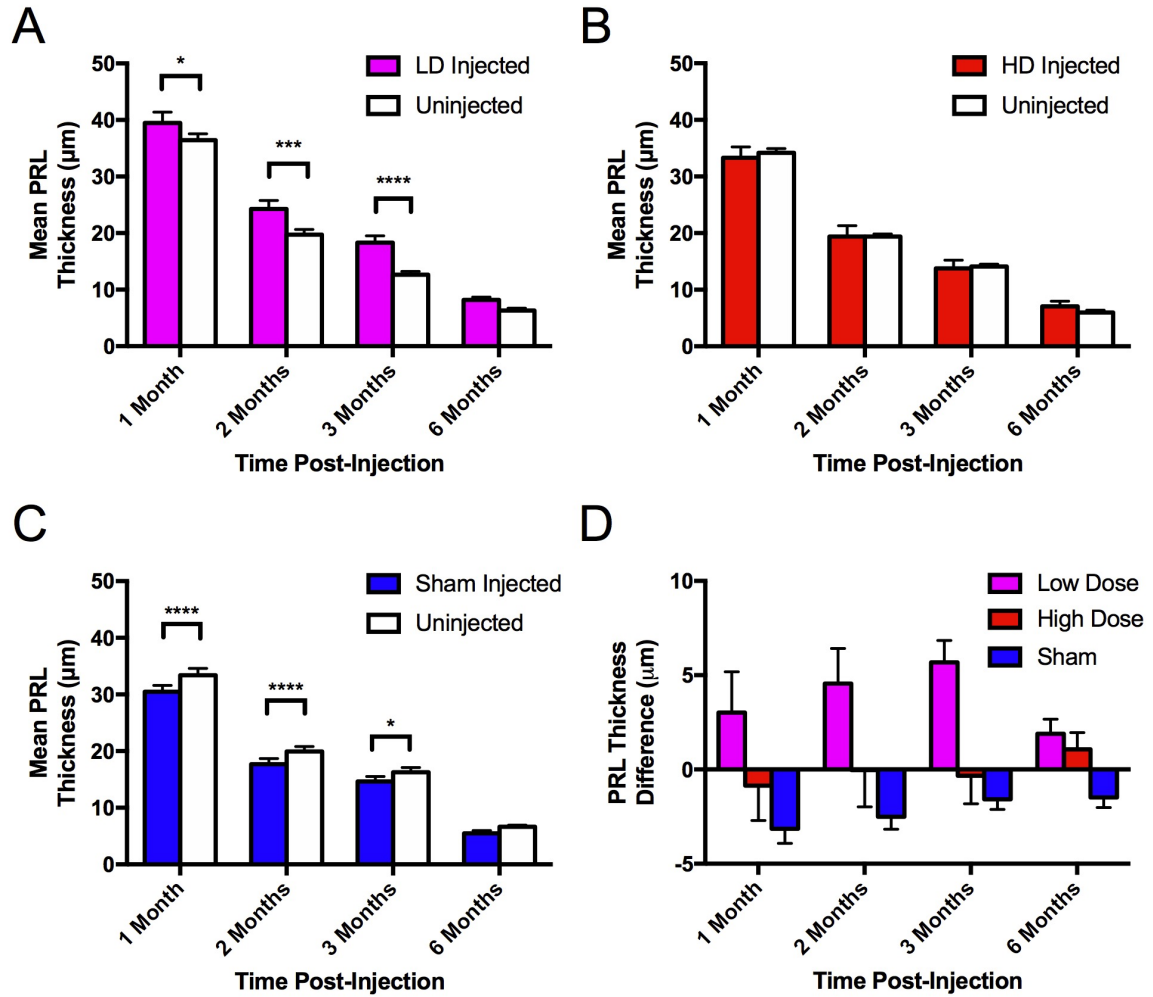


Figure 6.7. Effect of subretinal injection of AAV-M3.M5^H.WPRE on mean PRL thickness in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse. **(A)** Low dose cohort (n=17). **(B)** High dose cohort (n=17). **(C)** Sham cohort (n=14). **(D)** Difference in PRL thickness between paired injected and uninjected eyes for the three cohorts as a function of time post-injection. *p<0.05, ***p<0.001, ****p<0.0001, Sidak's multiple comparison test.

To detect regional effects, PRL thickness measured by SD-OCT was further analysed as a function of retinal location at eight cardinal points at a fixed eccentricity from the lateral margin of the optic disc (see Section 2.4.3). Mean PRL thickness of injected and uninjected eyes at these points is plotted in Figure 6.8, whilst the difference between paired treated and untreated eyes for the three cohorts is shown in Figure 6.9. This analysis revealed a thinning in the superior

outer retina in all groups, which may be attributed to the effect of surgical trauma. It is also clear that the greatest treatment effect in the low dose cohort was focussed along the horizontal meridian (temporal and nasal retina; representative SD-OCT images are shown in Figure 6.10). This is likely to reflect the fact that these are areas that received an adequate dose of vector from the superiorly-located subretinal bleb, but were subjected to a lesser degree of surgical trauma, being distant from the point of injection. No difference between the eyes was recorded for any cohort in the untreated inferior regions of retina. Representative BAF cSLO images which provide an en face representation of the effect of subretinal injection on surviving rod density are shown in Figure 6.11.

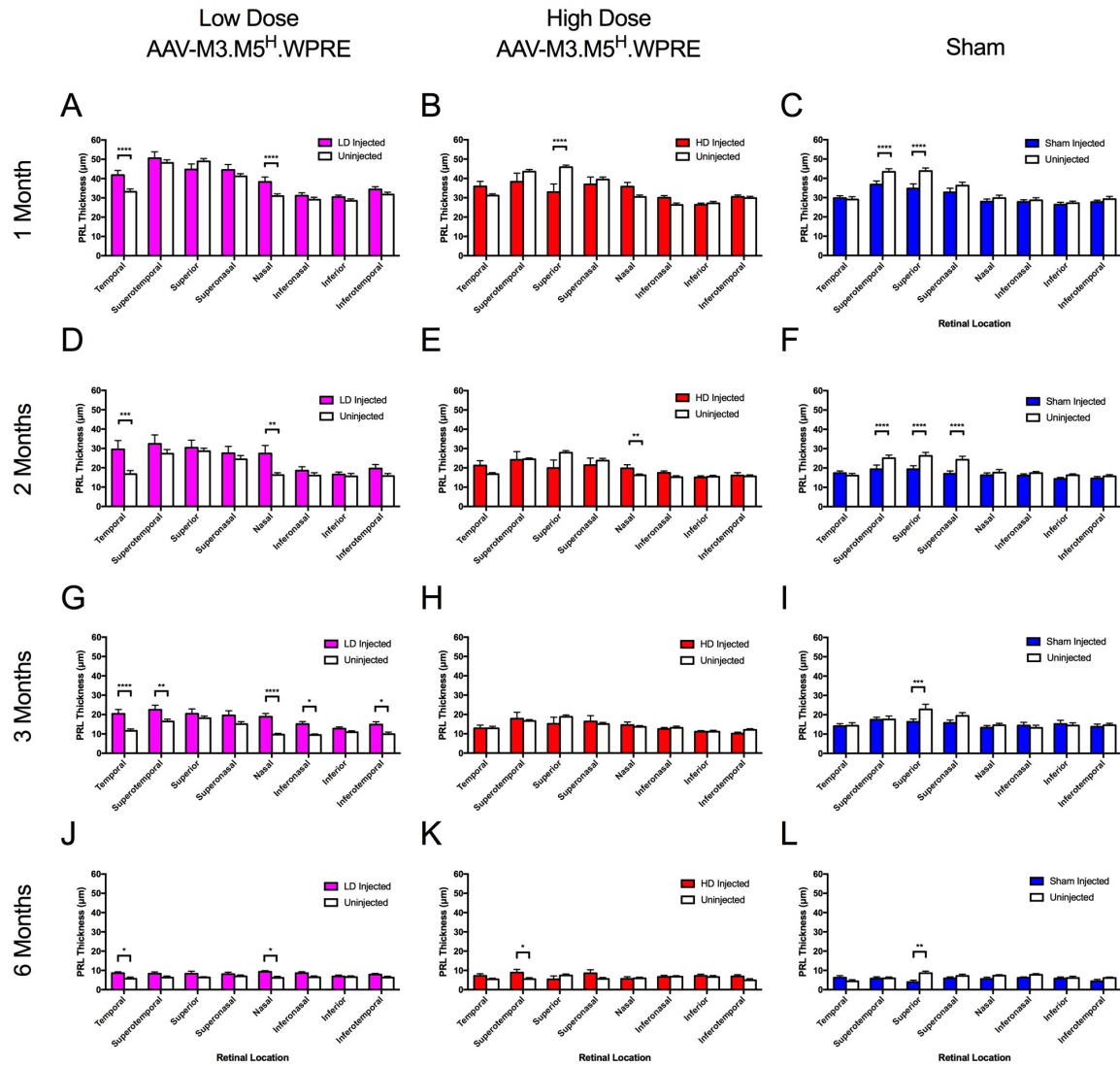


Figure 6.8. Effect of subretinal injection of AAV-M3.M5^H.WPRE on mean PRL thickness by retinal location in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse. All measurements were taken at a fixed eccentricity of approximately 22.5° from the optic nerve head in the stated direction. **(A, D, G, J)** Low dose (2x10⁸ gc) AAV-M3.M5^H.WPRE -injected cohort at 1 (A), 2 (D), 3 (G) and 6 (J) months post-injection. **(B, E, H, K)** High dose (2x10⁹ gc) AAV-M3.M5^H.WPRE -injected cohort at 1 (B), 2 (E), 3 (H) and 6 (K) months post-injection. **(C, F, I, L)** Sham (PBS)-injected cohort at 1 (C), 2 (F), 3 (I) and 6 (L) months post-injection. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, all other comparisons not statistically significant, Sidak's multiple comparison test.

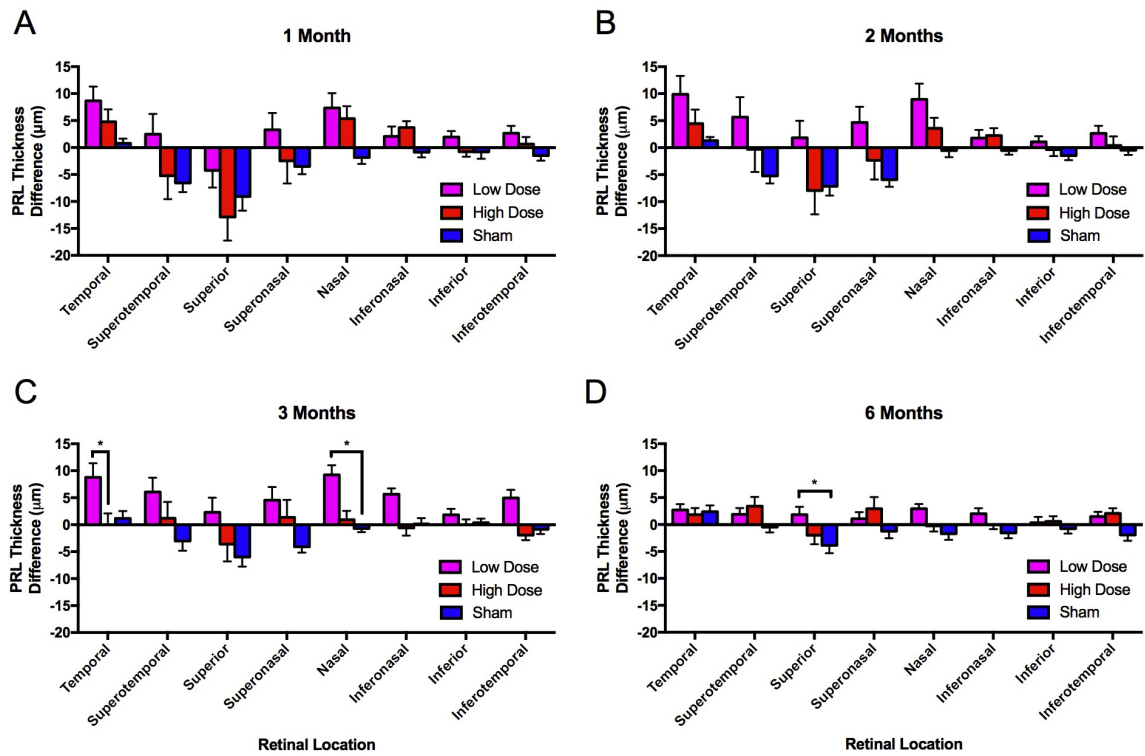


Figure 6.9. Difference in PRL thickness between AAV-M3.M5^H.WPRE-injected and fellow uninjected eyes as a function of retinal location. Values greater than zero suggest a benefit, whilst those below zero suggest damage as a result of subretinal injection. **(A)** One month post-injection. **(B)** Two months post-injection. **(C)** Three months post-injection. **(D)** Six months post-injection. * $p < 0.05$, all other comparisons not significant, Sidak's multiple comparison test.

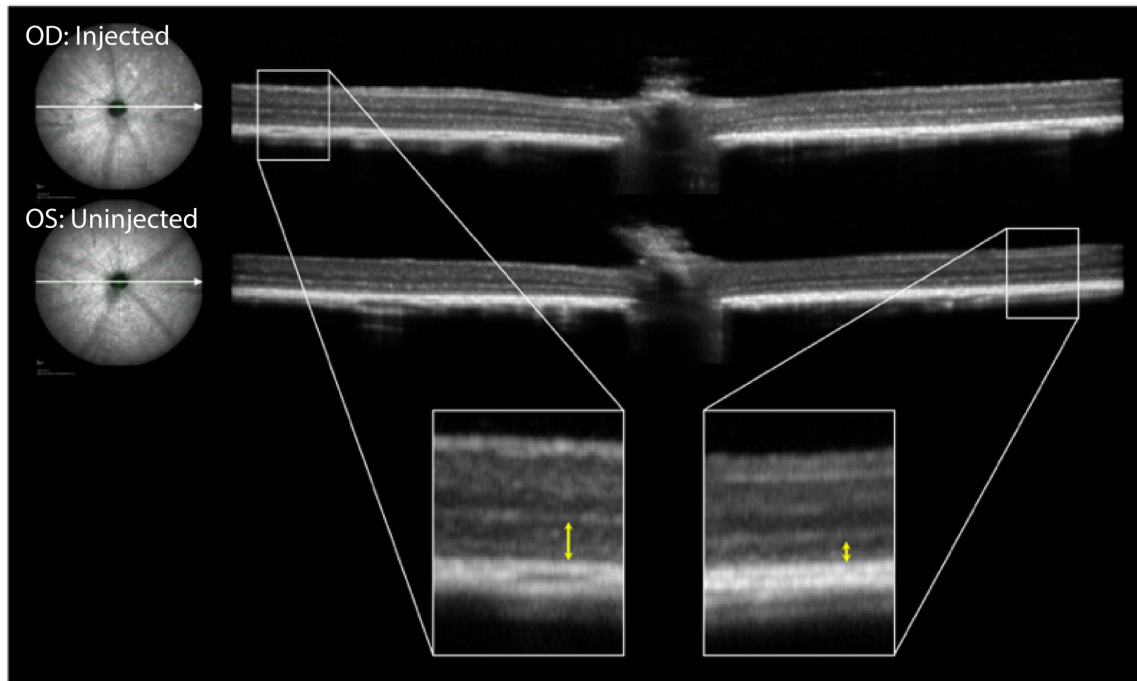


Figure 6.10. Subretinal injection of low dose AAV-M3.M5^H.WPRE in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse leads to relative preservation of the PRL along the horizontal meridian. Representative SD-OCT images of right treated (OD: injected), and left untreated (OS: Uninjected) eyes of single animal taken one month following superior subretinal injection of 2×10^8 gc AAV-M3.M5^H.WPRE. The orientation of each section is indicated by a white arrow on the corresponding en face IR image shown to the left. Magnified views of equivalent points of the temporal retina are shown in which the thickness of the surviving PRL is highlighted by yellow arrows.

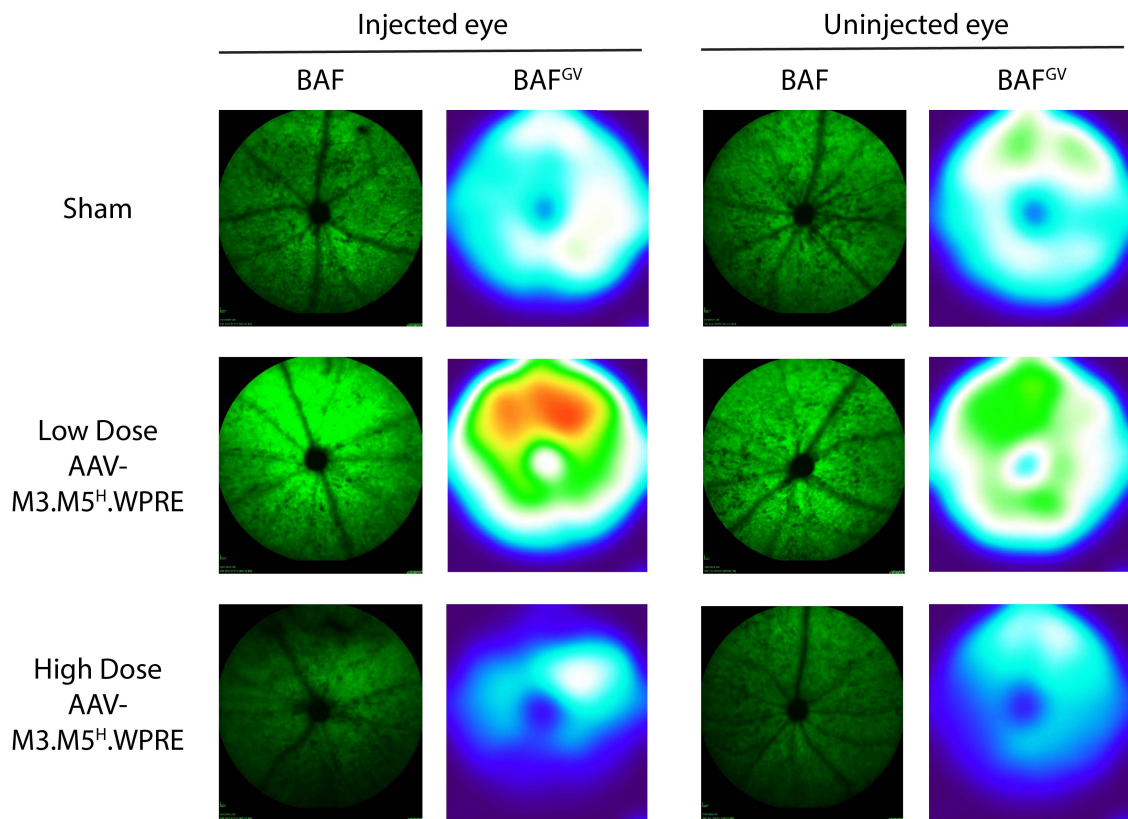


Figure 6.11. En face fluorescence imaging of *Nrl.GFP/+*, *Rho*^{P23H/+} retinas following subretinal injection of PBS (sham) or AAV-M3.M5^H.WPRE. Representative BAF images and BAF mGV surface plots taken four weeks following treatment. Paired right (injected) and left (uninjected) eyes are shown in each case.

6.4.5. Functional effect of delivery of AAV-M3.M5^H.WPRE to the *Nrl.GFP/+*, *Rho*^{P23H/+} retina

Retinal function following subretinal injection as determined by full-field ERG largely reflected the structural changes seen by OCT imaging. No significant difference was detected in either mean a- or b-wave amplitude between low dose AAV-M3.M5^H.WPRE-injected and fellow uninjected eyes at any time-point, although there was a trend towards a small beneficial overall response (Figure 6.12 and Figure 6.13; Table 6.2). Injection at high dose or sham injection however resulted in a significant reduction in ERG amplitude compared with untreated fellow eyes (Table 6.2).

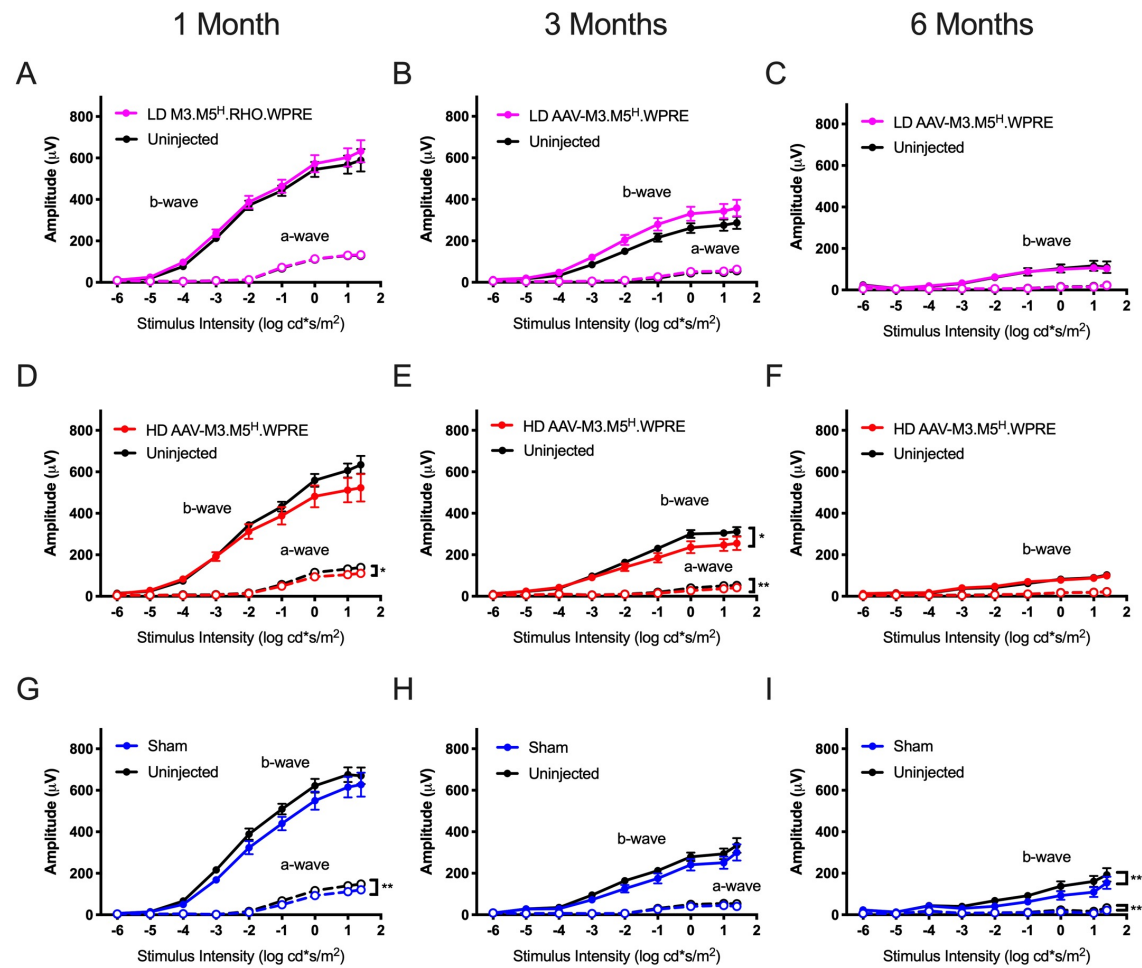


Figure 6.12. Full field dark-adapted ERG irradiance-response curves for low and high dose AAV-M3.M5^H.WPRE-injected and sham-injected cohorts. All data points represent mean signal $\pm$ SEM. **(A-C)** Low dose AAV-M3.M5^H.WPRE-injected cohort, n=17. **(D-F)** High dose AAV-M3.M5^H.WPRE-injected cohort, n=17. **(G-I)** Sham (PBS)-injected cohort, n=14. *p<0.05; **p<0.01, all other comparisons not significant, repeated measures two-way ANOVA for effect of injection.

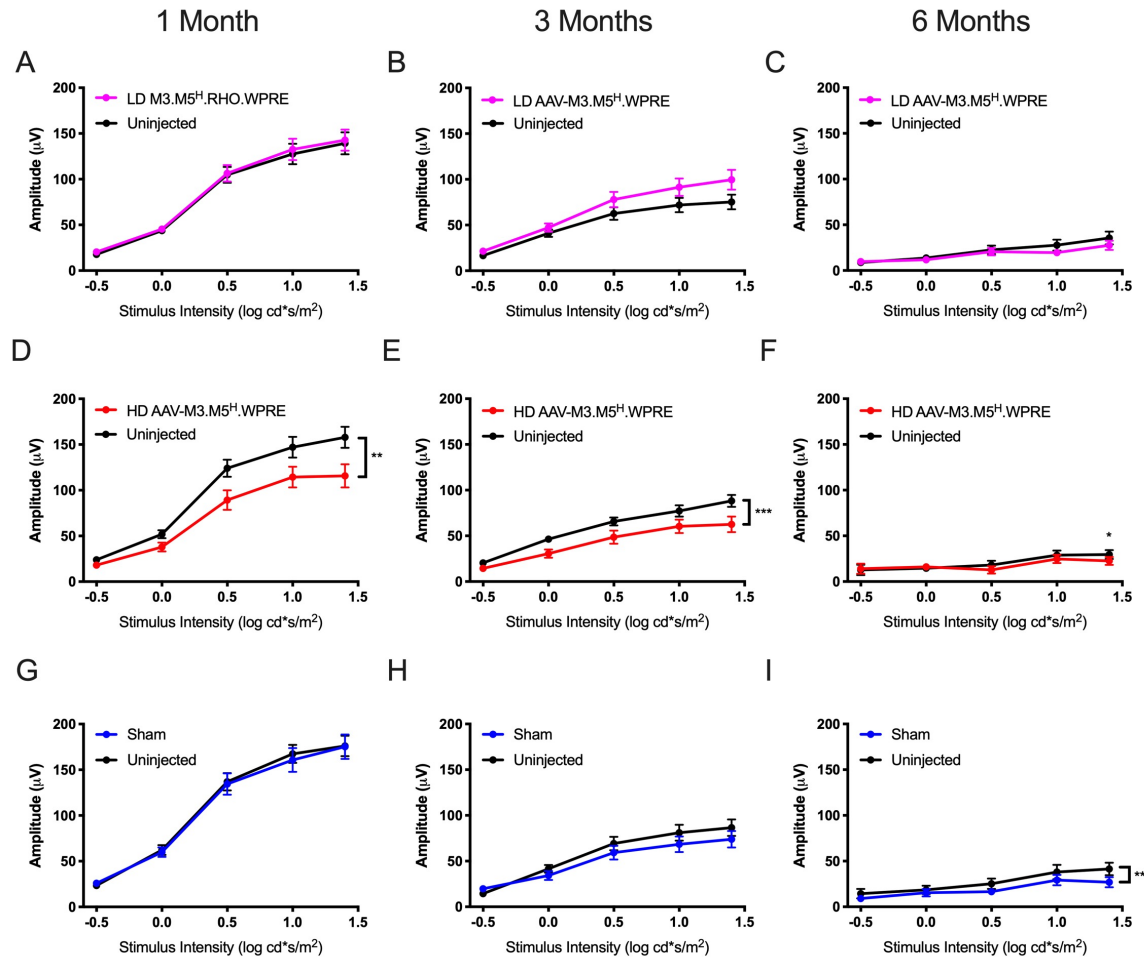


Figure 6.13. Full field light-adapted ERG irradiance-response curves for low and high dose AAV-M3.M5^H.WPRE-injected and sham-injected cohorts. All data points represent mean signal $\pm$ SEM. **(A-C)** Low dose AAV-M3.M5^H.WPRE-injected cohort, n=17. **(D-F)** High dose AAV-M3.M5^H.WPRE-injected cohort, n=17. **(G-I)** Sham (PBS)-injected cohort, n=14. *p<0.05; **p<0.01, ***p<0.001 for effect of injection, all other comparisons not significant, repeated measures two-way ANOVA.

Time Post-Injection				
Injection	Parameter	1 Month	3 Months	6 Months
High Dose	Dark-adapted a-wave	*0.0415	**0.0041	0.6131
	Dark-adapted b-wave	0.1806	*0.0418	0.8368
	Light-adapted response	**0.0058	***0.0007	0.1884
Low Dose	Dark-adapted a-wave	0.8324	0.3420	0.8922
	Dark-adapted b-wave	0.3523	0.1294	0.8318
	Light-adapted response	0.6299	0.1701	0.2470
Sham	Dark-adapted a-wave	**0.0037	***0.0009	**0.0022
	Dark-adapted b-wave	0.0901	**0.0056	**0.0022
	Light-adapted response	0.8241	0.0757	**0.0058

Table 6.2. ERG statistical analysis. Tabulated figures are p values from repeated measures two-way ANOVA testing for effect of injected substance on ERG amplitude. Statistically significant values are indicated according to the level of significance: * $p < 0.05$; ** $p < 0.01$. In all such cases, this represented a detrimental effect of injection on recorded response (see Figure 6.12 and Figure 6.13).

To allow comparison between groups, IRCs were plotted using the difference in ERG amplitude between injected and uninjected fellow eyes. This showed a beneficial effect of low dose AAV-M3.M5^H.WPRE injection when compared to either high dose or sham injection (Figure 6.14).

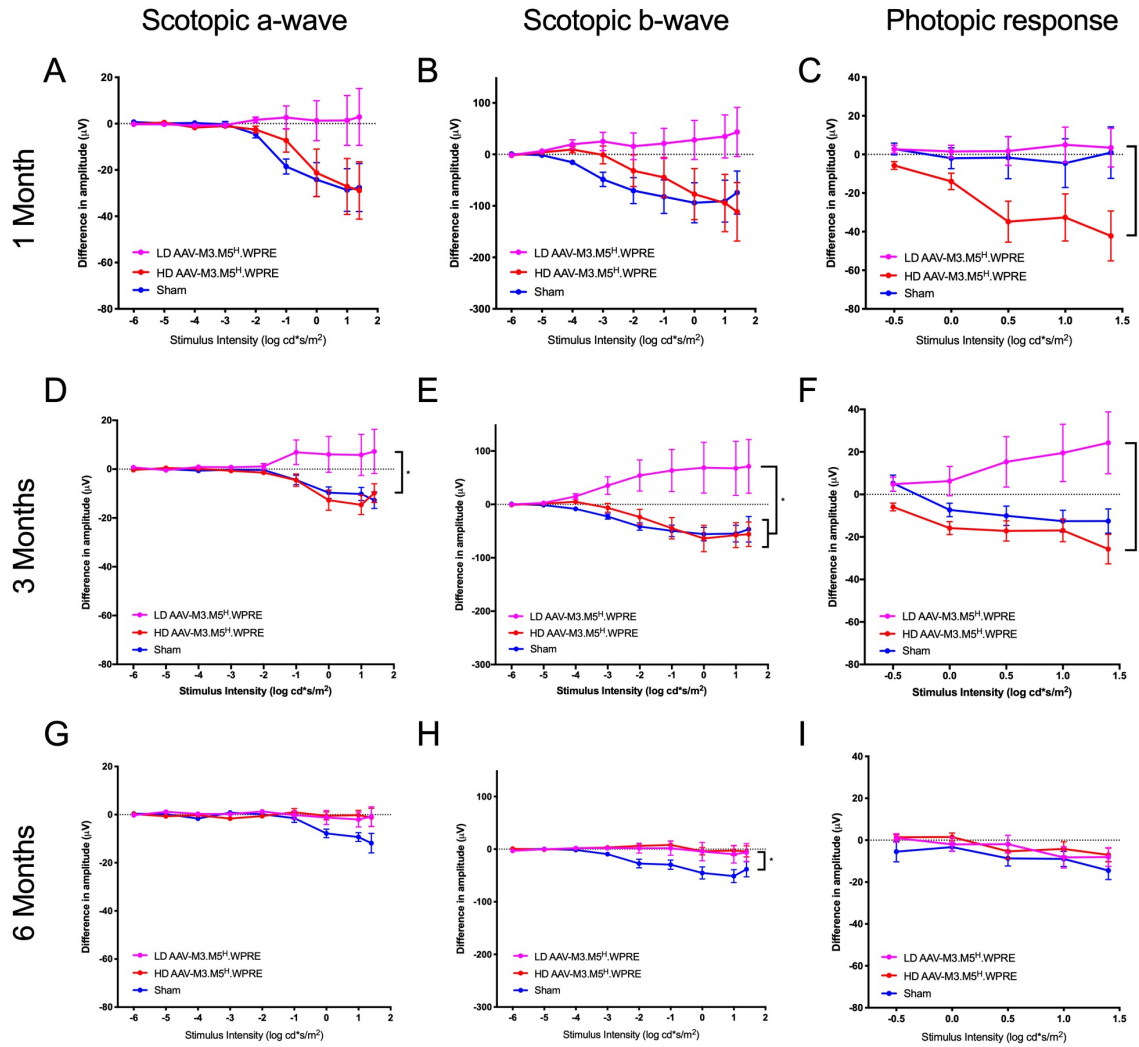


Figure 6.14. Difference in ERG amplitudes between treated and untreated eyes for low and high dose AAV-M3.M5^H.WPRE-injected and sham-injected cohorts. All data points represent mean signal difference $\pm$ SEM. Values greater than zero (dotted line) suggest a benefit whilst those below zero suggest damage as a result of the intervention. * $p < 0.05$; ** $p < 0.01$, Tukey's multiple comparison test.

In summary, subretinal injection of AAV-M3.M5^H.WPRE at a dose of 2×10^8 gc appeared to confer a small but significant rescue effect on the degenerative phenotype of the *Nrl.GFP/+*, *Rho^{P23H/+}* mouse. No such effect was however seen following injection at the higher dose of 2×10^9 gc, with the suggestion of a degree of retinal toxicity being apparent at those retinal loci subject to the highest effective viral multiplicity of infection (MOI). Since a similar superior

toxic effect was seen at this dose for the AAV-Ex/Int vector (Figure 4.18 - Figure 4.24), overexpression of mirtrons was unlikely to be the primary underlying cause. Very high global expression levels of transgenic human rhodopsin were observed following a single superior subretinal injection of 2×10^9 gc of either AAV-Ex/Int or AAV-M3.M5^H.WPRE (Figure 6.6). Given that an excess of wild type rhodopsin has been shown to induce rod cell death in wild type animals^{76,191}, it is possible that transgene overexpression may have contributed to the observed lack of efficacy, and even induced an acceleration of degeneration in the high dose cohort of mice in this study. To test this hypothesis, further mirtron-RHO AAVs were engineered in which the WPRE sequence had been removed, a modification that might be expected to reduce viral transgenic protein levels in the retina by 0.5-1 log units¹⁸¹. The first of these vectors, AAV2/8^{Y733F}.RHOp.Ex/Int.M3.M5^H (henceforth referred to as AAV-M3.M5^H), contained a viral genome as for AAV-M3.M5^H.WPRE less the WPRE sequence (Figure 6.1B). The second, AAV2/8^{Y733F}.RHOp.Ex/Int.M3.M5^M (henceforth referred to as AAV-M3.M5^M), contained a viral DNA genome that differed from that of AAV-M3.M5^H at only two nucleotide positions, such that its M5 sequence targeted mouse rather than human rhodopsin transcripts (Figure 6.1C). AAV-M3.M5^M would thus be expected to deliver two different functional mirtrons to the mouse retina which have previously shown to act in an additive fashion *in vitro* (see Section 5.4.9).

6.4.6. Rhodopsin mRNA replacement induced by AAV-M3.M5^M *in vivo*

To investigate the effect of co-delivery of two independently functional mirtrons on rhodopsin gene expression *in vivo*, 2×10^9 gc AAV-M3.M5^M were injected subretinally in *Nrl.GFP/+*, *Rho*^{P23H/+} mice, and four weeks later, whole retinas were harvested for gene expression analysis by RT-qPCR. This revealed a reduction in endogenous mouse rhodopsin gene expression of $41.8 \pm 9.5\%$ in AAV-injected relative to fellow sham-injected eyes (Figure 6.15). No significant difference in the magnitude of *in vivo* gene suppression was detected for the vector containing

one functional mirtron (AAV-M3.M5^H.WPRE) and the vector containing two (AAV-M3.M5^M),
 $p=0.5288$, Welch's t-test.

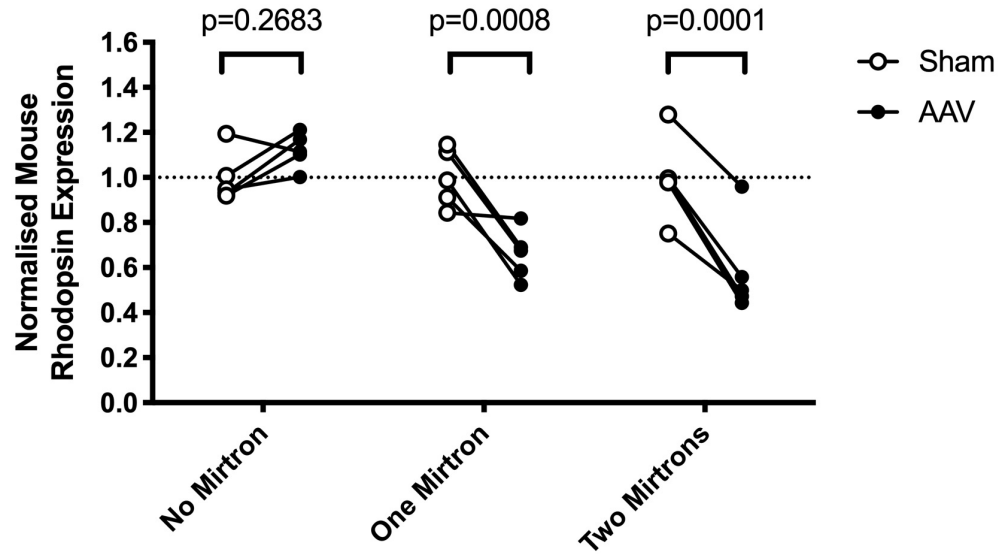


Figure 6.15. Effect of mirtron number on rhodopsin gene expression *in vivo*. Right eyes of *Nrl.GFP/+*, *Rho^{P23H/+}* cohorts designated ‘No Mirtron’, ‘One Mirtron’ and ‘Two Mirtrons’ were injected subretinally with 2×10^9 gc of AAV-Ex/Int, AAV-M3.M5^H.WPRE, and AAV-M3.M5^M respectively, whilst left eyes received isovolumetric sham injections of PBS ($n=5$ for all groups). Data is derived from RT-qPCR experiments in which *eGFP* was used as housekeeper gene i.e. $\Delta C_t = C_t(mRho) - C_t(eGFP)$. Calculated $2^{-\Delta C_t}$ values were normalised to the mean $2^{-\Delta C_t}$ of sham-injected fellow eyes for each cohort to generate normalised mouse rhodopsin expression values. Normalised *mRho* expression recorded in injected retinas was 1.120 ± 0.035 , 0.659 ± 0.050 and 0.587 ± 0.095 for ‘No Mirtron’, ‘One Mirtron’ and ‘Two Mirtrons’ cohorts respectively. Displayed p values were calculated from a two-way ANOVA Sidak’s multiple comparison test incorporating all data.

6.4.7. Structural effect of delivery of AAV-M3.M5^H and AAV-M3.M5^M to the *Nrl.GFP/+*, *Rho*^{P23H/+} retina

AAV-M3.M5^H or AAV-M3.M5^M were injected beneath the retina of right eyes of further cohorts of *Nrl.GFP/+*, *Rho*^{P23H/+} mice at a dose of 2×10^9 gc (high dose). As in previous experiments, left eyes did not receive an injection and acted as internal controls. The longitudinal changes in mean PRL thickness of treated and untreated eyes in these groups as measured by SD-OCT are shown in Figure 6.16. Mean PRL thickness in AAV-M3.M5^H-injected eyes was not significantly different from that of the untreated eyes (repeated measures two-way ANOVA: $F(1,8)=0.21$, $p=0.6579$ for effect of injection; Figure 6.16A), whilst injection of AAV-M3.M5^M led to a small reduction in mean PRL thickness ($F(1,16)=7.9$, $p=0.0125$; Figure 6.16B). The difference in PRL thickness between eyes was not significantly different for either AAV-M3.M5^H or AAV-M3.M5^M, and sham cohorts (Figure 6.16D).

A breakdown of PRL thickness by retinal location for injected versus uninjected eyes is shown in Figure 6.17, whilst the difference between treated and untreated as a function of retinal location is shown in Figure 6.18. This analysis suggested a damaging effect of both vectors in the superior regions of the retina above and beyond that induced by injection of PBS alone.

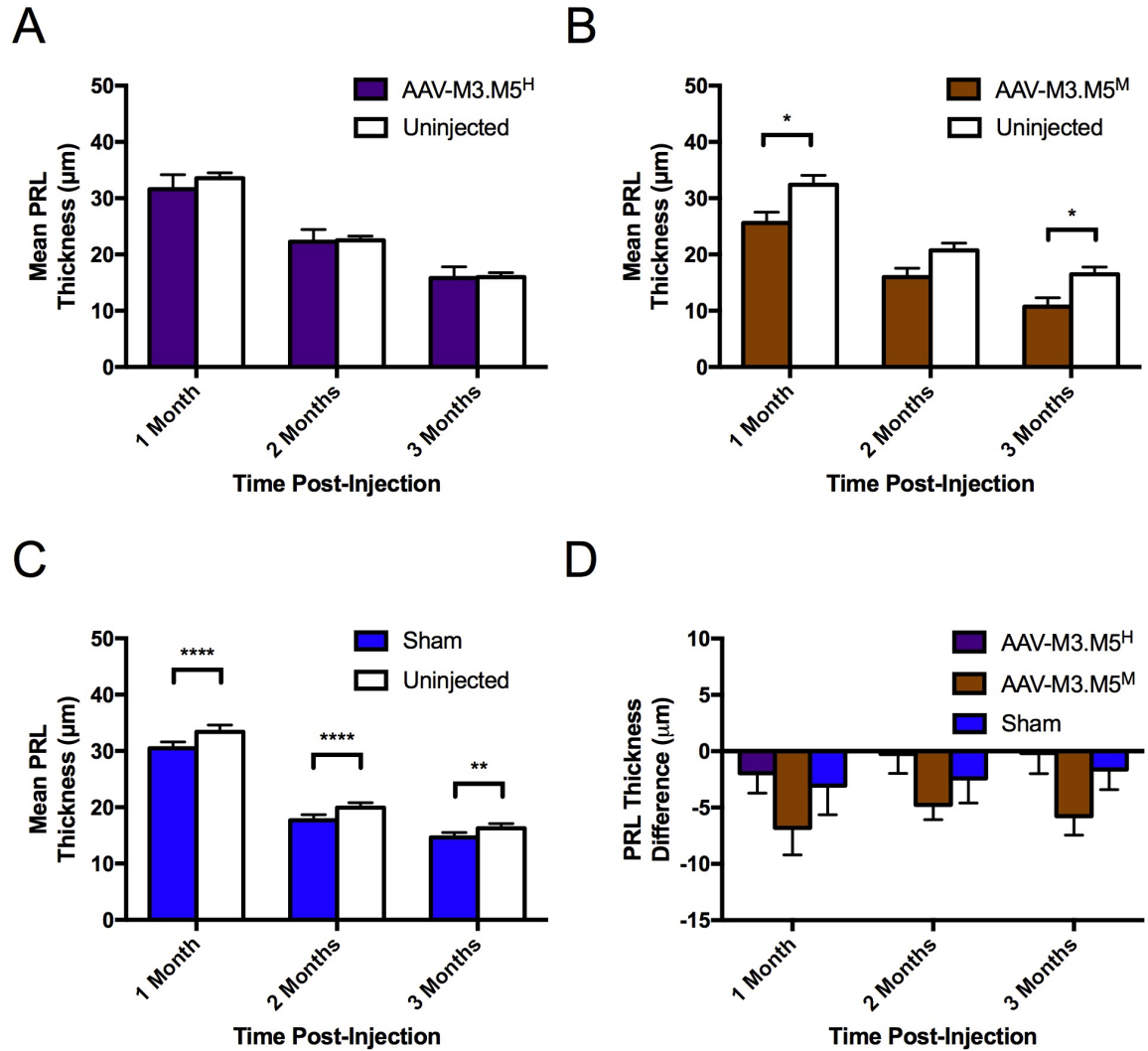


Figure 6.16. Effect of subretinal injection of 2×10^9 gc AAV-M3.M5^H and AAV-M3.M5^M on mean PRL thickness in the *Nrl.GFP/+*, *Rho^{P23H/+}* mouse. **(A)** AAV-M3.M5^H-injected cohort (n=9). **(B)** AAV-M3.M5^M-injected cohort (n=9). **(C)** Sham cohort (n=14). **(D)** Difference in PRL thickness between paired injected and uninjected eyes for the three cohorts as a function of time post-injection. *p<0.05, **p<0.01, ****p<0.0001, Sidak's multiple comparison test.

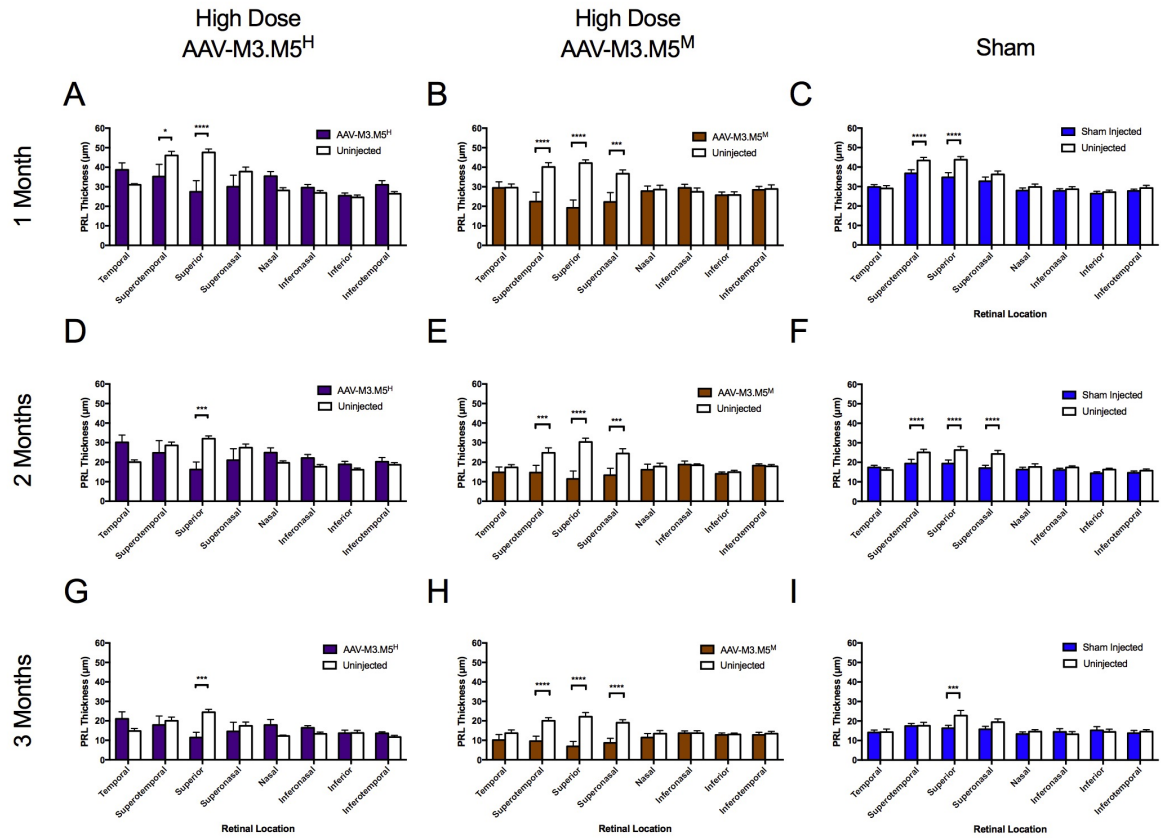


Figure 6.17. Mean PRL thickness as a function of retinal location following subretinal injection of AAV-M3.M5^H and AAV-M3.M5^M. All measurements were taken at a fixed eccentricity of approximately 22.5° from the optic nerve head in the stated direction. **(A, D, G)** AAV-M3.M5^H-injected cohort at one (A), two (D), and three (G) months post-injection. **(B, E, H)** AAV-M3.M5^M-injected cohort at one (B), two (E), and three (H) months post-injection. **(C, F, I)** Sham (PBS)-injected cohort at one (C), two (F), and three (I) months post-injection. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$, all other comparisons not significant, Sidak's multiple comparison test.

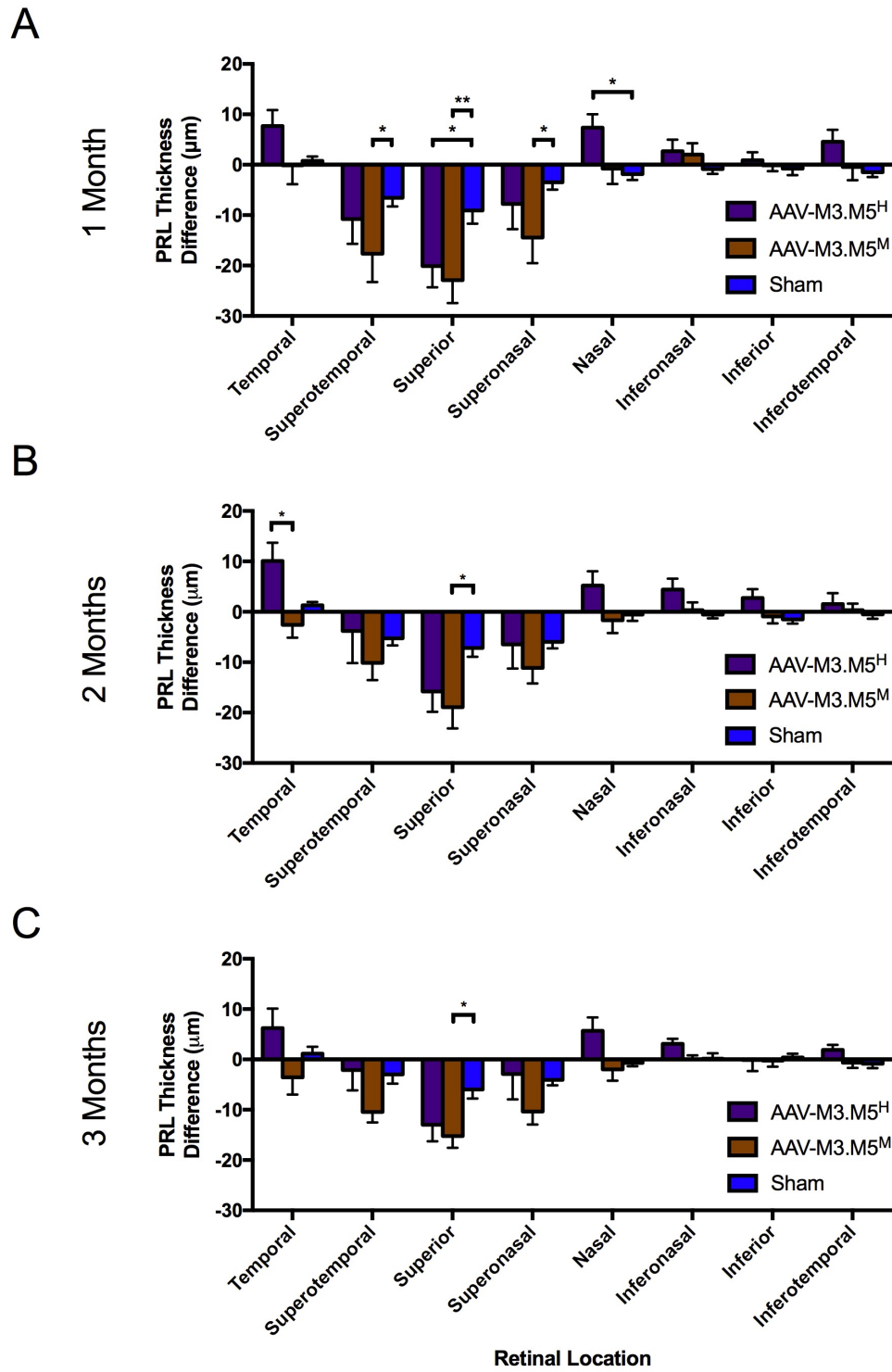


Figure 6.18. Difference in PRL thickness between AAV-M3.M5^H-, AAV-M3.M5^M-, or sham-injected eyes and uninjected fellow eyes as a function of retinal location. Values greater than zero suggest a benefit, whilst those below zero suggest damage as a result of subretinal injection. **(A)** One

month post-injection. **(B)** Two months post-injection. **(C)** Three months post-injection. * $p < 0.05$; ** $p < 0.01$, all other comparisons not significant, Sidak's multiple comparison test.

6.4.8. Functional effect of delivery of AAV2/8^{Y733F}.RHO_p.Ex/Int.M3.M5^H.RHO^{M3/M5R} and AAV2/8^{Y733F}.RHO_p.Ex/Int.M3.M5^M. RHO^{M3/M5R} to the *Nrl.GFP/+*, *Rho*^{P23H/+} retina

Full-field ERG was performed on *Nrl.GFP/+*, *Rho*^{P23H/+} mice one month after unilateral subretinal injection of 2×10^9 gc of either AAV-M3.M5^H or AAV-M3.M5^M. These interventions were associated with a significant reduction in both dark- and light-adapted ERG responses (Figure 6.19). The degree of observed retinal toxicity appeared greater for AAV-treated eyes than for sham-injected eyes (Figure 6.20).

In summary, the data presented above would suggest that at a dose of 2×10^9 gc, the AAV-M3.M5^H and AAV-M3.M5^M vectors induce a degree of structural and functional retinal damage resembling that caused by AAV-M3.M5^H.WPRE at this dose, and that this toxicity cannot be attributed to the effect of surgery alone.

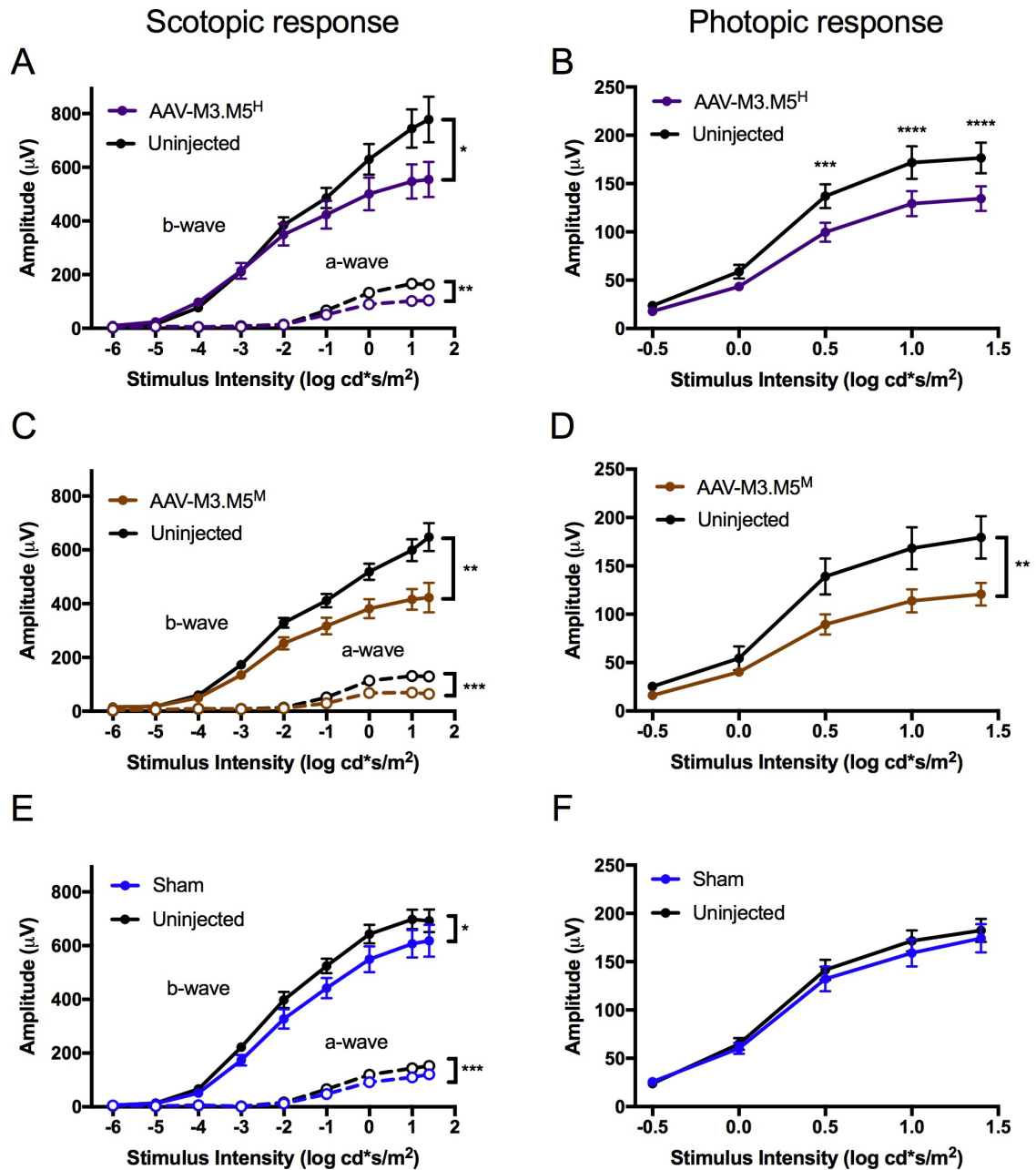


Figure 6.19. Dark- and light-adapted ERG IRCs one month following unilateral injection of AAV-M3.M5^H, AAV-M3.M5^M, or PBS (sham). All data points represent mean signal $\pm$ SEM. **(A & B)** AAV-M3.M5^H-injected cohort, n=9. **(C & D)** AAV-M3.M5^M-injected cohort, n=9. **(E & F)** Sham (PBS)-injected cohort, n=13. *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001, all other comparisons not significant, repeated measures two-way ANOVA for effect of injection.

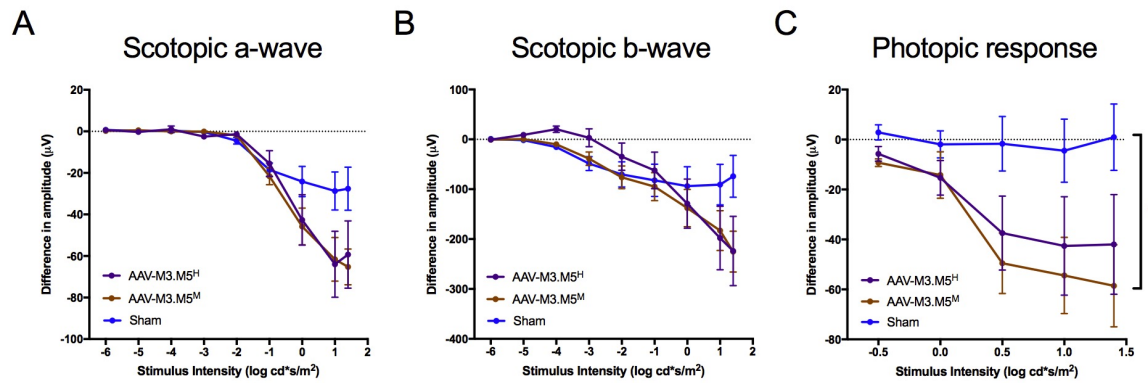


Figure 6.20. Difference in ERG amplitudes between treated and untreated eyes for AAV-M3.M5^H-, AAV-M3.M5^M-, and sham-injected cohorts one month post-injection. All data points represent mean signal difference $\pm$ SEM. Values greater than zero (dotted line) suggest a benefit, whilst those below zero suggest damage as a result of subretinal injection. * $p < 0.05$, Tukey's multiple comparison test.

6.5. Discussion

In this chapter, artificial mirtrons targeting rhodopsin mRNA have been packaged alongside a codon-modified resistant human rhodopsin CDS within AAV vectors, and tested *in vivo*. These vectors have been developed for the purpose of mutation-independent knockdown/replacement gene therapy for rhodopsin-related ADRP, where they may be expected to exert a treatment effect by exchanging mutant mRNA (and thereby protein) for wild type equivalents. Overexpression of rhodopsin protein in rods is known to induce rod cell death^{75,76,121}. Underexpression of the protein may however be similarly toxic, as evidenced by the degenerative phenotype observed in the rare recessively-inherited forms of rhodopsin-related RP caused by biallelic null/nonsense mutations in *RHO*^{81,82}, as well as in the *Rho*^{-/-} mouse^{73,195,283}. The balance between the gene suppressing and gene supplementing arms of any

rhodopsin knockdown/replacement vector or vectors, as well as the dose delivered, are thus likely to be critical factors in determining the safety and efficacy of any such therapeutic.

When injected subretinally in the rhodopsin null mouse, AAV-M3.M5^H.WPRE induced expression of rhodopsin protein which was appropriately trafficked to newly formed outer segments of rod photoreceptors (Figure 6.4). Further, treatment with this vector restored dark-adapted ERG responses to low-luminance flashes in these animals, confirming functionality of the transgenic protein (Figure 6.3). Rescue of rhodopsin expression in the *Rho*^{-/-} mouse has previously been demonstrated using recombinant AAV2/5¹⁹⁵, AAV2/8 and AAV2/rh10 vectors²⁸³ expressing human *RHO* under the control of a mouse rhodopsin promoter. If the histological sections presented in these reports are carefully studied, significant ectopic expression of rhodopsin can clearly be identified within the ONL in all cases, suggesting a degree of retrograde mistrafficking of transgenic protein from the point of translation in the IS to the region of the rod cell nuclei^{195,283}. This is of potential importance for the development of safe rhodopsin gene therapies, as mislocalization of protein is well described in itself as a cause of ADRP (Class I mutations- see Table 1.1)^{72,89}. Note that no such mislocalization was detected in this study following delivery of AAV-M3.M5^H.WPRE (Figure 6.4).

Switching synonymous codons has been commonly employed by researchers as a means of increasing protein expression, a process referred to as codon optimization³³⁰. This technique has been used here for the purpose of conferring resistance of the transgenic rhodopsin transcript to the suppressing effects of co-delivered mirtrons. Several studies have suggested that proteins translated from codon-optimised transcripts may differ in structure from those expressed from wild type mRNA, which could in theory have a downstream effect on their trafficking or function^{331,332}. This difference may occur in situations where the slowing or pausing of translation at rare codons, a process abolished in codon-optimised transcripts, is

required for the correct folding of a nascent peptide^{333,334}. The frequency of usage for the modified codons within the *RHO*^{M3/5R} CDS used in the RHO-Mirtron vectors discussed in this chapter was similar to that of the codons they replaced (see Table 5.7). The rhodopsin protein expression pattern observed by IHC following subretinal injection in *Rho*^{-/-} mice did not differ significantly following delivery of AAV-Ex/Int which includes the wild type human *RHO* CDS (Figure 4.13), and AAV-M3.M5^H.WPRE, which includes *RHO*^{M3/5R} (Figure 6.4A). It may thus be concluded that the transgenic *RHO*^{M3/5R} sequence leads to the translation of functional rhodopsin protein, with a retinal expression pattern comparable to that derived from transgenic wild type mRNA.

The data presented in this chapter represents the first demonstration of use of an artificial mirtron *in vivo*. Efficient splicing of mirtrons from transgenic mRNA was demonstrated by PCR amplification using cDNA template derived from injected retinas (Figure 6.5). It is interesting to note that the pattern of splice products generated from treated retinas differed from that derived from transfected HEK293 cells during the *in vitro* phase of this study, in which there was no suggestion of inter-mirtron exon skipping (Figure 5.15). This may reflect cell-specific variation in the splice machinery present in rod cells and the immortalised HEK293 cell line, or alternatively may be attributable to differences in the splicing contexts of the *in vitro* and *in vivo* constructs (i.e. in the number and location of ESEs). As the mirtrons used in this chapter were located within the 5'-UTR of, rather than nested within, the transgene CDS, the small degree of incorrect splicing observed here would not be expected to influence downstream gene expression. Indeed, locating mirtrons within the 5'-UTR overcomes the significant problem associated with placing them as artificial introns within a coding sequence, where incomplete or inaccurate splicing may result in poor gene expression in cases where the retained intron equates to an out-of-frame insertion³⁰², or generation of mutant protein when in-frame.

The RT-qPCR data presented in Figure 6.6 and Figure 6.15 clearly demonstrate that the artificial mirtrons developed in the previous chapter retain their functionality when delivered by rAAV to rod cells of the murine retina. Following subretinal injection with AAV-M3.M5^H.WPRE, a reduction in *Rho* gene expression of the order of 35% compared with fellow eyes was observed. Given that these data were derived from RNA extracted from whole retinal samples, which necessarily including untreated inferior regions, it is likely that the level of gene knockdown in any given cell that received an adequate MOI of vector was much higher. This level of overall gene knockdown is greater than, or at least comparable to, that observed in a recent study in which mouse rhodopsin was targeted using a CRISPR-based strategy⁵⁶. Cideciyan and colleagues reported more efficient suppression of canine rhodopsin following subretinal delivery of a construct including an H1 promoter-driven shRNA, but only treated areas of retina were sampled¹⁹⁸.

In this study, subretinal injection of AAV-M3.M5^H.WPRE at low dose (2×10^8 gc) was associated with a small but significant rescue effect in *Nrl.GFP/+*, *Rho*^{P23H/+} mice. Since no such benefit was observed following equivalent injection of AAV-Ex/Int (see Chapter 4), it is likely that this effect is attributable to the inclusion of the active mirtron M3 within the AAV genome. The same vector at high dose (2×10^9 gc) was however found to induce a degree of retinal damage comparable to that of sham injection. This would suggest a mild toxic effect of treatment at this dose that is similar in magnitude to any benefit conferred by RNA replacement. Possible causes of this toxicity include capsid-related immunogenicity or cytotoxicity (although this has not been observed in other studies using AAV2/8^{Y733F}³³⁵), or cell death induced by overexpression of the rhodopsin protein^{75,76,121}. Although miRNA/shRNA-related cytotoxicity has previously been reported as a result of saturation of RNAi processing pathways²⁸⁷, mirtron-related toxicity is an unlikely explanation for the accelerated degeneration observed in this study, as a comparable effect was apparent following injection of the mirtronless AAV-Ex/Int vector

(see Chapter 4). Similarly, an immune effect triggered by presence of human protein seems unlikely, given the structural similarity in the rhodopsin protein that exists between the two species; indeed, no antibody capable of distinguishing the two has been identified. Moreover, other researchers who have delivered the human *RHO* CDS by AAV to the murine retina at comparable doses^{120,196}, or to the canine retina¹⁹⁸, have not reported such an effect. To test the overexpression hypothesis, two vectors without the WPRE enhancer (AAV-M3.M5^H and AAV-M3.M5^M) were tested using the same experimental paradigm at high dose (2×10^9 gc). A similar degree of toxicity was however observed in both cases, with the implication that either rhodopsin overexpression is not the causal factor, or that removal of the WPRE sequence was insufficient to reduce levels of transgenic protein below the threshold for RHO-overexpression induced photoreceptor toxicity. In a recent study which specifically investigated the effect of WPRE on retinal gene expression, an increase in measured AAV-derived protein of the order of 0.5-1 log units was reported as a result of its inclusion when lower MOIs of virus were delivered¹⁸¹. No such increase was however observed at higher doses suggesting a lesser effect of WPRE at higher MOIs. This might explain why no reduction in toxicity was observed in this study after removal of the WPRE sequence from the AAV-M3.M5.WPRE vector construct (if indeed transgene overexpression was the responsible factor).

6.6. Conclusions

The work presented in this chapter demonstrates that functional artificial mirtrons directed against rhodopsin mRNA may be co-delivered in a recombinant AAV with a resistant version of the gene, which itself is able to traffic appropriately to the rod OS and support phototransduction. The resulting vector induced rhodopsin RNA replacement in the *Nrl.GFP/+*, *Rho*^{P23H/+} mouse model of ADRP and, when delivered at a dose of 2×10^8 gc, imparted a small but significant therapeutic effect on the degenerative phenotype. A degree of outer retinal toxicity that cannot be attributed solely to the effect of surgery was however

observed in all vectors tested (both with and without mirtrons) at the higher dose of 2×10^9 gc.

The exact cause of this remains elusive and warrants further investigation.

7. General Discussion

The broad objective of this thesis has been the development of treatment strategies for autosomal dominant RP caused by mutations in the rhodopsin gene. Together, these mutations represent one of the most common single genetic causes of non-syndromic RP¹⁶, and there is at present no approved treatment for such patients. Three approaches were considered: one optical therapy- filtration of short wavelength light, and two gene therapies- wild type rhodopsin supplementation, and mirtron-mediated rhodopsin RNA replacement.

7.1. Filtration of short wavelength light

Many forms of rhodopsin-related RP are characterised by a sectoral pattern of degeneration, where photoreceptors located in inferior regions of the retina appear to degenerate at a faster rate than those located superiorly¹⁶. It has been hypothesised that this distribution may be attributable to the modifying effect of light to which inferior regions of the retina are more intensely exposed^{29,89,234}. Indeed, animal models of rhodopsin-related RP that show the sector phenotype appear to have an increased susceptibility to light-induced damage^{107,110,112,235}, a phenomenon usually observed only at high light intensities in wild type animals³³⁶. Knockout of *Rpe65*³³⁷, or inhibition of the retinoid cycle with 13-cis retinoic acid (which acts as an antagonist of RDH5 and RPE65 enzymes)³³⁸, can confer resistance to high-intensity light-induced damage in mice. The L450M variant of Rpe65, which results in a slowing of rhodopsin regeneration, is similarly protective^{241,242}. Further, the rhodopsin knockout retina also appears to be immune to light-mediated damage³³⁷. These data support the hypothesis that regeneration of 11-cis retinal chromophore to form rhodopsin is required for light-induced damage to occur. Studies which investigated the spectral composition of light for induction of retinal damage concluded that blue light (403nm) was more damaging than green light (550nm) that had been

adjusted to the same energy. Taken together, these studies would suggest that filtration of shorter wavelength light may be of therapeutic benefit in sectoral RP, where the threshold for light-induced damage is lower. This hypothesis was tested in Chapter 3.

The P23H mutation in rhodopsin induces sector RP in both humans and animal models^{16,28,105}. The *Rho*^{P23H/+} heterozygous mouse investigated here showed a faster rate of outer retinal degeneration in inferior regions of the retina than in superior regions (Figure 3.3). Housing of these animals in red cages- which effectively acted as long-pass light filters- slowed the rate of photoreceptor loss as determined by both longitudinal OCT imaging (Figure 3.8) and ERG (Figure 3.7) when compared to littermates housed under standard conditions. Although the absolute increase in outer nuclear layer thickness (i.e. rod survival) conferred by red-rearing was modest, this was associated with a significant improvement in cone survival (see Figure 3.7 and Figure 3.8). Since much of the visual morbidity associated with RP is attributable to the secondary death of cones, prolongation of their survival would be expected to have a substantial impact on quality of life for these patients. The apparent disparity between observed rod and cone cell death rates for red- and clear-reared animals may be explained by the fact that cone death occurs as a secondary event once rod nuclear density is reduced beneath a critical threshold²⁶¹. This may be illustrated by way of mathematical modelling. The photoreceptor layer (PRL) survival curves for animals reared in clear and red cages from Figure 3.5A can be approximated to single-phase exponential decay functions with time constants (τ) of 2.43 and 4.07 months respectively (goodness to fit R square = 0.98 for both groups). Red-rearing was thus associated with a 67% increase in the time constant of degeneration. Figure 7.1 illustrates how this effect may be applied to a mathematical model of human RP. Here, the rate constant τ has been set at 20 years (black curve) simulating a slow exponential loss of rods as might occur in sector rhodopsin-related disease. The red curve represents the effect of increasing τ by 67% to 33.5 years. A threshold for secondary cone cell death (and thus significant visual

morbidity) has been set at 25% total rod survival, an estimate derived from data recorded from patients with rhodopsin-related ADRP²⁶¹. In this model, the age at which rod density reaches the critical threshold is delayed by 19 years when the rate constant is proportionally increased to the extent observed through red housing in Chapter 3.

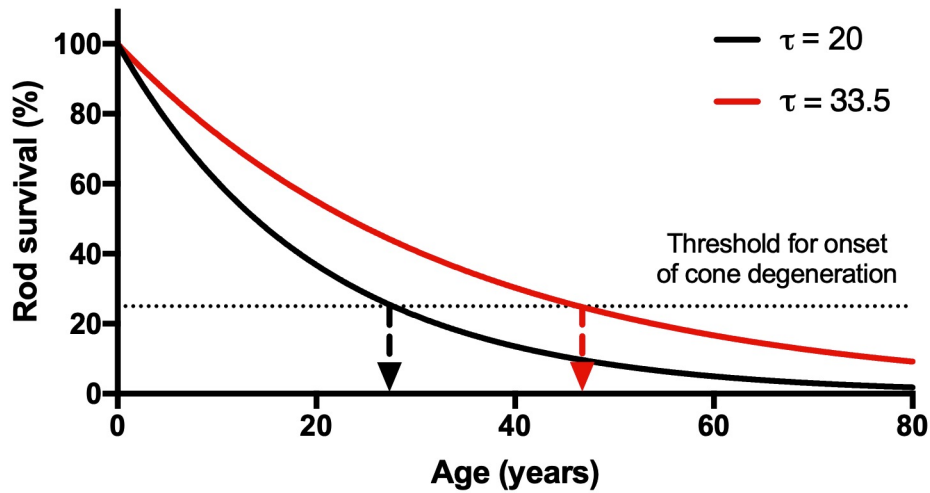


Figure 7.1. Mathematical modelling of the effect of short-wavelength filters explains the observed preservation of cones. Loss of rod photoreceptors has been modelled by the single-phase exponential decay function $R_t = 100e^{-t/\tau}$ where R_t represents percentage rod survival at time t , and τ is the rate constant. The red curve illustrates the change in decay function that would be expected if the rate constant were to be increased as a result of short-wavelength light filtration to the extent observed empirically in the *Rho^{P23H/+}* mouse model. A threshold based on patient data reported by Cideciyan and colleagues for the onset of cone degeneration has been set at 25%²⁶¹. Note how the increase in τ in this model results in a substantial delay in the onset of cone degeneration from 28 to 47 years. This phenomenon is likely to explain the relative preservation in cone structure and function observed for red-reared animals in Chapter 3.

The data presented in Chapter 3 would thus support the use of red optical filters as a treatment option for patients with rhodopsin-related RP in which a sectoral distribution is noted.

Introduction of short-wavelength light filters at later time points however led to a reduction in efficacy of the intervention, with no benefit being apparent when adult mice were placed into red cages (Figure 3.10). The contribution of short-wavelength light to the degeneration of photoreceptors in the *Rho*^{P23H/+} mouse model may thus occur early in its natural history. It is however difficult to extrapolate this finding to the human condition where in many cases, unlike the *Rho*^{P23H/+} mouse, degeneration progresses slowly, with many patients retaining reasonable visual function beyond their 4th decade⁹⁴. Nonetheless, given that the risks to patients associated with the use of long-pass light filters in the form of red-tinted spectacles are likely to be small and reversible, the recommendation for their clinical use may be justified. Predictable potential side effects of red-tinted spectacles include impairment of colour discrimination, and sleep disturbance secondary to disruption of the retinal melanopsin-driven central circadian clock. Investigation into the extent to which these occur in RP patients and healthy volunteers would be warranted as part of any future clinical investigation into the efficacy of light filters for the treatment of sector RP.

7.2. RHO supplementation

The second potential treatment for rhodopsin-related ADRP tested in this thesis was gene-therapy mediated overexpression of wild type rhodopsin without direct suppression of the dominant allele. This approach was based on experimental observations suggesting that P23H mutant rhodopsin might exert its detrimental effect, at least in part, via a dominant negative mechanism, through dimerization with wild type protein^{66,92,93}. Supplementation with wild-type rhodopsin in such cases might thus be expected to compete with the mutant version and thereby diminish its toxic effect²³¹. Although success of this approach has previously been reported in one particular *RHO*^{P23H} transgenic mouse line¹⁹¹, it has not been replicated in other animal models of ADRP^{120,121}. The initial aim of Chapter 4 was to develop a highly efficient rhodopsin-expression vector capable of inducing levels of transgenic protein in rods, that might be

sufficient to compete with genomically expressed mutant opsin, in order to overcome any dominant negative effect. Four AAV2/8^{Y733F} vectors were manufactured and compared for expression in the rhodopsin null mouse model. All four contained the human rhodopsin CDS driven by the human rhodopsin promoter. Of the four AAVs, the self-complementary vector and the single-stranded vector which included the exon-intron-exon sequence from the CAG promoter and the WPRE enhancer, were the most effective. It is interesting to note that inclusion of the WPRE in isolation appeared to confer no detectable increase in vector potency (AAV-RHO versus AAV-WPRE; Figure 4.13, Figure 4.14, Figure 4.15). Patrício et al. demonstrated that the WPRE could enhance expression of transgenic protein (eGFP or Rab escort protein 1 (REP1)) driven by the CAG promoter *in vitro*, and within both the mouse and human retinas¹⁸¹. The WPRE effect may thus be promoter specific, or alternatively may rely on the presence of an intron within the AAV-derived transcript. Context-dependency for the WPRE and other regulatory elements has been reported elsewhere and remains an area of controversy^{339,340}.

Protein expression levels achieved in the *Rho*^{-/-} retina following subretinal injection of these vectors were around 15-25% of that observed in age-matched wild type control animals (Figure 4.15). This may be considered a reasonable level of transgene expression, given the degenerate state of the ONL at the time of harvesting at PNW7, which is reduced to around half of that observed in wild type mice (Figure 4.8). Regional differences in transgenic rhodopsin expression would also be expected following the single superior subretinal injection.

In spite of the observed overexpression of total rhodopsin achieved in the *Nrl:GFP/+*, *Rho*^{P23H/+} by rhodopsin gene therapy, no beneficial effect on retinal structure or function was apparent. This may be attributable to a toxic gain of function (as opposed to a dominant negative) mechanism for rod cell death induced by the P23H mutation^{103,106,108}. Other possible

explanations include the relatively late delivery of treatment (at PND21) given the inherent rapidity of degeneration in this model¹⁰⁵, a potential negative effect of rhodopsin overexpression on rod survival^{75,76,191}, or a combination of these factors.

7.3. Mirtron-mediated RHO replacement

The strategies for the treatment of rhodopsin-related ADRP addressed in Chapters 3 and 4 cannot be considered mutation-independent since red coloured long-pass light filters would only be applicable to those mutations associated with a sector distribution of retinal degeneration, and wild type rhodopsin gene augmentation would only be of theoretical benefit for those mutations with a dominant-negative mechanism of action¹⁹⁰. Exchange of mutant for wild type mRNA using an RNA replacement gene therapy has the potential to be truly mutation independent, and the development of such an approach was the focus of Chapters 5 and 6. In the so-called knockdown/replacement paradigm for dominantly-inherited disease, all endogenous expression of the gene in question is suppressed, whilst additional wild type copies of the gene that are engineered so as to be resistant to the blocking mechanism are concurrently supplied by way of gene replacement. Although more elaborate in its scope, this technique obviates the requirement to develop a separate suppression system for each individual mutation within a given gene, making it more universal, and thus commercially viable. Several knockdown/replacement gene therapies are currently under development for rhodopsin-related ADRP using shRNAs^{121,193,194,196,198} or ribozymes^{198,203,204} as the mode of gene suppression at the level of RNA, and zinc finger endonucleases^{205,206} or CRISPR/Cas9⁵⁶ at the level of DNA.

Recently, successful treatment of a large animal model of rhodopsin-related ADRP using a single shRNA knockdown/replacement AAV vector has been demonstrated, which represents a significant milestone in the progression towards clinical translation of RHO-ADRP gene therapy¹⁹⁸. The authors first identified an shRNA which could efficiently silence the expression

of both wild type and mutant human rhodopsins *in vitro*. They then demonstrated that this reagent was capable of inducing near complete suppression of both rhodopsin mRNA and protein when packaged within a self-complementary AAV2/5 construct and injected subretinally in both wild type dogs, and in dogs heterozygous for the naturally occurring RHO^{T4R} dominant mutation. A codon-modified version of the human rhodopsin CDS, thereby rendered resistant to knockdown, was engineered and validated *in vitro*. The authors then compared the efficacy of a dual vector approach, where the shRNA and resistant transgene were delivered within separate AAV2/5 vectors, with that of a single vector strategy in which the two elements were packaged together as a single construct. Here the codon-modified *RHO* transgene was placed under the control of a short human rhodopsin promoter, whilst the shRNA was driven by a separate H1 RNA Pol-III promoter sequence. Following subretinal delivery in the RHO^{T4R} canine model, the authors reported a more favourable safety profile using the single vector approach, with significant inflammatory effects being observed in cases of dual vector treatment. This is likely to reflect the lower capsid load achieved when using a single rather than two separate AAVs to deliver the gene therapy. The T4R mutant dog undergoes an accelerated retinal degeneration in response to even moderate exposure to light²³⁵. Resistance to such light-induced damage was achieved in treated areas of retina following injection of the combined knockdown/replacement vector¹⁹⁸. The authors were further able to demonstrate long-term structural and functional benefit following one-off delivery of their combined vector in the RHO^{T4R} dog: after repeated light exposures, a dramatic preservation of the ONL was observed by SD-OCT, and longitudinal preservation of the dark- and light-adapted ERG responses was reported in treated eyes up until 37 weeks post-injection.

This impressive study indicates that the degeneration induced by dominantly-inherited mutations in rhodopsin is indeed amenable to treatment using a knockdown/replacement gene therapy strategy. The existence of recessive forms of RP caused by biallelic null mutations in

RHO^{81,82}, and the fact that the *Rho*^{-/-} mouse exhibits a rapid degeneration of the ONL⁷³, would suggest that too little expression of rhodopsin protein leads to accelerated rod cell death. Numerous studies have also demonstrated that overexpression of the protein may be similarly toxic to photoreceptors^{75,76,191}. The balance between rhodopsin suppression and replacement induced by a gene therapy vector is therefore likely to be critical, since both under- and over-expression may accelerate the degenerative process. Heterozygous carriers of null rhodopsin mutations appear to have a subclinical phenotype, exhibiting only marginal reductions in dark-adapted light sensitivity⁸², and borderline changes in the rod-derived ERG⁸¹. It may thus be concluded that around 50% of wild type protein expression is likely to be sufficient to maintain rod viability. In their canine study, Cideciyan and colleagues achieved near complete suppression of endogenous expression within treated areas of retina, and reported rhodopsin supplementation approximating 30% that of wild type levels¹⁹⁸.

The mirtron approach developed in this thesis represents a variation on the knockdown/replacement paradigm, whereby the two elements of the therapy may not only be delivered within a single vector, but under the control of the same cell-specific RNA Pol-II promoter. This imparts the theoretical benefits of more consistent matching of gene knockdown and replacement, along with cell-specific expression of the RNAi effector, which may reduce the risk of off-target effects that might otherwise occur in cell types other than rods. The mirtron approach does however impart additional target sequence constraints owing to the requirement of mirtrons to commence with the splice donor consensus motif. In Chapter 5, all possible mirtron guide sequences that target areas of homology between the human and mouse rhodopsin transcripts were tested *in vitro*. The most effective mirtron design, M3, achieved rhodopsin mRNA suppression of 67% against the human transcript, and 76% against the mouse transcript *in vitro* (Figure 5.13). This degree of potency is comparable to the 50% *RHO* mRNA knockdown achieved *in vitro* by Gorbatyuk and colleagues using an siRNA they

designated siRNA301¹⁹⁷. This sequence subsequently formed the basis for the combined knockdown/replacement AAV vector described by Mao and colleagues in their 2012 paper in which the authors demonstrated significant slowing of disease progression in a transgenic RHO^{P23H} , $Rho^{+/-}$ mouse¹²¹. The degree of RHO suppression achieved by M3 *in vitro* is also similar to that reported by O'Reilly and colleagues for their siRNA-BB construct (78%)¹⁹³, this sequence being later incorporated into a dual vector knockdown/replacement therapy which proved effective in slowing retinal degeneration in a transgenic RHO^{P347S} , $Rho^{+/-}$ mouse model¹²⁰.

Mirtron 3 induced a 34.1% suppression in total endogenous mouse rhodopsin mRNA *in vivo* (Figure 6.6). This figure cannot be directly compared with the level of mRNA knockdown reported by Cideciyan colleagues, since in their study, only the treated area of the dog retina was analysed¹⁹⁸. Palfi et al. reported over 90% knockdown of rhodopsin gene expression *in vivo*, following subretinal delivery of an AAV co-expressing a human rhodopsin-targeting shRNA together with eGFP. Again, this data is not directly comparable to that presented in Chapter 6, as in the Palfi study gene expression analysis was performed following fluorescence-activated cells sorting (FACS) to isolate transduced photoreceptors, and not on whole retinal cDNA. Tsai et al. reported a 25% reduction in total endogenous *mRho* expression as a result of subretinal injection of their CRISPR-based gene therapy in wild type mice⁵⁶. The authors subsequently demonstrated a treatment effect for this knockdown/replacement therapeutic in the $Rho^{P23H/+}$ knock-in murine model. The level of suppression achieved by mirtron 3 *in vivo* thus compares favourably with that reported by Tsai et al⁵⁶.

A rescue effect was demonstrated in the $Nrl:GFP/+$, $Rho^{P23H/+}$ mouse following subretinal injection of AAV.M3.M5^H.WPRE at low dose (2×10^8 gc). The magnitude of this benefit was however smaller than that reported by other researchers following equivalent treatment of mouse models of rhodopsin-related ADRP with knockdown/replacement gene therapies. This

may be explained by the relatively late delivery of treatment in the present study. All mice in this thesis were treated at the point of weaning at approximately PND21 by which point, given the exponential nature of rod loss in this model, significant outer retinal degeneration had already occurred (see Section 4.4.2 and Section 4.4.3). Other groups, by contrast, chose to deliver their gene therapies at much earlier timepoints, injecting animals as young as PND0-5^{56,194,196}. Treatment prior to the onset of significant photoreceptor loss may be expected to improve treatment efficacy, as a greater number of rods would be present at the point of intervention to rescue. It is also conceivable that by the time significant RNA replacement had taken place following injection at PND21, toxic levels of mutant Rho^{P23H} protein may already have accumulated in rods, making their subsequent degeneration inevitable. This concept is supported by data presented in Chapter 3, where delay in the introduction of long-pass light filters from PND0 to PNW3 or PNW7 resulted in a significant attenuation or complete abolition respectively of the associated treatment effect (Figure 3.10). Animals in this study were injected after the onset of retinal degeneration in order to reflect the state of the retina in patients undergoing any future gene therapy for rhodopsin-related ADRP, who would most likely be treated in adulthood. It is worth noting however that mice are imperfect model systems for RP due to their lack of a fovea, so the centripetal progression of degeneration seen in patients is not modelled in these animals. Indeed, parafoveal retinal anatomy in many patients with rhodopsin-related ADRP remains relatively normal until late in the disease course (Figure 1.5). Early injection of P23H knock-in mice at a time when their retinal architecture more closely resembles that of age-matched wild types may therefore be justified, and would be a natural next step in the pre-clinical evaluation of mirtron-rhodopsin vectors.

Another potential explanation for the poorer efficacy of knockdown/replacement vectors developed in this thesis, at doses equivalent to those used by other groups, may be sub-optimal RNA replacement. An imbalance between rhodopsin suppression and replacement may have

taken one of three forms. In the first scenario, rhodopsin suppression may have been insufficient to attenuate the negative effects of the endogenous mutant allele. Although, as discussed above, the magnitude of endogenous gene knockdown achieved in the present study both *in vitro* and at high dose (2×10^9 gc) *in vivo* appears comparable to that achieved by other researchers, a more accurate measure of the *in vivo* potency of these vectors could be achieved if gene expression analysis were to be performed using only retinal tissue overlying the initial subretinal bleb. Given that the effects of multiple active mirtrons in tandem within the 5'-UTR of an expression cassette may be additive (Figure 5.16), inclusion of additional mirtrons within the vector may represent a simple strategy by which the gene suppressing action of mirtron knockdown/replacement vectors may be augmented. In the second scenario, gene knockdown may have been sufficient, but replacement gene expression may have been inadequate to maintain rod OS structural integrity, thereby conferring an effective null degenerate phenotype. This situation however seems unlikely given the high levels of transgenic mRNA detected following injection with mirtron-RHO vectors: at low dose (2×10^8 gc) total retinal expression of transgenic rhodopsin in the treated eye was largely equivalent to that of endogenous rhodopsin measured in the fellow sham-treated eye, whilst at high dose (2×10^9 gc), transgenic *RHO* expression was over four times greater than that of mouse *Rho* recorded in the fellow eye (Figure 6.6). Furthermore, AAV-M3.M5^H.WPRE was capable of inducing both the de novo synthesis of rod OS organelles (Figure 6.4), as well as the rod-derived ERG (Figure 6.3) in the rhodopsin null retina, which would suggest expression of transgenic protein at least within the physiological range. In the third possible scenario, suppression of endogenous protein may have been sufficient, but may have been accompanied by overexpression of the replacement rhodopsin protein, with consequent toxicity. This idea was explored in the latter portion of Chapter 6. The qPCR data presented in Figure 6.6 would imply that treatment with low dose AAV-M3.M5^H.WPRE produced an appropriate degree of *RHO* supplementation, but insufficient endogenous gene knockdown, whilst treatment with high dose vector induced

potent mouse *Rho* suppression, but apparent overexpression of the replacement transcript. Toxicity owing to *RHO* overexpression at high dose might explain the lack of a dose-response relationship observed for treatment with AAV-M3.M5^H.WPRE, and could be responsible for the accelerated loss of photoreceptor observed in some cases in the superior regions of the retina which were exposed to the highest effective viral MOI. Removal of the WPRE enhancer from the mirtron-RHO AAV expression cassette however failed to attenuate this effect. This would suggest that either human RHO expression remains dangerously high even in the absence of the WPRE, or that an alternative factor such as transgene-related immunogenicity or capsid-related toxicity is responsible for the observed retinotoxicity. In support of the latter hypothesis, apparent toxicity induced by all vectors at high dose (both with and without mirtrons) seemed to disproportionately affect the light-adapted ERG response, which is derived exclusively from cones whose nuclei are located in the outermost regions of the ONL (see Sections 4.4.8, 6.4.5 and 6.4.8). Since transgene expression was driven in all cases by rod-specific promoters (as confirmed in Figure 6.4), this phenomenon cannot be attributed to rhodopsin overexpression, and may in fact have resulted from a direct toxic effect of the AAV. The potential role of the AAV capsid serotype could be explored in future studies by pseudotyping the mirtron-RHO expression cassettes developed in this thesis with alternative capsid serotypes for which safety in clinical trials has been established e.g. AAV8 without capsid mutation, or AAV5.

One method by which the balance between rhodopsin suppression and replacement in mirtron-RHO constructs could be adjusted to be more favourable might be through inclusion of additional mirtrons in the expression cassette. The presence of a single mirtron in the 5'-UTR had no discernible effect on downstream reporter gene expression *in vitro*, whilst the presence of a second in tandem reduced this by around 20% (Figure 5.18). Addition of a third mirtron might therefore both increase rhodopsin knockdown, and further reduce translation of the

replacement gene product, although this would need to be confirmed empirically in future studies.

7.4. Progress in retinal gene therapy

Tremendous progress has been observed in the field of gene therapy for IRDs over the past 20 years or so. The relative rapidity with which this has occurred owes much to the fact that the eye is particularly suited for the development of such novel therapeutics. The eye is relatively compartmentalised, and the subretinal space can be easily accessed for the purpose of gene delivery, using techniques commonly employed by vitreoretinal surgeons who perform subretinal procedures. Further, the eye exhibits a degree of immune privilege, and since it is a paired organ, the fellow untreated eye is able to act as an internal control in clinical trials.

The first pivotal human trials of retinal gene therapy in the United States and the UK involved delivery of AAV2/2 vectors packaging working copies of the *RPE65* gene beneath the retina of patients with Leber congenital amaurosis type 2 (LCA2) caused by biallelic missense, or null mutations in this gene. The RPE65 enzyme is responsible for the conversion of all-trans retinyl esters to 11-cis retinol within the RPE as part of the retinoid cycle (Figure 1.4). In its absence, rhodopsin cannot be regenerated, and so patients with LCA2 have profound visual impairment that is present from birth. The RPE and photoreceptors also undergo a slow degeneration. Initial experiments, in which an AAV2/2-hRPE65 vector was used to treat Briard dogs homozygous for a naturally occurring mutation in the *Rpe65* gene, were highly encouraging. These studies demonstrated robust rescue of the ERG, accompanied by improved pupillary responses to light, reduced nystagmus and convincing restoration of visually-guided behaviour in these animals as a result of treatment^{276,341}. Results of the first-in-man phase I/II retinal gene therapy trials in patients with *RPE65*-related LCA2 were published by three independent research groups in 2008^{129–131}. One study demonstrated an increase in the pupil light reflex

following treatment¹³⁰, whilst another reported a shift of the preferred retinal locus of fixation from the fovea to a treated area of retina, suggesting improvements in retinal light sensitivity as a result of gene therapy³⁴². Longer term data from these initial phase I studies have revealed an excellent safety profile for subretinal RPE65 AAV gene therapy, but efficacy results have been variable³⁴³. Initial visual gains reported in one trial appeared not to be sustained one to three years later, with ongoing retinal degeneration being observed in treated eyes³⁴⁴. The long-term data from another trial in which a cell-specific promoter was used, similarly showed declines in previously reported visual gains, which may have occurred as a result of declining transgene expression due to promoter silencing^{343,345}.

One concern surrounding gene replacement therapy in patients with biallelic null mutations was the possibility that an immune reaction to the transgenic protein may be mounted as a result of lack of prior immunological tolerance. These fears have, however, not been realised in patients who have received retinal gene therapy to date. Moreover, safety of second eye treatment with rAAV-hRPE65 has recently been demonstrated³⁴⁶.

In 2017, data from an industry-sponsored phase III open-label randomised-controlled trial of AAV2/2-hRPE65 for LCA2 was published¹³². In this landmark study, 31 patients were randomised to receive the gene therapy product, known as voretigene neparvovec, or allocated to an untreated control group. The authors reported significant improvements at one year in multi-luminance mobility testing in AAV-treated, but not in control patients, thus meeting the trial's primary endpoint criterion. On the back of this study, the FDA granted regulatory approval for voretigene neparvovec, which is now marketed in the USA under the trade name Luxturna by Spark Therapeutics, and is the first and only gene therapy treatment currently available to patients for any IRD.

RPE65 gene replacement therapy is directed towards correction of an inborn error of metabolism, and aims to restore normal retinal physiology in order to improve overall visual function. In contrast to LCA2, photopic visual function is frequently normal in the early stages of many forms of RP, including those attributable to dominant mutations in the rhodopsin gene. Although nyctalopia is a cardinal symptom of RP, much of the associated visual morbidity- namely visual field constriction and reduced visual acuity- is attributable to the secondary degeneration of cones. Although improvements in low luminance visual function might be expected, the primary aim of gene therapy for RP should thus be to slow or halt the degenerative process, in order to preserve useful photopic vision in patients. Several clinical trials are currently underway for the treatment of other degenerative IRDs including choroideremia and X-linked RP.

Choroideremia is an X-linked degeneration of the RPE and retina caused by mutations in the *CHM* gene. This encodes a protein known as Rab escort protein 1 (REP1), which is integral for the normal trafficking of vesicles within the retina and RPE³⁴⁷. Deficiency in REP1 results in the slow degeneration of RPE and photoreceptors. As for RP, this progresses in a centripetal fashion, and manifests in patients as a gradual constriction of the visual field. Long term data from the first 14 patients to be treated with a recombinant REP1-expressing AAV vector as part of a phase I/II clinical trial based in Oxford have recently been published, showing an excellent safety profile with suggestion of improved visual function³⁴⁸. Two years following subretinal gene therapy in this trial, visual acuity improved in treated eyes by a median of 4.5 Early Treatment Diabetic Retinopathy (ETDRS) chart letters, compared with a 1.5 letter loss in untreated fellow eyes. In one patient within this trial, a change in the preferred retinal locus of fixation from an untreated to a treated area of retina was reported two years after subretinal gene therapy¹⁸², an effect that was sustained in this individual three years later³⁴⁸. This shift in fixation is highly suggestive of an increase in sensitivity of the treated area of retina as a result

of the gene therapy. Whilst these results are encouraging, the question as to whether gene therapy can maintain the island of central vision in centripetal retinal degenerations such as choroideremia and X-linked RP remains to be answered, and is of great relevance for any future gene therapy for rhodopsin-related ADRP.

7.5. Future directions for the clinical translation of gene therapy for RHO-related ADRP

A number of specific challenges exist for the successful translation of rhodopsin gene therapy from the laboratory to patients with ADRP. Gene therapy clinical trials currently underway for IRDs are all directed towards the treatment of recessively inherited conditions resulting from loss of function or null mutations. Although variation exists in the natural history of disease progression for patients with recessive IRDs, they may nonetheless be generally considered, at least mechanistically, to form homogenous populations in which potential therapeutics may be trialled. By contrast, a large variety of missense mutations scattered throughout the gene are causative for dominantly-inherited rhodopsin-related RP⁸⁹. Each mutant form of the protein has the potential to cause rod cell death by a unique mechanism, or by a combination of such mechanisms, which have been classified based upon their biochemical characteristics^{72,89}. The clinical manifestations and disease course within an individual are as a result largely dependent upon the particular mutation present⁹⁴, making the population of patients with RHO-related ADRP as a whole highly heterogeneous^{245,349}. Consequently, a relatively large number of patients may need to be enrolled to provide adequate statistical power to any future interventional trial. Large scale natural history studies are made difficult by the fact that disease progression is slow for many such patients. The largest longitudinal observational study published to date followed 140 patients with rhodopsin-related ADRP for a mean of 8.7 years, and reported mean annual exponential rates of decline of 1.8% for visual acuity, 2.6% for visual field area and 8.7% for

ERG amplitude⁹⁴. Data from such natural history studies would be helpful for the planning of future therapeutic trials, in order to further inform power calculations, and guide appropriate timing of the intervention.

Given the variety of mechanisms thought to mediate photoreceptor degeneration in rhodopsin-related ADRP, it is likely that patients carrying certain mutations may respond more favourably to an RNA-replacement based gene therapy than others. For instance, in cases where toxicity is induced by accumulation of mutant protein, such as for Class I mutants (e.g. RHO^{P347S}) which appear to mislocalize to the IS, or for Class II mutants (e.g. RHO^{P231H}) where abnormal rhodopsin may be sequestered in the ER, intervention may be required at an early stage of disease, i.e. before abnormal protein has accumulated to toxic levels. There may therefore be a limited window of opportunity for certain patients, outside of which treatment, by gene therapy or otherwise, may be futile.

A further challenge in clinical trial design for therapeutics in rhodopsin-related ADRP, as for other retinal gene therapies, is the requirement for validated outcome measures that may be acceptable to regulatory authorities such as the US Food and Drug Administration (FDA). The only such metric relevant to ophthalmology for which the FDA has expressly stated a threshold it considers “visually significant”, is visual acuity, where this is defined as a gain of 15 letters or more on the ETDRS chart³⁴³. In ADRP, as for other retinal degenerations that progress in a centripetal fashion, visual acuity is likely to be a poor measure of therapeutic success, being largely preserved until late-stage disease when it may deteriorate more rapidly. Following successful RNA replacement gene therapy in rhodopsin-related ADRP, an early improvement in the symptom of nyctalopia might be expected as a result of a greater amount of functionally normal rhodopsin reaching rod outer segments. More clinically relevant outcome measures might therefore include those that reflect rod-mediated visual function, such as low-luminance

best corrected visual acuity and low-luminance contrast sensitivity³⁵⁰, or those which quantify rhodopsin kinetics such as dark adaptometry^{351,352}. Regional differences in function between treated and untreated areas might be detectable soon after retinal gene therapy using multifocal dark-adapted ERG³⁵³ or scotopic microperimetry³⁵⁴. Over a longer time period, it may also be possible to detect a structural rescue effect in treated compared with untreated fellow eyes, using validated SD-OCT metrics such as en face area of the surviving EZ^{355,356}, or through longitudinal measurement of the characteristic hyperautofluorescent ring seen in patients with RP on cSLO fundus autofluorescence imaging^{357,358}. In a recent retrospective study of patients with rhodopsin-related ADRP, EZ line width was found to decrease at a rate of $152 \pm 37 \mu\text{m}$ per year, whilst hyperautofluorescent ring area diminished by $0.6 \pm 0.2 \text{ mm}^2$ per year³⁵⁹. Effects of gene therapy on secondary cone loss in treated versus paired untreated eyes could additionally be quantified using adaptive optics biomicroscopy³⁶⁰, static visual field testing⁹⁴ or photopic microperimetry^{357,361}. It may however take many years to detect a significant difference between eyes given the slow expected decline in these measures in many patients⁹⁴. Although mean full field ERG signal appears to deteriorate at a relatively rapid rate in rhodopsin-related ADRP⁹⁴, it is unlikely that gene therapy would have a significant impact on this metric given the relatively small area of central retina that would be treated in early clinical trials. More novel measures of visual function such as object recognition and maze navigation have been included by the developers of the Argus II retinal implant and Luxturna RPE65 gene therapy as part of their successful submissions for regulatory approval^{132,343,362}. Performance of visually-guided tasks under scotopic light conditions might thus similarly represent an acceptable measure by which functional efficacy of gene therapy for ADRP may be assessed in any future clinical trial.

It must be acknowledged that subretinal gene therapy for rhodopsin-related RP may not be appropriate in all cases. Even if a highly effective therapeutic were to gain market approval, observation may be warranted as the preferred course of action, particularly for those patients

with very slowly progressive disease. Although recent refinements in gene therapy surgery technique have greatly improved its safety profile³⁶³, the procedure may nonetheless be associated with a number of potentially sight-threatening intra- and post-operative complications including macular hole formation³⁶⁴, retinal tear³⁴³ and bacterial endophthalmitis³⁴⁶. These risks must be carefully weighed against potential benefits on a case by case basis prior to any subretinal treatment.

7.6. Conclusions

Collectively, the data presented in this thesis would suggest that ADRP caused by mutations in the rhodopsin gene might indeed be amenable to treatment. Long-pass light filters which reduce retinal exposure to light of shorter wavelengths were associated with a significant treatment effect in the *Rho*^{P23H/+} knock-in mouse model, where a slowing in the rate of both primary rod and secondary cone degeneration was observed. Ocular filters in the form of tinted spectacles or contact lenses might therefore represent a low-risk and low-cost intervention, that could be protective for those patients with rhodopsin mutations presenting with the sectoral phenotype. Questions however remain with regards to the optimal timing of the intervention, applicability to other mutations, and the potential effects on short- and long-term colour perception and sleep that would need to be addressed prior to any future interventional trial.

A novel mirtron-based method for the treatment of dominant disease has been presented, and this work represents the first published use of an artificial mirtron to affect gene knockdown *in vivo*. Although evidence for a small rescue effect of a mirtron-based knockdown/replacement vector for rhodopsin-related ADRP has been presented, further research is required to optimise the construct prior to translation to clinical trial.

Two patents have been filed on behalf of the University of Oxford by Oxford University Innovations Ltd. to protect the intellectual property associated with the data presented in this thesis. These pertain to the two key areas of innovation presented that may be of commercial interest, namely the use of artificial mirtrons located within the 5'-UTR of a transgene for gene therapy application, and the use of artificial mirtrons as part of a knockdown/replacement AAV vector for the treatment of ADRP caused by rhodopsin mutations. These applications may help in attracting the further funding that will be required for the ongoing development of mirtron-based gene therapies, as well as for a potential future phase I/II clinical trial in patients.

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Appendix

List of Primers

Rpe65 Genotyping

1. Rpe65 FW: 5'-ACCAGAAATTTGGAGGGAAAC
2. Rpe65 RC: 5'-CCCTTCCATTCAGAGCTTCA

RHO Plasmid Cloning

3. KpnI RHO_p FW: 5'-AGGTACCCAGATCTTCCCCAC
4. RHO HindIII RC: 5'-ATAAGCTTTTAGGCCGGGGCCA
5. CAG RHO_p RC: 5'-AGCGACTCCGGATGACTCTGGGTTCCTGAC
6. RHO_p CAG FW: 5'-GAGTCATCCGGAGTCGCTGCGCGCTGCCT
7. Kozak CAG RC: 5'-ATGGTGGCGCTGCAGGAATTCGATATCA
8. CAG Kozak FW: 5'-CCTGCAGCGCCACCATGAATGGCACAGA

qPCR Primers for RHO AAV Titration

9. pA FW: 5'-CCAGCCATCTGTTGTTTGCC
10. pA RC: 5'-GAAAGGACAGTGGGAGTGGC

qPCR Primers for Transgene Expression in Dissociated Retina

11. qRHO FW: 5'-CCTCTCTGCATGGATACTTCGTC
12. qRHO RC: 5'-ATGGGCTTACACACCACCAC

Mirtron Cloning

13. Mirt FW: 5'-CACAAATTTTCTGTCAGC

14. XhoI RHO FW: 5'- ATCTCGAGATGAATGGCACAGA
15. RHO NotI RC: 5'- ATGCGGCCGCTTAGGCCGGGGCCA
16. AsiSI mRho FW: 5'-ATGCGATCGCATGAACGGCACAGA
17. mRho NotI RC: 5'-ATGCGGCCGCTTAGGCTGGAGCCA
18. PC Seq FW: 5'- TCGAGTCCGACCCTGGGTCT

Mirtron Splice Assay

19. Mirt Splice FW: 5'-GACGGGAACTACAAGACCCG
20. Mirt Splice RC: 5'-CTGCACGGATCCATCCTCAA

Short M3 Target PsiCHECK2 Vector Cloning

21. Xho1 M3 FW: 5'-ATCTCGAGACATGTTTCTGCTGATCGTGC
22. NotI M3 RC: 5'-ATGCGGCCGCGTCGACCTTGTGCTGGAC

RHO Fusion Plasmid Cloning

23. Nhe1 RHO FW: 5'-AGGCTAGCCACCATGAATGGCACAGAAG
24. APVAT RHO RC: 5'-TGTAGCCACAGGAGCGGCCGGGGCCAC
25. APVAT Luc FW: 5'-GCTCCTGTGGCTACAATGGCTTCCAAGGTG
26. AsiS1 Luc RC: 5'-GAGCGATCGCCTAGAATTACTGCTCG
27. XbaI CAG FW: 5'- GGCGGCTCTAGAGCCTCTGCTAA
28. APVAT GFP FW: 5'-GCTCCTGTGGCTACAATGAGCAAGGGCG
29. HindIII GFP RC: 5'-GATAAGCTTTCACCTGTACAGCTCGTCC

5'-UTR Mirtron Plasmid Cloning

30. UTR-Mirt FW: 5'- TGACGGGAACTACAAGACCC

31. UTR-Mirt RC: 5'- ATCCTCCTTAAAGTCAATGCCC

5'-UTR Mirtron Splice Assay

32. UTR Splice FW: 5'-GCTGGTTATTGTGCTGTCTC

33. UTR Splice RC: 5'-GGGCTGCAGGAATTTCGAT

Codon Modified RHO Cloning

34. M3CO-RHO FW: 5'- ATTTCCAATTAATTTTCTGACGCTCTACGTCACCG

35. M3CO-RHO RC: 5' CAGAAAATTAATTGGAAATCCCAGCACGATCAGCAG

36. M5CO-RHO FW: 5'- TGAATCCTTCGTGATTTATATGTTTCGTGGTCCAC

37. M5CO-RHO RC: 5'- ATAAATCACGAAGGATTCATTGTTGACCTCCGGC

Mirtron AAV Plasmid Cloning

38. EcoRI RHO FW: 5'-TCGAATTCCGCCACCATGAATGGCAC

In Vivo Splice Assay

39. RHO Splice RC: 5'- CCAGCACGATCAGCAGAAAC