



Developing gene therapy for inherited retinal degenerations

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

This thesis is entirely my own work except where otherwise stated. All figures are original; where adapted from another source, this has been appropriately referenced. The development of a genotyping assay for the *Cdhr1*^{-/-} mouse was undertaken in collaboration with John Hunyara (PhD student), and c-terminal antibodies to Cdhr1 were gifted by Dr. Amir Rattner, both of Johns Hopkins, USA. Electron microscopy sectioning and imaging was undertaken by Dr Thomas Burgoyne, UCL. At the time of submission, data from Chapter 3 had been published in *Experimental Eye Research* (2021) and a major review on retinal cadherinopathies published in *Progress in Retinal Eye Research*. No other data have been submitted elsewhere. Plasmids were either acquired from lab members or acquired commercially, with sources stated at the time of first mention. All DNA sequencing was subcontracted to Source Bioscience, UK or Eurofins, Germany.

The word count for this thesis is approximately 48,832 (excluding table of contents, lists of figures and tables, figure legends and references). No material presented in this thesis has been previously submitted to the University of Oxford, or to any other academic institution, for another degree or qualification.



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Robert is still, to this day, awaiting confirmation of segregation.

Abstract

Developing gene therapy for inherited retinal degenerations

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The aim of this thesis was to evaluate the efficacy and safety of retinal gene therapy in a pre-clinical model of *CDHR1*-associated retinal degeneration – a hitherto untreatable, blinding disorder.

Deep phenotyping of the *Cdhr1*^{-/-} murine model demonstrated severe early deficits in cone and rod photoreceptor function followed by progressive photoreceptor cell death – recapitulating *CDHR1* cone-rod dystrophy. Two AAV8 vectors expressing the full-length human *CDHR1* coding sequence were designed, manufactured, validated and titrated *in vivo*.

Sub-retinal injection of AAV8.GRK1.CDHR1.pA (1.5×10^8 vg) in *Cdhr1*^{-/-} mice at 3-4 weeks of age resulted in functional rescue of cone and rod photoreceptors with improved response amplitudes and decreased implicit times to 12-months post-injection; a slowing of photoreceptor cell death with regeneration of full-length photoreceptor outer segments confirmed on ultrastructural analysis to 21-months post-injection; and a behavioural rescue effect on photopic and scotopic optomotor testing, sustained to 21-months post-injection. AAV8.GRK1.CDHR1.pA at $1.5 \times$

10⁸ vg appeared safe in *C57BL/6J* mice by the same outcome measures to 22-months post-injection.

Genetic prevalence estimates presented herein suggest more than 200,000 affected individuals worldwide. We characterised 146 individuals with biallelic variants in *CDHR1*, describing the retinal phenotype, natural history, genotype-phenotype associations, with 25 novel pathological sequence variants.

This is the first therapy shown to improve retinal structure and function in a pre-clinical model of *CDHR1*-associated retinal degeneration. A patent to protect the vectors described herein has been filed on behalf of the University of Oxford, with commercial agreements obtained in preparation for an onward phase 1 clinical trial.

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List of Abbreviations

A	Adenine
aap	Assembly-activating protein
AAV	Adeno-associated virus
ABCA4	ATP Binding Cassette Subfamily A Member 4
ABE	Adenine Base Editor
ACMG	American College of Medical Genetics
AD	Autosomal dominant
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
AR	Autosomal recessive
ART	Automatic real-time
ARVO	Association for Research and Vision in Ophthalmology
BAF	Blue autofluorescence (short wavelength)
bp	Base pairs
BM	Bruch's membrane
BSA	Bovine serum albumin
C	Cytosine
CAG	<u>C</u> MV early enhancer element, chicken beta- <u>a</u> ctin & the splice acceptor of the rabbit beta- <u>g</u> lobin, synthetic promoter
Cap	Capsid protein
CBA	Chicken beta-actin
CC	Connecting cilium
CDHR1	Cadherin-related family member 1
cDNA	Complementary DNA
CDS	Coding sequence
CEP290	Centrosomal Protein – 290kDa
CI	Confidence Interval

CMO	Cystoid macular oedema
CMV	Cytomegalovirus
CRD	Cone-rod dystrophy
CRISPR	Clustered regularly-interspersed short palindromic repeats
<i>Crx</i>	Cone-rod homeobox gene
cSLO	Confocal scanning laser ophthalmoscopy
CSNB	Congenital Stationary Night Blindness
DA	Dark-adapted
DAPI	4',6-diamidino-2-phenylindole
dB	Decibels
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDI	Enhanced depth imaging
EDTA	Ethylenediaminetetraacetic acid
ELM	External limiting membrane
EMA	European Medicines Agency
EORD	Early-onset retinal dystrophy
ERG	Electroretinography
ERM	Epiretinal membrane
ES	Embryonic stem cell
ESCS	Enhanced S-cone syndrome
ETDRS	Early Treatment Diabetic Retinopathy Study
Ex	Exon
ExAC	Exome Aggregation Consortium
EZ	Ellipsoid zone (inner segment-outer segment junction)
FAF	Fundus autofluorescence
FBS	Fetal bovine serum
FDA	Food and Drug Administration (U.S.)
g	Gravitational force

G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gc	genome copies
GCL	Ganglion cell layer
GFP	Green fluorescent protein
GMP	Good Manufacturing Practices
GnomAD	The Genome Aggregation Database
GPCR	G-protein-coupled receptor
GRK1	Human Rhodopsin Kinase Promoter
HEK293	Human Embryonic Kidney 293
HR	High-resolution
Hz	Hertz
ICGA	Indocyanine green angiography
IHC	Immunohistochemistry
ILM	Internal limiting membrane
IMP	Investigational Medical Product
In	Intron
INL	Inner nuclear layer
IPL	Inner plexiform layer
iPSC	Induced pluripotent stem cells
IRD	Inherited retinal disease
IS	Inner segment
ITR	Inverted terminal repeat
IVC	Individually ventilated cage
kb	Kilobase
kDA	Kilodaltons
LA	Light-adapted
LB	Luria Broth
LCA	Leber Congenial Amaurosis

LED	Light-emitting diode
logMAR	Logarithm of the minimal angle of resolution
LoVD	Leiden Open Variation Database
MHRA	Medicines and Health Regulatory Agency (U.K.)
MOI	Multiplicity of Infection
mRNA	Messenger ribonucleic acid
MTA	Material Transfer Agreement
MZ	Myoid zone
NDS	Normal donkey serum
NGS	Normql goat serum
NICE	National Institute of Clinical Excellence (U.K.)
NIR-AF	Near infra-red autofluorescence
NCT	Non-template control
<i>nrl</i>	Neural retina-specific leucine zipper
ns	Not significant
OCT	Optical coherence tomography
OCT-C	Optimal cutting temperature compound
OD	Oculus dexter
OKN	Optokinetic nystagmus
OMR	Optomotor reflex
ONL	Outer nuclear layer
OPL	Outer plexiform layer
ORF	Open reading frame
OS	Oculus sinister
OS	Outer segment
P	Probability
pA	Polyadenylation tail
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline – Tween 20

PCR	Polymerase chain reaction
PCT	Positive control
PDE	Phosphodiesterase
PEI	Polyethylenimine
PF-68	Pluronic™ F-68 Surfactant
PFA	Paraformaldehyde
PI	Protease inhibitor
PIL	Personal licence
PNA	Peanut agglutinin
PPL	Project licence
PRL	Photoreceptor layer
PVDF	Polyvinylidene difluoride
RCS	Royal College of Surgeons
Rep	Replication
RGCs	Retinal ganglion cells
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RPGR	Retinitis pigmentosa GTPase regulator
rpm	revolutions per minute
rSAP	Shrimp alkaline phosphatase
scAAV	Self-complementary AAV
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SLO	Scanning laser ophthalmoscopy
SNP	Single nucleotide polymorphism
SOC	Super Optimal broth with Catabolite repression

SW-AF	Short-wavelength autofluorescence
T	Thymine
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TEM	Transmission electron microscopy
T_m	Melting temperature
U	Uracil
USH	Usher syndrome
UTR	Untranslated region
V	Volts
vg	Vector genomes
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WHO	World Health Organization
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
WT	Wildtype
XL	X-linked
XLRS	X-linked retinoschisis

1. General Introduction

1.1 The Retina

1.1.1 Structure

The human retina forms the neurosensory inner lining of the eye, derived from the optic cup of the developing embryonic diencephalon (Figure 1.1). The retina forms a trilaminar structure (Figure 1.2) with the outer retina composed of rod and cone photoreceptors which synapse with ON-bipolar cells, and thereafter to retinal ganglion cells which relay signals to the primary visual cortex.

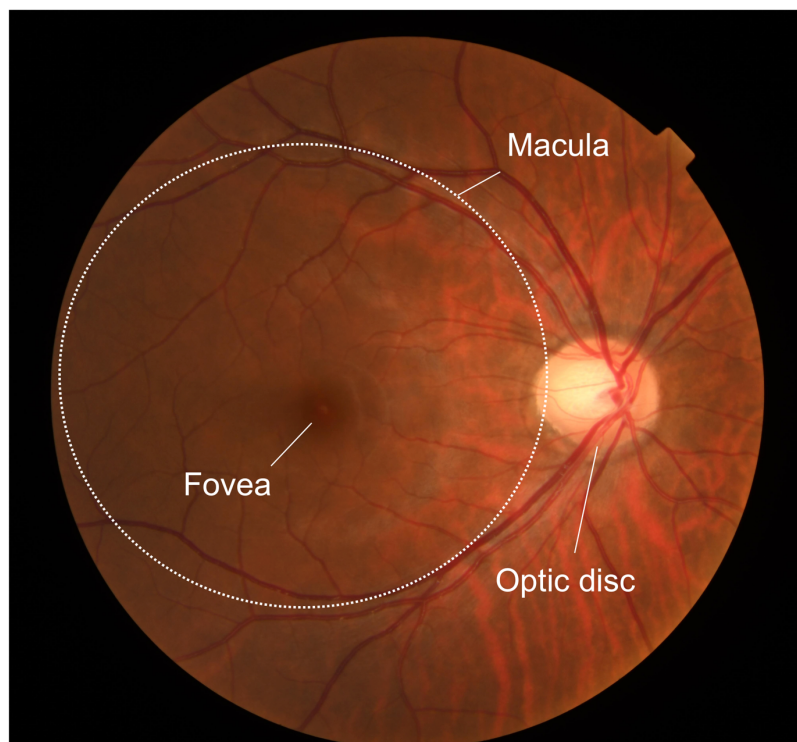


Figure 1.1 Colour fundus photograph of the human retina

A colour fundus photograph of the right eye of the author is presented, captured by Lee Davidson at the Oxford Eye Hospital. The macula is not precisely defined, although is generally considered to exist within the major vascular arcades. The fovea appears as a darker spot due to macular pigment with a Purkinje light reflex at its centre. The optic disc is formed from the axons of ~1.2 million retinal ganglion cells. A reflex from the internal limiting membrane is apparent.

The human retina contains approximately 110-130 million rods and 5-7 million cones.¹ Rod photoreceptors express rhodopsin which is able to detect a single photon of light for vision under scotopic conditions. Cone photoreceptors relay colour vision through three different opsins (S-, M- and L-opsins) in primates. The central 200µm is exclusively populated by cones where the inner retinal layers are displaced laterally for high spectral, temporal and spatial resolution.

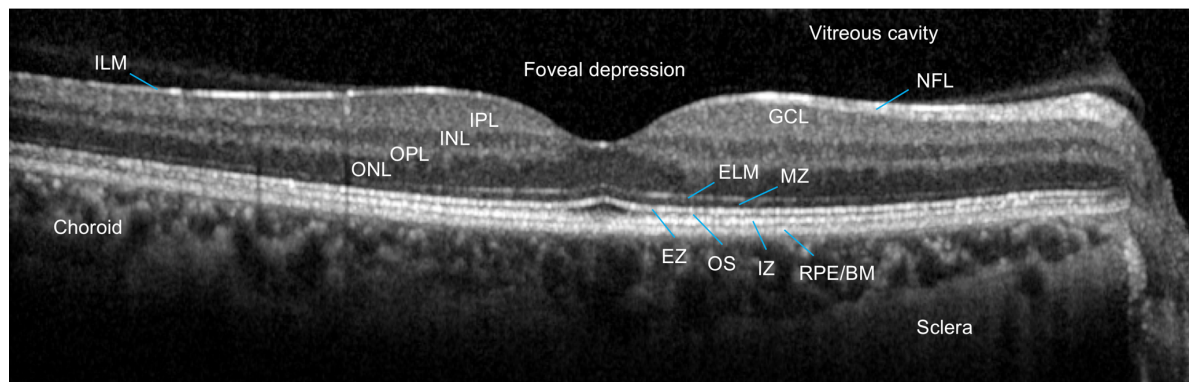


Figure 1.2 Optical coherence tomography imaging of the human retina Heidelberg Spectralis OCT imaging of the right eye of the author is shown, captured by Amanda Harbour at the Oxford Eye Hospital. RPE/BM – retinal pigment epithelium/Bruch’s membrane complex; IZ – interdigitation zone; OS – outer segments; EZ – ellipsoid zone; MZ – myoid zone; ELM – external limiting membrane; ONL – outer nuclear layer; OPL – outer plexiform layer; INL – inner nuclear layer; IPL – inner plexiform layer; GCL – ganglion cell layer; NFL – nerve fibre layer.

The retina is supported by the retinal pigment epithelium (RPE) - a monolayer of polarised cuboidal cells which serve a number of functions critical for photoreceptor function and survival.² The RPE phagocytoses shed outer segment membrane discs, forms the outer blood retinal barrier and regenerates 11-*cis*-retinal as part of the canonical visual cycle. Bruch’s membrane is an acellular basement membrane underlying the RPE.

This laminar retinal structure is preserved throughout the mammalian kingdom, such as the house mouse, *Mus musculus*. However, key differences exist between species. As nocturnal animals, 98% of photoreceptors in the murine retina are rods (versus ~94% in primates).³ Moreover, mice do not possess an anatomical macula. Macroscopically, the murine retina is generally radially symmetrical around the optic disc. However, S-cones (10% of all cones) clustered in the ventral (lower) retina, enable the perception of the blue spectrum in the upper visual field – a probable evolutionary adaptation to evade predators. M/L-cones concentrated in the dorsal (upper) retina detect the green spectrum in the lower visual field.^{4, 5}

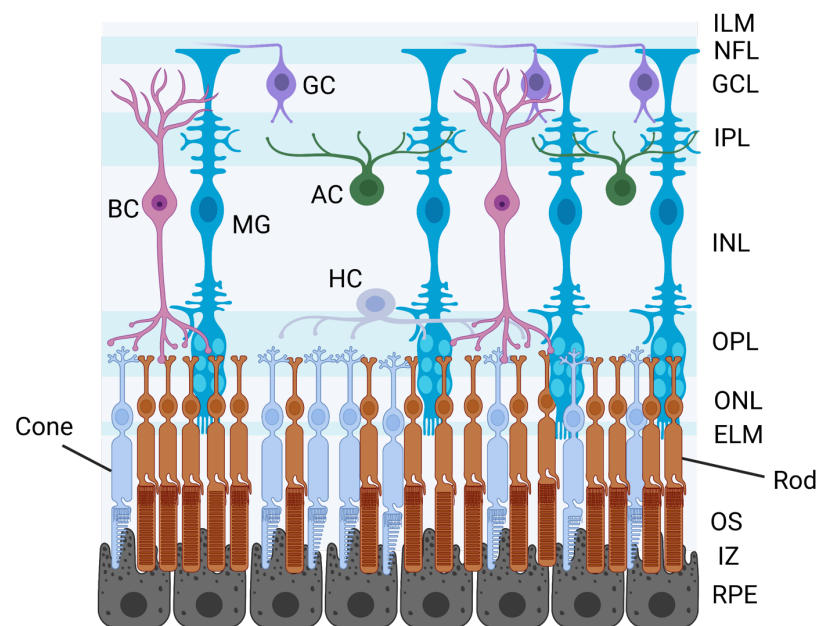


Figure 1.3 Schematic representation of the cellular architecture of the mammalian retina

Seven major cell types populate the mammalian retina. Rod and cone photoreceptors relay electrical signals to ON-bipolar cells (BC) and subsequently to retinal ganglion cells, both of which are involved in initial processing of visual stimuli. Müller-glia (MG) cells have a supporting role; their footplates form the ELM and ILM. Horizontal (HC) and amacrine cells (AC) are involved in lateral processing at the level of bipolar and retinal ganglion cells (GC), respectively. Figure drawn using BioRender.

1.1.2 Function

Photons of light, focussed onto the outer segments by the cornea and lens anteriorly, initiate phototransduction in rod and cone photoreceptors and intrinsically photosensitive retinal ganglion cells (ipRGCs) through their respective opsins - rhodopsin in rods; S- M- and L-opsins in primate cones; and melanopsin in ipRGCs. In photoreceptors, a single photon of light may trigger the isomerization of 11-*cis*-retinal into all-*trans* retinyl esters, with conformational change in the relevant opsin.⁶⁻⁸ This is followed by amplification through the phototransduction cascade with photoreceptor membrane hyperpolarisation through cGMP-gated ion channels, and subsequent depolarisation of the ON-bipolar cells.⁹ The retinal ganglion cells synapse in the lateral geniculate nucleus of the thalamus, and thereafter to the primary visual cortex where images are perceived. Visual reflexes (e.g. pupil responses, vestibular-ocular reflexes) are mediated through synapses in the brainstem. Horizontal cells and amacrine cells (4-20, depending on species) are interneurons that form powerful parallel processing circuits within the retina involved in visual processing, lateral inhibition, and inhibitory surrounds.

The retina is able to perceive light over more than eight log units (base 10). Electrical responses to light stimulation may be measured objectively at the corneal surface by Ganzfeld, full-field electroretinography (Figure 1.4). Rod responses may be isolated following dark-adaptation with low intensity flashes

(e.g. 0.01 cd.s/m²). Cone responses may be isolated by photobleaching of rod photoreceptors through light-adaptation, high light-intensities and high-frequency repetition beyond the temporal resolution of rods.¹⁰

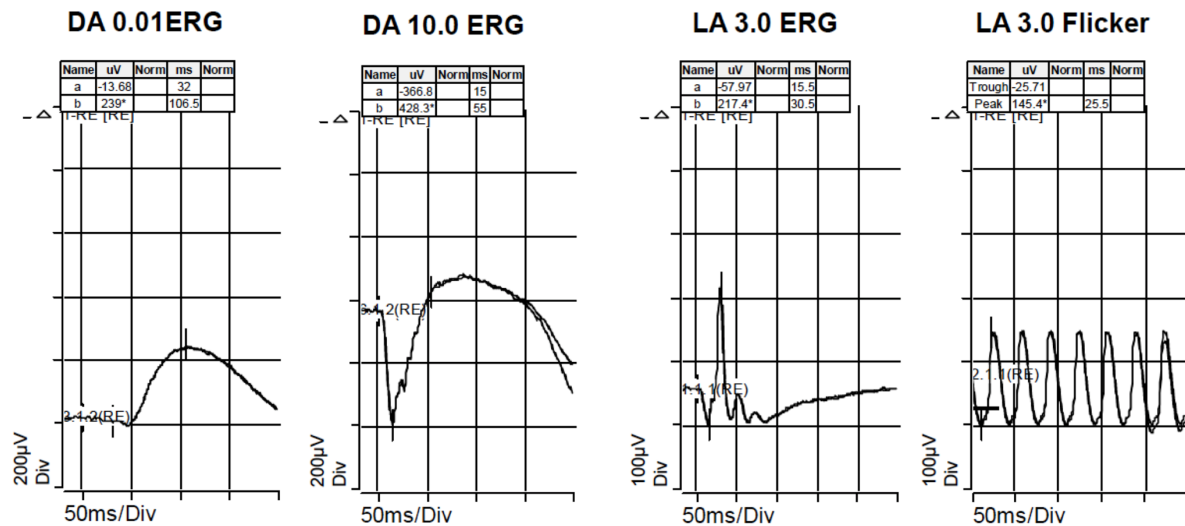


Figure 1.4 Full-field electroretinography in healthy human subject. Dark-adapted (DA) traces illustrate rod-isolating B-wave responses (at 0.01 cd.s/m²), and maximal (i.e. combined rod-cone) responses at 10 cd.s/m², with a demonstrable A-wave. Following light-adaptation (LA), single-flash cone responses are demonstrated, and further isolated using a 30Hz flicker test at 3 cd.s/m² above background (30 cd.s/m²). ERG testing is performed in accordance with International Society for the Clinical Electrophysiology of Vision (ISCEV).

1.2 Inherited Retinal Degeneration

Inherited retinal degeneration (IRD), is the leading cause of visual loss in the working population in the United Kingdom.¹¹ IRDs encompass numerous monogenic disorders in which the primary degeneration affects one or more retinal cell types. At the time of writing, 280 genes have been identified in association with retinal degeneration from 316 identified loci (RetNet, 2021). The inheritance of these genes may be autosomal dominant, recessive, X-linked or mitochondrial.

Alternatively, mutations may occur *de novo* - occurring in a parental germ cell or during early embryogenesis, where approximately 60 new mutations occur per generation.¹² The large number of genes underlying IRDs relates to the highly-specialised function of the photoreceptor, exclusive gene expression, high-metabolic turnover, and a non-motile cilium which traffics and maintains a huge concentration gradient of proteins between the inner and outer segments.

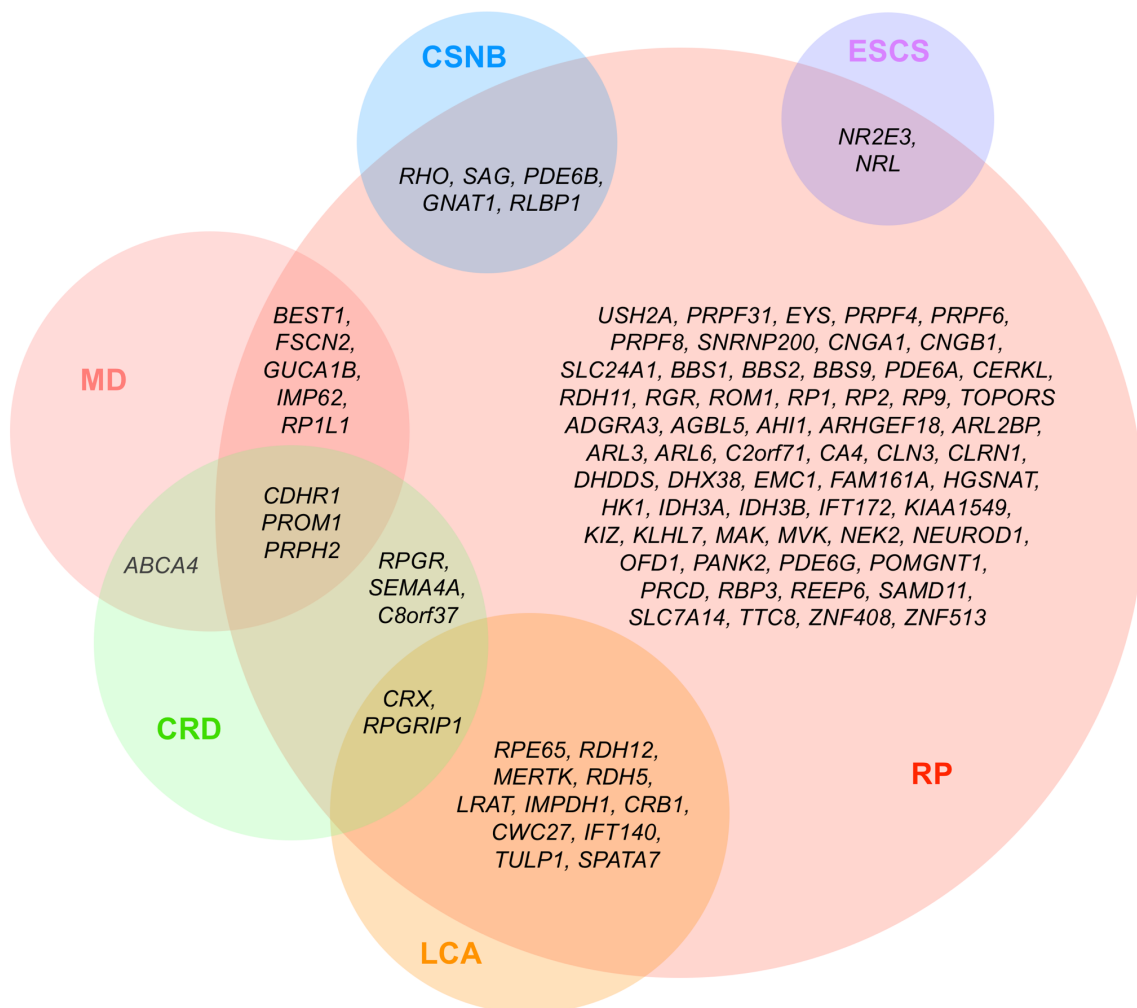


Figure 1.5 Genetic overlap between forms of retinal degeneration

Venn diagram is adapted from Verbakel *et al*, *Prog Ret Eye Res*.¹³ RP – retinitis pigmentosa; CRD – cone or cone-rod dystrophy; MD – macular dystrophy; ESCS – enhanced S-cone syndrome; CSNB – congenital stationary night blindness; LCA – Leber Congenital Amaurosis. Note that *CDHR1* may result in CRD, MD or RP.

The most common form of retinal degeneration is retinitis pigmentosa (RP), which describes primary rod photoreceptor degeneration with concurrent or later degeneration of cones, through a variety of mechanisms.¹⁴ Cone- and cone-rod (CRD) dystrophies describe disorders characterised by primary degeneration of cones with concurrent or later degeneration of rods.^{15, 16} Macular dystrophies (MD) describe predominant degeneration of the macula, with primary degeneration occurring at the level of the photoreceptor or RPE. Severe, early onset degeneration may result in Leber Congenital Amaurosis (LCA), typified classically by severe visual impairment, oculodigital sign and nystagmus. Congenital stationary night blindness (CSNB) and achromatopsia describe usually non-syndromic functional deficits of rods and cones, respectively, without photoreceptor degeneration. The genetic overlap between these forms of retinal degeneration may be explained by the severity of the underlying variants, and/or modifiers which underlie phenotypic variability.

The majority of cases (70-80%) exhibit isolated retinal degeneration, with 20-30% demonstrating an associated systemic/syndromic manifestation.¹³ Rod-cone and cone-rod dystrophies may be a manifestation of generalised ciliopathies (i.e. Bardet-Biedel). Usher syndrome (first described by Charles Usher, 1914) describes a syndrome of RP with deafness. Clinically absent systemic manifestations in syndromic genes may be explained by hypomorphic variants, alternatively spliced isoforms, or other tissue-specific factors.¹⁷

1.2.1 Epidemiology

RP has been described in populations throughout the world with an approximate overall prevalence of ~1 in 4,000,¹⁸⁻²⁰ although reports vary from 1 in 750²¹ to 1 in 9,000.²² Cone dystrophies have an estimated prevalence of approximately ~1 in 40,000 individuals.¹⁶ However, these figures are likely to underestimate the true prevalence of disease since access to ophthalmic services, misclassification of disease and access to genetic testing form barriers to diagnosis. The genetic prevalence of may be estimated more precisely using the Hardy-Weinberg equilibrium if the population frequency of confirmed pathological variants are known,²³ on the assumption that the disease is fully penetrant. A higher prevalence of retinal degeneration may be found in older,²⁴ genetically isolated populations,²⁵ or in geographic locations with founder mutations.²⁶

1.2.2 Clinical presentation

The initial clinical presentation of RP is typified by night blindness and generalised visual field constriction due to dysfunction and degeneration of rod photoreceptors. Late in the disease, central vision becomes affected as cone photoreceptors degenerate. The classical fundus appearance of peripheral pigment migration with retinal thinning, arteriolar attenuation and optic disc pallor may be absent or subtle in early disease. Outer nuclear layer thinning is evident on optical coherence tomography (OCT) imaging, although the most central foveal and perifoveal architecture may be preserved until late. Fundus autofluorescence

imaging often detects mid-peripheral RPE loss, and in some cases, a perifoveal ring at the leading edge of degeneration as rod photoreceptor outer segments are lost whilst inner segments are retained (Figure 1.6). Dark-adapted electroretinography (ERG) may confirm rod photoreceptor degeneration, and visual field testing can be used to document the degree of visual field constriction.

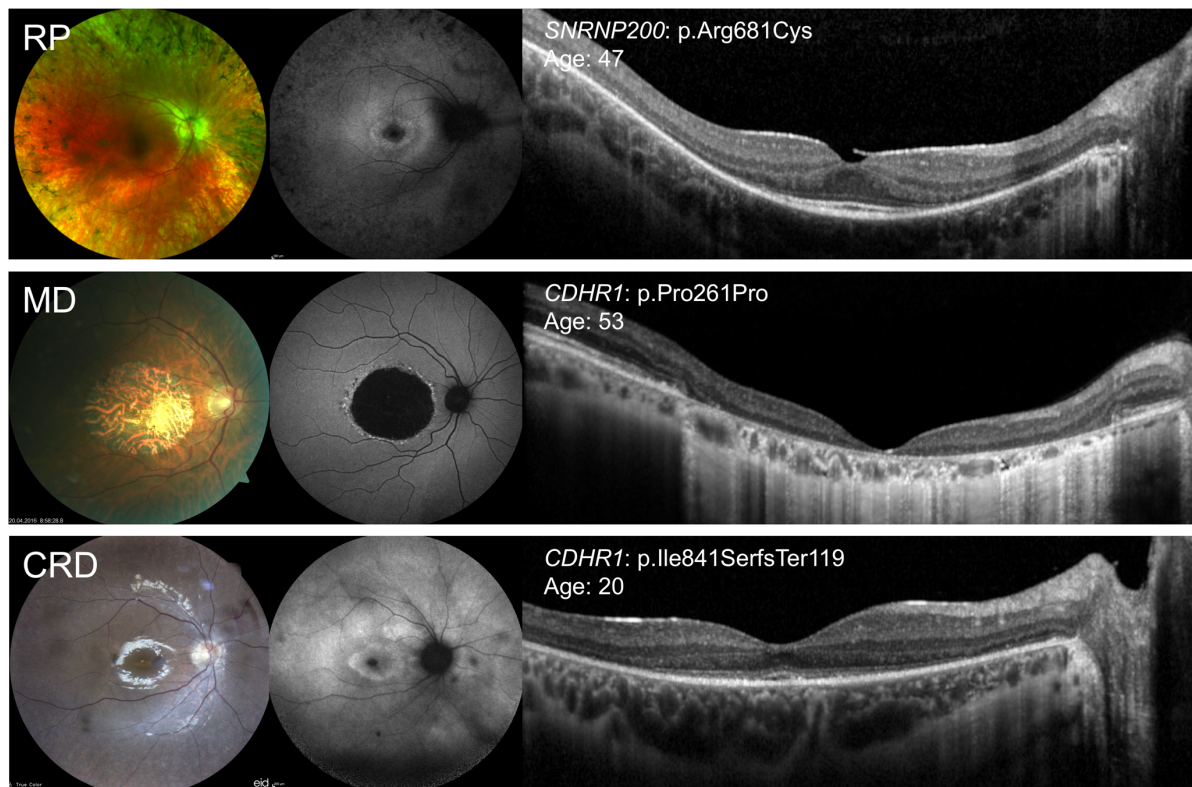


Figure 1.6 Multimodal retinal imaging findings in retinal dystrophies
Colour/pseudocolour, fundus autofluorescence and OCT imaging findings in RP, MD, and CRD. In RP, peripheral pigment migration is evident on pseudo-colour retinal and FAF imaging with arteriolar attenuation. Outer nuclear layer retinal thinning seen on OCT imaging with relative preservation of the fovea. In MD, total RPE loss is evident with choroidal hypertransmission on OCT imaging; more peripheral retina appears normal on OCT and FAF imaging. In CRD, generalised ONL thinning is seen, involving the fovea in early disease with disruption of the sub-foveal ellipsoid zone and external limiting membrane. The *SNRNP200* patient is heterozygous, whereas the *CDHR1* patients are homozygous.

In cone dystrophies, glare, impaired colour vision, reduced acuity or difficulty reading may be the presenting features. In severe disease, features of LCA may be apparent. In cone-rod dystrophies (CRD), symptoms of rod degeneration may be present at presentation or later in the disease course. Macular dystrophies typically present with difficulties in reading and/or reduced visual acuity. Full-field ERG may be used to distinguish CD, CRD and MD; in the latter, classically, a normal ERG may be demonstrable since most of the ~6.4 million cones exist outside of the macula, although pattern ERG and multifocal ERG are usually abnormal.²⁷ However, conversion to RP or CRD may occur in late disease.²⁸

For all forms of primary photoreceptor degeneration, the age at which symptoms develop and rate of retinal degeneration is highly variable between genes, genetic variants, and often within families.

1.2.3 Molecular Genetic Testing & Management

Establishing a genetic diagnosis in IRD has four main advantages: 1) molecular confirmation of a clinical diagnosis; 2) to facilitate genetic counselling regarding the mode of inheritance; 3) inform the natural history of disease; and 4) to determine eligibility for ongoing clinical trials. In modern healthcare systems, a molecular genetic diagnosis is typically established using next-generation sequencing (NGS) that examines the coding sequence and intron-exon boundaries of a defined set of IRD genes, with variants confirmed by Sanger sequencing. This

method may detect a causative variant in 60-80% of cases,²⁹ although this may be lower in sporadic (~40%)³⁰ and/or later-onset cases.³¹

General management strategies for confirmed RP include smoking cessation, avoidance of strong light sources where possible, and management of co-existent cataract, macular oedema and other associated pathologies (i.e. retinal detachment in X-linked retinoschisis). Supportive care through registration of visual impairment, low vision aids/visual rehabilitation and genetic counselling is essential.³² Appropriate referral and management of possible syndromic manifestations is paramount. Patients eligible for ongoing clinical trials for gene or mutation specific therapies should be notified as appropriate. Progress in the field of retinal gene therapy is summarised in section 1.4., although anti-sense oligonucleotides (ASOs) and CRISPR/Cas9 constructs are also in clinical trial for this group. Mutation independent therapies include the delivery of neurotrophic factors shown to be of benefit in animal models, although none have yet demonstrated benefit in RP.³³ The evidence-base for vitamin A and/or omega-3 fatty acids supplementation is poor.³⁴ Experimental approaches for end-stage retinal degeneration include electronic retinal implants,³⁵⁻⁴⁰ optogenetics^{41, 42} and stem cell therapies.⁴³⁻⁴⁵

1.3 *CDHR1*-associated retinal degeneration

Cadherins form a superfamily of cell adhesion molecules ubiquitously expressed throughout the animal kingdom. Their classical role is in calcium-dependent cell-to-cell adhesion. Three non-classical cadherins have been identified in primate photoreceptors which form highly specialised intercellular adhesions around the outer segments, critical for photoreceptor function: *CDHR1*, *CDH23* and *PCDH15*; the latter two genes underlie subtypes of Usher syndrome type 1. *CDH3* is a classical cadherin expressed in the RPE that underlies hypotrichosis with juvenile macular dystrophy. Thus, four monogenic retinal cadherinopathies have been identified.

CDHR1 was originally identified as a non-classical cadherin in the cDNA of the olfactory bulb of rats; designated KIAA1775 in the original study.⁴⁶ In the same year, *CDHR1* was identified in a bovine retinal cDNA library (designated “photoreceptor-specific cadherin”), and localised to the base of the outer segment discs of rods and cones.⁴⁷ Targeted disruption of the murine homologue, *Cdhr1*, resulted in progressive photoreceptor cell death, dysmorphic outer segments and reduced rod and cone function on electroretinography. These observations identified a highly specialised role for *CDHR1* in outer segment disc morphogenesis and as a candidate gene for human retinal degeneration.⁴⁸

1.3.1 Discovery of *CDHR1* as a cause of human retinal degeneration

CDHR1 was identified as a cause of retinal degeneration in humans in 2010 in two near co-incident reports (originally designated as *PCDH21*).^{49, 50} Autozygosity mapping was used identify *CDHR1* as a cause of cone-rod or rod-cone dystrophy in three families from the Faroe Islands, South Asia and the Middle East.^{49, 50} In both studies, observations made in the *Cdhr1*^{-/-} mouse supported *CDHR1* as the causative gene in pedigrees with evidence of both rod and cone dysfunction. Over the ensuing 10 years, further clinical and genetic characterisation of 27 families with *CDHR1*-associated retinal degeneration were published; as summarised in Table 1.1. Across these reports, cone-rod dystrophy appeared to be the predominant phenotype, although classic RP and MD were also described.

1.3.2 *CDHR1*: function and structure

Observations in the *Cdhr1*^{-/-} mouse identified that CDHR1 is essential for outer segment disc morphogenesis and photoreceptor survival.⁴⁷ Biochemical analysis revealed the post-translational modification of CDHR1 through a single cleavage event, releasing the ectodomain from the cytosolic domain which remains associated with the outer segment.⁵¹ Later experiments using immunoelectron microscopy identified that nascent, evaginating OS discs form cadherin-based contacts with the inner segment;⁵² contacts that are presumably severed through proteolysis. Targeted disruption of *Cdhr1* and *Prom1* in *X. laevis* identified that *Cdhr1* is not necessary for horizontal disc elongation, although appears essential

for the higher order organisation of the outer segment.⁵³ Although co-localisation of *Cdhr1* and *Prom1* has been demonstrated in murine tissues,⁵⁴ the inner segment binding partner and the enzyme catalysing the proteolytic cleavage of *CDHR1* are unknown.

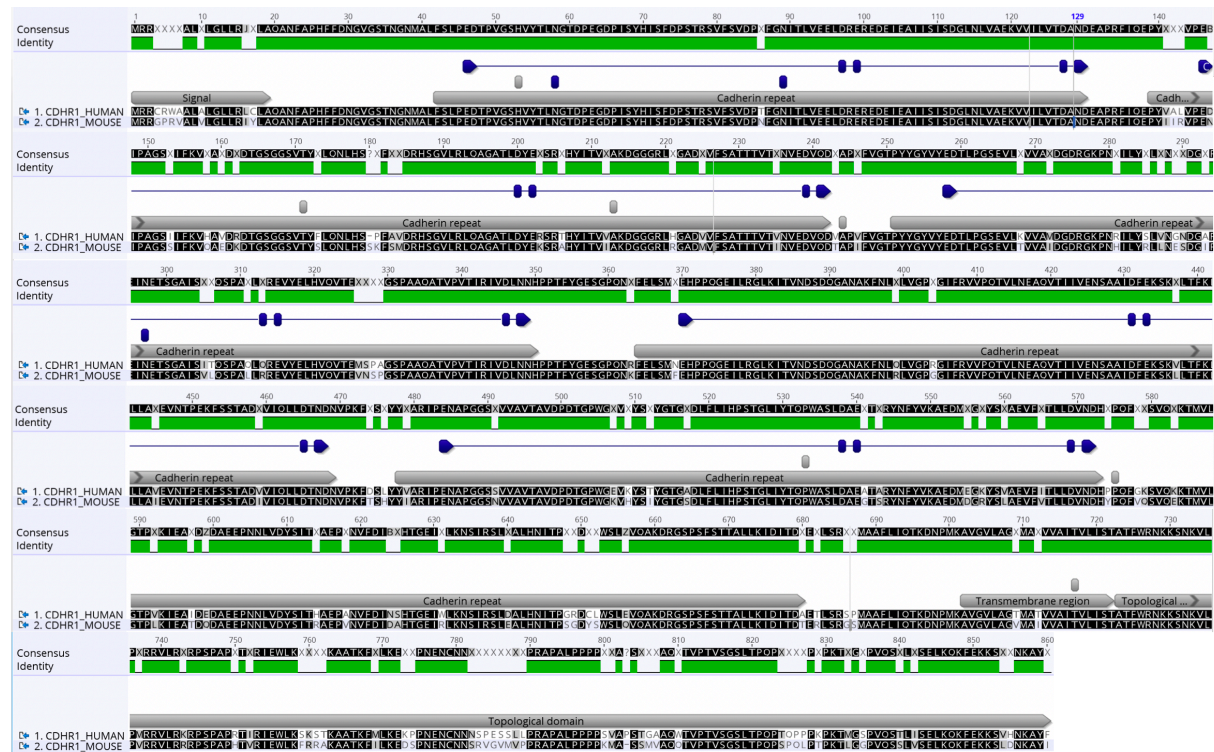


Figure 1.7 Structure of human *CDHR1* and the murine *Cdhr1* orthologue. Green bars indicate homologous residues between human and murine *CDHR1* orthologues. Blue markings indicate calcium-binding sites within the cadherin repeats. Note the high degree of sequence homology within cadherin repeats, and relatively low homology in the cytoplasmic domain.

The *CDHR1* gene is located on 10q23.1 in humans, containing 17 exons with a transcript length of 2,580bp (NM_033100.3). The translated *CDHR1* protein is a non-classical cadherin with 6 extracellular cadherin-like repeats containing calcium-binding sites, a transmembrane and cytoplasmic domain (Figure 1.7 &

1.8). The extracellular domain is more highly conserved than the cytoplasmic domain (63.4% sequence identity versus 31.2%), suggesting a more important biological function. The murine *Cdhr1* homologue shares 85.1% identity at the amino acid level (87.4% identity across extracellular domains; 74% across transmembrane and cytosolic domains) (Figure 1.7).

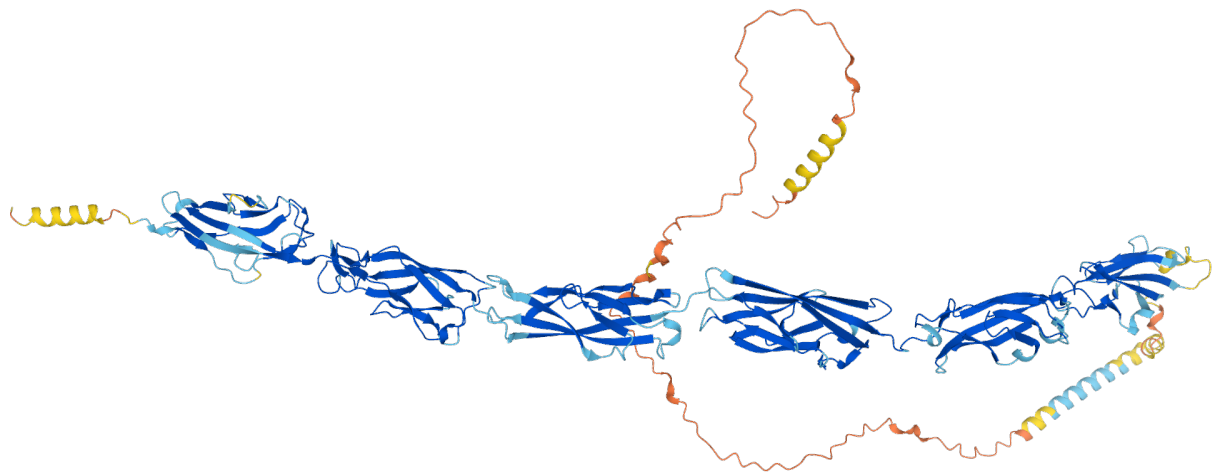


Figure 1.8 CDHR1 protein structure in humans (Q96JP9) as modelled by AlphaFold®

The structure of the six extracellular cadherin domains (dark and light blue) are predicted with high confidence, although the cytoplasmic domain (orange and yellow) are not confidently predicted.

Accessed at: <https://alphafold.ebi.ac.uk/entry/Q96JP9>.

1.3.3 Genetics of *CDHR1*-associated retinal degeneration

Table 1.1 lists all previously published pathological variants in *CDHR1* with their protein consequence, minor allele frequency, associated retinal phenotype and reference. The genotype-phenotype associations are somewhat unclear. Individuals with CRD appear to harbour biallelic null variants, or what are assumed to be functional nulls. Certain hypomorphic variants appear to be associated with macular dystrophy (e.g. c.783G>A), although it is unclear as to

which missense variants are associated. RP has been described; however associated variants are not well defined. Moreover, phenotyping is non-standardised with some reports avoiding specific descriptive terms that define the retinal phenotypes. Detailed, systematic evaluation of a large number of patients is required to describe phenotypes and unravel genotype-phenotype associations.

<i>CDHR1</i> nucleotide variant	Protein consequence	MAF	Phenotype	Year of initial report
c.1A>G	p.Met1Val	0.00002024 rs794726954	-	2020 ⁵⁵
c.18G>A	p.Try6Ter	0.00001651 rs1220602138	-	2019 ⁵⁶
c.56-1G>A	Splice acceptor	0.00001198 rs751972593	RCD	2017 ⁵⁷
c.152-2A>G	Splice acceptor	-	CRD	2017 ⁵⁸
c.296A>G	p.Glu99Gly	-	CD	2017 ⁵⁷
c.297+1G>T	Splice donor	0.000007953 rs765924534	-	2020 ⁵⁵
c.338del	p.Gly113AlafsTer2	0.000003979 rs747425652	CRD	2010 ⁴⁹
c.386A>G	p.Asn129Ser	0.00001277 rs1424495418	CD	2016 ⁵⁹
c.398C>G	p.Pro133Arg	-	-	2016 ⁶⁰
c.438+1G>A	Splice donor	-	-	2019 ⁵⁶
c.439-17G>A	Intron	0.000008672 rs771510913	-	2016 ⁶⁰
c.524dup	p.Gln176GlnfsTer48	-	CRD	2010 ⁵⁰
c.562G>A	p.Gly188Ser	0.00006820 rs748412274	-	2020 ⁵⁵
c.639+1del	Splice donor	0.000004173 rs745932099	-	2017 ⁵⁷
c.783G>A	p.Pro261=	0.003052 rs147346345	MD	2014 ⁶³ 2019 ^{56, 61}
c.838C>T	p.Arg280Ter	0.00001988	CRD	2015 ⁶²

<i>CDHR1</i> nucleotide variant	Protein consequence	MAF	Phenotype	Year of initial report
		rs191858417		
c.928C>T	p.Gln310Ter	-	-	2017 ⁵⁷
c.1311_1316del	p.Leu437_Thr438del	0.000003983 rs1257781536	-	2017 ⁵⁷
c.1373T>A	p.Val458Asp	0.000003976 rs760942217	-	2020 ⁵⁵
c.1381C>T	p.Gln461Ter	-	RCD	2012 ⁶³
c.1448A>G	p.Glu483Gly	-	-	2017 ⁵⁷
c.1463del	p.Gly488fsTer 20	0.00005966 rs756678484	CRD	2013 ⁶⁴ 2010 ⁴⁹
c.1485+2T>G	Splice donor	0.000003980 rs767366723	CRD	2012 ⁶⁵
c.1485+2T>C	Splice donor	0.000007959 rs767366723	RCD	2015 ⁶⁶
c.1503_1507del	p.Gly502LeufsTer32	0.00001061 rs1266986282	CRD	2019 ⁵⁶
c.1527T>G	p.Tyr509Ter	0.000003976 rs1477733493	CRD	2016 ⁶⁷
c.1554-2A>C	Splice acceptor	-	RCD	2017 ⁶⁸
c.1570_1592del	p.Ser524AlafsTer4	0.000003976 rs751597954	CRD	2019 ⁵⁶
c.1700T>C	p.Leu567Pro	-	RCD	2017 ⁵⁷
c.1720C>G	p.Pro574Ala	-	CRD	2015 ⁶⁶
c.2040+5 G>T	Splice donor	0.000003984 rs1446751435	CRD	2017 ⁵⁷
c.2108G>A	p.Gly703Asp	-	RCD	2017 ⁵⁷
c.2203C>A	p.Pro735Thr	0.000007975 rs780447091	-	2020 ⁵⁵
c.2522_2528del	p.Ile841SerfsTer119	0.00003313 rs752341696	RCD	2016 ⁶⁷

Table 1.1 Reported disease-associated variants in *CDHR1*

Variants are presented in order of nucleotide sequence. MAF – Minor allele frequency, derived from GnomAD annotated with Reference SNP cluster ID (rs) accession number. Phenotypes are annotated where reported in an individual homozygous for the relevant variant. This table is current as of the time of submission for published, clinically validated *CDHR1* variants.

1.4 Gene therapy in inherited retinal disease

Monogenic inherited retinal degenerations remain at the frontier of somatic gene transfer therapies in humans for several reasons. Firstly, monogenic retinopathies may be phenotyped in detail through clinical assessment, multimodal retinal imaging, functional and psychophysical tests prompting appropriate genetic testing and aiding in the interpretation of variants. For example, ACMG criteria for pathogenicity may be relaxed for variant calling when a clinical phenotype is pathognomonic, such as Best's vitelliform dystrophy. Secondly, numerous reproducible, non-invasive clinical tests that examine different components of the visual system are available as outcome measures for clinical trials (discussed further below). Thirdly, the eye is a paired organ, allowing the untreated fellow eye to serve as an internal control, enabling the detection of smaller treatment effects within subjects (demonstrated in Chapters 5-6). Fourthly, the eye is both generally self-contained by the corneo-scleral coat and relatively immune privileged through the blood-retinal barriers. The former property supports the retention of therapeutic molecules, thereby minimizing the risks of adverse systemic effects: the first in human *in vivo* delivery of active CRISPR-Cas9 enzymes was by sub-retinal injection to treat retinal degeneration secondary to a deep intronic variant (c.2991+1655A>G) in *CEP290* (NCT03872479).⁶⁹ The latter property minimizes inflammatory responses to the AAV capsid and/or transgene following delivery of AAV vector, enabling efficient transduction and stable long-term gene expression at relatively low doses. Additionally, photoreceptors and

RPE cells are terminally differentiated, post-mitotic cells in which stable transgene expression may be achieved following a single intervention. The final benefit of the retina as a target organ is that its laminar structure enables the precise surgical delivery of vectors into potential spaces adjacent to target cells in order to favour a high transduction efficiency at relatively low doses (i.e. in the subretinal space). This cannot be easily achieved in solid organs, such as the liver.

The field has capitalised on these inherent advantages, with vast progress over the past 20 years in retinal gene therapies.⁷⁰ These advances have been supported in parallel by the rapid evolution and decreasing cost of molecular genetic testing, enabling the identification of more than 340 associated genes and loci underlying inherited retinal degeneration (RetNet: <https://sph.uth.edu/retnet>, accessed 8th November 2021). Moreover, genome aggregation (ExAC, GnomAD etc.) and disease-associated variant (LoVD, ClinVar etc.) databases, sequencing of genomes throughout the animal kingdom, *in silico* prediction tools together with published reports have supported the interpretation of disease-associated sequence variants, and consequently, unravelled the molecular basis of disease for many genes. Protein modelling and the development of *in vitro* tools (e.g. splicing assays) have further contributed. The development and application of gene therapies depend upon precise gene and variant identification and interpretation.

Against this backdrop, the first *in vivo* demonstration of a rescue effect following somatic gene transfer was in 1996 in the *rd* mouse (*Pde6b*), where it was shown that subretinal injection of a recombinant adenoviral vector slowed photoreceptor degeneration by 6 weeks.⁷¹ AAV emerged as the leading vector for gene transfer to photoreceptors and RPE, showing ultrastructural rescue of the *rd*s (*Prph2*) mouse.⁷² Due to the relatively early discovery of *RPE65* as a genetic cause of LCA (1997),^{73, 74} the severe clinical phenotype, short AAV-encodable cDNA sequence (1,602bp), and the availability of naturally occurring disease models (Briard dog),⁷⁵ *RPE65* gene replacement became the first retinal gene therapy shown to rescue a disease phenotype in a large animal model using recombinant AAV (2001).⁷⁶ *RPE65* gene therapy (Luxturna®; voretigene neparvovec) showed benefit in Phase 3 clinical trials (2017), and became the first retinal gene therapy vector to receive received FDA (2017) and EMA approvals (2018) shortly thereafter. Luxturna® was approved by the National Institute of Clinical Excellence (NICE) in the UK for treatment of *RPE65*-associated LCA on the NHS (2019), a decisive precedent for the clinical application of retinal gene therapies.

1.4.1 Methods of gene transfer

The replacement of genes within mammalian cells is dependent on a safe and efficient mechanism for delivery of the relevant DNA or RNA molecules. This can be achieved using chemical transfection reagents, electrical currents (electroporation)⁷⁷, or using vectors which may be viral or non-viral.^{78, 79} In the

mammalian retina, transfection reagents are predictably toxic to photoreceptors; the densely packed photoreceptor outer segment membrane discs are vulnerable to lysis at concentrations of reagent necessary for the transfection of supercoiled plasmid DNA. Moreover, low transfection efficiency, lack of cell specificity, poor nuclear localisation and long-term stability of transgene expression further limit the use of non-viral gene transfer methods.^{78, 79} However, optimisation of nanoparticle-mediated gene transfer may help to overcome some of these limitations.^{78, 80} Electroporation has been shown to be effective in the transfection of plasmid DNA in rodent retina *in vitro* and *in vivo*, although is typically only effective in transfecting photoreceptors in newborns or pups.⁷⁷ Recombinant adeno-associated viral vector mediated gene transfer remains the safest and most effective method for transgene expression in primate photoreceptors; those currently used in ongoing clinical trials are listed in Table 3.1.

1.4.2 Adeno-associated viral vector

Adeno-associated virus (AAV) is a naturally occurring, non-enveloped single-stranded DNA virus (parvovirus) which is relatively non-pathogenic in humans. It cannot self-replicate in the absence of adenoviral co-infection. The AAV genome of 4.7kb contains three genes: *Rep* (replication), *Cap* (Capsid) and *aap* (assembly proteins), which through alternate transcriptional start sites, promoters and alternative splicing, encode numerous viral proteins. The AAV genome is flanked by inverted terminal repeats (ITRs) essential for viral packaging and genome

replication. The capsid proteins (VP1/VP2/VP3) are important in the cellular adhesion of AAV to host cells and subsequent transduction. AAV variants were originally distinguished on the basis of serotypes, although are now categorised on the basis of their genomic sequences. AAV has evolved over millions of years to infect eukaryotic cells and replicate with adenoviral co-infection.

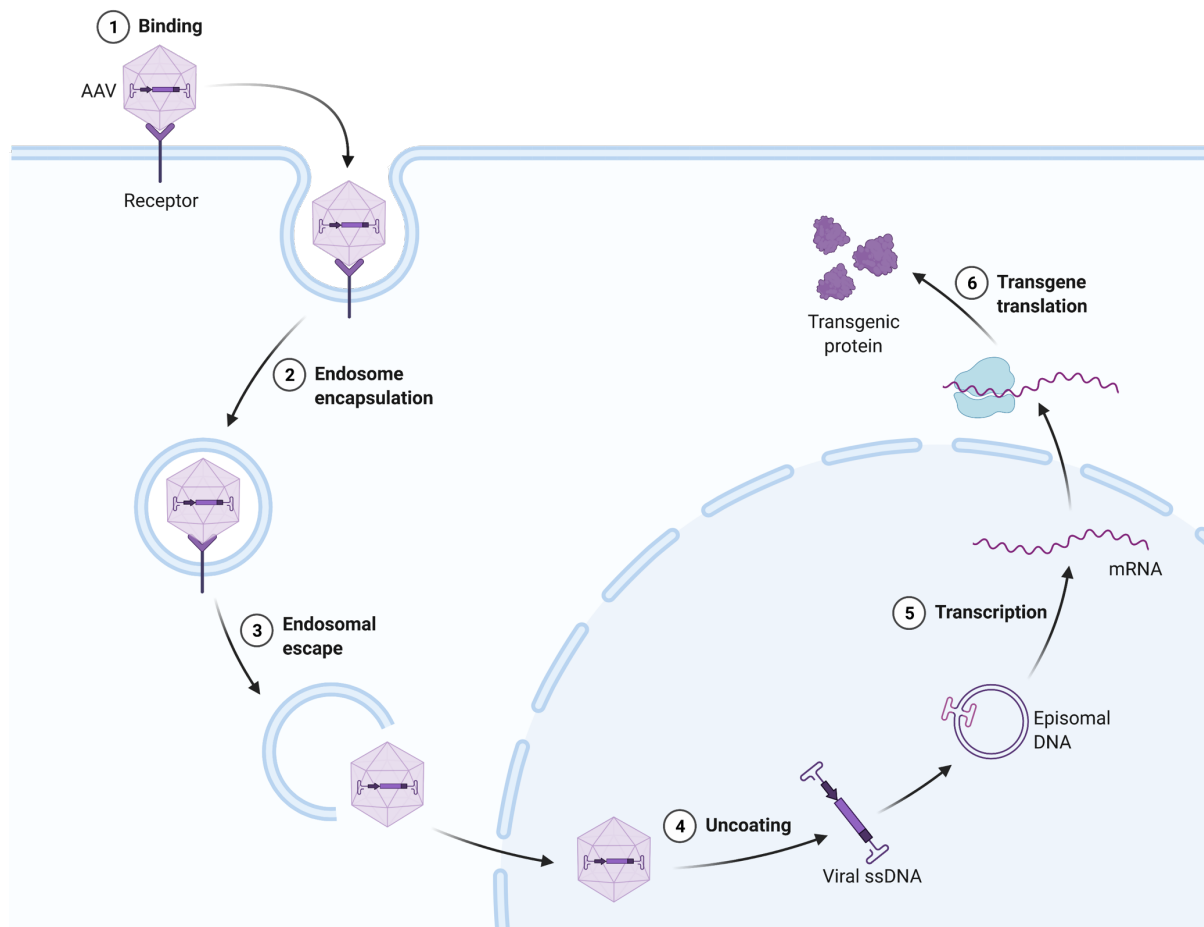


Figure 1.9 AAV infection in a host cell

Viral capsid proteins interact with specific cell surface receptors, which differ by serotype, followed by receptor-mediated endocytosis, cytosolic escape with subsequent nuclear localisation and uncoating. The AAV genomic DNA commonly persists within the nucleus through concatemerization, forming circular episomes within the nucleus, driving transgene expression for the life of the host cell. Created using BioRender.

Since AAV is non-pathogenic and does not integrate with the host genome, AAV has been used as a vector for somatic gene transfer and supplementation, principally in the treatment of monogenic disorders. Recombinant AAV describes the sequence modification of wildtype AAV, replacing the *rep* and *cap* genes of the AAV genome with other nucleotide sequences designed to express one or more genes within a eukaryotic target cell, and render the AAV particle replication deficient. AAV capsid serotypes have variable cellular tropism which is the basis for cellular specificity together with the relevant promoter which may be cell-specific or generalised. Synthetic capsid sequence modification has been undertaken in order to further improve the efficiency of viral transduction in target cell types within the retina, principally by substitution of tyrosine residues which may lead to ubiquitination and subsequent degradation within the host cell (discussed in Chapter 4).⁸¹⁻⁸⁵

Viral capsid proteins interact with specific cell surface receptors, which differ by serotype, followed by receptor-mediated endocytosis, cytosolic escape with subsequent nuclear localisation and uncoating (Figure 1.9). The AAV genomic DNA commonly persists within the nucleus through concatemerization, forming circular episomes within the nucleus, although rarely integration occurs at the preferred locus on chromosome 19 (at a rate of less than 0.1%). Following transduction, transgene expression may persist for the lifetime of the host cell through episomal expression. However, the limited size of the AAV genome (4.7kb)

limits the size of the transgene that can be delivered to a host cell, together with a relevant promoter and regulatory sequences. The rational design of recombinant AAV molecules produced in this thesis are described in Chapter 4.

1.5 Thesis aims and overview

The overall aim of this thesis was to develop and evaluate the first therapy for *CDHR1*-associated retinal degeneration, a currently untreatable form of blindness, that may be a candidate for onward clinical trial. The design of the vector and all experimental work were undertaken in order to support and simplify onward regulatory approval.

1. Validation of the *Cdhr1*^{-/-} mouse model by deep phenotyping

Although a murine model generated from the targeted disruption of the murine *Cdhr1* homolog has been described, deep phenotyping had not been undertaken.⁴⁷ In Chapter 3, longitudinal deep phenotyping using multimodal retinal imaging techniques, electroretinography and behavioural testing is undertaken to validate the *Cdhr1*^{-/-} mouse model for *in vivo* development and downstream assessment of retinal gene therapy vectors.

2. AAV.CDHR1 vector production and *in vivo* validation

The rational design, cloning and AAV packaging of at least two constructs expressing the full-length human CDHR1 transgene are presented in Chapter 4.

Confirmation of cell specificity and expression of CDHR1 in rod and cone photoreceptors is assessed following sub-retinal delivery of the candidate vectors in the *Cdhr1*^{-/-} mouse model.

3. AAV8.CDHR1 vector dosing study

A formal pre-clinical open-label, dose-reduction study of two candidate vectors is presented in Chapter 4 in both the *Cdhr1*^{-/-} and *C57BL/6J* strains. A paired, active-controlled experimental design using retinal thickness measurements on OCT imaging as a primary endpoint is used to determine the maximum tolerated dose of each vector. Electroretinography is used to assess early therapeutic signals in the *Cdhr1*^{-/-} group to identify the optimum therapeutic dose and select the lead vector construct for a longitudinal gene rescue experiment.

4. Evaluation of AAV8.CDHR1 gene replacement

Two selected doses of the lead AAV8.CDHR1 vector construct are evaluated in both *Cdhr1*^{-/-} and *C57BL/6J* mice to 22-months post-injection in an open-label, active-controlled, prospectively powered gene therapy rescue experiment in Chapter 6. Retinal structure was evaluated by OCT imaging to 18-months post-injection and rod and cone function by dark- and light-adapted electroretinography to 12-months post-injection, with measurement of response amplitudes and implicit times. Behavioural testing using optomotor testing was

undertaken to 22-months post-injection. The efficacy (*Cdhr1*^{-/-}) and safety (*C57BL/6J*) of the chosen doses of AAV8.CDHR1-gene therapy are assessed.

5. Human *CDHR1*-associated retinal degeneration

A key requirement for the design and approval of a phase 1/2 clinical trial is a clear understanding of the natural history of the disease for which an investigational medical product (IMP) is intended. The largest previously reported clinical series of *CDHR1*-associated retinal degeneration is 7 patients.⁵⁷ In Chapter 7, an international retrospective natural history study of 146 individuals with retinal degeneration attributed to biallelic variants in *CDHR1* is presented, with descriptions of retinal phenotype(s), natural history of visual decline, multimodal retinal imaging characteristics, quantitative analysis of retinal thickness measurements on OCT and growth of RPE atrophy on FAF imaging, and electrophysiological studies. Genotype-phenotype associations are presented, with 24 novel pathological sequence variants identified. The global genetic prevalence of *CDHR1*-associated retinal degeneration is calculated, with refined estimates according to predicted phenotype. The significance and relevance of the data presented in this thesis in support of the *CDHR1* programme is discussed in Chapter 8 with, with specific reference to the Food and Drug Administration (FDA) guidance on the development of investigational gene therapy products, translation into clinical trial and subsequent approval.⁸⁶⁻⁸⁹

2. Materials & Methods

2.1 Molecular Biology

All molecular biology experiments described in this thesis were undertaken in controlled laboratory conditions using calibrated equipment according to standardised protocols. Separate workspaces were used, where possible, for DNA, RNA and protein work. Cloning experiments and plasmids described in this thesis were designed *in silico* using Geneious v.11.0.3 (Biomatters Ltd., Auckland, NZ).

2.1.1 Polymerase chain reaction

Polymerase chain reaction (PCR) is an experimental technique in which a thermostable polymerase enzyme is used to amplify a DNA template using flanking primers and a thermocycler (Multigene, Labnet International Inc.). For sequence sensitive applications, such as amplification of the *CDHR1* coding sequence, the high-fidelity KOD Hot Start Mastermix was used (Sigma-Aldrich, UK), containing a polymerase derived from *Thermococcus kodakarensis*.

Primers were designed (Thermo Fischer Scientific, UK) using the following desirable criteria as stated by the manufacturer:

1. Length: 18-30 bp
2. GC content: 40-60%

3. Primer melting temperature (T_m): 65-75°C, with primer pair T_m values within 5°C of each other to promote annealing
4. A 3-bp overhang beyond restriction sites to facilitate enzyme binding
5. Primers starting or ending in a G or C nucleotide to promote annealing
6. Avoiding complementary sequences to prevent secondary structures

All primers used in experimental work are listed in the Appendix. PCR reactions undertaken as part of cloning were performed according to manufacturer's instructions. Briefly, KOD Hot Start Mastermix was thawed on ice. Primers were diluted to 10 μ M and added to the reaction mixture to achieve a final primer concentration of 0.3 μ M. Template DNA was added to a final concentration of 0.02 ng/ μ l. The reaction mixture was made up to the total desired volume with molecular biology grade water. The cycling conditions were modified according to primer T_m (annealing temperature) and amplicon length (duration of extension). Polymerase activation was at 95°C for 2 minutes, followed by 20-40 cycles of [denaturation at 95°C for 20 seconds, annealing at X° C for 10 seconds, and extension at 70°C for Y seconds]. Annealing temperature (X) was set at 5°C below the lowest of the two primer T_m values. Extension time (Y) was for 10 seconds for an amplicon length of less than 500 bp, 15 seconds for an amplicon of 500-1000 bp, and 20 seconds per kilobases for longer amplicons. PCR reaction components and thermocycling conditions used for genotyping were undertaken using Immomix, described in section 2.4.2.

2.1.2 Restriction Digests

Restriction digestion involves the specific cleavage of DNA sequences using restriction endonucleases. Restriction digests were planned *in silico*, and performed according to manufacturer's instructions (New England Biolabs, UK). Briefly, 1 unit of restriction enzyme will digest 1 µg of DNA in a 50 µl reaction in 60 minutes. Endonucleases were thawed on ice, and 1µl used (around 10 units) in each reaction, together with reaction buffer, the template DNA and molecular biology grade water to make up the reaction volume. All substances were kept on ice, and flicked to mix before brief microcentrifugation. The restriction digest was undertaken at 37°C for 1 hour, although Time-Saver™ enzymes were incubated for 15 minutes. An undigested plasmid sample and a non-template control were always included in each restriction digest and assessed by 2% agarose gel electrophoresis. Occasionally, heat inactivation or a stop solution was used to terminate the activity of an endonuclease. The NEB Double Digestion tool was used to identify the correct reaction conditions and buffer for double digests. Single digest controls were used to confirm the activity of each endonuclease.

2.1.3 Agarose gel electrophoresis

The size and quantity of PCR products were generally confirmed on agarose gel electrophoresis. Agarose gel was made using 2 g of Agarose (Thermo Fisher Scientific) in 200 mls of TAE (Tris base, Acetic acid and EDTA) buffer, and boiled. 10µl of Ethidium Bromide (0.05µg/ml; Thermo Fisher Scientific) was added, and

the solution poured into a gel cast and left to set. Loading buffer was added to PCR samples before loading into the gel in TAE buffer. The Ultra Ranger 1kB DNA ladder (Norgen BioTech) produced markers from 300-24,000 bp and was used to identify the size of DNA fragments. 2% agarose gels were run at 100V for up to 2 hours depending on the application. The gels were imaged using a UV light source and digital images captured.

2.1.4 PCR purification and agarose gel extraction

PCR purification was undertaken to remove salts and/or other contaminants as necessary for downstream applications (QIAquick PCR purification kit, Qiagen), according to the manufacturer's instructions. Briefly, 5 volumes of buffer PB was added to 1 volume of reaction mix. In the event of base excess, 10 µl of 3M Sodium acetate pH 5.0 was added. DNA was bound to the membrane in a QIAquick column and centrifuged at 17,900g for 60 seconds. 750 µl of wash buffer PE was added and centrifuged at 17,900g for 60 seconds. The QIAquick spin column was placed in a clean microcentrifuge tube and DNA eluted with centrifugation at 17,900g for 60 seconds using 30-50 µl of molecular biology grade water. Sample DNA concentration and quality was measured using the Nano-Drop® 1000 Spectrophotometer (Thermo Fisher Scientific, Northumberland, UK) with 1.5 µL of sample. The spectral image of all samples was inspected for quality and purity of DNA, with maximal absorbance of nucleic acids at 260nm. A 260/280 ratio of around 1.8 and a 260/230 ratio of around 1.8-2.2 was accepted as a high purity

yield. DNA samples were stored at -20°C, or kept on ice for immediate experimental use.

In the case of sub-cloning where specific restriction digest products were required for downstream ligation, gel extraction of the desired bands was undertaken using the QIAquick Gel Extraction Kit (QIAGEN). Briefly, desired gel bands were excised under blue light using a surgical scalpel (Swann-Morton, UK) and placed into pre-weighed microcentrifuge tubes. Three gel volumes of buffer QC were added and the samples heated to 50°C for 10 mins with intermittent vortexing. In the event of base excess, 10 µl of 3M Sodium acetate pH 5.0 was added. 1 gel volume of molecular grade isopropanol was added. The method of DNA elution was thereafter identical to that described for PCR purification.

2.1.5 DNA ligation

Ligation a process in molecular biology of joining one or more nucleic acid sequences using a ligase enzyme to create recombinant molecules. The ligase catalyses the formation of a phosphodiester bond between the 3' hydroxyl group of one nucleic acid terminus and the 5' phosphoryl group of another. Ligation reactions were set up in a PCR tube on ice. 1 µl of T4 DNA Ligase (New England Biolabs, UK) was used as part of a 10 µl reaction, with 1 µl of DNA Ligase Buffer (10x), varying molar ratios of DNA backbone and DNA insert, made up to 10 µl with molecular biology grade, nuclease-free water. The ratio of DNA insert to DNA

backbone was calculated according to the DNA length and DNA mass of each sequence, using NEB Ligation Calculator. Insert to backbone molar ratios ranging from 1:1 to 7:1 were typically used, in addition to a NCT and ligation control reaction. The reactions were mixed by gentle pipetting on ice. For blunt-ended ligation, incubation was at room temperature overnight, and for cohesive ends, ligation incubation was at room temperature for one hour. Samples were chilled on ice until bacterial transformation.

2.1.6 Bacterial transformation

Ultracompetent *E. Coli* Cells (XL10-Gold®, Stratagene, Agilent technologies) were used for bacterial transformation due to their efficiency, according to manufacturer's instructions with modifications. *E. Coli* cells were thawed on ice, and apportioned into 50 uL aliquots. 4 uL of β-mercaptoethanol was added to each aliquot and incubated on ice for 10 minutes, whilst gently swirled every 2 minutes. 5 μL of ligation reaction, and 1 μL of pUC18 positive control plasmid was added to each aliquot and incubated on ice for 30 minutes. 5 μl of molecular biology grade water was added to the NCT aliquot. All samples were heat-pulsed at 42°C for 30 seconds in a water bath, before incubation on ice for a further 2 minutes. 900 μL of S.O.C. medium (15544-034, Thermo Fisher Scientific) was added to each reaction and incubated on a bacterial culture shaker (10 x g) at 37°C for 1 hour. Each aliquot was spun down on a table-top centrifuge at 2,500 rpm for 2 minutes to concentrate cells. 700 μL of excess S.O.C. mediate was removed, and the cells

resuspended in the remaining 200 μ L volume and plated onto LB-agar culture dishes (created by reconstituting 12.5g of LB in 500mls of distilled water, and adding 7.5g of agar before autoclaving, and once cooled, impregnating with filtered Ampicillin (0.22 μ m filter following reconstitution in RO water) at a concentration of 0.1 μ g/ml). All plates were incubated for 17 hours at 37°C, to ensure bacteria were in the logarithmic growth phase following which approximately 50-200 individual colonies were typically present.

2.1.7 Colony selection

Following overnight culture, all plates, including positive and negative control plates, were inspected for the presence and density of colonies. A sterile pipette was used to touch selected colonies in test plates and placed immediately into a sterile 15ml falcon tube containing 3mls of autoclaved LB medium with Ampicillin; created by mixing Luria Broth with distilled water according to the manufacturer's instructions and autoclaved before the addition of filtered Ampicillin to a final concentration of 100 μ g/ml. Each sample was incubated on a bacterial shaker at 37°C for 17 hours at 10 x g.

2.1.8 Plasmid preparation and measurement of DNA concentration

Following bacterial culture, pipette tips were removed using forceps treated with 70% ethanol for 30 minutes. 15ml falcon tubes containing 3ml culture products were centrifuged at 4,500g for 20 minutes at room temperature. Mini plasmid

preparation was undertaken using the Zyppy® endotoxin-free mini-prep kit according to the manufacturer's instructions (D4015, Zymo Research). Briefly, pelleted *E. Coli* were resuspended in 600µL of molecular biology grade water in a 1.5 ml microcentrifuge tube. 100 µL of 7x lysis buffer was added to each sample and inverted, followed by addition of 350 µL of cold neutralisation buffer and inverted again. Samples were centrifuged at 11,000g for 4 minutes, and the resulting supernatant transferred to the Zymo-Spin™ IIN column. The samples were centrifuged at 11,000g for 15 seconds, followed by addition of 200 µL of Endo-wash buffer and Zyppy® wash buffer with centrifugation after each. The column was transferred to a clean microcentrifuge tube and plasmid DNA eluted by centrifugation after addition of 30-50 µL of molecular biology grade water.

For larger preparations, the Qiagen® EndoFree® Mega Prep Kit was used, which typically yielded 2 x 1ml samples of 1 µg/ml. Starter cultures were undertaken in 15ml falcon tubes for 8 hours at 37°C, at 10 x g, and then transferred to 8 x 500ml flasks of LB-ampicillin for culture under identical conditions. Cells were pelleted in 500 ml Nalgene flasks in a Beckmann Coulter centrifuge for 20 mins at 8,000 x g for 20 minutes. A single freeze-thaw cycle (1 hour at -20°C) was undertaken following removal of the supernatant to encourage cell lysis. Cell lysis, plasmid extraction and purification were undertaken according to the manufacturer's protocol with 1.5ml of elution buffer.

2.1.9 DNA sequencing

All DNA sequencing was sub-contracted to two commercial companies, Source Bioscience, UK and Eurofins, Germany using appropriate sequencing primers. Results of Sanger sequencing were provided through electronic chromatograms (*.ab1), which were imported and inspected using Geneious®, software version 11.0.3. Sequences were then aligned with relevant plasmid maps to determine coverage and identity.

2.2 Cell culture techniques

All cell culture techniques were undertaken in a designated tissue culture laboratory in a Class II laminar flow cabinet under aseptic conditions. Cell incubation was at 37°C in a humidified incubator with 5% CO₂. All media was pre-warmed in a water bath at 37°C unless otherwise stated.

2.2.1 Cell culture

Human embryonic kidney (HEK293) cells (#85120602, Culture Collections, Public Health England) were acquired from designated cell banks and stored in liquid nitrogen. Following resuscitation, cells were passaged at least three times prior to transfection. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (Complete DMEM; Gibco, Thermo Fisher Scientific, UK) supplemented with 10% filtered fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and phenol red pH indicator. Cells were passaged when

70-90% confluent on microscopy and cell counting using standard protocols. Briefly, excess media was removed with a serological pipette, cells washed using sterile PBS which was removed before trypsinization with TrypLE and incubation at 37°C for 5 minutes. A further 3mls of DMEM was added after confirmation of cell detachment and resuspension achieved by pipetting. Following centrifugation and discarding of the supernatant, the cells were resuspended in fresh media, before 1-2mls of the cell suspension was added to a new T75 flask with 13mls of fresh DMEM for continued cell culture to achieve a seeding density of ~20-40%. Any remaining cells were discarded or used for transfections.

2.2.2 *In vivo* cell transfection

Transfection of HEK293 cells were used for positive controls for western blot experiments (CAG.CDHR1.WPRE.pA), or negative controls using a lab plasmid (CAG.coRPGR.WPRE.pA; gift Dr. Cristina Martinez-Fernandez de la Camara). Transfection was undertaken using a positive control plasmid (CAG.GFP.WPRE.pA; gift Dr Michelle E. McClements). Transfections were optimised (i.e. DNA:reagent, seeding density) from the manufacturer's protocols. Cells were pelleted by centrifugation at 300g for 5 minutes, the supernatant discarded, and resuspended in 10mls of fresh DMEM, before cell counting using a haemocytometer using trypan blue to determine cell viability in order to estimate the cell density of the original suspension. Cells were seeded into wells of a 12-, 24-, 48- or 96- well sterile culture plates using 1 mls, 500 µl, 250 µl or 100µl of cell

suspension at 10^5 cells/ml. Transfections were undertaken 24 hours after cell seeding, and performed using *TransIT*®LT-1 (Mirus Bio, USA). The transfection reagent was warmed to room temperature and vortexed, and added in a ratio of 3 μ l to 1 μ g of DNA mixed in a volume of Opti-MEM™ serum-free medium appropriate to the number of wells and the culture plate. The transfection medium was incubated at room temperature for 30 minutes, and then added directly to the wells of the culture plate, with gentle swirling to encourage distribution of the complexed DNA. The plates were incubated at 37°C for up to 72 hours.

2.3 AAV vector production

All work was conducted, unless otherwise stated, in a class II safety cabinet. All disposable equipment was treated with 1% Virkon (Anachem) for 1 hour before discarding. This protocol describes the simultaneous production of two AAV vectors using four HyperFlasks® (Corning).

2.3.1 Culture of HEK293T cells and seeding of HyperFlasks

Two T175 flasks were seeded with HEK293T cells in standard culture media (500 mls of Dulbecco's Modified Eagle's Medium (DMEM; high glucose) with 10% Foetal Bovine Serum (FBS; Sigma-Aldrich), 5 mls L-Glutamine (Sigma-Aldrich) and 5 mls Penicillin-Streptomycin (Sigma-Aldrich)). Cells were cultured at 37°C in 5% CO₂ for 72 hours until flasks were 80-90% confluent. The culture media was removed and cells washed with 10 mls of PBS. 5 mls of PBS was added and cells

incubated at 37°C in 5% CO₂ for 10 minutes. Cells were loosened with 15 mls of culture media and a further 180 mls of culture media was added to each T175 flask. The cell suspensions from each T175 flask were divided between two HyperFlasks (Corning), which were placed on their sides to allow an even distribution of cells, which were then filled with media (~560 mls). Air bubbles were removed and the HyperFlasks were incubated at 37°C in 5% CO₂ for 72 hours.

2.3.2 Transfection of HEK293T cells in HyperFlasks

A total of 500 µg of DNA was prepared for each HyperFlask. A single plasmid containing AAV8 wildtype viral capsid DNA and helper sequences (pDP8.ape, Plasmid Factory) was used in addition to the CDHR1 expression plasmids, both GRK1.CDHR1.pA and GRK1.In.CDHR1.WPRE.pA. The ratio of plasmid quantities was proportional to their relative size: for the unenhanced CDHR1 expression plasmid, 114.9 µg was used and for the enhanced CDHR1 expression plasmid, 128.6 µg was used with DNA quantity made up to 500 µg with pDP8.ape. The AAV and CDHR1 expression plasmids were added to a 50 ml falcon tube and volume made up to 10 ml with NaCl 150 mM. 1.125ml of PEI transfection reagent (1 mg/ml) was added to a separate 50ml falcon tube and volume made up to 10 ml with NaCl 150 mM. The DNA and PEI transfection reagent were complexed by slowly adding the PEI solution to the DNA, and incubated for 20 minutes at room temperature. The PEI/DNA complex was added to 500 mls of transfection media

(as per culture media, with 2% FBS). The media was removed from the HyperFlask and transfection media containing PEI/DNA complexes was added, and HyperFlasks incubated at 37°C in 5% CO₂ for 72 hours.

2.3.3 Harvesting and lysing of transfected HEK293T cells

The HyperFlasks were shaken vigorously after removal of 200 mls of media to loosen cells, confirmed on microscopy. The cell suspension was poured into an autoclaved 500 ml Nalgene centrifuge flask (Thermo Fisher Scientific), and centrifuged at room temperature for 10 minutes at 1,200g. 1ml of lysis buffer (1M Tris(hydroxymethyl)aminomethane, with 150 nM NaCl and molecular biology grade, DPEC-treated and filtered water which was autoclaved and pH adjusted to 8.5) and protease inhibitor tablet (Roche) were mixed for each pair of HyperFlasks. The media was removed from the pelleted HEK293T cells, and cells resuspended in 30mls of media. This volume was transferred to a 50 ml falcon tube, and centrifuged at room temperature for 10 minutes at 1,200g. The media was removed with a serological pipette. 15mls of lysis buffer was added to the cell pellet and mixed, with the addition of 500 µL of the prepared protease inhibitor cocktail and mixed again. The falcon tubes were placed in a -80°C freezer for 1 hour, thawed in a 37°C water bath for 15 minutes, and the freeze-thaw process repeated twice before leaving the samples at -80°C.

2.3.4 Isolation of AAV particles using an Iodixanol gradient

The cell lysate was thawed at 37°C for 15 minutes. 5 µl of Benzonase (Merck Millipore) was added and the lysate was incubated at 37°C for 45 minutes, with intermittent agitation. The lysate was centrifuged at room temperature for 20 minutes at 3,700g. Iodixanol gradients were prepared in Beckman-Coulter ultracentrifuge tubes by layering the fractions described in **Table 2.1**. Six gradients were prepared for two AAV preparations. Fractions of increasing iodixanol density were layered underneath those with lower density with a Pasteur pipette in sequence. The lysate supernatant was placed on top of the prepared gradients, and lysis buffer used to fill the container which was sealed with a centrifuge cap. All six samples were centrifuged at 59,000rpm for 1 hour and 30 minutes at 20°C. Following centrifugation, the AAV particles were removed at the interface between the 60% and 40% iodixanol fractions with an 18-gauge needle and 5 ml syringe for each sample and stored overnight at 4°C.

Fraction	Iodixanol	5M NaCl	5x PBS-MK	H ₂ O	Phenol Red
15%	6 ml	4.8 ml	4.8 ml	8.4 ml	-
25%	7.5 ml	-	3.6 ml	6.9 ml	30 µl
40%	10.05 ml	-	3 ml	1.95 ml	-
60%	15 ml	-	-	-	30 µl

Table 2.1 Constituent fractions for three iodixanol gradients

2.3.5 AAV purification and concentration

5 mls of PBS were added to an Amicon Ultra 100K filter and centrifuged at 20°C for 15 minutes at 3,000g. PBS was added to each collected iodixanol fraction to make up the volume to 15 mls, mixed, and the total volume added to the filter. Samples were filtered at 3,000g at 20°C until the volume was reduced to 500 µl. 15 mls of PBS was added to the filter and centrifuged at 3,000g at 20°C, and flow through discarded. This process was repeated for a total of three washes, and on the final spin, the volume allowed to reduce to 250 µl. The filter cartridge was rinsed and AAV separated into 10 µl aliquots as “concentrated” AAV samples. 500 µl of PBS was used to wash each filter and 50-100 µl aliquots were prepared, designated as “wash” AAV samples.

2.3.6 Assessment of AAV purity

AAV purity was assessed by SDS-PAGE. A pre-cast 10% TGX gel (BioRad) was placed in half a tank of running buffer. AAV concentrate and AAV wash samples from each AAV preparation were heated to 95°C for 10 minutes after addition of 5 µL of protein loading buffer. The full quantity of all 4 AAV samples were loaded into the wells of the gel with appropriate molecular weight protein ladder. The gel was run for 1 hour and 45 minutes at 100V. The gel was removed and rinsed with RO water in a tray for 5 minutes with gentle shaking, changing the water for 2 more cycles. The gel was covered in EZBlue™ gel staining reagent (Sigma-Aldrich) and shaken for 1-2 hours at room temperature. Background staining was removed

with three further washes with RO water. Viral purity was assessed by the presence and strength of three protein bands corresponding to viral capsid proteins VP1 (87kDa), VP2 (72kDa) and VP3 (62kDa) in an approximate ratio of 1:1:10, and the absence of other bands which would suggest contamination.

2.3.7 AAV quantification

The copy number of the selected plasmid for generating the standard curve for 1 ng of GRK1.CDHR1.pA was 1.41×10^8 (<http://cels.uri.edu/gsc/cndna.html>), based on the assumption that the average weight of 1bp is 650 Daltons. Two primer pairs were designed against the CDHR1 transgene, suitable for simultaneous amplification of both AAV vectors. Both primer pairs were 16 bp in length, generating amplicons of 232 bp and 205 bp, respectively. The AAV samples were DNase digested for 30 minutes at 37°C in a water bath before heat inactivation at 95°C for 10 minutes. AAV samples were diluted to 1:10 and 1:100. A 1:10 serial dilution of GRK1.CDHR1.pA was prepared for the standard curve from 1 ng/μl to 0 ng/μl. 1 μl of each diluted plasmid, and each diluted AAV sample was added in triplicate to designated wells of the qPCR plate. Primers (specified in the Appendix) were diluted from 100 μM stock to 2 μM, and both forward and reverse primers (1 μl each) were added to each well with 5 μl of iTaq reagent and 2 μl of molecular biology grade water. The plate was sealed and briefly spun down whilst protected from light. The plate was loaded into the CFX real time PCR detection system (BioRad). The run settings were 95°C for 3 minutes then [95°C for 10

seconds, 60°C for 30 seconds] for 40 cycles. The melt curve was inspected for a single peak (single amplicon) to confirm primer specificity, and primer efficiency between 95-105% with no amplification in NCT wells. The plasmid copy number was applied to the standard curve. The AAV titre for each of 4 samples was calculated relative to the standard curve. A mean of 3 values for each triplicate was used to determine the AAV titre.

2.3.8 AAV dilution

AAV samples were thawed on ice from -80°C stock on the morning of sub-retinal injections and prepared in a sterile fashion using a class II biological safety cabinet. AAV samples were diluted using sterile PBS and mixed with 0.01% PF68 such that the final concentration of PF68 was 0.001%. PF68 is a non-ionic surfactant that prevents AAV particles adhesion to the surgical needle and syringe during sub-retinal delivery, and has been shown to enhance stability of recombinant AAV titre.⁹⁰ For paired control sub-retinal injections, PF68 was diluted in PBS to a final concentration of 0.001%. Diluted AAV samples and control samples were stored on ice until sub-retinal delivery.

2.4 Licencing and Animals

2.4.1 Licensing and experimental authority

All experimental animal work undertaken as part of this thesis was performed in accordance with UK Home Office guidelines and ARVO Statement for the Use of

Animals in Ophthalmic and Vision Research. Experimental work was performed at a licenced establishment (University of Oxford), under the project licence PPL 30/3363, with the personal licence PIL I451EA232 held by the author since June 2017. Furthermore, the project licence was checked before any experimental design and was scrutinised further by senior members of the local Animal Users Group (AUG) through a formal experimental authority form. This authority form made explicit the specific skills that were required for the proposed experiment. This was checked against a personal record of animal procedural competency.

Animals were housed in a specialised, pathogen-free housing unit with 12-hour light (<100 lux) and dark cycles, with food and water provided *ad libitum*. Mice were housed in individually ventilated cages (IVC) with bedding, and environmental enrichment. Sentinel mice were used to regularly confirm the pathogen-free status of all experimental mice. Breeding cages housed a single male with one or two females, and pups were weaned at 3 weeks of age and housed separately according to sex at a density of 6-7 mice per cage.

2.4.2 Genotyping

Mice were identified by ear notching undertaken at the time of weaning; ear notch tissue was used for PCR-based genotyping using the alkali lysis method.⁹¹ 100 µl of 0.05 M NaOH was added to each ear notch in a microcentrifuge tube and incubated at 95°C for 60 minutes, followed by vortexing and centrifugation at

10,000 rpm for 30 seconds. 10µl of Tris-EDTA (TE) buffer was added for neutralisation before vortexing and centrifugation at 10,000rpm for 30 seconds. 100 µl of supernatant was removed and DNA kept on ice. PCR amplification using 2 x ImmoMix™ (Bioline Reagents Ltd., London, UK), 0.5 µl of forward and reverse primers (0.5µM final concentration), 2 µl of DNA (50-200ng) and made up to 25 µl with molecular biology grade water. Thermocycling conditions were 35 cycles of 95°C for 1 minute, 60°C for 30 seconds, 72°C for 1 minute, with a final step of 72°C for 5 minutes. Products were separated by 1% agarose gel electrophoresis for 1 hour at 100V. DNA was stained with ethidium bromide (0.05µg/ml), imaged under UV light. PCR amplicon sizes were estimated using a standard low molecular weight ladder (UltraRanger 1kb DNA ladder, Norgen Biotek Corp).

2.4.3 *C57BL/6J*

The *C57BL/6J* strain was acquired from the original JAX™ colony (Jackson Laboratories), distributed through Charles River (Margate, Kent, UK). This colony are genetically identical within each strain, preserving uniformity in the genetic background over the course of experimental work. *C57BL/6J* mice were used as experimental controls in the *Cdhr1*^{-/-} natural history study, AAV8.CDHR1 vector dosing and longitudinal rescue experiments and as positive controls in western blots, immunohistochemistry and genotyping assays.

2.4.4 *Cdhr1*^{-/-} (B6;129-*Cdhr1*^{tm1Nat/J})

The homozygous mutant mouse derived from the original *B6;129-Cdhr1*^{tm1Nat/J} line will be referred to as the *Cdhr1*^{-/-} mouse throughout this thesis for simplicity. The *Cdhr1*^{-/-} mutant mouse was created in 2001 through targeted deletion of exons 10-17 in the mouse *Cdhr1* gene, the ortholog of human *CDHR1*.⁴⁷ Six *B6;129-Cdhr1*^{tm1Nat/J} heterozygous mice were acquired from the Jackson Laboratory through Charles River following cryorecovery of embryos, and interbred to generate *Cdhr1*^{-/-} mice. However, the sequencing primers provided were not able to differentiate homozygous and heterozygous *Cdhr1* mice, as the targeted DNA sequence within exon 17 had reinserted elsewhere in the genome (personal correspondence with original authors), and consequently, a wildtype primer band was present following PCR in the *Cdhr1*^{-/-} mice. Following the development of a genotyping assay, the *Cdhr1* mutant allele was backcrossed to the *C57BL/6J* over six generations as described in Chapter 3.

2.5 *In vivo* techniques

2.5.1 General anaesthesia and pupil dilation

Animals selected for an experimental procedure were first inspected for signs of general and ocular health. Induction of general anaesthesia was by intraperitoneal injection of the NMDA-receptor antagonist, Narketan® (Ketamine Hydrochloride, Vetoquinol, UK) at a dose of 80 mg/kg of body weight and the alpha-2 adrenergic receptor agonist, Rompun® (Xylazine Hydrochloride, Bayer,

Germany, 10 mg/kg of body weight) diluted in sterile water, delivered using a 30g needle. This achieved general anaesthesia within 5 minutes, with an approximate duration of 45 minutes. Pupil dilation was achieved by topical 2.5% phenylephrine and 1% Tropicamide (Bausch & Lomb, UK) sequentially to both eyes.

Recovery from general anaesthesia was assisted by use of intraperitoneal alpha-2 adrenergic receptor antagonist Antisedan® (Atipamezole Hydrochloride, Zoetis, USA, 2 mg/kg of body weight). Viscotears was applied to both eyes to prevent the onset of desiccation-related cataract and the mouse was placed supine in an incubator set to ~35°C to aid in thermoregulation during recovery. Once mice were able to correct their posture to a prone position and demonstrated clear signs of recovery based on observations of respiration, movement and responses, they were replaced in their original cages and observed intermittently.

2.5.2 Sub-retinal injection

Mice underwent sub-retinal injection in the first week after weaning. AAV dilution prior to subretinal injection is detailed in section 2.3.8. Subretinal injection was delivered using a very low dead-space NanoFil 10 µl syringe attached to a 35-gauge NanoFil bevelled reusable needle (both manufactured by World Precision Surgical Instruments, Hitchin, UK). The syringes were first checked for patent flow and resistance using sterile PBS prior to aspiration of the test substance. 1.5 µl of AAV or PBS was drawn into the syringe ready for injection. Guttae

Proxymetacaine hydrochloride 0.5% (Bausch & Lomb, UK) was applied to both eyes for topical anaesthesia, and Viscotears (Bausch & Lomb, UK) was applied to the fellow eye. The mouse was positioned on a customised foam pad and pillow such that the iris plane was parallel to the microsurgical operating microscope which was under foot control (Leica Biosystems, UK). A cotton-tipped applicator was used to dry the cornea, and the eyelids were pushed back to expose the globe.

A self-sealing paracentesis was achieved using a 33-gauge hypodermic needle (Steriject, TSK, USA) inserted at the limbus, with resulting efflux of a small quantity of aqueous, in order to achieve a temporary hypotony and reduce the risk of reflux of vector delivered into the sub-retinal space. Viscotears was applied to the ocular surface and a 6mm circular glass slide (VWR International, East Grinstead, UK) was placed on top in order to permit visualisation of the retina and posterior pole. The superior rectus was grasped with cupped titanium forceps to maximise surgical countertraction and indented into the globe to allow visualisation of the retina anterior to the equator. The NanoFil needle was inserted bevel-up with syringe parallel to the vector of force exerted in the opposite direction by the forceps in order to prevent rotation of the globe and ensure the force of needle entry was applied to the ocular coat. The needle was advanced *ab externo* into the subretinal space exposing the bevel, after which the superior rectus was released and the non-dominant hand used to depress the syringe

plunger in order to deliver 1.5 μ l into the subretinal space with formation of a superior, bullous hemi-retinal detachment.

The needle was withdrawn and eye was inspected to determine the nature, location and size of any intraocular haemorrhage. Small epiretinal haemorrhages limited to the retinal surface which were non-progressive were tolerated. However, any vitreous haemorrhage or sub-retinal haemorrhage, which were always progressive, resulted in Schedule 1 sacrifice and the mouse was not included in the study. The size of the bleb was also assessed. 1.5 μ l of fluid delivered into the sub-retinal space, in the absence of reflux, resulted in a large bleb which obscured the optic disc in the primary position (Figure 2.1). Smaller blebs indicated reflux into the supra-choroidal space or vitreous body (in the case of a retinotomy) led to Schedule 1 sacrifice. Care was taken not to touch the crystalline lens which would result in post-operative cataract. Following successful injection, g.Chloramphenicol 1% (Bausch & Lomb, UK) was applied to the eye followed by Viscotears to prevent desiccation cataract formation. A digital operative record was completed, and the mouse repositioned for injection of the fellow eye, if required.

Animals were recovered as detailed in section 2.5.1. Following the completion of a surgical session, needles and syringes were cleaned and disinfected according to a protocol which involves flushing with an enzymatic cleaner to remove organic

debris and soaking in disinfectant followed by rinsing in sterile water and air drying overnight. Separate needles and syringes were used for each vector or control and cleaned separately to avoid cross-contamination.

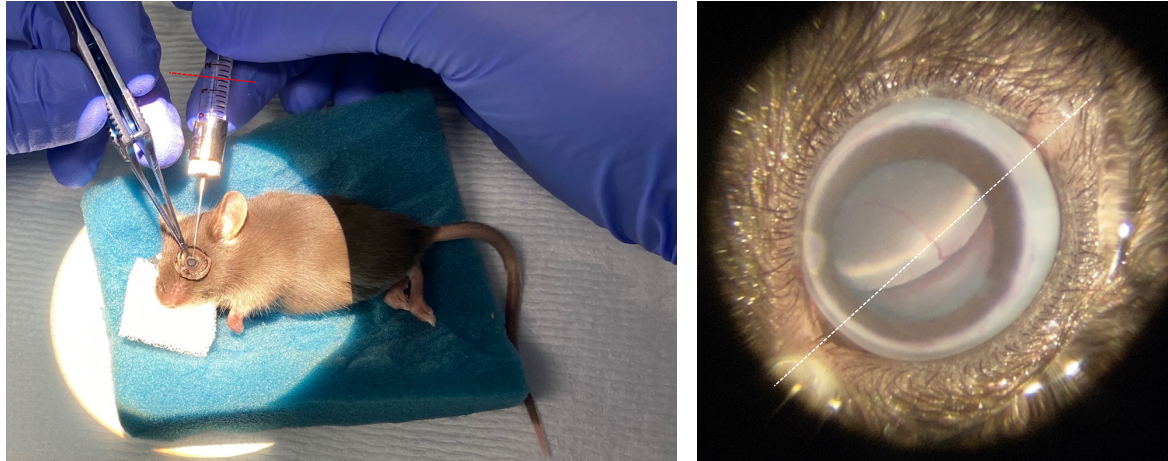


Figure 2.1 Technique of sub-retinal injection and post-operative appearance of sub-retinal bleb

Left: Surgical positioning of a mouse, placement of coverslip, and hand position for subretinal injection by the author. Dashed red line indicates 1.5 μ l of PBS for control injection to the left eye. *Right:* Post-operative appearance of surgically induced superior hemi-retinal detachment with bleb obscuring the optic disc. Dashed white line indicates the horizontal meridian between the canthi.

2.5.3 *In vivo* retinal imaging techniques

Retinal imaging was performed using the Heidelberg Spectralis® HRA2 retinal imaging system (Heidelberg Engineering, Heidelberg, Germany), which houses three laser light sources: 488nm, 790nm and 830nm. Barrier filters remove reflected light, depending on the application. Following general anaesthesia and pupil dilation, 0.5% Hypromellose (Isopto plain 0.5%, Alcon, Camberley, UK) was applied to both eyes. A custom-made rigid (polymethylmethacrylate) contact lens

eyes (diameter: 3.2mm, back optic zone radius: 1.7mm, central thickness: 0.4mm) was applied to both eyes (Cantor & Nissel, Brackley, UK) and centered on the pupil and limbus.

Each mouse was positioned on a specialised raised platform to enable image capture and registered on the Heidelberg software using unique identifiers: colony name, individual mouse designation, ear notch number and date of birth. The c-curve default of 7.7 was left unchanged for all experiments. Prior to retinal imaging, an image of the anterior segment was taken to confirm pupil dilation, centration of the contact lens and to later confirm the laterality of subsequent images.

2.5.3.1 Scanning Laser Ophthalmoscopy (SLO)

Infra-red reflectance confocal Scanning Laser Ophthalmoscopy (SLO) uses an 830nm laser light source. NIR reflectance imaging was used first to attain confocality on the midperipheral retina, and centration of the optic disc. The IR laser intensity was reduced to eliminate over-exposure before IR reflectance image capture with a detector sensitivity of around 65%. Images were recorded using automatic real-time (ART) to reduce the effect of small movements from respiration. ART increases the signal:noise ratio (SNR), whereby the SNR equals the square root of the number of images averaged. The resolution of single averaged images was 1536 x 1536 pixels using the 55° confocal imaging lens.

2.5.3.2 *Fundus autofluorescence (FAF)*

Short-wavelength autofluorescence imaging (SW-AF) uses a laser wavelength of 488nm, and exploits the autofluorescent properties of lipofuscin. With minimal adjustment of the dioptric focus, the detector sensitivity setting was increased to 95. Normalisation was disabled, and Automatic Real Time (ART) function was activated to average ~60 scans with improved resolution of image capture. If an image was not attainable at a sensitivity of 95, the FAF sensitivity was increased to 100, and then to 107 (maximal sensitivity). Near-infra red autofluorescence imaging (NIR-AF) exploits the autofluorescent properties of melanin/ melano-lipofuscin, and was undertaken using an ART averaging 60 scans, and a sensitivity of 100.

2.5.3.3 *Optical coherence tomography (OCT)*

The OCT device used in the described experiments houses a near infra-red laser light source (830nm) to provide high-resolution, cross-sectional images of the retina and other structures at the posterior pole. OCT images were acquired following vertical and horizontal centration on the optic disc. The OCT service screen was accessed to allow adjustment of the reference arm in order to focus on the retinal plane. OCT images were captured in “High Speed” mode with enhanced depth-imaging (EDI) (8 bits per pixel with 1536 x 1536 pixel resolution). Four radial line scans were captured with ART averaging set at 60 images per line scan,

enabling retinal thickness measurements at 8 peripheral retinal locations (i.e. at 45-degree intervals).

In order to analyse the quantitative and qualitative differences between *Cdhr1*^{-/-} and *C57BL/6J* mice, OCT images were exported as uncompressed TIF files, and analysed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, USA) in order to convert the composite B-scan image into A-scans to determine grey values against distance (pixels).

2.5.4 Electroretinography (ERG)

Mice were dark adapted overnight (>12 hours) using a specially designed, ventilated light-tight cabinet. All experiments were undertaken in a dark room with dim head-mounted red-light illumination where required. Mice underwent general anaesthesia and pupil dilation as described above. Electroretinography (ERG) was performed in a custom-made light-tight Faraday cage using the Espion system and software (V6.0.56, Diagnosys LLC Cambridge, UK). The Ganzfeld full field ERG ColourDome™ was used to deliver light stimuli. A heated platform using a circulating water pump was to support thermoregulation of mice during anaesthesia (set to 35°C). Contact lens electrodes were created as previously described⁹² from achromatic ACLAR (Honeywell International, USA) to create custom contact lenses of suitable radius of curvature for the mouse ocular surface. Two small notches were created opposite each other using a scalpel and a DTL-

type silver-coated nylon electrode (Dawson, Trick & Litzkow (DTL) (Diagnosys Ltd., Cambridge, UK) was inserted across the convex surface of the contact lens.

Step	Log	Flash intensity [cd.s/m²]	Repetitions	Inter-stimulus interval [sec]	Interval after intensity step [sec]	
0		Dark adaptation: 600 seconds				
1	-6	0.000001	16	0	10	Scotopic testing
2	-5	0.00001	16	0	30	
3	-4	0.0001	9	1	30	
4	-3	0.001	9	1	30	
5	-2	0.01	9	5	30	
6	-1	0.1	4	13	30	
7	0	1	4	29	60	
8	1	10	4	61	60	
9	1.4	25	1	20	60	
10	0.5	3	20	20Hz flicker		
		Pre-exposure: Rod-saturating steady full-field white background illumination (30 cd/m²) for 10 min				
11	0.5	3	10	2 (0.5Hz)		Photopic testing
12	0.5	3	20	20Hz flicker		
13	1	10	10	2 (0.5Hz)		
14	1	10	20	20Hz flicker		

Table 2.2 Full field electroretinography test protocol

Mice were dark-adapted overnight in a light-tight cabinet for a minimum of 12-hours. A further 10 mins of dark-adaptation was performed prior to recordings.

Following instillation of 0.5% Hypromellose (Isopto plain 0.5%, Alcon, Camberley, UK), the mouse was placed on the pre-warmed platform. Two subcutaneous platinum needles were placed: an earth electrode above the tail, and a reference electrode in the midline of the scalp between the eyes. The contact lens electrodes were placed on each eye. The platform was then engaged with the Ganzfeld

ColourDome™ and the light cabinet was closed. An infrared camera allowed the position and anaesthetic status of the mouse to be monitored throughout the recording. Signals were amplified and digitized at a rate of 5kHz. AC interference was removed with a 50Hz filter where necessary.

A pre-defined ERG protocol (Table 2.2) was programmed into the Espion software to ensure repeatability of presented stimuli and recordings. The baseline trace allowed to stabilise over 5-10 minutes. Dark-adapted, single-flash responses were recorded to light stimuli of increasing intensity, followed by a dark-adapted flicker test. Light adaptation was achieved following exposure to a steady full-field white background illumination of 30 cd.s/m² for 10 minutes, to achieve complete rod saturation. Single-flash cone responses and cone flicker responses at two light intensities were superimposed on this background illumination. Following ERG recordings, electrodes were removed, Viscotears was applied to both eyes and a recovery from general anaesthesia was undertaken as previously stated. Data were exported into MS Excel for Mac (v16.16.27), with custom macros to automate the extraction of data on the mean amplitudes and implicit times of a- and b-wave responses for single-flash recordings, and amplitudes of flicker responses. Data were imported in GraphPad Prism (v.9.1.2) for statistical analysis.

2.5.5 Optomotor reflex behavioural testing

The OMR was examined using a custom designed (University of Oxford, Department of Physics) programmable rotating drum in which an alternating high-contrast black and white vertical grating, optimised and fixed at 0.1 cycles per degree⁹³⁻⁹⁵ (i.e. fixed spatial frequency) was presented (Figure 2.2 & Table 2.3).

Step	Time (s) for 360° rotation	Duration (seconds)	Direction of rotation of OKN drum	Phase & Duration
1	-	180	-	Habituation (3 minutes)
2	15	30	Clockwise	Test protocol (6 minutes)
3	15	30	Anti-clockwise	
4	15	30	Clockwise	
5	15	30	Anti-clockwise	
6	15	30	Clockwise	
7	15	30	Anti-clockwise	
8	30	30	Clockwise	
9	30	30	Anti-clockwise	
10	30	30	Clockwise	
11	30	30	Anti-clockwise	
12	30	30	Clockwise	
13	30	30	Anti-clockwise	

Table 2.3 Test protocol for optomotor reflex responses

The protocol generates data in triplicate for each experimental mouse for head tracks per minute in both clockwise and anti-clockwise rotation at speeds of 15 (fast) and 30 seconds (slow) per rotation.

For photopic testing, mice were exposed to ambient room light for at least 10 minutes, placed on a clean, sterile petri dish on a raised platform in the centre of the drum with 3 minutes for acclimatisation. A fluorescent white light source was

placed above the drum, measured at 1,000 lux at the level of the platform to bleach rods and isolate the cone system. The rotation of the drum was programmed using Autostep software (v3.2, PC control Ltd., UK) with alternating periods of clockwise and anticlockwise rotation to produce 3 technical replicates for each direction. Two speeds were tested: 15s and 30s per 360° rotation. Video recordings (Logitech C920 webcam) of each animal were examined by two observers blinded to the genotype and treatment allocation. Head turns that matched the direction and approximate speed of the grating were counted in each 30 second interval in both directions to produce an output value of mean head tracks per minute across three replicates. Responses from AAV- and PBS-injected eyes were isolated simply by changing the direction of rotation of the OKN drum, as described previously.⁹⁶ 70% ethanol was used to clean the floor of the drum unit with a new petri dish for each animal.

For scotopic testing, mice were dark-adapted overnight in a light-tight cabinet. Sequential neutral density filters were used to reduce the white light source to 0.01 lux to isolate the rod system. A near-infra red USB webcam was used to record mouse behaviour during the test protocol, which was otherwise identical to photopic recordings.

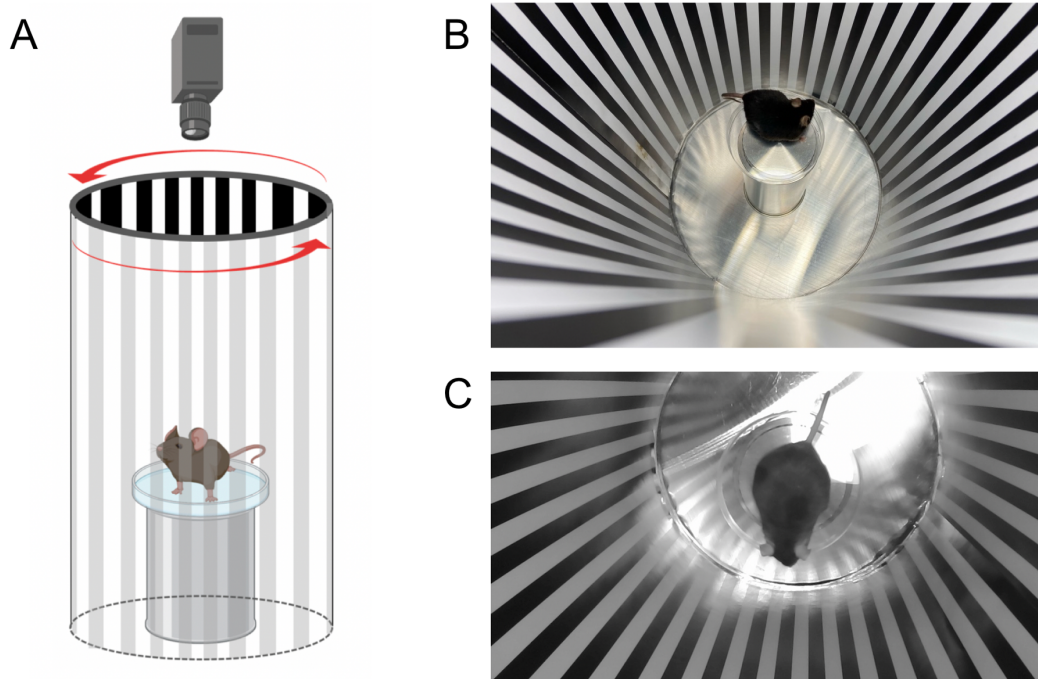


Figure 2.2 Optomotor reflex response testing in mice

(A) Schematic illustration of the optomotor reflex testing apparatus with (B) Photopic and (C) Scotopic capture. A fixed spatial frequency vertical grating optimised at 0.1 cycles per degree was used for all recordings. The mouse was placed on a raised platform on a clean petri dish with white light illumination at 1,000 lux for photopic and 0.01 lux for scotopic optomotor testing. A near infra-red camera was used for recording under scotopic conditions (C).

2.5.6 Light avoidance behavioural testing

Mice are nocturnal animals and naturally prefer dark environments that confer protection against predators. This behavioural preference may be used as a rudimentary examination of vision or light perception in mice. Following overnight dark adaptation in a light-tight box, mice were placed in a custom designed Perspex box containing an aperture between clear and occlusive compartments, allowing a single murine subject to move freely between them. Recordings were undertaken in a dark room with dim red-light illumination where

necessary. An LED array with a green filter (~500nm) to target the peak in spectral sensitivity of mouse rhodopsin was placed above the light compartment, and a light meter used to ensure identical illuminance (lux) for each mouse. A video camera mounted to the LED array was used to record the behaviour of the mouse over 10 minutes (600 seconds), following an initial 60 second period for habituation. The box was meticulously cleaned between individual animals and the room was otherwise vacated for the period of recording. The video recordings were analysed retrospectively by a masked observer to determine the time each mouse spent in the light and dark compartments over the 600 second interval.

2.6 Tissue processing

2.6.1 Immunohistochemistry

Eyes were dissected in 4% paraformaldehyde (PFA) immediately after schedule 1 sacrifice using a 30-gauge needle to puncture the cornea, with subsequent removal of cornea and lens. Eye cups were left in 4% PFA for 30 mins and dehydrated through sequential immersion in increasing concentrations of sucrose (10%, 20% & 30%), with overnight incubation in 30% sucrose at 4°C. Following a single PBS wash, eyes cups were placed into optimal cutting temperature compound (OCT-C) and flash frozen in a metal container containing isopentane placed on dry ice. Eyes were stored at -80°C, and sectioned in the axial plane using a cryostat (Leica Biosystems, UK) at -20°C into 18µm sections onto poly-lysine-D slides, dried overnight at room temperature and then frozen at -20°C.

Slides were thawed and moisture removed before application of hydrophobic pen (Thermo Fisher Scientific, UK) around the sections. Slides were washed three times in 0.01 M PBS for 5 minutes, and permeabilised with 0.2% Triton-X 100 in 0.01 M PBS for 20 minutes. A further three washes with 0.01 M PBS were undertaken. Blocking buffer (10% Normal Donkey Serum (NDS), 0.1% Triton X-100 in 0.01 M PBS) was applied for 1 hour to reduce non-specific binding. Primary antibody was diluted at the specified concentration, diluted in 1% NDS, 0.1% Triton X-100 in 0.01 M PBS, applied to the sections and incubated for at least 2 hours at 4°C; PBS was applied for secondary antibody only controls. Two washes in PBS Tween-20 (0.01 M PBS, 0.05% Tween-20) were performed for 5 minutes, followed by a single PBS wash for 5 minutes. Secondary antibody was applied for 2 hours at room temperature, diluted in 0.1% Triton X-100 in 0.01 M PBS. Slides were washed twice in PBS Tween-20 (0.01 M PBS, 0.05% Tween-20) for 5 minutes. Coverslips were mounted with ProLong™ Diamond Antifade mountant with DAPI (P36962, Thermoscientific), and dried overnight.

Details of the primary and secondary antibodies used are presented in the methods sections of the relevant experimental chapters.

2.6.2 Microscopy

All sections were viewed and captured using the Zeiss confocal laser scanning microscope (LSM-710) which houses 350-nm UV, 488-nm argon and 543-nm HeNe excitation laser light sources. Laser wavelengths were selected on the basis of the absorbance and emission spectra of fluorophores linked to the labelled epitopes, which were located within the section using epifluorescence illumination before image capture. The DAPI channel, Alexa-488 and Alexa-568 channels were typically used to capture nuclear staining and fluorophore-labelled epitopes with white light differential interference contrast (DIC; Normarski) to capture the retinal pigment epithelium. Slides were imaged at 20x magnification, and 63x magnification following oil immersion to reduce spherical aberration where stated. Z-stacks were captured and processed following image acquisition in ImageJ.

2.6.3 Protein extraction and quantification

Whole mouse retinae were dissected from eye cups following Schedule 1 sacrifice. Corneas were punctured with a 30-gauge needle, followed by removal of the cornea, lens and iris. Retinae were exposed by stretching the sclero-choroidal cup and removing the retina by the optic nerve stalk with minimal direct handling of tissue. Whole retinae were placed into 50µl of RIPA lysis buffer (Sigma-Aldrich, UK) in which a protease inhibitor tablet had been added (Roche Diagnostics, UK) and placed immediately on dry ice. Retinal lysates were disrupted by homogenisation using sterile, RNase-free pestles for 15 seconds, placed on a tube

rotator at 20rpm at 4°C for 30 minutes, and then centrifuged at 14,000g for 10 minutes at 4°C following which the supernatant was transferred into a new microcentrifuge tube and kept on ice.

Cell pellets were also analysed by western blot following transfection as positive and negative controls as appropriate. Media was removed and cells washed twice with 1ml of cold sterile PBS, following which cells were removed and wells washed with an additional 500 µL of PBS. Centrifugation at 1000g was undertaken for 5 minutes at 4°C. One further wash with PBS was undertaken and cell pellets stored at -80°C if required. Cell pellets were then resuspended with RIPA buffer/protease inhibitor tablet cocktail and homogenised as per whole retinal samples.

Protein quantification was achieved using the Quickstart™ Bradford Dye Kit which assesses protein concentration through colorimetric measurements against a standard serial dilution of bovine serum albumin (BSA) of known concentration. A standard dilution series of stock BSA at 2 mg/ml was undertaken using RIPA buffer/protease inhibitor solution as the diluent. A 1:10 dilution of the test samples was also undertaken. 5 µL of all samples and standards were loaded in duplicate onto a clear-bottomed 96-well plate. 250 µL of Bradford dye reagent was added to each well, incubated at 20°C for 5 minutes, and read using the iMark™ microplate reader (BioRad, UK) with absorbance read at 595nm using Galapagos software.

The protein concentration each sample was calculated in Excel by interpolating from the BSA standard curve.

2.6.4 Western blot

Western blot was used for detection of Cdhr1 protein in wildtype murine retinae and of human CDHR1 isoforms following transgene expression. Equivalent quantities of protein, typically 30-60 µg were diluted as appropriate with RIPA-protease inhibitor together with protein loading buffer (National Diagnostics, USA) before loading into the wells of a pre-cast 10% Criterion® TGX™ Tris-glycine extended gel (BioRad, UK) for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Following submersion in tris/glycine/SDS running buffer (National Diagnostics, USA) in a BioRad western blot gel tank, samples were added separately, with 5 µL of BLUeye pre-stained protein ladder (5671024, GeneFlow, UK). Samples were run at 100V for 1-2 hours to allow protein bands to separate according to electrophoretic mobility. Thereafter, proteins were transferred to a LF-PVDF membrane using the Trans-Blot Turbo transfer system (Bio-Rad, UK) in accordance with the manufacturer's instructions. The LF-PVDF membrane was soaked in tris-buffered saline (TBS) for 2 minutes. 50mls of blocking buffer (5% donkey serum (D9663, Sigma-Aldrich) in TBS-T) was then applied with incubation and gentle rotation for 1 hour. The buffer was then discarded and the membrane submerged in primary antibody solution (i.e. 1:500 of rabbit anti-CDHR1 (c-terminal, Johns Hopkins, gifted) and 1:2,000 mouse anti-

GAPDH (ab8245, Abcam) in TBS-T and 1% donkey serum) with incubation and gentle rotation for at least 1 hour. The membrane was then washed three times in 30mls of TBS-T, with application of fluorescent-tagged secondary antibodies in solution (i.e. 1:10,000 of donkey anti-rabbit IRDye 800CW (926-32212) and donkey anti-mouse IRDye 680RD (926-68072; both LICOR, NE, USA) in TBS-T) for 1 hour under dark conditions. Three further washes with TBS-T were then undertaken, the PVDF membrane dried, and imaged using the Li-Cor Odyssey Fc imaging system (LI-COR Inc., USA) using the 700nm and 800nm detection channels.

2.7 Statistical analysis

Data were collated in spreadsheets using Excel for Mac (v16.16.27) prior to statistical analysis and graphical presentation in GraphPad Prism (v9.1.2). Quantitative measures are presented as mean $\pm$ 95% CI unless otherwise stated. Datasets were analysed independently for normality using Shapiro-Wilk, and parametric tests applied, if proven. Parametric datasets were analysed using paired or unpaired t-tests if comparing two or more groups, with Welch's correction applied if the standard deviation was unequal between groups. For comparisons of three or more groups, two-way ANOVA was used, with correction for multiple testing undertaken as appropriate using Tukey's, Dunnett's, or Šídák's multiple comparison tests depending on the dataset. Non-parametric datasets were analysed using Wilcoxon's test if more than one variable was present, otherwise Mann-Whitney test was used as appropriate. Kaplan-Meier

survival data was analysed using both Mantel-Cox and Gehan-Breslow-Wilcoxon tests to evaluate differences between groups. Statistical significance was defined as $P < 0.05$ for all tests.

Power calculations (*a priori* and *ad hoc*) were undertaken using G*Power (software version 3.1.9.3).

3. Phenotyping the *Cdhr1*^{-/-} mouse

3.1 Introduction

Animal models continue to serve useful roles in expanding the understanding of human retinal disease, and support the development and translation of novel therapies. The constitutive disruption (knock-out) or sequence modification (knock-in) of specific genes enables the characterisation of molecular, biochemical, histological/morphological, functional and behavioural effects of genetic modifications. The expression of fluorophores under the promoters of photoreceptor transcription factors (i.e. *Nrl* or *Crx*) enables further characterisation of specific photoreceptors in the disease model following cross breeding. Further control of gene expression may be achieved using conditional knockout models or inducible promoters. Animal models enable the *in vivo* evaluation of the efficacy and safety of therapeutic intervention as part of proof-of-concept pre-clinical studies and support subsequent regulatory approval.

Small animal models (i.e. rodents) have particular advantages over larger animals (i.e. canine, pig, non-human primate) in that a shorter lifespan enables the longitudinal characterisation of slowly progressive retinal degeneration. The experimental use of mice is relatively time- and cost-efficient compared to larger animals, enabling the inclusion of larger numbers of experimental animals to increase statistical power. The retinal cell biology of rodent models may be

considered closer to humans when compared to lower order vertebrates (i.e. *Danio riero*, *Xenopus laevis* etc.). However, some notable exceptions exist, such as the absence of retinal degeneration in USH1 murine models due to the absence of calyceal processes in rodents where the USH1 proteins are expressed.⁹⁷ Numerous mutant and transgenic murine models of retinal disease are available. However, validation of a chosen model is required in order to demonstrate suitability for downstream assessments in any experiment. Murine models of monogenic retinal degeneration do not necessarily recapitulate the human phenotype, signifying important inter-species differences in retinal cell biology and anatomy. For example, rodents lack an anatomical macula – a key limitation in the characterisation of inherited maculopathies, such as *ABCA4* retinopathy or Best's disease, or acquired maculopathies such as age-related macular degeneration (AMD) or hydroxychloroquine retinopathy.

Mice homozygous for the targeted *Cdhr1* allele (henceforth designated *Cdhr1*^{-/-}), were shown to lack *Cdhr1* mRNA and protein on western blot, immunohistochemistry and in situ hybridization. Outer segments were shown on immunoelectron microscopy at 1-month to be shortened, misaligned and disorganised, associated with a 50% reduction in rhodopsin.⁴⁷ Histological analysis at 6-months of age indicated that the outer nuclear layer, representing the cell bodies of rod and cone photoreceptors was thinned by approximately 50%. Furthermore, electroretinography undertaken at 2-months showed an

approximate 2-3-fold reduction in rod and cone responses.⁴⁷ However, longitudinal assessment of retinal function was not undertaken in order to determine the natural history of rod- and cone-function in the disorder. Moreover, *in vivo* retinal imaging techniques (such as OCT and fundus autofluorescence imaging) were not available at the time, and longitudinal morphological data are therefore lacking. Naturally occurring models of *Cdhr1*-associated retinal degeneration have not yet been described in any animal species.⁹⁸ However, this may in part relate to the relatively recent discovery of human *CDHR1*-associated retinal degeneration.

Molecular therapies which aim to modify the genetic sequences of living organisms (i.e. gene therapy, gene editing) can be studied *in vivo* using small animal models to permit the longitudinal assessment of the effects of an intervention. This allows for an assessment of the safety and efficacy of test vectors in pre-clinical studies, beyond that which can be provided from cell-based assays or retinal organoids where cellular immune responses are absent. Moreover, observed dose-response relationships and toxicity studies in small animal models may predict responses in human studies and aid in the determination of an approximate dosing range in early clinical trials through allometric scaling. In pre-clinical studies, the evaluation of functional and behavioural responses following intervention may support the case for regulatory approval towards clinical trial. Consequently, murine models have been crucial in the pre-clinical development of gene therapy vectors that are now under evaluation in clinical trials in humans (Table 3.1).

Disease	Gene	Rodent models ^(ref)	Vector construct	Clinical Trial
Leber congenital amaurosis	<i>RPE65</i> (LCA2)	<i>Rpe65</i> ^{-/-} (99)	AAV2/2.CMV.RPE65 AAV2/2.CMV.RPE65 AAV2/2.CMV.RPE65 AAV2/2.CBSB.RPE65 AAV2/2.CBA.RPE65 AAV2/2.CBA.RPE65 AAV2/2.hRPE65p.RPE65	NCT00999609 NCT01208389 NCT00516477 NCT00481546 NCT00749957 NCT00821340 NCT00643747
	<i>CEP290</i> (LCA10)	<i>Cep290</i> ^{rd16} (100) <i>Cep290</i> ^{rd16/Nrl} ^{-/-} (101)	AAV2/5.GRK1.CRISPR (EDIT-101)	NCT03872479
Choroideremia	<i>CHM</i>	<i>Chm</i> ^{null} (102)(101, 103)	AAV2/2.CAG.CHM AAV2/2.CAG.CHM AAV2/2.CAG.CHM AAV2/2.CAG.CHM AAV2/2. CHM AAV2/2.CAG.CHM AAV2/2.CAG.CHM AAV2/2.CAG.CHM	NCT03496012 NCT03507686 NCT02671539 NCT02407678 NCT02341807 NCT01461213 NCT02077361 NCT02553135
X-linked RP	<i>RPGR</i>	<i>Rpgr</i> ^{-/y} (104) <i>C57BL/6J</i> ^{Rd9/Boc} (104)	AAV2/8.GRK1.coRPGR AAV2tYF.GRK1.RPGR AAV2/5.GRK1.RPGR	NCT03116113 NCT03316560 NCT03252847
X-linked retinoschisis	<i>RS1</i>	<i>Rs1h</i> ^{-/y} (105)	AAV2/8.RS/IRBP.RS1(sc) AAV2tYF.CBA.RS1	NCT02317887 NCT02416622
Achromatopsia	<i>CNGA3</i>	<i>Cnga3</i> ^{-/-} (106)	AAV2tYF.PR1.7.CNGA3 AAV2/8.hCARp.CNGA3 AAV2/8.hG1.7p.coCNGA3	NCT02935517 NCT02610582 NCT03758404
	<i>CNGB3</i>	<i>CnGB3</i> ^{-/-} (107)	AAV2tYF.PR1.7.CNGB3 AAV2/8.hCARp.CNGB3	NCT02599922 NCT03001310
Leber's hereditary optic neuropathy	<i>ND4</i>	<i>ND4</i> ^{R340H} (108)	AAV2/2.CMV.ND4 AAV2/2.CMV.ND4 AAV2/2.CMV.ND4 AAV2/2.CMV.ND4 AAV2/2.CMV.ND4 AAV2/2.CMV.ND4 AAV2tYF.CAG.ND4	NCT03293524 NCT03153293 NCT02064569 NCT02652767 NCT02652780 NCT01267422 NCT02161380
Retinitis pigmentosa	<i>MERTK</i>	RCS rat ⁽¹⁰⁹⁾	AAV2.2.VMD2.MERTK	NCT01482195
	<i>PDE6B</i>	<i>Pde6b</i> ^{rd1} (110) <i>Pde6b</i> ^{rd10} (111)	AAV2/5.GRK1.PDE6b	NCT03328130
	<i>RLBP1</i>	<i>RlbP1</i> ^{-/-} (112)	AAV8.scRLBP1.pRLBP1	NCT03374657

Table 3.1 AAV gene therapy vectors in clinical trial that were developed using murine models

Murine models were pivotal in the pre-clinical development of retinal gene therapies that are now in clinical trials. Note the wide variation in mode of vector delivery, target cell and severity of the clinical phenotype. The information in this table has been aggregated from Clinicaltrials.gov and the EU clinical trials register, current as of November 2021. co – codon optimised; RP – retinitis pigmentosa; XLRs – X-linked retinoschisis; sc – self-complementary; RCS – Royal College of Surgeons; CMV – cytomegalovirus; CBA – chicken beta-actin; GRK1 – human rhodopsin kinase; CAG – synthetic promoter composed of the CMV early enhancer element, the promoter, the first exon and intron of chicken beta-actin gene and the splice acceptor of the rabbit beta-globin; VMD2 – bestrophin gene promoter. CRISPR indicates unpublished active editing constructs.

Deep phenotyping of murine models of human retinal degeneration is necessary in order to validate them for the development and evaluation of candidate therapeutics. Moreover, longitudinal structural and functional observations from animal models may provide further insights into the human disease, particularly for slowly progressive forms of retinal degeneration. Such data may inform the design of natural history studies for the human disorder, and in the identification of relevant endpoints for pre-clinical and clinical studies.

3.2 Aims

The aims of this chapter were:

1. To determine whether the retinal phenotype of the *Cdhr1*^{-/-} murine model is consistent with human *CDHR1*-associated cone-rod dystrophy (i.e. to validate the *Cdhr1*^{-/-} mouse as a disease model).
2. To define the structural, morphological, electrophysiological and visual behavioural characteristics of retinal degeneration in *Cdhr1*^{-/-} mice to inform pre-clinical study design, and against which the effect of any therapeutic intervention can be evaluated in pre-clinical and clinical studies.
3. To identify novel observations that might be relevant to further the understanding of human *CDHR1*-associated retinal degeneration.

3.3 Materials and methods

3.3.1 Experimental mice

B6;129-Cdhr1^{tm1Nat}/J mice were obtained from Jackson laboratories (Jackson Laboratory, Bar Harbor, ME, USA) as detailed in Chapter 2. The wildtype strain for comparison were *C57BL/6J* mice acquired from Charles River (Margate, Kent, UK) to which the *Cdhr1*^{-/-} mice were backcrossed. This colony is maintained according to the Genetic Stability Program in which cryopreserved embryos are used to maintain colonies every five generations to reduce genetic drift.

3.3.1.1 *Development of a PCR-based genotyping assay*

The Jackson Laboratory genotyping primers were not able to differentiate heterozygous and homozygous mice from the *Cdhr1* line. Personal communication with Dr. Amir Rattner (PhD, Johns Hopkins), suggested that genomic DNA from the *Cdhr1* gene had reinserted elsewhere in the genome during the targeting process; southern blotting was consequently used by their laboratory for genotyping litters. Amplification of this genomic sequence occurred on PCR using wildtype primers in the homozygous mouse, which could not therefore be distinguished from heterozygous littermate controls (*Cdhr1*^{+/-}).

New wildtype primer pairs were designed in collaboration with John Hunyara, PhD student, Johns Hopkins), exploiting a single nucleotide polymorphism in the *C57BL/6J* genome (NCBI Gene ID:170677) located ~1,000bp downstream of the

final coding exon (Table 3.2). This accurately genotyped *Cdhr1* mice bred on a *C57BL/6J* background (Figure 3.1). The SNP was sufficiently close to the targeted region to reduce the risk of genetic recombination during meiosis between itself and the *Cdhr1* gene. DNA was extracted from mouse ear notches or tail by alkaline lysis and PCR amplified using ImmoMix (Bioline Reagent Ltd, UK). PCR cycling conditions were as follows: 95°C for 3 mins; 35 cycles of [95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds], 1 cycle of 72°C for 10 mins, 4°C hold.

CDHR1_WT_FWD1	5' - ATCTGGTCTGAGAGCCCCCAT - 3'
CDHR1_WT_RV1	5' - CAAACACAGACTGGGGGCGGTA - 3'
CDHR1_KO_FWD1	5' - GGAAGGTGCCACTCCCCTGTCC - 3'
CDHR1_KO_RV1	5' - CTTACCTGTTTGTATCGCTACATCC - 3'

Table 3.2 Primers used to genotype *Cdhr1*^{-/-} mutant mice by PCR

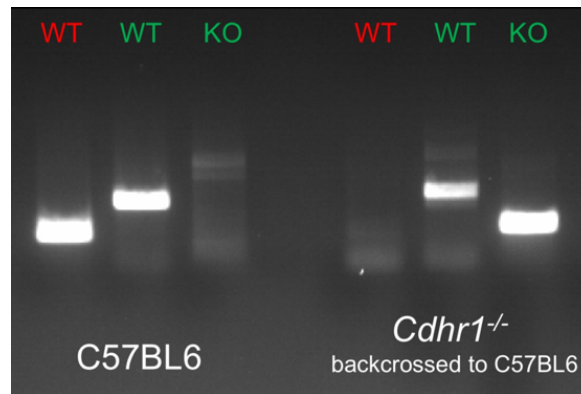


Figure 3.1 PCR-based genotyping of *Cdhr1*^{-/-} mice

1% agarose gel electrophoresis shows amplicons produced by wildtype primer pairs provided by Jackson Laboratories (green label) and new primer pairs (red label). An amplicon is absent in *Cdhr1*^{-/-} mice backcrossed to the *C57BL/6J* strain using the new primer pairs. Amplicon size on 1% agarose gel electrophoresis were ~300bp for both wildtype and knockout primer pairs.

3.3.1.2 Backcrossing the *Cdhr1*^{-/-} allele to the *C57BL/6J* strain

Six generations of backcrossing of the mutant *Cdhr1*^{-/-} allele to the *C57BL/6J* background was undertaken prior to experimental work, in order to reduce the risk of unknown genetic variants producing variability between mice. It is notable that the original study describing the *Cdhr1*^{-/-} mouse found no difference between ERG responses between those bred on a *C57BL/6J* or *129* background.⁴⁷ The hypothetical genetic identity between pups of the final backcross was approximately 98.38%. Furthermore, only one other gene on murine chromosome 14 was known to influence visual function: *Opn4* which is encoded 3.5 million base pairs 3' of *Cdhr1* on the reverse strand. This was not sequenced directly.

3.3.2 CDHR1 immunohistochemistry

Four littermate mice of a *Cdhr1*^{+/-} heterozygous cross were selected (one wildtype, one *Cdhr1*^{+/-} and two *Cdhr1*^{-/-}), all 5 months of age at tissue harvesting. Tissue was harvested and sectioned as per methods detailed in Chapter 2.

Three anti-CDHR1 primary antibodies (Johns Hopkins (gifted), Abcam (ab36669) and Sigma-Aldrich (HPA036819)) were optimised and validated using immunohistochemistry in both *C57BL/6J* and *Cdhr1*^{-/-} mice, with an absence of published data with commercially available antibodies (Table 3.3). Four additional anti-CDHR1 primary antibodies were tested without signal at high concentrations on immunohistochemistry (i.e. Santa Cruz (sc-514574)), or were unable to detect

Cdhr1 in wildtype retinal lysates on western blot analysis (Sigma Aldrich (HPA036819), Thermo Scientific (PA5-43510), St Johns Laboratory (STJ119835)).

Manufacturer	Antigenic epitope (aa)	Homology	CDHR1 domain	Clonality & Conjugation	Host Species	Concentration
Abcam	50-100	98%	1 st cadherin repeat	Polyclonal Unconjugated	Chicken	IHC: 1:100 – 1:500
Sigma-Aldrich	250-325	84%	3 rd cadherin repeat	Polyclonal Unconjugated	Rabbit	IHC: 1:100 – 1:1,000
Johns Hopkins*	844-858	87%	C-terminus	Polyclonal**	Rabbit	IHC: 1:1000 – 1:20,000 WB: 1:500

Table 3.3 Anti-CDHR1 primary antibodies

Optimised anti-CDHR1 primary antibodies used for immunohistochemistry (IHC) and western blot (WB). Homology specifies the percentage identity between human CDHR1 and murine Cdhr1 amino acid sequences across the antigenic epitope. Concentrations indicate those used to identify Cdhr1 in the specified assays. aa – amino acid; * - unpurified whole serum, gifted by Jeremy Nathans' laboratory, Johns Hopkins, USA.

3.3.3 CDHR1 western blot

Four age-matched mice (one wildtype, one *Cdhr1*^{+/-} and two *Cdhr1*^{-/-}) all 8-9 weeks of age were selected. The retinal phenotype was confirmed using OCT and fundus autofluorescence imaging under non-recovery terminal anaesthesia before tissue recovery. Retinae were harvested in cold PBS, placed in 50µl of RIPA buffer and flash frozen on dry ice. Untransfected HEK293T cells were used as a negative control and those transfected with CAG.CDHR1.WPRE.pA and recombinant human CDHR1 protein missing the c-terminus (Abcam; ab164895; amino acids 1-

745) were used as a positive control. All samples were processed as detailed in Chapter 2. 30µg of protein was loaded into each lane of a 10% tris TGX® gel.

Western blot undertaken using the gifted Johns Hopkins anti-CDHR1 antibody successfully detected full-length and c-terminally cleaved CDHR1 in wildtype retinal lysates. Sigma-Aldrich, Thermo Scientific, St. John's Laboratory and Abcam anti-CDHR1 primary antibodies were unsuccessful at detecting CDHR1 in wildtype retinal lysates, although detected a high-concentration of recombinant human CDHR1 protein (data not shown). Anti-CDHR1 primary antibody gifted by Johns Hopkins was used at a concentration of 1:500, which was used with loading control primary antibody anti-mouse GAPDH (Origene) at a concentration of 1:2,000. Secondary antibodies linked to fluorophores were used at a concentration of 1:10,000; donkey anti-rabbit (800cW) (against anti-CDHR1 primary antibody) and donkey anti-mouse (680cW) (against anti-GAPDH primary antibody).

3.3.4 Optical coherence tomography imaging

OCT imaging was performed as detailed in Chapter 2, according to a standardised protocol for all animals. All retinal thickness measurements were taken at 8 identical mid-peripheral retinal loci in each mouse using the callipers on the Heidelberg Spectralis imaging software with measurements recorded along the A-scan.

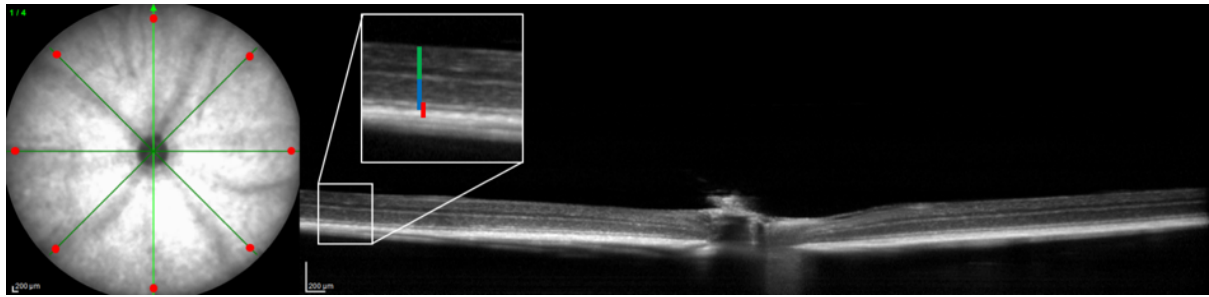


Figure 3.2 Retinal measurements derived from OCT imaging

Red dots on the SLO NIR reflectance image identify the retinal loci at which measurements are taken in each eye. The bars on the OCT B-scan (*C57BL/6J*) label the limits of measurement for inner retinal thickness (green bar), photoreceptor layer thickness (blue bar), and ellipsoid zone to RPE (red bar). Note that measurements were recorded along the axis of the A-scan.

3.3.4.1 OCT image analysis

Outer retinal thickness measurements were the photoreceptor layer thickness (outer plexiform layer to the interdigitation zone) and ellipsoid zone (where present) to the RPE to capture photoreceptor outer segments which were too short to measure directly in *Cdhr1*^{-/-} mice. A pilot study identified loss of the ellipsoid zone band before 6 months of age in *Cdhr1*^{-/-} mice. Inner retinal thickness was defined as the distance between the outer plexiform layer and the internal limiting membrane. Definitions of retinal laminae on SD-OCT imaging were as those described in humans.¹¹³ Consistent retinal locations for manual retinal thickness measurements were ensured by exact centration on the optic disc (Figure 3.2).

ImageJ (NIH, Bethesda, USA) software was used to convert OCT B-scan images into greyscale values, plotted against distance in pixels to produce an approximation of the original A-scan for analysis. This allowed a direct

comparison of traces between genotype groups, retinal locations and retinal imaging modes in order to detect the presence or absence of retinal laminae, and to identify differences in retinal thickness measurements. Briefly, OCT images from the Spectralis were imported to ImageJ, normalised to the signal from the vitreous cavity. A-scans were aligned along their length (pixels) to the internal limiting membrane. Grey values were plotted using GraphPad Prism.

3.3.5 Fundus autofluorescence imaging

Fundus autofluorescence imaging was undertaken according to a standardised protocol for all animals (Chapter 2). Short-wavelength fundus autofluorescence (SW-AF) imaging was undertaken with detector sensitivity settings of 95, and then 100, without normalisation using 488nm excitation light. Long-wavelength autofluorescence imaging (NIR-AF) was acquired at a sensitivity setting of 100 using 790nm excitation. Each SW-FAF image was graded based on the presence or absence of autofluorescent dots, and whether they were localised (occupying less than half of the imaging field), or generalised (more than half of the imaging field). Semi-quantitative FAF imaging analysis at did not detect a difference in autofluorescent signal between age-matched *Cdhr1*^{-/-} and *C57BL/6J* mice at 6-months ($P=0.82$; $n=3$) or 12-months of age ($P=0.35$; $n=4$).

3.3.6 Electroretinography

Electroretinography was undertaken as described in Chapter 2.¹¹⁴ ERG a- and b-wave amplitudes were measured using Espion v6.0 (Diagnosys LLC). Technically poor replicates were excluded rarely, due to the drying of coupling fluid which was replenished and protocol continued. Trials within the same step were averaged. A-waves amplitudes were measured from baseline to the trough of the initial negative deflection and B-wave amplitudes were measured from the trough of the A-wave where present, or from the amplitude at stimulus onset when absent, to the peak of the B-wave. All A-wave and B-wave markers were inspected and adjusted manually where automated designations were inaccurate.

3.3.7 Behavioural testing: optomotor reflex and light avoidance testing

Photopic (1000 lux) OMR testing was undertaken in both *Cdhr1*^{-/-} and *C57BL/6J* mice at 20-months of age ($n=4$ in each group), and scotopic (0.01 lux) OMR testing at 24-months of age using the protocol outlined in section 2.5.5. Light avoidance behaviour was assessed in *Cdhr1*^{-/-} and *C57BL/6J* mice at 18- and 20-months of age ($n=4$ in each group) as detailed in section 2.5.6.

3.3.8 *Cdhr1*^{-/-} natural history study design

Deep phenotyping of *Cdhr1*^{-/-} and *C57BL/6J* wildtype mice was performed over 24 months, using retinal imaging studies and dark- and light-adapted electroretinography, supported by behavioural testing at late timepoints (Figure

3.3). Female mice were used in order to reduce the risk of attrition from group-housed male mice which may have reduced the power of the study at later timepoints.¹¹⁵ Data from the right eye of each animal was included for analysis, since paired eyes were not independent. Separate groups of mice were assigned to each timepoint with ERG performed one week prior to retinal imaging studies. This enabled the experiment to fall within the maximum number of anaesthetic sessions permissible under the licence. Furthermore, the statistical power of the experiments would remain unaffected with attrition.

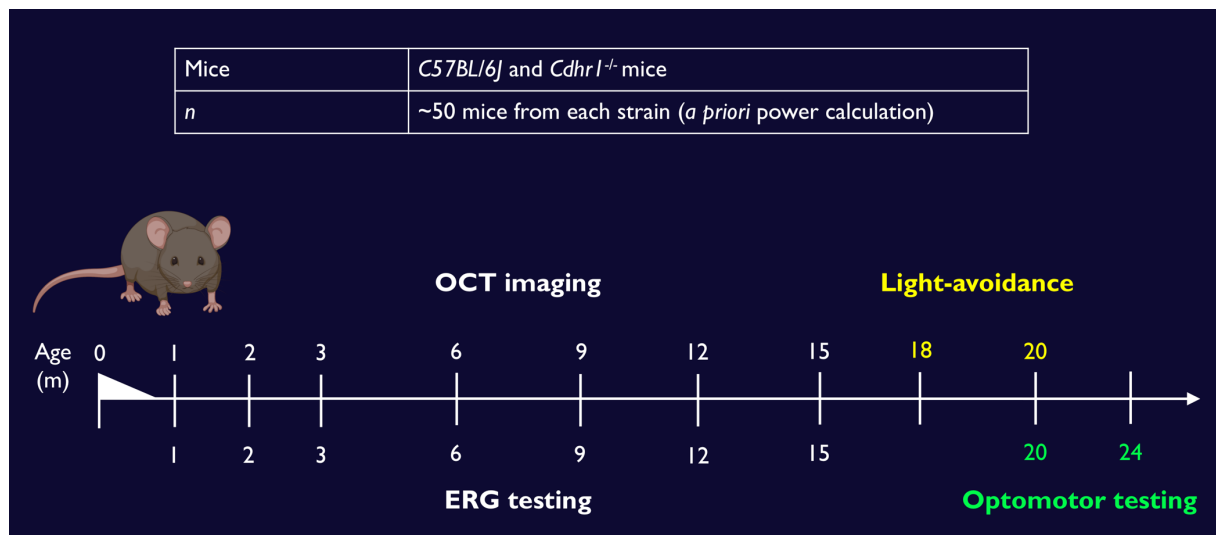


Figure 3.3 Experimental design of *Cdhr1*^{-/-} natural history study

Separate cohorts of aged *Cdhr1*^{-/-} and *C57BL/6J* mice were used for each timepoint for ERG and OCT assessments, which were undertaken 1-week apart (white font). The size of experimental groups was determined by an *a priori* power calculation (99% power) based on both ERG and OCT data in a pilot study. Behavioural testing assessing light-avoidance (yellow font) and optomotor responses (green font) were undertaken at later timepoints as indicated.

3.3.8.1 *A priori power calculation for Cdhr1^{-/-} natural history study*

In order to determine the number of mice required to detect statistically significant differences between ERG assessments and OCT measurements in *Cdhr1^{-/-}* mice and *C57BL/6J* controls, an *a priori* power calculation was undertaken based on the effect size determined in a pilot study (Table 3.4). The number of mice chosen in each group was appropriate to the housing density in the animal facility (i.e. 6 mice per cage).

Age (months)	Effect size (<i>d</i>) ERG*	Effect size (<i>d</i>) OCT**	Mice required in each group to achieve 99% Power (<i>n</i>)		Final number of mice in each group
			ERG	OCT	
1	0.15	0.143	12	3	12
2	0.59	0.16	2	2	6
3	0.6	0.20	2	2	6
6	0.62	0.34	3	3	6
9	-	0.51	-	2	6
12	0.953	0.61	2	2	6
15	-	-	-	-	6

Table 3.4 *A priori power calculation for Cdhr1^{-/-} natural history study*
Power calculations were determined from the effect size on ERG and OCT assessments in a pilot study. *based on dark-adapted, A-wave amplitudes at 10cd/cm²/sec; ** based on outer retinal thickness measurements (OPL-RPE) on OCT imaging; *d* – effect size (calculated as the difference between the means, divided by the standard deviation of the group with the greater variation). Two-tailed test; $\alpha = 0.05$. Multiples of 6 were to match the housing density of animals.

3.4 Results

3.4.1 *Cdhr1* protein is absent in *Cdhr1*^{-/-} mice on immunohistochemistry

Immunohistochemistry with three primary anti-*Cdhr1* antibodies identified a relatively low abundance of *Cdhr1* protein at the junction of the inner and outer segments in *C57BL/6J* mice using Johns Hopkins c-terminal antibody (at all concentrations up to 1:20,000), Sigma-Aldrich (>1:1,000) and Abcam antibodies (>1:1,000). There did not appear to be a difference in staining pattern or approximate abundance between antibodies. *Cdhr1* protein was not present in *Cdhr1*^{-/-} retinas with any anti-*Cdhr1* antibody (Figure 3.4), nor in secondary antibody only controls (data not shown).

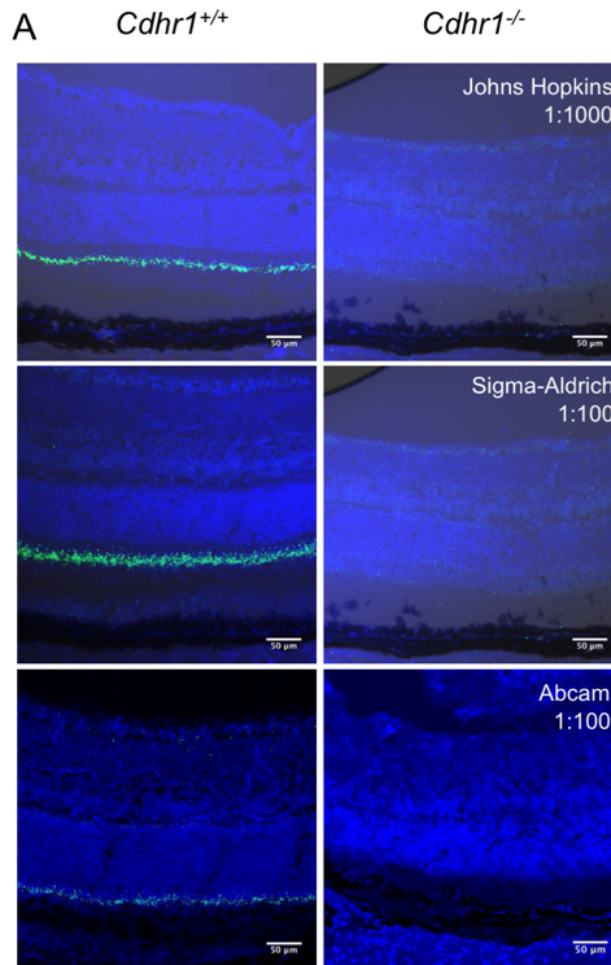


Figure 3.4 Immunohistochemistry in *C57BL/6J* and *Cdhr1*^{-/-} mice

Immunostaining with three anti-CDHR1 antibodies (green fluorescent signal) identifies *Cdhr1* at the base of the photoreceptor outer segments in *C57BL/6J* mice, not present in *Cdhr1*^{-/-} mice. Nuclei are stained with DAPI within the mounting medium. Concentrations of primary antibodies used for each micrograph are indicated. Scale bars represent 50µm.

3.4.2 *Cdhr1* protein is absent in *Cdhr1*^{-/-} mice on western blot

Western blotting using the Johns Hopkins antibody identified two CDHR1 protein fragments, a full-length fragment of around 120kDa and a c-terminal fragment of around 25kDa in both *Cdhr1*^{+/+} and *Cdhr1*^{+/-} mice. The c-terminal fragment appears more abundant, which may in part relate to the increased solubility of the

N-terminal fragment.⁵¹ *Cdhr1*^{+/-} mice produce 50% of the protein of wildtype mice; Figure 3.5 demonstrates bands of reduced intensity of the same molecular weight in *Cdhr1*^{+/-} littermates. *Cdhr1*^{-/-} retinal lysates did not demonstrate *Cdhr1* protein, as per the negative control sample.

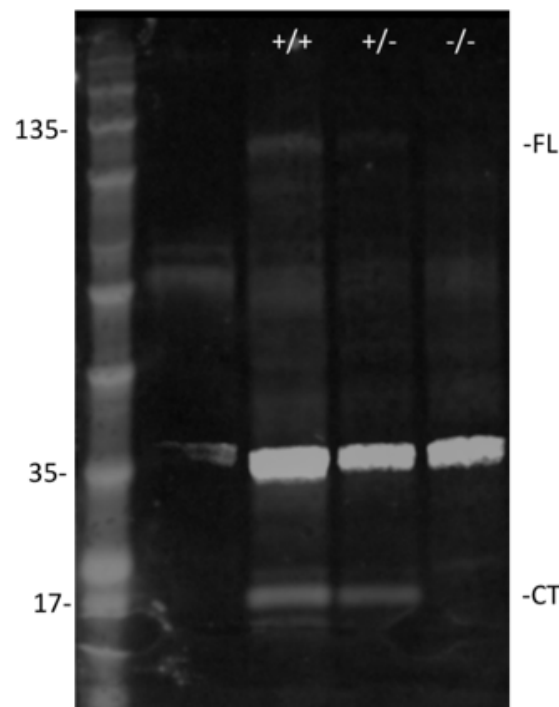


Figure 3.5 Western blot analysis in *C57BL/6J* and *Cdhr1*^{-/-} mice

Full-length (FL) *Cdhr1* ~120kDa, and a C-terminal (CT) fragment ~25kDa are present in *C57BL/6J* whole retinal lysate. Less abundant bands of the same size are seen in *Cdhr1*^{+/-} mice. *Cdhr1* protein is absent in *Cdhr1*^{-/-} retinal lysates and lysate of cultured HEK293T cell, negative control, lane 1. The loading control labels mouse GAPDH.

3.4.3 Attenuation of the ellipsoid zone and shortening of photoreceptor segments is seen on OCT imaging at 1-month in *Cdhr1*^{-/-} mice

OCT imaging identified qualitative and quantitative abnormalities of the outer retina in *Cdhr1*^{-/-} mice compared to age-matched wildtype controls (Figure 3.6).

In *Cdhr1*^{-/-} mice, the ellipsoid zone was present but with reduced reflectivity (i.e. similar reflectivity to the ELM) at 1-month of age with associated shortening of the layer representing the photoreceptor outer segments. These morphological differences are evident on reflectivity profiles compared to age-matched *C57BL/6J* controls (Figure 3.6). In *Cdhr1*^{-/-} mice, the ellipsoid zone, and therefore the band representing the photoreceptor outer segments, were no longer identifiable at 6 months of age. The ELM was identifiable up to the age of 6 months, but was not clearly discernible at later time-points.

3.4.4 *Cdhr1*^{-/-} mice exhibit progressive outer retinal degeneration

Photoreceptor layer thickness measurements identified significant thinning in 1-month old *Cdhr1*^{-/-} mice versus *C57BL/6J* controls (mean 84.4 μm vs 102.6 μm at all retinal locations; $P < 0.0001$). Degeneration of the outer retina was progressive in *Cdhr1*^{-/-} mice at all retinal locations: $P < 0.0001$ for 1-month vs 3-15 month time-points) (Figure 3.6). In *C57BL/6J* mice, outer retinal thinning was identified at 12 ($p = 0.0006$) and 15 months ($P < 0.0001$) but not at earlier timepoints compared to baseline measurements at 1 month.

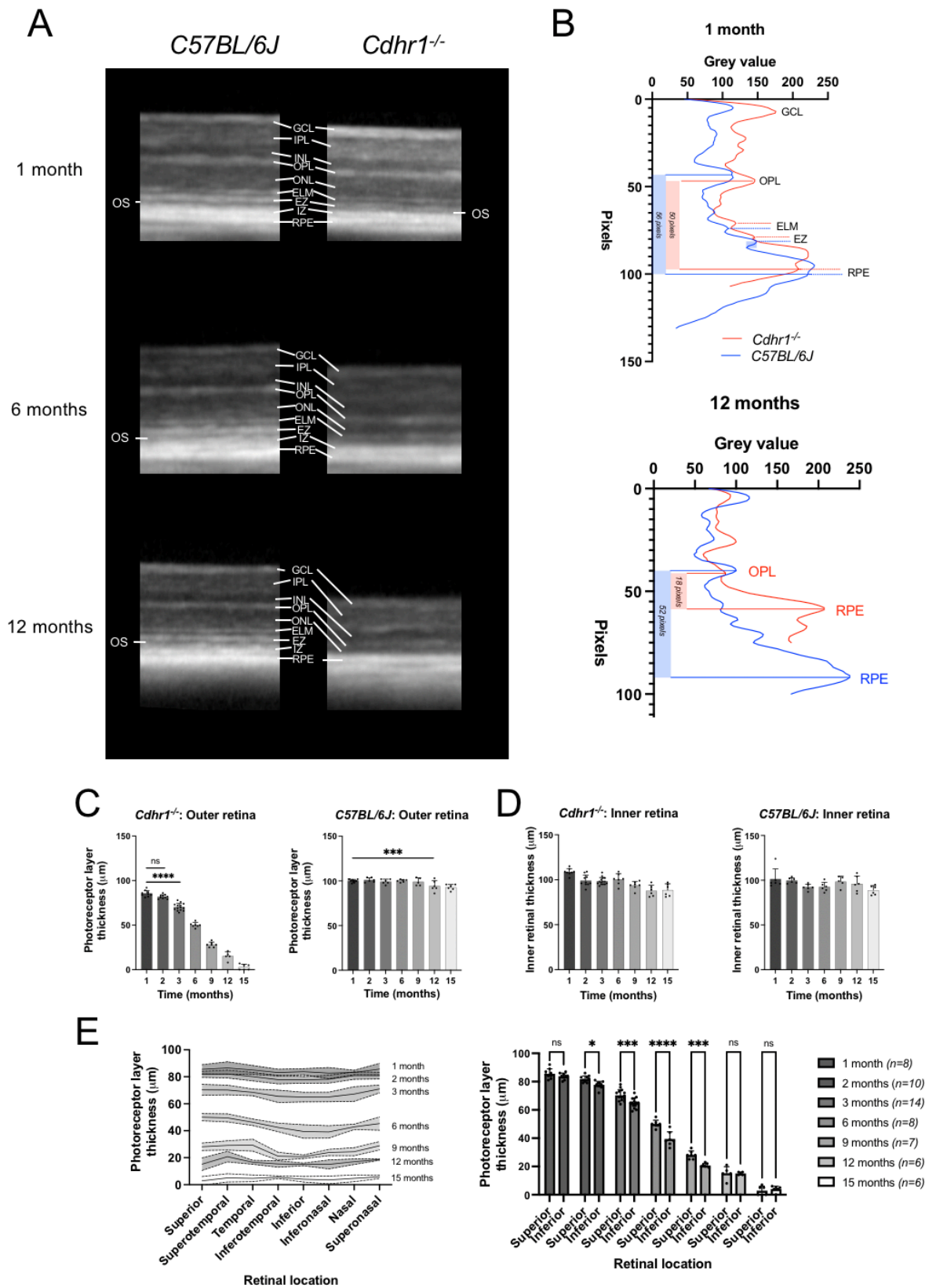


Figure 3.6 Longitudinal *in vivo* OCT retinal imaging characteristics in *Cdhr1*^{-/-} and *C57BL/6J* mice

[Continued] **(A)** Optical coherence tomography (OCT) retinal imaging studies identified an indistinct ellipsoid zone and thinner photoreceptor outer segments in *Cdhr1*^{-/-} mice at 1 month; neither are discernible at 6 months. Progressive thinning of the outer retina is seen at 6 and 12 months. Thickening of the innermost hyperreflective band is seen in *Cdhr1*^{-/-} mice at 1 month, also shown in panel B. **(B)** OCT reflectivity profiles confirm outer retinal thinning at 1 month in *Cdhr1*^{-/-} mice. The EZ reflection is diminished with reduced width and depth of the adjacent OS band, indicated by coloured boxes on the reflectivity profile. At 12 months, the outer retina is thinner and ELM and EZ are absent in *Cdhr1*^{-/-} mice. **(C)** Photoreceptor layer thickness measurements on OCT imaging in *Cdhr1*^{-/-} mice are reduced at 1 month compared to wildtype controls (mean 100.2µm vs 85.6µm). Data points represent measurements from the superior retina only. Repeated measures two-way ANOVA for overall effect of genotype on photoreceptor layer thickness, $P<0.0001$. Progressive outer retinal degeneration is seen in *Cdhr1*^{-/-} mice to 15 months ($P<0.0001$, two-way ANOVA for effect of time). In *C57BL/6J* mice, the outer retina is not significantly thinned until 12 months of age ($P=0.0006$; two-way ANOVA for effect of time). **(D)** The inner retina is thickened in *Cdhr1*^{-/-} mice at 1-month of age compared to wildtype with mild progressive thinning to 15 months. **(E)** Photoreceptor layer thinning progresses more quickly at inferior when compared to superior retinal locations in *Cdhr1*^{-/-} mice as shown across 8 retinal loci. Repeated measures two-way ANOVA for overall effect of retinal location on photoreceptor layer thickness in *Cdhr1*^{-/-} mice: $P<0.0001$. A direct comparison of superior and inferior retinal thickness measurements across age groups *Cdhr1*^{-/-} cohort identifies significant inferior retinal thinning from 2-9 months ($p<0.05$ at all timepoints; two-way ANOVA for effect of retinal location).

Analysis of the distance between ellipsoid zone and RPE (Figure 3.7) was performed in both genotype groups at 1 to 3-month timepoints before the ellipsoid zone was lost in *Cdhr1*^{-/-} mice. *Cdhr1*^{-/-} exhibited a significantly reduced distance compared to wildtype (mean 49.0 versus 31.2µm; $P=0.0001$, unpaired, two-tailed t-test), which did not change from 1 to 3 months in *Cdhr1*^{-/-} mice ($P>0.05$ for all retinal locations). A mild degree of inner retinal thinning was evident in both genotype groups but was more pronounced in *Cdhr1*^{-/-} mice (Figure 3.6) ($P<0.0001$

overall for both groups). The innermost hyper-reflective line appeared thicker at 1 month in *Cdhr1*^{-/-} mice.

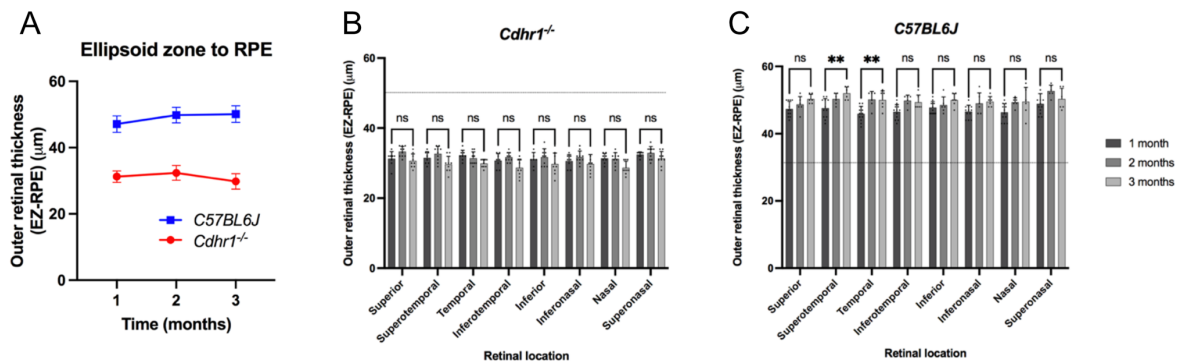


Figure 3.7 Ellipsoid zone to RPE measurements in *Cdhr1*^{-/-} and *C57BL/6J* mice to 3 months

(A) Mean and SD of measurements for all retinal locations identified a significant difference between *Cdhr1*^{-/-} and *C57BL/6J* mice over 3 months ($P < 0.0001$; two-tailed, unpaired t-test). (B) Outer retinal thickness measurements plotted by retinal location show no significant change in retinal thickness measurements over 3 months in *Cdhr1*^{-/-} mice on multiple comparison two-way ANOVA testing for the effect of time. The dashed line indicates average retinal measurements in *C57BL/6J* mice at 3 months for comparison. (C) An increase in ellipsoid zone to RPE measurements are seen in *C57BL/6J* mice which was not statistically significant at most retinal locations. Dashed line indicates the mean outer retinal thickness in *Cdhr1*^{-/-} mice.

3.4.5 Inferior retinal degeneration occurs more quickly in *Cdhr1*^{-/-} mice

Outer retinal degeneration occurred at a greater rate at inferior retinal locations versus superior, at 2 months ($P = 0.034$) persisting to 9 months ($P < 0.0002$ at all intervening timepoints) (Figure 3.6 & 3.8). Beyond this timepoint, comparisons of retinal thickness measurements by location were limited by a floor effect due to progressive retinal degeneration. In *C57BL/6J* mice, there was no difference in outer retinal thickness between superior and inferior retinal locations at the same timepoints (data not shown).

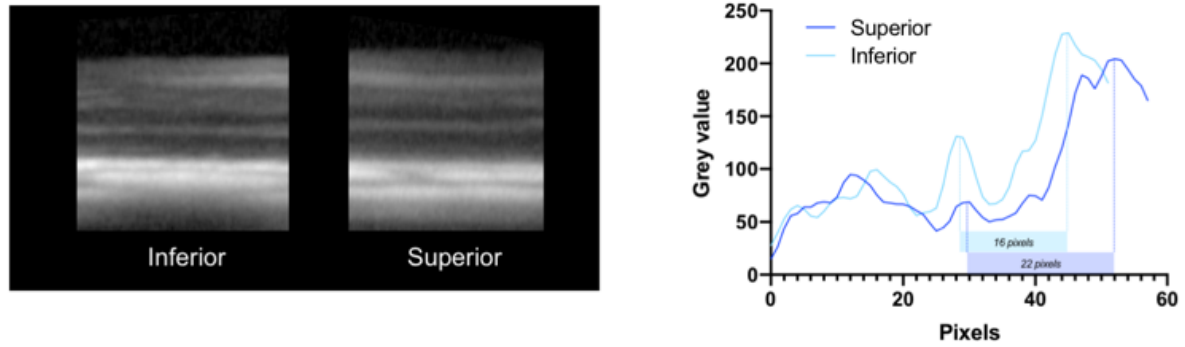


Figure 3.8 Retinal degeneration in *Cdhr1*^{-/-} mice progresses more quickly at inferior retinal locations

Representative OCT images and reflectivity profiles from the same eye at 6 months showing that inferior outer retinal thinning occurs at a greater rate than superior retinal thinning in *Cdhr1*^{-/-} mice.

3.4.6 Fundus autofluorescence imaging demonstrates autofluorescent dots in *Cdhr1*^{-/-} mice

Short-wavelength fundus autofluorescence imaging (SW-AF) revealed generalised punctate autofluorescent dots in *Cdhr1*^{-/-} mice from 2 months of age (Figure 3.9) which varied in size and location on longitudinal imaging of the same eye (data not shown). At 6 months of age and at all later timepoints, autofluorescent dots were seen in 100% of *Cdhr1*^{-/-} mice (Table 3.4). In *C57BL/6J* mice, autofluorescent dots were absent to 6 months of age, although were present in some animals at later timepoints. In *Cdhr1*^{-/-} mice, SW-AF imaging excluded generalised RPE atrophy to 15 months. SW-AF revealed constriction of the retinal arterioles by 6 months of age, which progressed to severe, generalised constriction of major retinal arterioles and venules by 15 months of age in *Cdhr1*^{-/-} mice.

	<i>C57BL/6J</i>	<i>Cdhr1</i> ^{-/-}
1 month	0% (<i>n</i> =12)	16% (<i>n</i> =8)
2 months	0% (<i>n</i> =6)	80% (<i>n</i> =10)
3 months	0% (<i>n</i> =6)	83% (<i>n</i> =14)
6 months	33% (<i>n</i> =6) (localised)	100% (<i>n</i> =8)
9 months	33% (<i>n</i> =5) (localised)	100% (<i>n</i> =7)
12 months	66% (<i>n</i> =6) (2 localised, 1 generalised)	100% (<i>n</i> =6)
15 months	83% (<i>n</i> =6) (4 localised, 1 generalised)	100% (<i>n</i> =6)

Table 3.5 Frequency of autofluorescent dots on SW-AF imaging in *Cdhr1*^{-/-} and *C57BL/6J* mice.

The reported figures relate to mice, using the right eye of each animal. Localised – 2 of fewer quadrants; Generalised – 3 or more quadrants

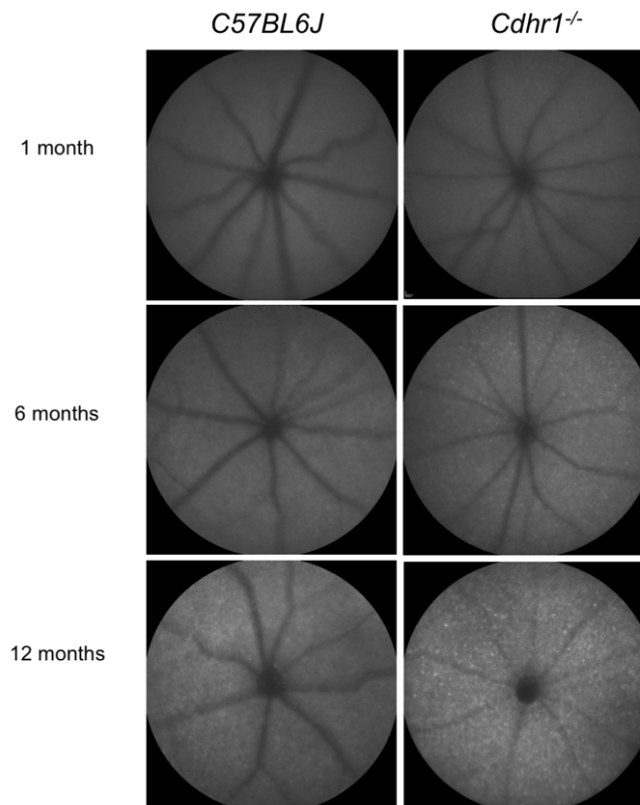


Figure 3.9 Fundus autofluorescence imaging in *Cdhr1*^{-/-} mice

SW-AF imaging demonstrates generalised punctate autofluorescent dots in *Cdhr1*^{-/-} retinas from 2 to 15 months which are usually absent at 1-month of age.

3.4.7 Dark-adapted electroretinography demonstrates progressive loss of rod function in *Cdhr1*^{-/-} mice over 15 months

In 1 month old *Cdhr1*^{-/-} mice, mean dark-adapted rod-specific responses (0.01 cd.s/m²), as well as maximum A-wave and B-wave amplitudes (10 cd.s/m²) were significantly reduced by 27%, 63% and 41%, respectively, compared to *C57BL/6J* mice ($P<0.0001$; Figure 3.10 & 3.11). Responses decreased with ageing; at the age of 15 months, responses were extinguished in *Cdhr1*^{-/-} mice and approximately 50% of baseline amplitudes in *C57BL/6J* mice.

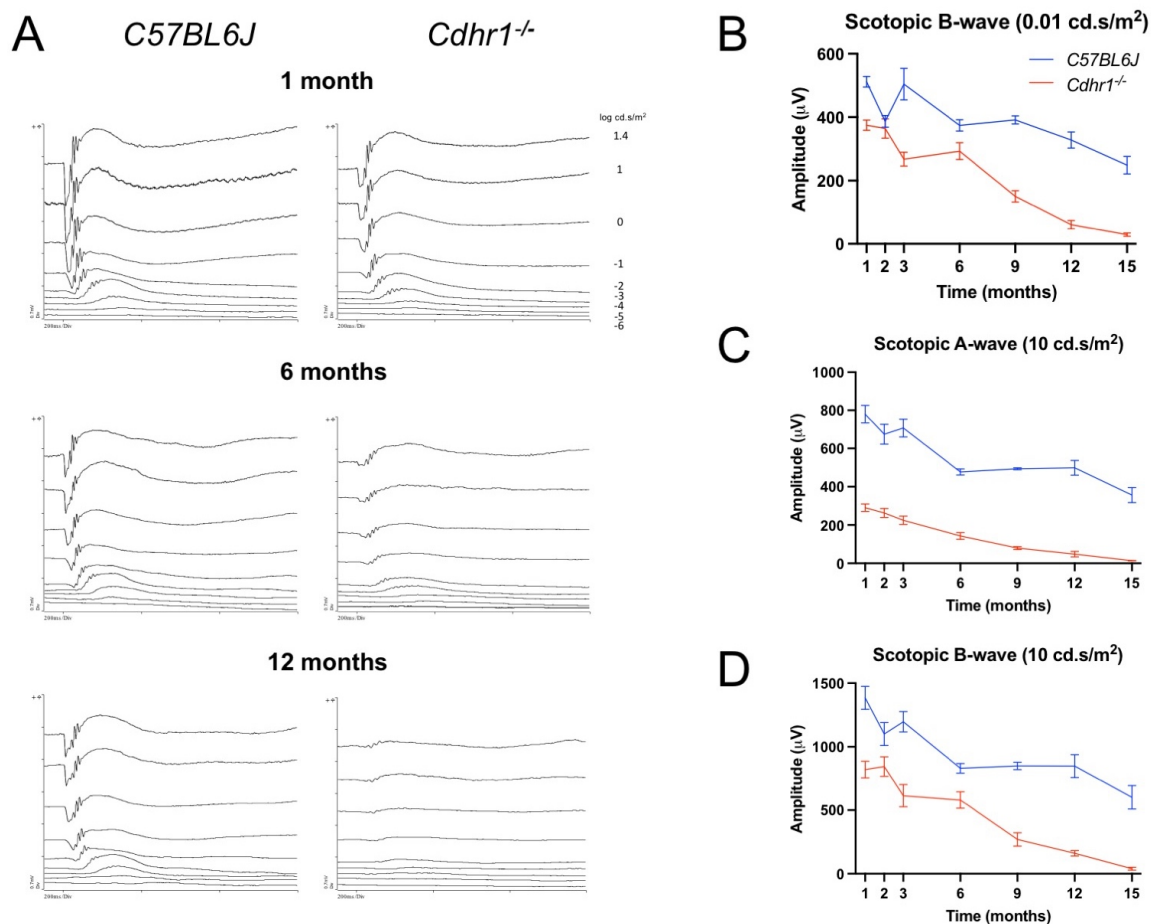


Figure 3.10 Functional testing by longitudinal dark-adapted ERG in *Cdhr1*^{-/-} and *C57BL/6J* mice

[Continued] (A) Representative raw dark-adapted ERG traces at 1, 6 and 12 months. (B) Scotopic B-wave responses at 0.01 cd.s/m² were significantly reduced in *Cdhr1*^{-/-} mice. Repeated measures two-way ANOVA for overall effect of genotype; $P<0.0001$. Responses did not differ significantly at 2- and 6- month timepoints. (C) Scotopic A-wave responses at 10 cd.s/m² were significantly reduced in *Cdhr1*^{-/-} mice ($p<0.0001$ for all timepoints). Repeated measures two-way ANOVA for overall effect of genotype on A-wave amplitude: $P<0.0001$. (D) Scotopic B-wave responses at 10 cd.s/m² were significantly reduced in *Cdhr1*^{-/-} mice ($P<0.0001$, except at 2- and 6- months). Repeated measures two-way ANOVA for overall effect of genotype on B-wave amplitude: $P<0.0001$. Mean and SEM across representative flash intensities are presented.

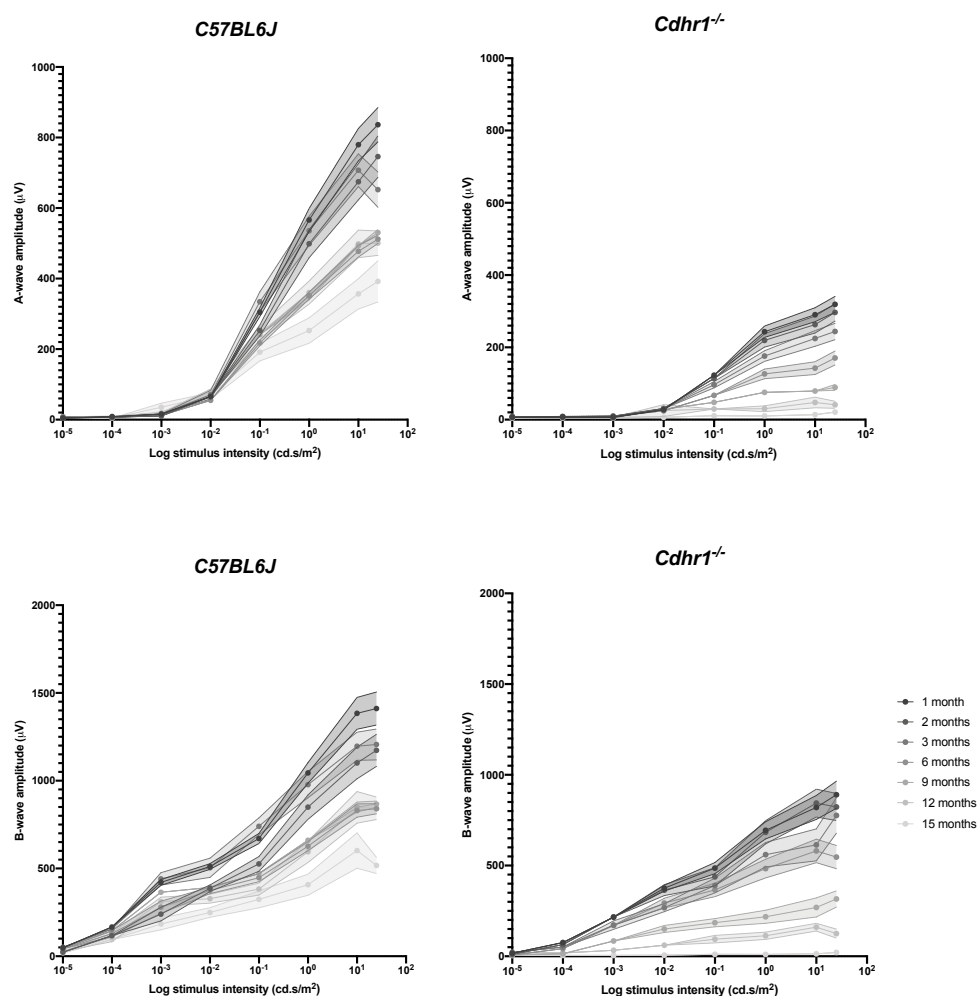


Figure 3.11 Dark-adapted electrophoretography dose-response curves in *Cdhr1*^{-/-} and *C57BL/6J* mice

Dark-adapted ERG responses across all cohorts over 15-months. Plots represent mean +/- SEM indicated by shaded areas for each aged cohort.

3.4.8 Light-adapted electroretinography demonstrates progressive loss of cone function in *Cdhr1*^{-/-} mice over 15 months.

Cdhr1^{-/-} mice also exhibited significantly reduced responses overall across light-adapted ERG tests compared to *C57BL/6J* mice ($P<0.0001$; Figure 3.12 & 3.13).

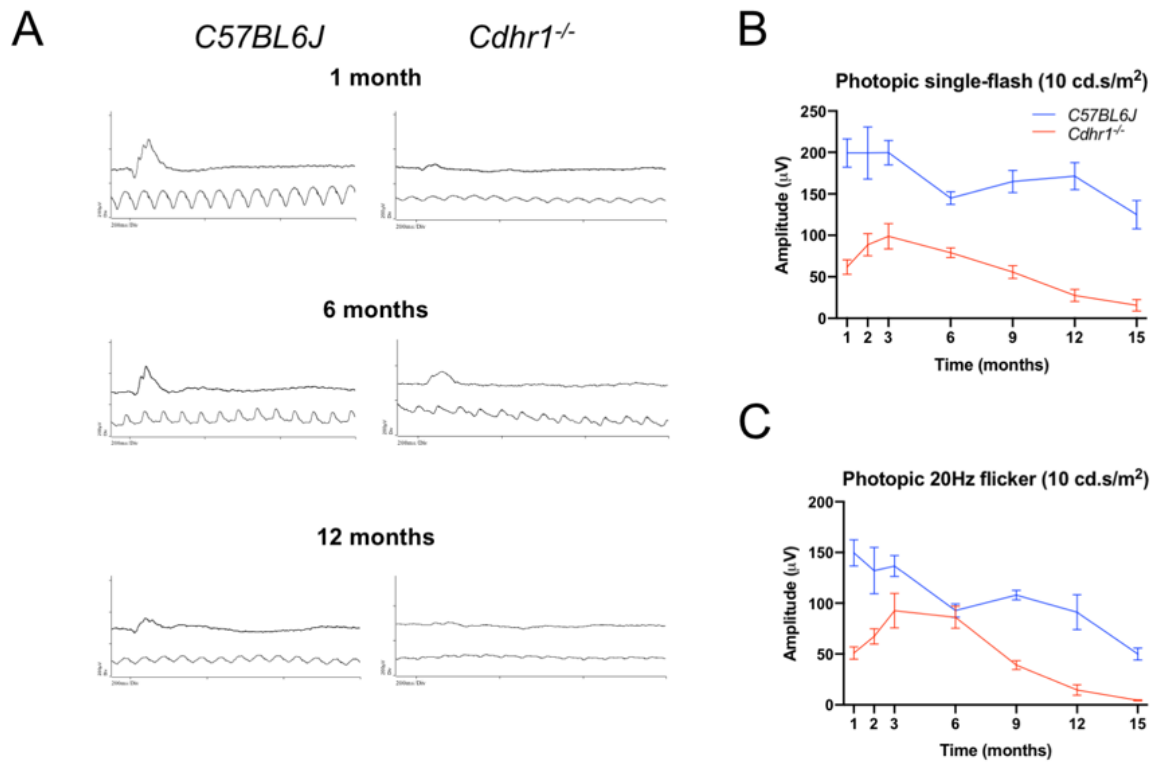


Figure 3.12 Functional testing by longitudinal light-adapted ERG in *Cdhr1*^{-/-} and *C57BL/6J* mice

(A) Representative raw ERG traces at 1, 6 and 12 months showing single-flash (above) and 20Hz flicker responses (below) at 10 cd.s/m² (B) Photopic single-flash response responses were significantly reduced in *Cdhr1*^{-/-} mice ($P<0.05$ for all timepoints). Repeated measures two-way ANOVA for overall effect of genotype on photopic single-flash amplitude: $P<0.0001$. (C) Photopic 20Hz flicker responses were significantly reduced overall in *Cdhr1*^{-/-} mice. Repeated measures two-way ANOVA for overall effect of genotype on photopic flicker responses: $P<0.0001$. However, responses did not differ by genotype at the 3- and 6-month timepoints on Šidák's multiple comparisons test at both flash intensities. Mean and SEM across representative flash intensities are presented.

Responses in *Cdhr1*^{-/-} mice increased in amplitude across all cone tests between 1 and 3-months after which responses declined, and were extinguished by 15 months. In *C57BL/6J* mice, there was a gradual, continuous decline in cone responses with age, which appeared more pronounced in flicker responses compared to single-flash responses (Figure 3.12 & 3.13). Whilst single-flash, light-adapted responses at 10 cd.s/m² were significantly different between *Cdhr1*^{-/-} and wildtype mice at all timepoints ($P<0.0001$), light-adapted flicker responses were not significantly different at 3 and 6 months between genotype groups.

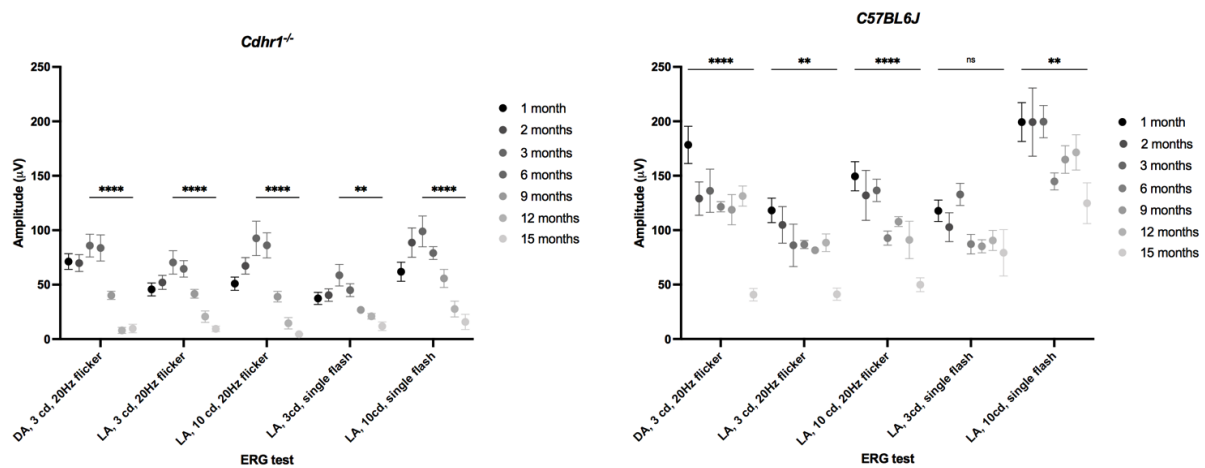


Figure 3.13 A comparison of light-adapted and flicker ERG in *Cdhr1*^{-/-} and *C57BL/6J* mice

(A) *Cdhr1*^{-/-} mice exhibited increased light-adapted responses to 3 months across all tests, followed by progressive reduction to 15 months where traces were extinguished. Repeated measures two-way ANOVA for overall effect of age on light-adapted and flicker responses in *Cdhr1*^{-/-} mice: $P<0.0001$. Dunnett's multiple comparison test results compare maximal responses at 3-months with later timepoints. **(B)** *C57BL/6J* mice exhibited a milder reduction in response amplitudes. Repeated measures two-way ANOVA for overall effect of age on light-adapted and flicker responses in *C57BL/6J* mice: $P<0.0001$. Flicker responses amplitudes were diminished at the 15-month timepoint. Plots represent mean $\pm$ SEM.

3.4.9 Early deficits in rod and cone function in *Cdhr1*^{-/-} mice are not explained by photoreceptor cell death alone

In *Cdhr1*^{-/-} mice, retinal function as measured by both mixed rod-cone and cone ERG responses were more severely affected than structural measures of outer retinal thickness compared to wildtype at early timepoints. Normalised to wildtype measurements at 1-month, ERG responses were 0.38 for mixed rod-cone (max. scotopic A-wave) and 0.3 for cone (20Hz photopic flicker) measurements, compared to 0.85 for outer retinal thickness on OCT imaging (Figure 3.14). The thinning of the outer retina at the earliest time point (1 month) seemed to be explained by the reduced distance between ellipsoid zone and RPE (Figure 3.7), which remained stable from 1-3 months whereas subsequent thinning was mainly of the outer nuclear layer indicative of progressive photoreceptor cell death.

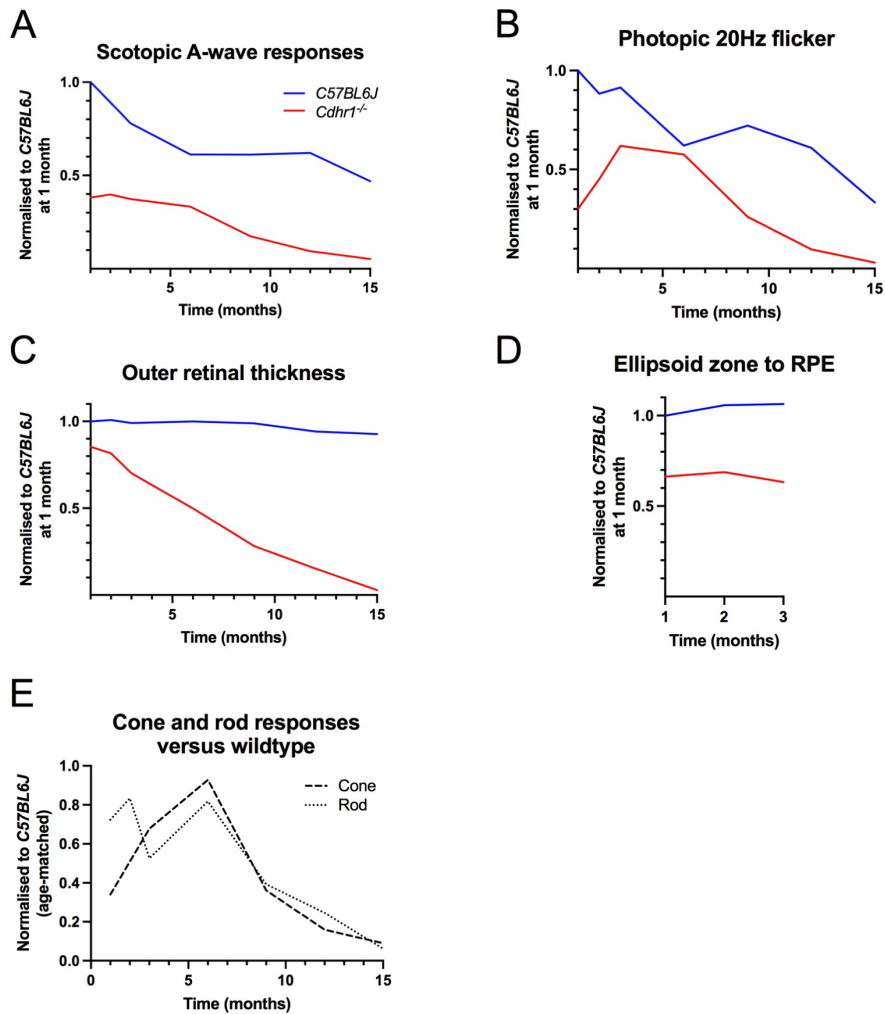


Figure 3.14 Longitudinal structural and functional decline in *Cdhr1*^{-/-} mice compared to *C57BL/6J* mice

(A) Mixed rod-cone (10cd.s/m²) A-wave responses on dark-adapted ERG normalised to *C57BL/6J* responses at 1 month shows severe functional deficits at 1-month (62% reduction) when compared to outer retinal thickness measurements on OCT imaging in (C). (B) Normalised light-adapted 20Hz flicker responses exhibited severe deficits in cone function (70% reduction) in *Cdhr1*^{-/-} mice at 1 month, with improvement of responses to 3 months, followed by decline. (D) OCT measurements of ellipsoid zone to RPE, normalised to *C57BL/6J* at 1 month shows stable retinal measurements to 3 months. The ellipsoid band is lost in *Cdhr1*^{-/-} mice at later timepoints. (E) Longitudinal cone (photopic 20Hz flicker at 10.cd/s/m²) and rod (scotopic B-wave responses at 0.01cd/s/m²) responses in *Cdhr1*^{-/-} mice, normalised to age-matched *C57BL/6J*, shows progressive increase in cone responses to 3 months followed by decline to 15 months. Rod responses are less affected at 1 month although the rate of functional loss beyond 6 months appears to match that of cones.

3.4.10 *Cdhr1*^{-/-} mice exhibit abnormal photopic and scotopic optomotor reflex responses at 20 and 24 months of age

Cdhr1^{-/-} mice demonstrated near extinguished photopic and scotopic optomotor reflex responses compared to age-matched *C57BL/6J* controls at 20- and 24-months of age ($n=4$ in each group; Figure 3.15).

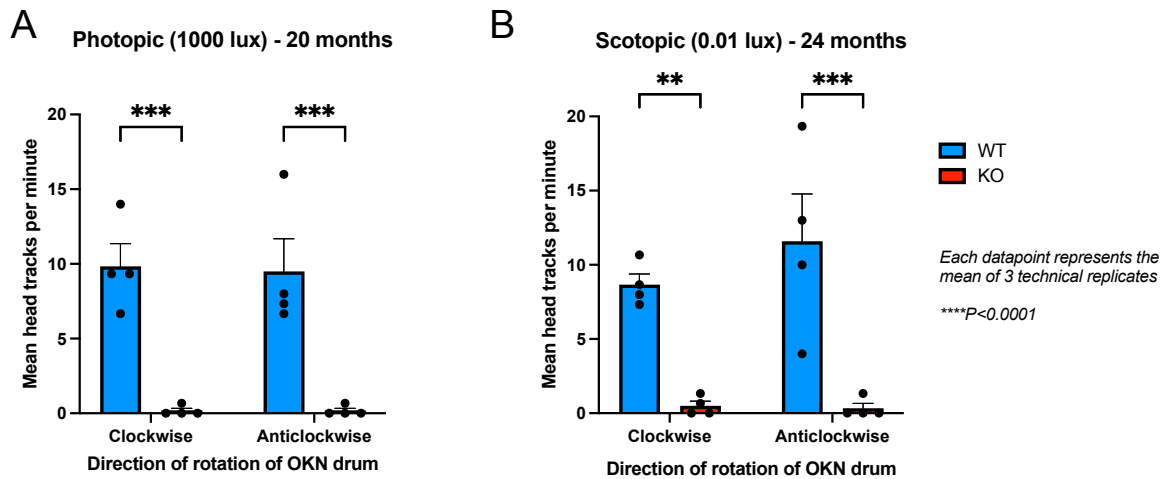


Figure 3.15 Scotopic and photopic optomotor behavioural testing in *Cdhr1*^{-/-} and *C57BL/6J* mice at 20- and 24-months of age

(A) Photopic and (B) Scotopic optomotor responses are absent in *Cdhr1*^{-/-} mice, and significantly different from age-matched *C57BL/6J* wildtype mice ($P < 0.0001$ for both tests), tested at a fixed spatial frequency of 0.1 cycles per degree at 30 seconds per rotation ($n=4$ animals in each group) at 20- and 24-months, respectively. This effect was independent of the direction of rotation, indicating similar responses from right and left eyes. Each datapoint represents a mean of three technical replicates, compared using two-way ANOVA for effect of genotype.

3.4.11 *Cdhr1*^{-/-} mice do not exhibit abnormal light avoidance behaviour to 20 months of age

Despite near-absent dark-adapted ERG responses at 15-months of age, there were no observable differences in light-avoidance behaviour in a cohort of *Cdhr1*^{-/-} and

age-matched *C57BL/6J* controls at 18- or 20-months of age at two different levels of illuminance (Figure 3.16).

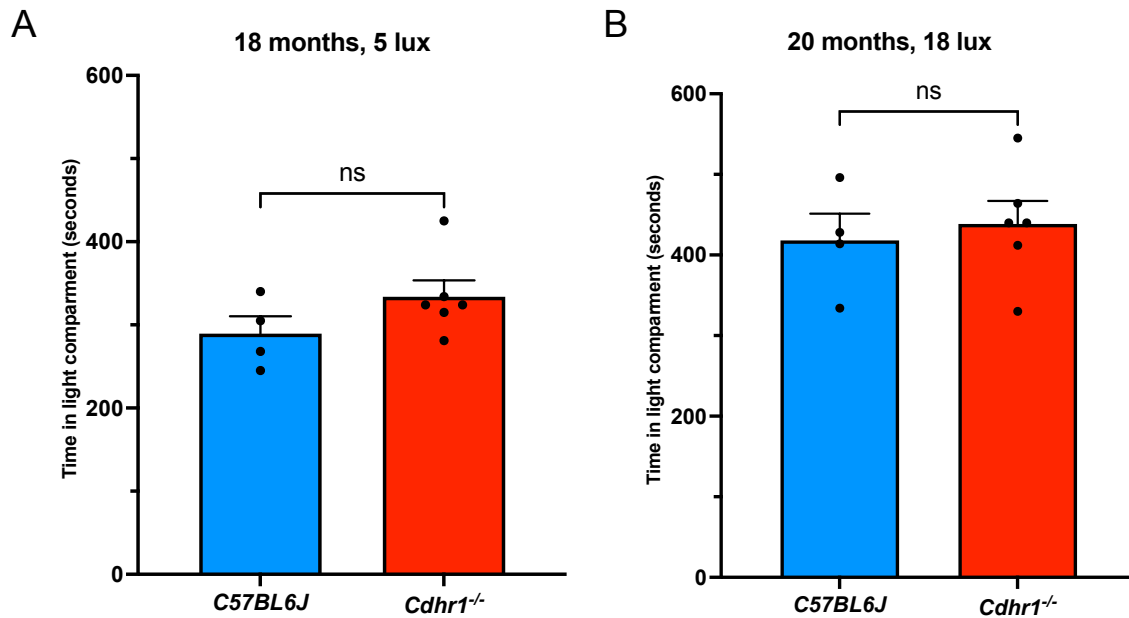


Figure 3.16 Light-avoidance behavioural testing in *Cdhr1^{-/-}* mice at 18 and 20-months of age

No difference was detected in light avoidance behaviour between *Cdhr1^{-/-}* mice ($n=6$) and age-matched *C57BL/6J* mice ($n=4$) at (A) 18- and (B) 20-months of age at two different levels of illuminance. Data were normally distributed on Shapiro-Wilk testing; Welch's t-test (two-tailed) was used to compare groups.

3.5 Discussion

Longitudinal deep phenotyping of the *Cdhr1^{-/-}* mutant mouse over 15 months revealed evidence of slowly progressive outer retinal degeneration on OCT imaging and loss of rod and cone function on electroretinography. These findings recapitulate the major structural and functional characteristics of the human phenotype of *CDHR1*-associated cone-rod dystrophy, validating the use of the

Cdhr1^{-/-} mouse model for downstream gene therapy assessments. Moreover, these observations provide insights into the phenotype and natural history of human *CDHR1*-associated retinal degeneration, which is less well characterised, and will be further discussed in Chapter 7.

Functional deficits at 1-month affecting rod and cone photoreceptors, out of proportion to structural photoreceptor degeneration in *Cdhr1*^{-/-} mice, are likely explained by shortened photoreceptor outer segments. The latter has previously been confirmed by electron microscopy⁴⁷, associated with a 50% reduction in rhodopsin at 1-month.⁴⁷ Accordingly, *in vivo* OCT imaging showed that more severe early alterations (34% reduction versus wildtype) are observed in layers representing the photoreceptor inner and outer segments (reduced reflectivity of the ellipsoid zone and thinning of the adjacent layer representing photoreceptor outer segments), compared to layers representing photoreceptor cell bodies at 1-month (15% reduction versus wildtype). Moreover, further thinning of photoreceptor outer segments was not observed to 3 months in *Cdhr1*^{-/-} mice. The decline in rod and cone photoreceptor function across this interval is likely to result from photoreceptor cell death as determined by photoreceptor layer thinning, which progressed to 15 months. Since outer segments offer only a small contribution to outer retinal thickness measurements, the rate of functional decline logically exceeds structural degeneration. Beyond 6 months, the rates of functional decline affecting rods and cones are closely matched relative to wildtype

controls, and are likely explained by continued photoreceptor cell death as measured by outer nuclear layer thinning to 15-months.

Longitudinal outer nuclear layer thinning forms a structural correlate for progressive functional decline at a rate similar to estimates based on the counting of nuclei on histological sections, further validating the experimental approach.⁴⁷ Histological analyses are limited by artefacts related to tissue processing and sectioning. OCT imaging has been validated in previous study as a reliable biomarker of retinal degeneration; identifying toxicity¹¹⁴ and rescue effects.^{82, 116-118} The rate of outer retinal degeneration of the *Cdhr1*^{-/-} mouse model, based on OCT data, appears comparable to *Prom1*^{rd19}, *Rpe65*^{rd12} and *Prph2*^{rd2} mouse models.¹¹⁹ *Rpe65*^{-/-} mice exhibit diminished scotopic photoreceptor responses (<25% of wildtype) at early timepoints, which are nearly extinguished at 6 months.^{118, 120} Cone responses were diminished by ~80% at early timepoints, similar to the *Cdhr1*^{-/-} mice although did not improve in the *Rpe65*^{rd12} mouse to 3 months.¹²¹ In the 1-month-old *Prph2*^{rd2} mouse, a ~90% reduction in scotopic B-wave amplitudes was demonstrated which further declined over 6 months,¹²² with abolished photopic responses at all timepoints.¹²³ There are no longitudinal data for the *Prom1*^{rd19} mouse. Thus, despite the similar rates of degeneration based on OCT data, the *Cdhr1*^{-/-} mouse exhibits milder early functional deficits on both the rod and cone systems than *Rpe65*^{rd12} and *Prph2*^{rd2} mouse models. These deep

phenotyping data render the *Cdhr1*^{-/-} mouse as a useful additional model of slow retinal degeneration for investigators in the field.

Cdhr1 is expressed at the leading edge of nascent outer segment discs opposite the connecting cilium, and through its ectodomains, forms physical connections with the periciliary ridge of the inner segment.⁵² These connections appear crucial for horizontal disc elongation and outer segment morphogenesis.⁵³ On review of published reports of human *CDHR1*-associated cone-rod dystrophy, reduced ellipsoid zone reflectivity and shortened outer segments are evident on OCT imaging in early disease.⁶⁴ Moreover, progressive outer retinal degeneration is a universal finding on OCT in individuals with *CDHR1*-associated cone-rod dystrophy, although this has not yet been quantified. Hence, the human retinal phenotype on OCT imaging seems to parallel these observations in *Cdhr1*^{-/-} mice.

An unexpected, novel finding on light-adapted electroretinography in *Cdhr1*^{-/-} mice was an increase in cone-related ERG responses to 3 months, consistent across all cone ERG assessments. Possible explanations for this observation include altered higher-level processing within the retina (i.e. lateral inhibition affecting light-adaptation or processing of cone signals)¹²⁴, a primary effect of null *Cdhr1* variants on cone development, or an upregulation of an unknown factor secondary to rod cell death. S-opsin has been shown to correctly localise in *Cdhr1*^{-/-} mice, with rhodopsin and other outer segment proteins; M-opsin has not been

examined.⁴⁷ However, expression of genes relevant to rod and cone development (i.e. *Crx*, *Nrl* etc.) have not been evaluated in *Cdhr1*^{-/-} mutants versus controls. Further studies are required to determine the mechanism behind this observation; in particular, examination of light-adapted flicker responses at lower frequencies (i.e. 5-7Hz) may further clarify the decrease in cone function.

A further novel finding in this study which may have clinical implications is the observation of an increased rate of retinal degeneration in the inferior retina in *Cdhr1*^{-/-} mice, suggesting that light toxicity may be a modifier in *CDHR1*-associated retinal degeneration. *Sfrp1*, a regulator of Adam10 which may catalyse the cleavage of the *Cdhr1* ectodomain, releasing the connections between nascent discs and the inner segments, has been shown to significantly enhance light toxicity through an unknown, but perhaps related, mechanism.¹²⁵ This hypothesis may be proven by housing *Cdhr1*^{-/-} mice in cages which filter short-wavelength light, as shown to be beneficial in a model of RP.¹¹⁴ The maximal effect size of inferior versus superior retinal thinning in *Cdhr1*^{-/-} mice is smaller than seen in the knock-in *Rho*^{P23H/+} mouse model (Cohen's *d*: 2.75 versus 7.07).¹¹⁴ A predominant inferior pattern of retinal degeneration has been shown in some individuals with biallelic null variants in *CDHR1*.¹²⁶ However, this requires further clinical investigation. The *Rpe65* variant Met450Leu, which has been independently associated with light-toxicity,¹²⁷ and the *Crb1*^(rd8) mutation which has been shown to occur naturally in *C57BL/6N* mouse lines,¹²⁸ but not

C57BL/6J, were not sequenced in either the wildtype or the knockout groups in this study. However, the *Cdhr1*^{-/-} retinal phenotype does not resemble the *Crb1*^{-/-} (rd8) phenotype; for example, retinal oedema and retinoschisis was not observed.

The *Cdhr1*^{-/-} retinal phenotype identified in this study validates it as a suitable model for the pre-clinical development of a candidate therapeutic for this disorder. Current AAV-delivered retinal gene therapy vectors in clinical trials¹²⁹⁻¹³¹ were enabled by the pre-clinical validation of murine disease models – such as RPE65,¹³² choroideremia^{102, 133} and X-linked rod-cone dystrophy due to variants in retinitis pigmentosa GTPase regulator (RPGR) - prior to proof-of-principle pre-clinical studies.¹⁰⁴ The characterisation and validation of the *Cdhr1*^{-/-} mouse model represents a key step towards a therapy for *CDHR1*-associated retinal degeneration.

Phenotypic data presented in this study further support *CDHR1* as an attractive candidate for gene therapy. Firstly, a wide therapeutic window exists for intervention given the slow rate of retinal degeneration. Outer retinal structure is relatively well preserved despite significant functional deficits at early time-points. Early intervention through gene therapy may improve retinal function by restoring photoreceptor outer segment length, and increasing the cellular capacity for proteins critical for phototransduction. Regrowth of photoreceptor outer segments on OCT imaging has been shown to be associated with improvements in

visual function following intervention in both *CEP290*¹³⁴ and *RPGR*-associated photoreceptor degeneration,¹²⁹ both of which are also associated with shortened photoreceptor outer segments. Accordingly, the three characteristic hallmarks on OCT imaging of progressive outer nuclear layer thinning, attenuation of the ellipsoid zone and shortened photoreceptor outer segments in *Cdhr1*^{-/-} mice may be valuable and relevant structural outcome measures in pre-clinical and clinical studies.

3.6 Conclusions

Data presented in this chapter demonstrate that the *Cdhr1*^{-/-} mouse model recapitulates the key features of human *CDHR1*-associated cone-rod dystrophy due to biallelic null variants. Severe, early deficits in cone and rod function are attributable to congenitally shortened photoreceptor outer segments with progressive functional decline attributable to degeneration of cone and rod photoreceptors. These observations appear to reflect those seen in *CDHR1*-associated cone-rod dystrophy from limited published data.⁶⁴ Photoreceptor layer thickness measurements, outer segment length measurements and A-scan profiles on OCT imaging may be useful structural endpoints for pre-clinical evaluation of the efficacy and safety of gene therapy. Dark- and light-adapted ERG confirm progressive rod and cone photoreceptor cell death at a similar rate after 6 months when normalised to wildtype responses. The natural history data presented in this chapter will permit comparisons with structural and functional outcome measures following gene therapy (Chapters 5 & 6). The morphological features on OCT imaging and functional data identified in *Cdhr1*^{-/-} mice will be examined in the context of phenotypic data from human subjects with *CDHR1*-associated retinal degeneration in Chapter 7.

4. Development of *CDHR1* gene therapy

4.1 Introduction

Therapeutic strategies for inherited retinal degeneration may intervene in the disease process prior to cell death, or replace cellular function after cell death has occurred. Strategies to prevent, or slow, degeneration may include general measures, such as smoking cessation (smoking has been shown to correlate with poorer visual acuity and reduced central retinal thickness measurements in patients with retinitis pigmentosa¹³⁵), dietary modification (e.g. avoidance of, or interference in the metabolic pathways of, vitamin A in *ABCA4* retinopathies¹¹⁶), light avoidance (e.g. avoidance of short-wavelength light in dominant RP due to rhodopsin mutations¹¹⁴), the delivery of systemic drugs (e.g. aminoglycosides or biosimilar molecules to promote translational readthrough of in-frame nonsense or stop gain variants¹³⁶), or locally delivered drugs or factors which may intervene in retinal degeneration.¹³⁷

Gene therapy strategies include the supplementation of deficient genes or editing of genetic (DNA or RNA) sequences, which therefore require administration prior to target cell death and are variant or gene specific. Dominantly inherited diseases may occur due to haploinsufficiency or a dominant negative (toxic) disease mechanism. Recessive disorders occur due to deficiency, which may be absolute or

relative, or functional deficiency (i.e. hypomorphic alleles). The optimal therapeutic approach in any given monogenic retinopathy is dependent on the underlying genetic mechanism, the rate of degeneration/availability of target cells, the primary affected cell type(s) (i.e. dividing or terminally differentiated), the availability of suitable promoters to drive expression in the relevant cell types, and the size and sequence stability of the deficient transgene. Furthermore, the availability of suitable disease models – cellular (*in vitro/ex vivo*), retinal organoids, and animal models – may further support the development of therapeutics for individual variants, genes and disorders.

For recessive disorders, such as *CDHR1*-associated retinal degeneration, gene supplementation strategies are appealing as they aim to treat the underlying cause of the disease. Gene supplementation carries an advantage in terminally differentiated cells, such as photoreceptors, in that a single intervention may result in stable transgene expression for the life of the cell. Moreover, in monogenic disorders, the functional roles of a given protein need not be characterised in detail, since the sequence conservation of key functional protein domains across species should permit interactions with as yet unidentified binding partners. This is an advantage for structural proteins whose functional characterisation is more challenging; for *Cdhr1*, only *Prom1* has direct evidence of interaction on immunolocalization studies.⁵⁴ The other two likely binding partners are as yet

unknown (inner segment binding partner) or unconfirmed (Adam10¹³⁸⁻¹⁴⁰; the putative catalyst involved in cleavage of the *Cdhr1* ectodomain).

The coding sequence of *CDHR1* is 2.58kb and is therefore encodable within AAV with sufficient packaging capacity for a promoter, polyadenylation tail and various *cis*-acting enhancing elements. Other viral vectors were not considered to be as efficacious or safe (i.e. lentivirus) for *CDHR1* transgene delivery. In Chapter 3, we presented clear evidence for the recapitulation of human *CDHR1*-associated cone-rod dystrophy by the *Cdhr1*^{-/-} mouse and relatively slow retinal degeneration, both of which support the rationale and development of *CDHR1* gene therapy. Gene supplementation was considered the optimal therapeutic approach for *CDHR1*-associated cone-rod dystrophy.

4.2 Aims

The aims of this chapter were to:

- Design two or more vectors expressing a synthetic copy of the human *CDHR1* transgene which efficiently target rod and cone photoreceptors
- Create one vector with *cis*-acting enhancing elements for *in vivo* comparison
- Package both *CDHR1* expression plasmids in AAV with demonstration of sufficient AAV purity and titre

- Demonstrate expression and localisation of CDHR1 protein in both rod and cone photoreceptors of the *Cdhr1*^{-/-} mouse by immunohistochemistry following subretinal injection of AAV.CDHR1 vector

4.3 Materials, methods and rational basis of vector design

The rational basis of vector design, specific methodology used in the production of all plasmids and sequence verification are presented in this section. All cloning techniques used in the development of four *CDHR1* expression plasmids, and methods used for AAV production, are described in detail in Chapter 2.

4.3.1 Selection of AAV capsid serotype and ITR

Several AAV serotypes have been used as rAAV vectors developed for monogenic retinal degeneration; AAV phylogeny based on VP1 sequences is presented by Boye et al.¹⁴¹ AAV variants exhibit varied cell surface receptor binding preferences which underpins cellular tropism. AAV2, for example, has been used to target the RPE in retinal degeneration caused by biallelic variants in *RPE65*. AAV capsid serotypes used in ongoing clinical trials for retinal disorders are summarised in Table 3.1. For photoreceptor cells, transduction has been demonstrated using AAV2, AAV5, AAV8, AAV8^{Y733F} (i.e. mutant capsid) and AAV9 *in vitro*.¹⁴² Assessment of tropism for retinal cell types is typically evaluated using animal models. However, human iPSC-derived retinal organoids now offer an additional method of verification of cellular tropism in human cells.¹⁴³ Non-human primate

studies identified that AAV8 confers superior tropism for photoreceptor cells compared to AAV2.¹⁴⁴ However, novel AAV capsid variants – typically involving one or more amino acid substitutions - are continually sought through targeted evolution or rational design, to identify those with greater tropism for a given target cell with less immunogenicity.^{141, 145} For example, AAV44.9^{E531D} demonstrated greater efficiency of transduction compared to AAV8, although this novel capsid mutant has not been approved for clinical trials.¹⁴¹ Synthetic AAV capsid alterations have mostly been driven by attempts to transduce rod and cone photoreceptors using intravitreal delivery of AAV where the effective dose delivered to the outer retina is low.¹⁴⁶ However, mutant capsid modification may affect cellular tropism: off-target expression of the inner retina has been shown with the AAV2 quad capsid-mutant requiring an additional knockdown mechanism to mitigate against these effects.¹⁴⁶

A single capsid mutant, rAAV8^{Y733F}, has been shown to improve photoreceptor transduction in the *Abca4*^{-/-} mouse.⁸⁴ A triple capsid mutant, AAV8^{Y447F, Y733F, T494V}, has been shown to improve the persistence of transduction in photoreceptor cells of a mouse model of *CNGA3* achromatopsia.¹⁴⁷ However, novel capsid mutants were not selected for use in this study since regulatory approval and manufacturing at scale of novel mutant capsids were likely barriers to onward clinical trial. Furthermore, for a relatively low-abundance protein, such as

CDHR1, wildtype AAV8 capsids were considered sufficient for photoreceptor transduction.

Inverted Terminal Repeats (ITRs) are necessary *cis*-acting elements for AAV production which act to rescue, replicate and package the AAV genome. ITRs generally cross-complement with capsid proteins from other AAV serotypes.¹⁴⁸ Since AAV2 was the first serotype shown to be capable of replicating in human cells with a helper plasmid *in trans*, the AAV2 ITR became consolidated in AAV constructs; all current clinical trials in retinal gene therapy, where details of the AAV vector are published, incorporate AAV2 ITRs.¹⁴⁸ AAV2 ITRs are 145 base-pairs in length with the first 125 bases forming a T-shaped hairpin structure which is inherently unstable. AAV2 ITRs used in this study featured flanking 15bp sequences with <40% GC content, shown to improve stability of the construct.¹⁴⁹

4.3.2 Self-complementary versus single-stranded AAV

Self-complementary AAV (scAAV) describes the packaging of double-stranded DNA which is complementary to itself, providing two copies of genomic DNA on sense and anti-sense strands. This is achieved by removing the terminal resolution site (*trs*) from one of the two ITRs in a vector genome, preventing Rep68/78 from cleaving the sequence, resulting in duplicated vector genomes which self-anneal in the target cell. This obviates the need for second strand synthesis with subsequent rapid transgene expression.¹⁴⁹ Self-complementary AAV encoding is of

particular advantage in the context of rapid photoreceptor degeneration where the rate of transduction is critical. Secondly, scAAV packaging is only possible with small genes since the coding sequence requires the duplication of its sequence in reverse complement. scAAV encoding has been shown to improve photoreceptor responses and functional vision in a null model of early onset retinal degeneration caused by variants in *Aip1l1* (CDS, 1,152bp).⁸³ Moreover, scAAV encoding of RP2 was found to be toxic due to possible overexpression.¹⁵⁰ However, since the natural history of retinal degeneration in *Cdhr1*^{-/-} mice (Chapter 3) and patients with biallelic null *CDHR1* variants (Chapter 7) is relatively slow, and given the size of the *CDHR1* coding sequence (2,580bp), scAAV encoding was neither necessary, nor possible.

4.3.3 Selection of promoter

Promoters are *cis*-acting DNA sequences that drive gene expression. In general, promoters may be cell-specific or broadly active and are selected for gene therapy applications on the basis of the efficiency and specificity with which they promote transgene expression in the intended cellular target(s). In retinal gene therapy, a number of promoters have been selected for use in AAV vectors in pre-clinical studies and clinical trials (Table 3.1). Assessment of retinal toxicity following sub-retinal injection of AAV in mice demonstrated that broadly active, and RPE-specific promoters were more toxic than photoreceptor-specific promoters.¹⁵¹

Rhodopsin kinase is an enzyme expressed in cone and rod photoreceptors that protects the cells against light-induced toxicity. The promoter driving human rhodopsin kinase expression (GRK1) was isolated, mapped and sequenced as a highly-active photoreceptor-specific promoter.¹⁵² It lacks a TATA box, which normally contributes to transcription initiation in eukaryotic cells. The expression characteristics of the GRK1 promoter and its derivatives were further investigated using the all-cone *Nrl*^{-/-} mouse (blue cone rich) and the Nile rat (green cone rich) finding that the expression characteristics were more uniform in rods in comparison to the mouse opsin promoter.¹⁵³ The basal, condensed GRK1 promoter (199bp; from -112 to +87 position) drove efficient expression in rods and cones, excluding the conserved 5' untranslated region,¹⁵³ which is included in full-length promoter (292bp; -112 to +180).¹⁵³

Subretinal delivery of transgenes delivered by this version of the GRK1 promoter, were shown effective in the transduction of rod and cone photoreceptors in non-human primates,¹⁵⁴ following on from studies demonstrating efficacy of photoreceptor-specific GRK1-driven transgene expression in rodent models.¹⁵⁵⁻¹⁵⁹ The 199bp condensed derivate of the GRK1 promoter was cloned into the *CDHR1* expression plasmids used in this thesis, originally described by Khani in 2007, independent of a TATA box for transcription initiation.¹⁵³

Two RNA-Seq studies have identified *CDHR1* expression in retinal ganglion cells.¹⁶⁰ Retinal ganglion cell specific promoters have been described, such as *Thy1* (~6kb) and *Nefh*. However, since there is as yet no direct evidence of structural or functional effects on the RGCs from either human or murine forms of *CDHR1*-associated retinal degeneration, RGC-specific promoters were not used. Moreover, a promoter which expresses transgenes exclusively in both rod and cone photoreceptors and RGCs has not yet been described. Inducible gene expression systems were not used in this thesis, since stable, long-term expression of *CDHR1* was presumed necessary in both the murine *Cdhr1*^{-/-} model and in human *CDHR1*-associated retinal degeneration.

4.3.4 Selection of *cis*-acting enhancing elements

Numerous *cis*-acting enhancing elements have been identified and developed which independently increase the expression of a given transgene within eukaryotic cells. The inclusion of one or more non-coding *cis*-acting enhancing elements within the expression construct may allow an equivalent level of transgene expression with fewer viral particles, thereby reducing the potential adverse effects of dose-dependent inflammation. Selection of one or more appropriate *cis*-acting enhancing elements would allow a comparison of an enhanced with an unenhanced AAV.CDHR1 vector for *in vivo* analysis of safety and efficacy in the *Cdhr1*^{-/-} and *C57BL/6J* mouse models.

4.3.4.1 *Exon-Intron-Exon splice enhancer from the chicken beta-actin promoter*

The human cytomegalovirus CMV promoter features a distal enhancer (-550 to -300) and a proximal component (-300 to -39, relative to the transcriptional start site). Without the distal enhancer, CMV replication is slower. The major immediate-early promoter and enhancer of CMV is a powerful driver of recombinant gene expression in mammalian cells. The hybrid promoter, CAG, is formed in part from the CMV early enhancer. Inclusion of the first exon and intron from the CMV-IE has been shown to increase expression in mammalian cells.¹⁶¹
¹⁶² This sequence can be cloned immediately downstream of a promoter to promote transcription of the relevant transgene in an expression plasmid.

4.3.4.2 *Woodchuck hepatitis post-transcriptional regulatory element (WPRE)*

The woodchuck hepatitis post-transcriptional regulatory element (WPRE) was first described in 1998 as a naturally occurring post-transcriptional tripartite *cis*-acting enhancer, that is 2-3 fold more active than the bipartite hepatitis-B virus post-transcriptional regulatory element (HBVPRE).¹⁶³ The WPRE has been used to enhance transgene expression at the post-transcriptional level following AAV delivery, exhibiting a 2-50 fold increase in expression, depending on target tissue¹⁶⁴, which may increase further in combination with other *cis*-acting elements.¹⁶⁵ Specifically, WPRE-enhanced constructs packaged in AAV8 capsids exhibited improved transgene expression in liver, muscle and brain tissue.¹⁶⁶

Moreover, transgenes encoded by AAV exhibited significantly greater expression under WPRE enhancement following subretinal delivery, an effect also seen in *ex vivo* human retina.¹⁶⁷ A WPRE sequence has been included in the AAV2.REP1 gene therapy vector in clinical trial for choroideremia, the pre-clinical study used a modified WPRE sequence, with modification of the We1 promoter and deletion of the We2 promoter.^{103, 168}

4.3.5 Selection of mRNA-stabilising polyadenylation sequence

The polyadenylation sequence, or tail, is present downstream (3') of a coding sequence and is important in the stability, nuclear export and translation of mRNA sequences. Almost all eukaryotic mRNA sequences are polyadenylated since this modification protects transcripts from cytoplasmic degradation. The bovine growth hormone polyadenylation sequence was shown to result in 3-fold higher expression when compared to the early SV40 or human collagen polyadenylation signals.¹⁶⁹ This effect may be attributable, in part, to the formation of an extensive hairpin loop by the bovine growth hormone polyadenylation tail which prevents cleavage of transcripts by cytoplasmic nucleases.¹⁷⁰ The bovine growth hormone polyadenylation sequence was first patented in 1984 to support the expression of genes in eukaryotic cells.

4.3.6 *CDHR1* transgene sequence

The human *CDHR1* coding sequence (cDNA) is 2,577bp in length, encoding a peptide of 859 amino acids - the murine *Cdhr1* homolog is of identical length. The *CDHR1* cDNA is of “low complexity” (IDT codon optimisation tool score 4.7 (*Homo sapiens*) and 4.4 (*Mus musculus*) with no repetitive or unstable sequences (<7 signifies low complexity) with GC content 57.1% (ideal: 30-70%), and percentage of low frequency codons is 1%, based on the relevant target organisms. All parameters predicted efficient *CDHR1* expression in murine models. Analysis of the wildtype human *CDHR1* coding sequence did not identify any negative *cis*-acting elements such as cryptic splice sites or negative repeat elements. Consequently, there was no justification for the synthetic modification of the *CDHR1* transgene sequence for the purposes of AAV gene therapy in humans.

The *CDHR1*-expressing constructs featured a classic six nucleotide Kozak consensus sequence “GCCACC” to promote the initiation of translation of the transgene, identical to sequences typically used in clinical trials. A recent study identified a modest increase in expression (<10%) of transgenes expressed in the retina through Kozak optimisation in a luciferase assay using retinoblastoma cell lines (WERI-RB1 and Y-79), featuring a 9 nucleotide Kozak consensus sequence “ACCGAGACC”.¹⁷¹ However, the sequence of nucleotides in the -7 to -9 positions were not further modified.

4.3.7 Surgical method of AAV vector delivery

Several methods of surgical AAV vector delivery have been described and evaluated for the transduction of retinal cells. From anterior to posterior, these include intra-vitreous, sub-internal limiting membrane (sub-ILM), subretinal and suprachoroidal.^{172, 173} The preferred method depends on the target cell type for transduction, the dose required to achieve a therapeutic effect, the surgical equipment and viewing/imaging systems, target species and age of subject, and limitations related to dose-related intraocular inflammation.

The ideal method of surgical vector delivery aims to use the minimal amount of AAV vector to achieve the maximum therapeutic effect, with the smallest risk of eliciting inflammatory responses. For this reason, subretinal delivery is the most logical for photoreceptor transduction since high titres of vector are delivered immediately adjacent to photoreceptors. Smaller AAV doses delivered into a relatively immune privileged space reduces the risk of inflammation. The first FDA (USA) and NICE-approved (UK) retinal gene therapy product targets the RPE and is administered by subretinal injection following pars plana vitrectomy. However, iatrogenic photoreceptor detachment is implicit in the subretinal injection vector delivery technique which may lead to outer retinal morphological changes, such as loss of outer segments, thinning of the outer nuclear layer and focal RPE loss – all of which may affect measures of visual function in patients (microperimetry, visual acuity etc.). Surgical risk can be mitigated somewhat by

introducing the subretinal cannula outside the macula, allowing blebs to progress into the macula without foveal detachment; thereby sparing the outer segments of foveal cones. This is less of a concern in the radially symmetrical murine retina without an anatomical macula or analogous cone-rich region. Furthermore, the accurate anatomical application of vector following subretinal delivery permits the comparison of treated and untreated areas, and therefore the utility of an internal control. This is not possible with intravitreal vector delivery.

Ab externo subretinal injection was selected as the preferred surgical method for AAV delivery in experimental mice for the pre-clinical development and evaluation of *CDHR1*-gene therapy. Moreover, the translational relevance of this technique further supports its use; further non-human primate studies are not required prior to clinical trial.

4.3.8 Vector dosing study: experimental design

There are currently no suitable photoreceptor cell lines which express transgenes driven by the GRK1 promoter; *in vitro* validation of the AAV8.CDHR1 vector constructs was not possible. *Cdhr1* protein expression was not detected in the *Cdhr1*^{-/-} mouse by immunohistochemistry and western blotting (Chapter 3), supporting its use for the *in vivo* validation of the two AAV8.CDHR1 vector constructs (Figure 4.1). Four *Cdhr1*^{-/-} mice were injected with each vector to ensure adequate tissue for immunohistochemistry/western blotting. Prior to

tissue harvesting, OCT imaging was undertaken to determine if retinal thinning was present at the selected dose, or varied between vector groups.

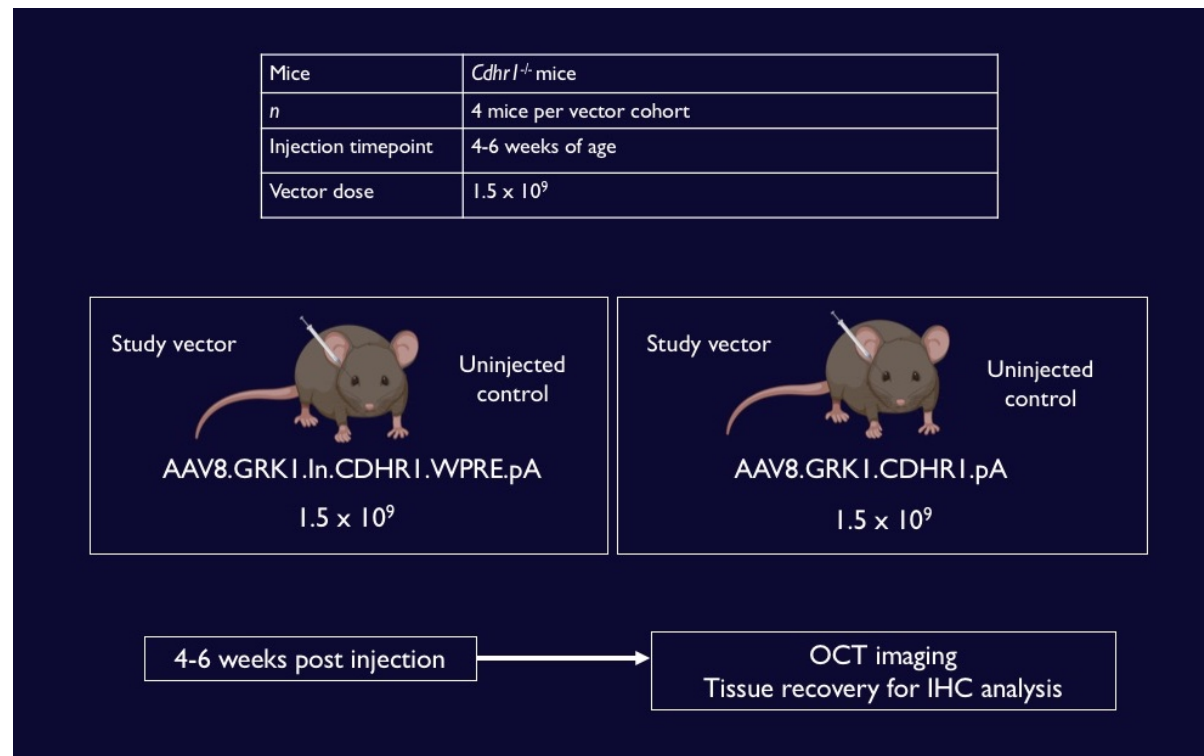


Figure 4.1 Experimental design of the AAV8.CDHR1 *in vivo* vector validation study

Assessment 4 weeks post-injection was considered sufficient for retinal transduction following AAV8.CDHR1 vector delivery.¹⁷⁴ Moreover, a high vector dose was selected (as permitted by the achieved AAV titre for both vectors) to maximise CDHR1 expression.¹⁷⁴ Control eyes were left un-injected since it was shown in Chapter 3 that *Cdhr1* expression was not present under these conditions. CDHR1 immunohistochemistry was undertaken according to protocols detailed in Chapter 2 & 3.

4.4 Results and vector selection

4.4.1 Cloning of the *CDHR1* transgene

The human *CDHR1* coding sequence (cDNA; NM_033100) was amplified from 10ng of plasmid DNA (GenScript, Hong Kong) with KOD polymerase, using flanking primers containing suitable restriction sites (*HpaI* and *HindIII*) for downstream sub-cloning. The reaction conditions were: Polymerase activation - 95°C for 2 mins, Denaturing – 95 °C for 20s, Annealing - 48 °C for 10 seconds (based on theoretical T_m of primers of 53 °C and 57 °C, Extension – 70°C for 52s [25 cycles]. The products of the PCR reaction produced an appropriate sized amplicon on 1% agarose gel electrophoresis (Figure 4.4).

4.4.2 Cloning of *CDHR1* expression plasmids

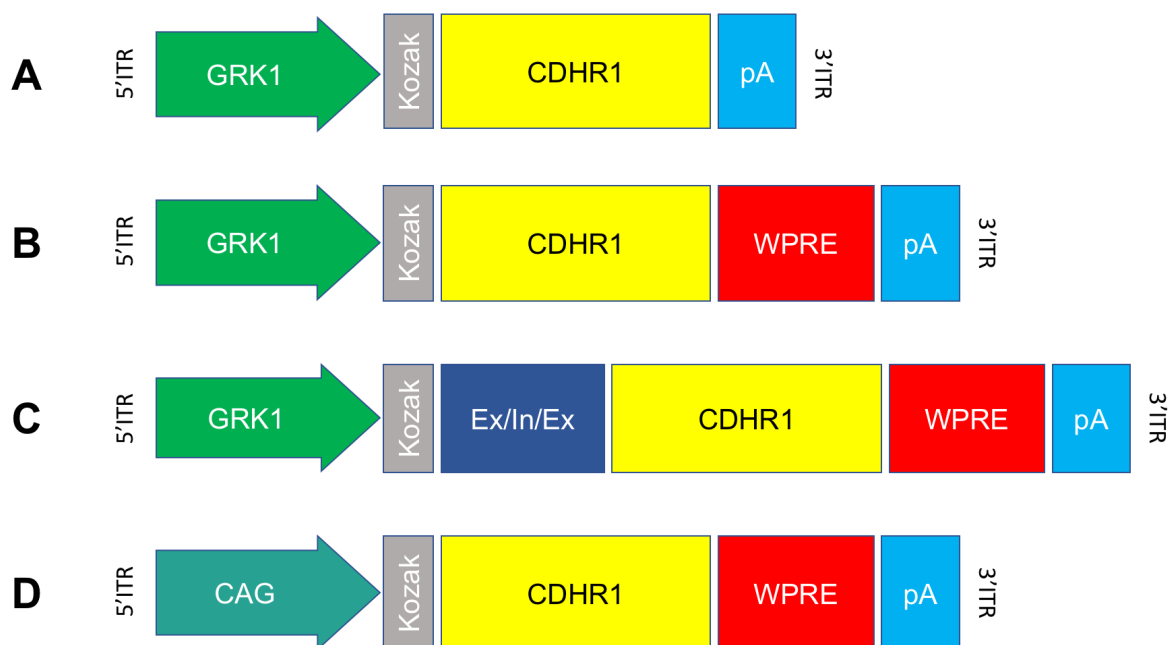


Figure 4.2 Design of four constructs expressing the *CDHR1* transgene

[Continued] GRK1 – human rhodopsin kinase; Ex/In/Ex – enhancer sequence from the CMV promoter; CAG – CMV early enhancer/first intron and exon from chicken beta actin promoter/splice acceptor site from rabbit beta-globin gene; WPRE – woodchuck hepatitis post-transcriptional regulatory element; pA – bovine polyadenylation sequence

Four plasmids expressing the human *CDHR1* transgene were designed and created (Figures 4.2 & 4.3). Three were driven by the GRK1 promoter for *in vivo* evaluation, and one by the CAG promoter for *in vitro* assay validation (i.e. positive control on western blot).

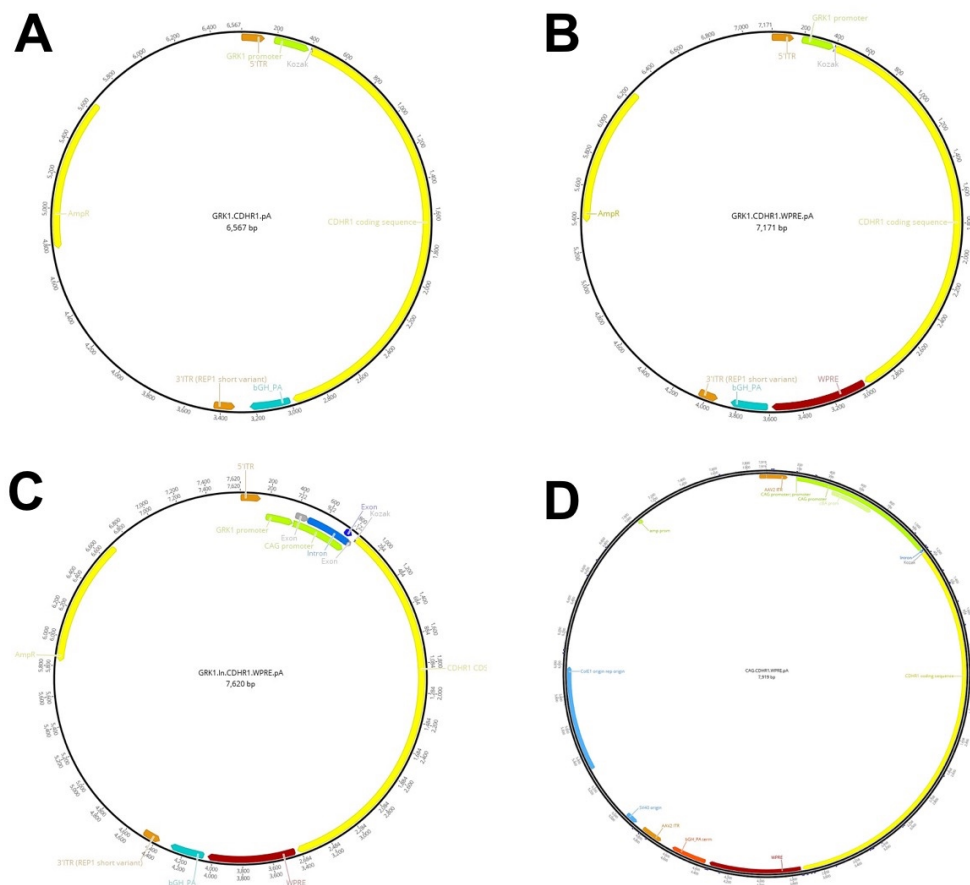


Figure 4.3 Plasmid maps of four vector constructs expressing the *CDHR1* transgene
(A) GRK1.CDHR1.pA (B) GRK1.CDHR1.WPRE.pA
(C) GRK1.In.CDHR1.WPRE.pA (D) CAG.CDHR1.WPRE.pA

4.4.2.1 *GRK1.CDHR1.WPRE.pA*

The GRK1.GFP.WPRE.pA expression plasmid was modified to excise the GFP sequence and replace it with the full-length human CDHR1 sequence. The cloning steps in CDHR1 coding sequence amplification, sub-cloning, sequencing and confirmation of ITRs are presented in Figure 4.4.

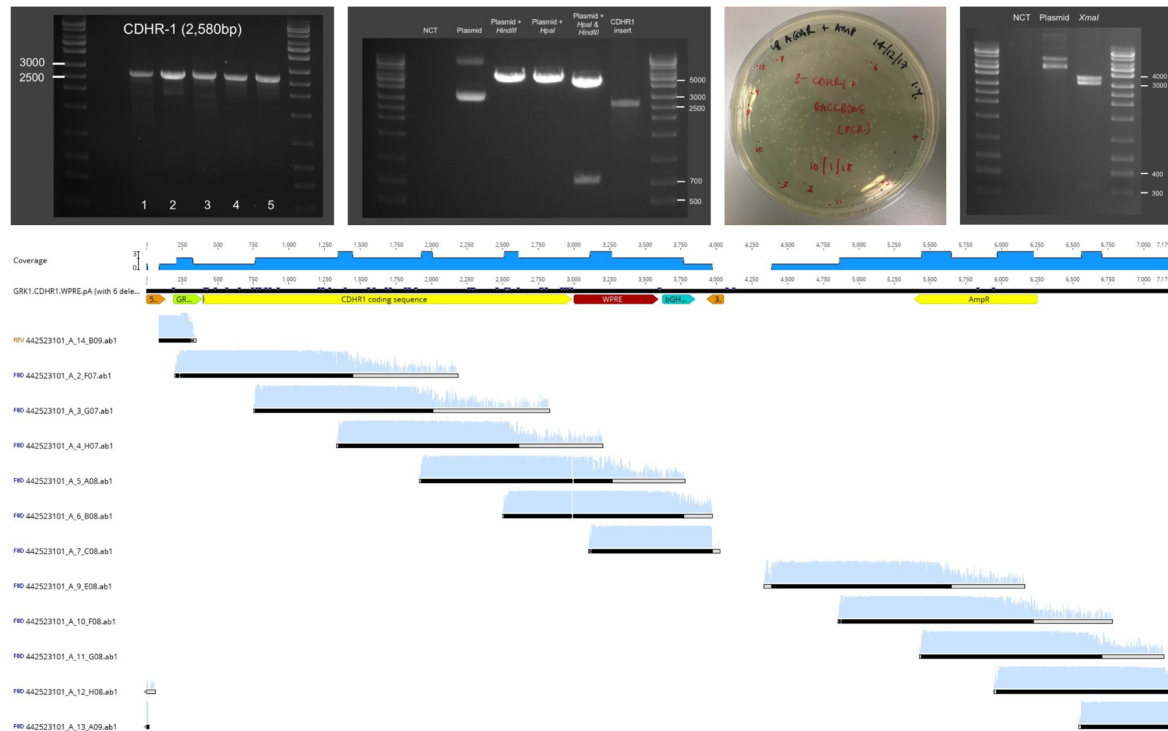
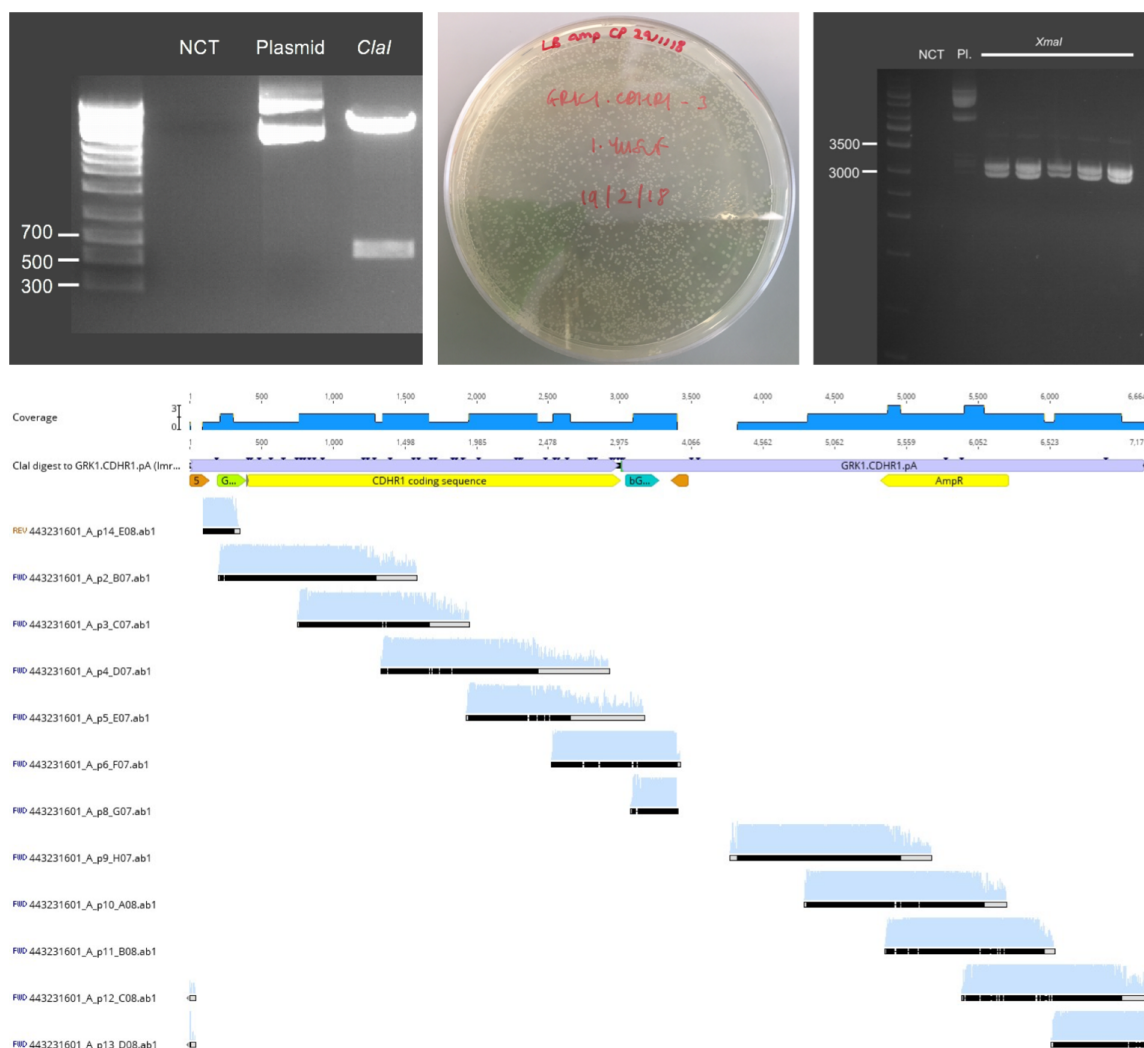


Figure 4.4 Cloning and sequencing of the *CDHR1* transgene and GRK1.CDHR1.WPRE.pA construct

The human CDHR1 sequence was amplified by KOD polymerase-based PCR reaction. This produced a 2,580bp amplicon as expected (**A**). The GRK1.GFP.WPRE.pA plasmid was digested with *HpaI* and *HindIII* which successfully linearized the plasmid as expected (**B**). Gel extraction of the double digested plasmid backbone and *CDHR1* amplicon was undertaken, followed by ligation, transformation and plating, showing colonies (**C**). *XmaI* digest reveals the 3' and 5' AAV2 ITRs are present as expected with bands ~3,600, 3,200 and 340bp in size (**D**). Full sequencing of GRK1.CDHR1.WPRE.pA is shown in (**E**). The vector sequence annotations are presented as coloured bars, with sequenced nucleotide sequences shown below in blue.

The GRK1.CDHR1.WPRE.pA expression plasmid was modified to remove the WPRE *cis*-acting enhancing element (Figure 4.5), before confirmation of the presence of both AAV2 ITRs by *XmaI* digest, and Sanger sequencing.



The GRK1.CDHR1.WPRE.pA sequence was digested with *ClaI* to excise the WPRE sequence, demonstrated on 1% agarose gel electrophoresis. (A). Ligation of the backbone to itself with numerous colonies (B). *XmaI* digest reveals the presence of 3'ITR and 5'ITR as expected (C) Sequencing revealed correct excision of WPRE and no errors in CDHR1 coding sequence with excellent coverage (D). NCT – negative control; Pl – plasmid.

4.4.2.3 GRK1.In.CDHR1.WPRE.pA

The exon-intron-exon sequence from the CAG promoter was PCR amplified from a parent plasmid using flanking primers which introduced suitable restriction sites (*HpaI*) and subsequently ligated into the GRK1.CDHR1.WPRE.pA backbone downstream of the GRK1 promoter (Figure 4.6).

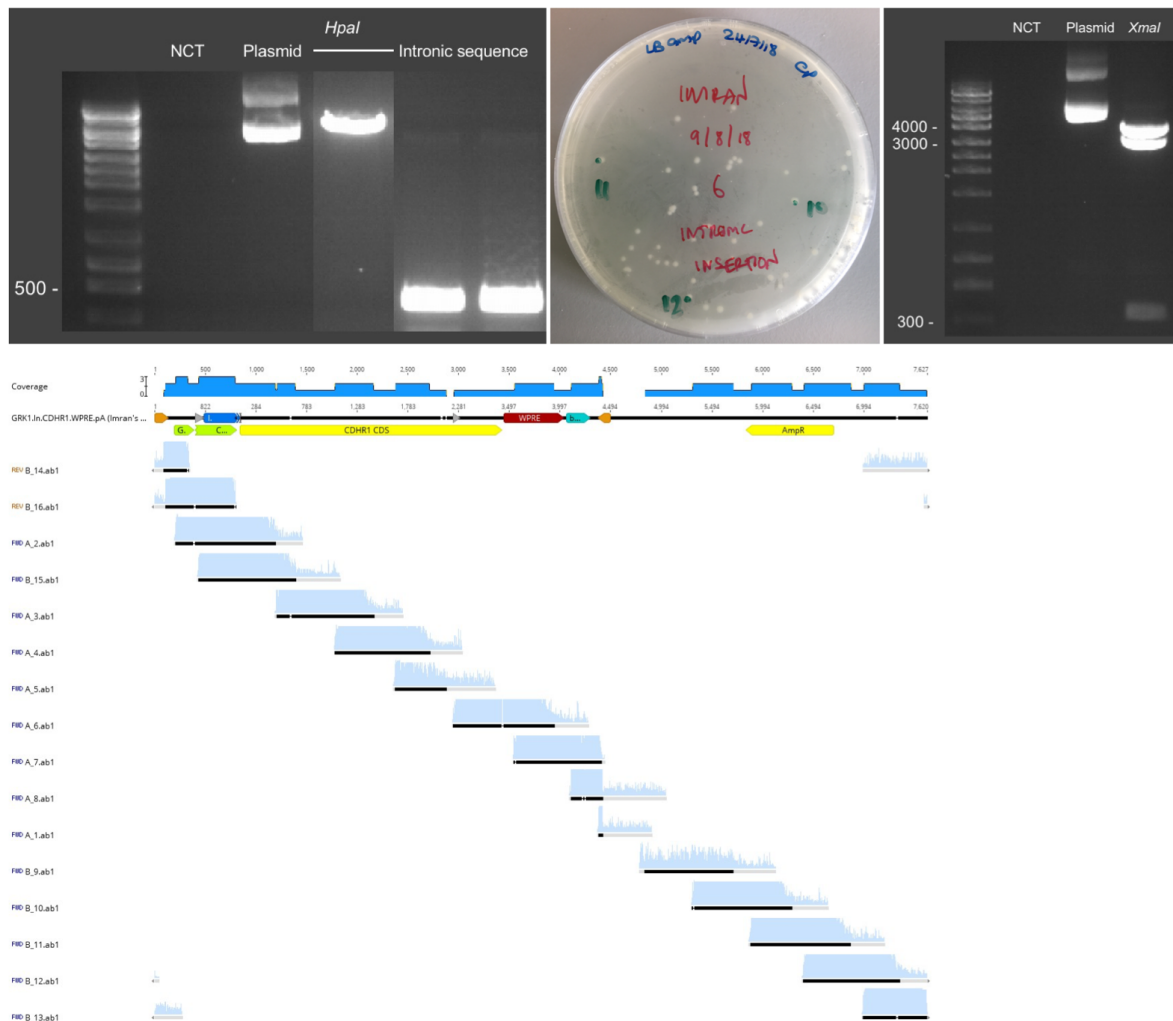


Figure 4.6 Cloning and sequencing of GRK1.In.CDHR1.WPRE.pA construct

The intronic sequence of the CAG promoter (Exon/Intron/Exon) was amplified using flanking primers (see Appendix) using KOD polymerase PCR amplification. This generated a 435bp amplicon which was gel extracted and purified (A). The GRK1.CDHR1.WPRE.pA backbone was digested with *HpaI* and successfully linearized (A: gel cropped to remove superfluous information). The intronic

[Continued] amplicon was ligated into the linearised backbone with colonies on the LB-agar-ampicillin plate **(B)**. Sanger sequencing revealed successful incorporation of the intronic sequence. *XmaI* digest revealed bands consistent with the presence of 3' and 5'ITRs (341, 3193, 4075bp) **(C)**. Sanger sequencing reveals good sequencing coverage of the plasmid with intronic sequence inserting 5' of the CDHR1 coding sequence with preservation of the Kozak consensus sequence **(D)**. NCT – negative control.

4.4.2.4 CAG.CDHR1.WPRE.pA

In the absence of a photoreceptor cell line that reliably expresses transgenes driven by the human rhodopsin kinase promoter (GRK1), a CDHR1 expression plasmid was created driven by the ubiquitous CAG promoter created from the chicken beta CMV early enhancer/ Chicken β -actin (promoter, first intron and exon)/ Rabbit β -globin gene (splice acceptor). Transfection of CAG.CDHR1.WPRE.pA into cell lines (such as HEK293) enabled the expression of human CDHR1 protein for positive controls. The plasmid was created from CAG.GFP.WPRE.pA through amplification of the *CDHR1* transgene using primers with HindIII and EcoRV restriction sites, which were double digested into the plasmid backbone following excision of the GFP sequence by gel extraction following 1% agarose gel electrophoresis. Dephosphorylation of the plasmid backbone was achieved with shrimp alkaline phosphatase (rSAP, NEB, USA), to reduce the probability of re-ligation of the backbone, incubated at 37.C for 30 mins, with heat inactivation at 65.C for 5 mins. *XmaI* digest confirmed the presence of ITRs, and the sequence confirmed by Sanger sequencing following megaprep plasmid purification.

4.4.3 Selection of vector constructs for *in vivo* assessment

The ideal CDHR1 vector construct is one that leads to expression of a therapeutic quantity of transgene without AAV- or transgene-related inflammation or transgene overexpression. However, the level of CDHR1 expression (mRNA or protein) necessary in each rod and cone photoreceptor in order to rescue photoreceptor structure and function in *CDHR1*-associated retinal degeneration was unknown. Furthermore, it was unknown as to whether overexpression of CDHR1 is toxic to photoreceptors, although dose-dependent inflammation has been shown following intra-vitreous and sub-retinal delivery of AAV8 vectors expressing other transgenes.^{129, 175, 176} Dose-toxicity studies are therefore necessary, both in small animal models and in patients (i.e. phase 1 trials) in order to determine the maximum tolerated dose of AAV8.CDHR1 vector.

Immunohistochemical analysis of *C57BL/6J* mouse retina suggests a relatively low level of expression of CDHR1 at the base of the photoreceptor outer segments (Chapter 3), of similar magnitude to RPGR.¹⁰⁴ A strongly enhancing vector may permit a reduction in the dose of AAV required to achieve a given level of transgene expression, thereby reducing the risk of dose-related inflammation related to AAV capsids. However, if transgene overexpression results in toxic effects, a less efficient vector may be preferable, particularly for CDHR1 which is not highly expressed in photoreceptors relative to other genes (i.e. rhodopsin etc).

Two CDHR1 expression constructs were selected in order to compare the effects on retinal structure and function of a “unenhanced” (GRK1.CDHR1.pA) with an “enhanced” construct (GRK1.In.CDHR1.WPRE.pA) in which two *cis*-acting enhancing elements were sub-cloned. The two vector constructs were selected for downstream AAV packaging and *in vivo* assessment.

4.4.4 AAV8.CDHR1 vector production

AAV8 vector production was performed according to the protocol outlined in Chapter 2 to package two CDHR1 expression plasmids cloned within AAV2 ITRs:

- GRK1.CDHR1.pA
- GRK1.In.CDHR1.WPRE.pA

Viral purity of the two AAV8.CDHR1 vector constructs was assessed by SDS-PAGE using EZ blue dye, showing three protein bands corresponding to the viral capsid proteins (VP1/VP2/VP3) without contaminants in both the concentrate and the wash from both preparations. Viral titre was assessed by qPCR, using primers targeted against the CDHR1 transgene (Appendix), using a standard curve generated using serial dilution of the original plasmid and SYBR green. The AAV titre of AAV8.GRK1.CDHR1.pA was 9.11×10^{12} gc/ml and AAV8.GRK1.In.CDHR1.WPRE.pA was 3.09×10^{12} gc/ml (Figure 4.7). A higher titre for the unenhanced vector may be explained by a shorter recombinant viral

genomic sequence. The AAV empty capsid ratio and endotoxin levels were not assessed.

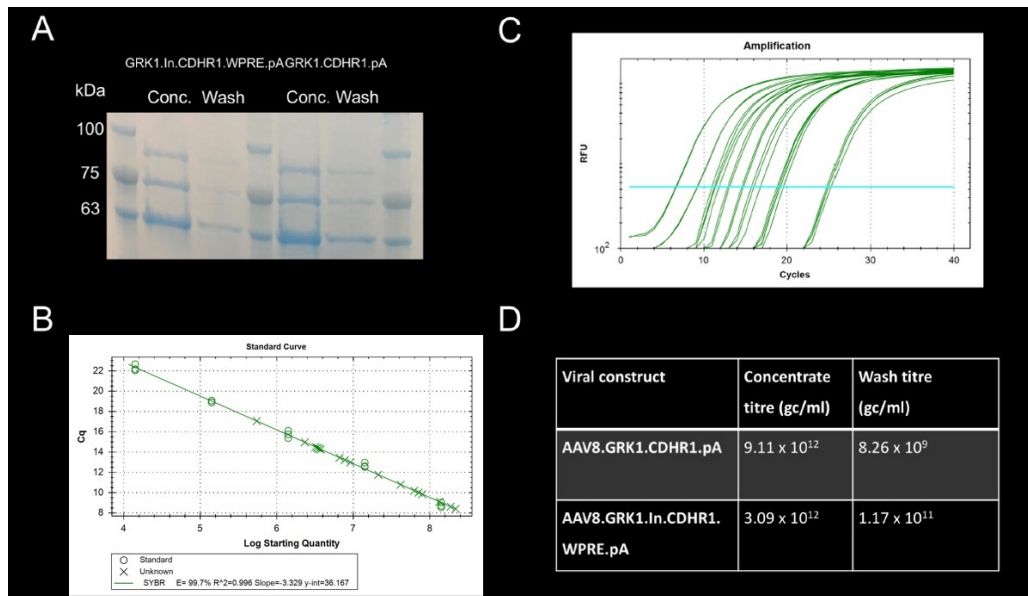


Figure 4.7 Assessment of AAV purity by SDS-PAGE and titre by qPCR (A) SDS-PAGE of concentrate and wash samples of the enhanced and unenhanced AAV8 preparations. Three bands are evident, representing the three major viral capsid proteins (VP1/VP2/VP3). No contaminants are identified. (B) A standard curve, derived from qPCR of serially diluted CDHR1 expression plasmids. (C) Amplification plot showing the derivation of the Cq values plotted in (B). Note that distortion of amplification curves at high concentrations may lead to inaccuracies due to a fixed background correction from cycles 1 to 15. (D) Viral titres for both AAV8.CDHR1 constructs for both concentrate and wash samples.

Subretinal delivery of 1.5µl of solution is sufficient to detach the superior hemiretina in mouse eyes when injected post-weaning, such that the optic nerve is obscured in the primary position immediately after surgery. This translated to a maximum deliverable dose of 1.37×10^{10} vg for AAV8.GRK1.CDHR1.pA and 4.64×10^9 vg for AAV8.GRK1.In.CDHR1.WPRE.pA. A dose of 1.5×10^9 vg, delivered in 1.5µl, was therefore selected as the maximum dose used to determine expression of CDHR1 following subretinal delivery of each AAV8.CDHR1 vector construct.

4.4.5 Sub-retinal delivery of AAV8.GRK1.CDHR1.pA results in CDHR1 expression in rod and cone photoreceptors in the *Cdhr1*^{-/-} retina

Immunohistochemistry using anti-CDHR1 primary antibodies directed against both the N-terminal ectodomains (Abcam) and the C-terminus (Johns Hopkins), identified CDHR1 protein in photoreceptor cells of *Cdhr1*^{-/-} mice following subretinal injection of AAV8.GRK1.CDHR1.pA. Immunostaining with GFP-conjugated peanut agglutinin, which stains cone sheaths, confirmed CDHR1 expression in cone photoreceptors following vector delivery (Figure 4.8). Perinuclear staining with accumulation of CDHR1 within the photoreceptor inner segments was identified in the superior retina; features which may be consistent with overexpression. In the inferior retina, a more diffuse, speckled pattern of CDHR1 immunostaining as observed in wildtype mice (Chapter 3) was demonstrable (Figure 4.8). CDHR1 was not identified in uninjected control eyes, or in control sections treated only with secondary antibodies (data not shown).

4.4.6 Sub-retinal delivery of AAV8.GRK1.In.CDHR1.WPRE.pA results in CDHR1 expression in rod and cone photoreceptors in the *Cdhr1*^{-/-} retina

Subretinal injection of the enhanced vector, AAV8.GRK1.In.CDHR1.WPRE.pA resulted in a similar pattern of CDHR1 expression to the unenhanced vector with perinuclear staining of CDHR1 and accumulation within the photoreceptor inner segments. CDHR1 immunostaining was not evident in uninjected control eyes or secondary antibody only control sections.

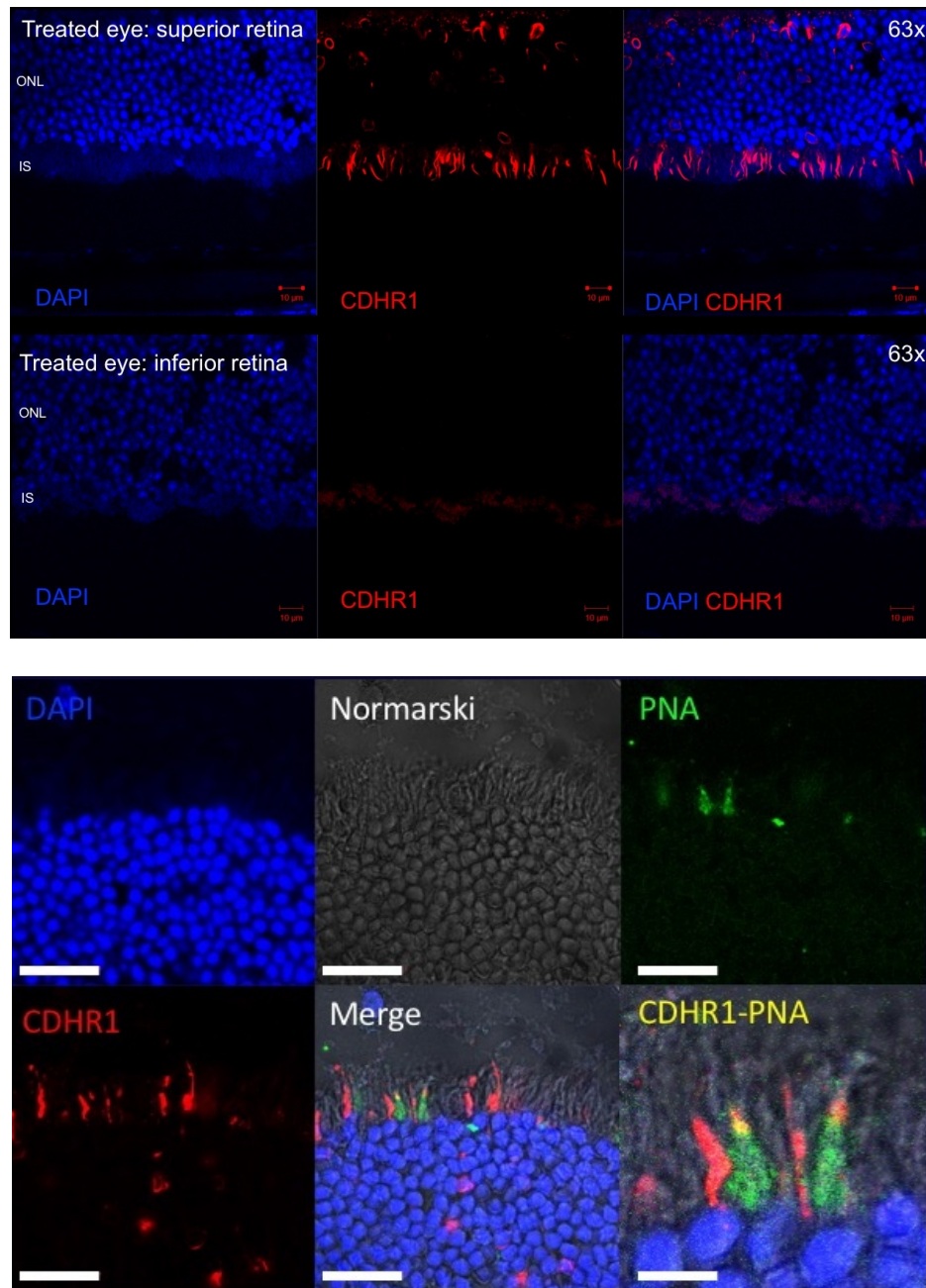


Figure 4.8 CDHR1 immunohistochemistry following sub-retinal injection of AAV8.GRK1.CDHR1.pA at a dose of 1.5×10^9 vg in *Cdhr1*^{-/-} mice

Upper CDHR1 expression is demonstrated in the superior retina in AAV-injected retinas around the photoreceptor cell nuclei and within the inner segments. A lower level of expression is demonstrated in the inferior retina. *Lower* Counterstaining with GFP-conjugated peanut agglutinin (PNA) which stains cone sheaths confirms CDHR1 expression in cone photoreceptors (yellow fluorescence). CDHR1 is not expressed in PBS-injected control eyes (data not shown). Scale bars represent 10μm, upper panel, and lower panel, and 5μm in lower right image.

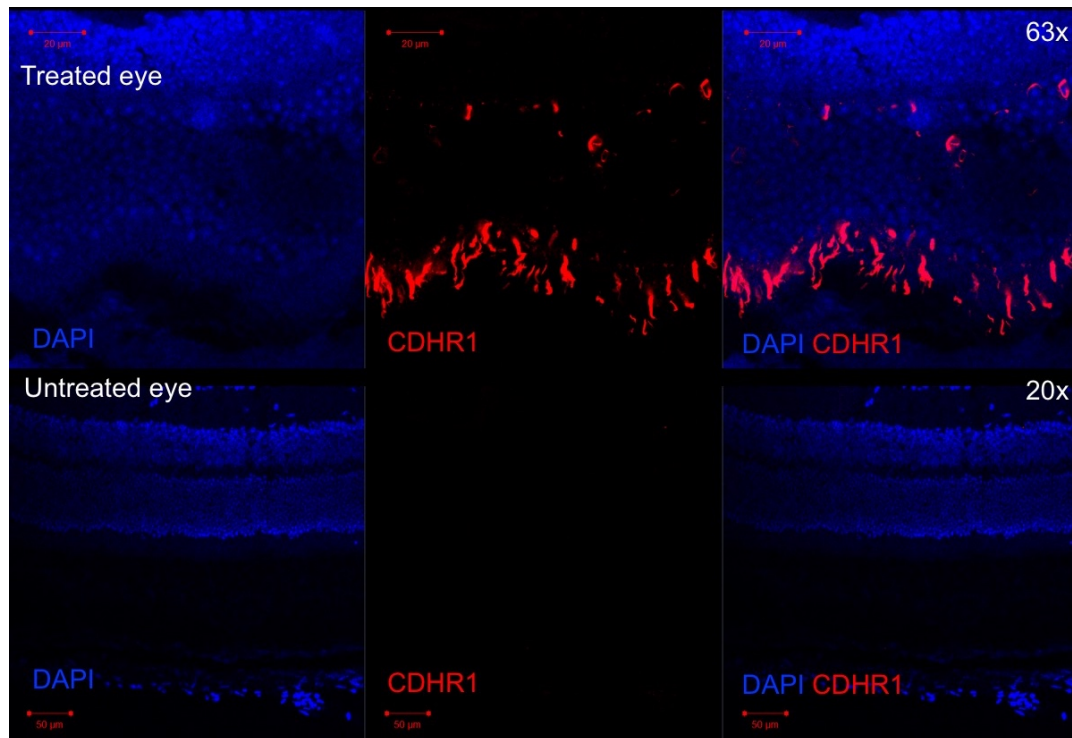


Figure 4.9 CDHR1 immunohistochemistry following sub-retinal injection of AAV8.GRK1.In.CDHR1.WPRE.pA at a dose of 1.5×10^9 vg in *Cdhr1*^{-/-} mice

CDHR1 expression is demonstrated around photoreceptor nuclei and within the inner segments in the superior retina of AAV-injected eyes. CDHR1 expression is not present in uninjected control eyes (lower). Scale bars represent 20μm in the upper panel and 50μm in the lower panel.

4.4.7 Retinal thickness measurements on OCT imaging following sub-retinal injection of AAV8.CDHR1 vectors at 1.5×10^9 vg per eye.

Since the effects of sub-retinal injection of AAV8.GRK1.CDHR1.pA were unknown, OCT imaging was undertaken under anaesthetic without recovery followed by tissue harvesting. Total retinal thickness measurements were recorded (ILM to RPE) in AAV injected and untreated paired control eyes, in both superior and inferior retinal locations for both vectors (at loci indicated as per methodology in Chapter 3). This analysis showed significant superior retinal

thinning in AAV-injected eyes (Figure 4.10). Since the control group was untreated, the observed superior retinal thinning may have been due to the effect of retinal detachment and/or toxicity related to the AAV capsid or transgene. However, there was a significant trend towards retinal thinning at equivalent AAV doses with the enhanced vector construct ($P=0.047$), which also produced inferior retinal thinning in AAV-injected eyes. For the unenhanced vector, mild retinal thinning may have been due to the effect of retinal detachment alone.

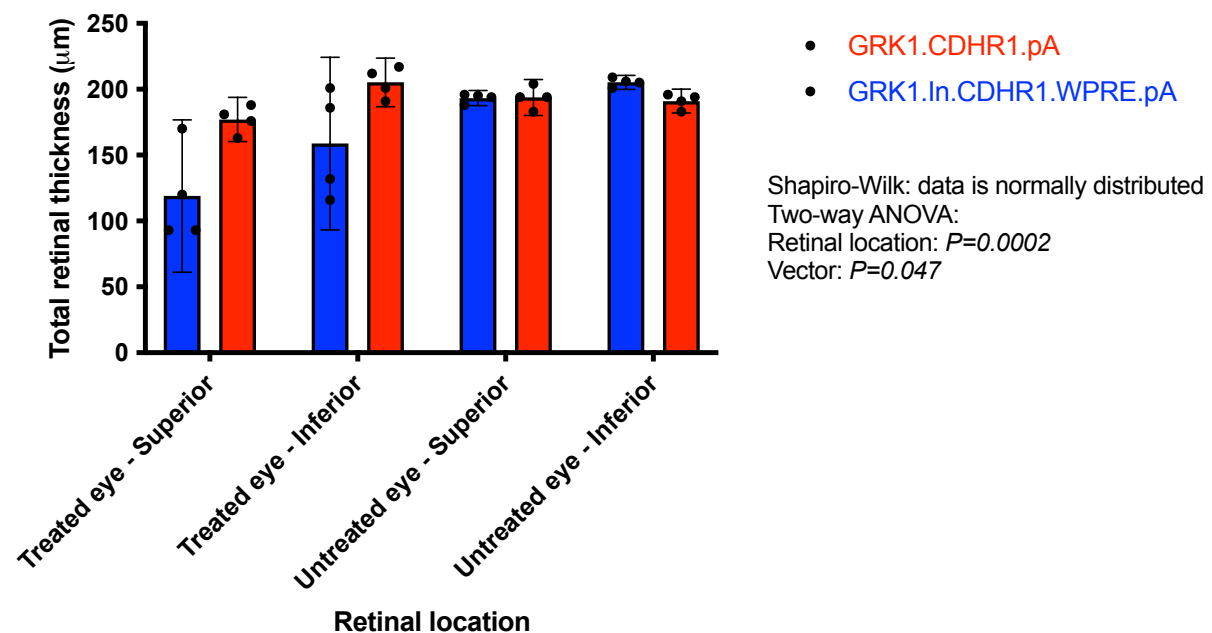


Figure 4.10 Total retinal thickness measurements on OCT imaging 4-weeks post-injection with AAV8.CDHR1 vectors at a dose of 1.5×10^9 vg
At equivalent doses, retinal thinning is shown in the superior and inferior retina following AAV-injection of the enhanced vs. unenhanced AAV8.CDHR1 vector construct ($P=0.047$). The experimental design did not control for the effect of surgically induced retinal detachment, since control eyes were untreated.

4.5 Discussion

In this chapter, the rational design, cloning of expression plasmids, AAV vector production and *in vivo* expression of two *CDHR1* vector constructs have been demonstrated. The efficient expression and correct localisation of *CDHR1* following sub-retinal delivery of a dose of 1.5×10^9 vg justifies the rational design of the *CDHR1* expression plasmids as they relate to the selection of promoter, polyadenylation sequence, *cis*-acting enhancing elements, and AAV capsid without the use of mutant capsid variants or codon optimisation. The rational design of CDHR1 vectors was influenced by the possibility of future regulatory approval of any validated vector for onward clinical trial; vector components already approved for clinical trial were favoured. In particular, confirmation of *CDHR1* expression in cone photoreceptors was crucial in the support downstream assessments (i.e. light-adapted ERG and photopic optomotor testing). The validation of two AAV constructs expressing the *CDHR1* transgene enables the downstream *in vivo* comparison of vector dosing, safety and efficacy.

Overexpression of CDHR1 following subretinal delivery of both AAV8.CDHR1 vectors at a dose of 1.5×10^9 vg is likely based on equivalent immunohistochemical analysis in *C57BL/6J* mice (Chapter 3). Furthermore, retinal imaging studies post-injection identified a greater degree of retinal thinning following subretinal injection of the enhanced AAV8.CDHR1 vector when compared to the unenhanced vector at an equivalent dose, at both superior and inferior retinal locations, albeit

in a small pilot cohort. This observation supports the hypothesis of retinal toxicity due to transgene overexpression with the enhanced vector at 1.5×10^9 vg, although other mechanisms may be relevant. Overexpression of proteins that are trafficked to the photoreceptor outer segments may produce toxicity by overloading the cilium or inner segment, competing with the transcription, translation, post-translational modification and/or transport of other critical outer segment proteins (rhodopsin, cone opsins etc).¹⁷⁷ This is supported by the apparent accumulation of CDHR1 within the inner segments and the outer nuclear layer of transduced photoreceptors at a dose of 1.5×10^9 vg with both vector constructs. Retinal thinning on OCT imaging may be a biomarker for retinal toxicity following sub-retinal AAV delivery. Moreover, the use of superior and inferior retinal thickness measurements serves as a useful internal control for vector dosing in AAV-injected eyes. However, in the absence of an active control intervention in paired eyes, the effect of surgically-induced retinal detachment on retinal thickness measurements could not be evaluated. Consequently, it could not be determined from these experiments whether the unenhanced vector was toxic at a dose of 1.5×10^9 vg.

The mechanism of dose-dependent toxicity with GRK1.In.CDHR1.WPRE.pA is unclear. Potential mechanisms include immunogenicity to AAV particles or encoded genetic sequences, immunogenicity to the membrane-bound transgene, overexpression of the CDHR1 transgene, or others. These mechanism(s) could be

further elucidated through the production of a null vector packaged in identical wildtype AAV8 capsids, introducing a single nucleotide change in the GRK1 promoter sequence, or a premature stop codon in the transgene. This experimental approach would control for dose-dependent responses to AAV, permitting an evaluation of the effect of the transgene expression on retinal structure and function. However, other mechanisms may exist: for example, specific nucleotide sequences within AAV genomes (such as unmethylated CpG dinucleotides) can trigger cellular immune responses and photoreceptor toxicity. It is notable that a similar hybrid promoter to that used in the enhanced vector was found to be associated with dose-dependent toxicity in pre-clinical studies of RP2 gene therapy.¹⁵⁰

Multiplicity of infection (MOI) may be used as a parameter to estimate or model the number of viral particles per target cell in experimental models. Histological analysis of the *C57BL/6J* mouse retina revealed an estimated 6.4 million rod photoreceptors and 180,000 cone photoreceptors per retina (average rod density of 437,000/mm² and cone density of 12,400 cells/mm²). A dose of 1.5 x 10⁹ vg of GRK1.In.CDHR1.WPRE.pA delivered under the superior hemiretina corresponds to mean MOI of 450 viral particles per photoreceptor, associated with superior retinal toxicity. However, an equivalent MOI did not appear to be as toxic with the unenhanced vector. Moreover, an approximate MOI of 600 was found to be therapeutic following AAV gene therapy in a mouse model of RPGR-related

XLRP.¹⁰⁴ The effect of an equivalent MOI is likely influenced by the transgene, other elements of the vector (promoter, cis-acting enhancers, capsid), delivery system or surgical technique etc. and the disease model and disease stage. One limitation of the model is that transduction may not occur uniformly. This can be refined using a Poisson distribution which can estimate the number of cells that are transfected more than once, once or that remain untransfected. A MOI of 10 or greater generally restricts the number of untransfected cells. A further limitation is that post-operative migration of the bleb under the inferior retina with gravity may also occur, which may also be dependent on dose and capsid.¹⁴¹ Moreover, the photoreceptor cell density is likely to be reduced in *Cdhr1*^{-/-} mice with ~14% photoreceptor cell loss at 1-month of age based on data in Chapter 3.

The most rational therapeutic strategy is the transduction of the maximum number of rod and cone photoreceptors within the constraints of the experimental design and what is technically achievable with subretinal vector delivery. Treatment of a hemi-retina is both technically more achievable (as it requires a single *ab externo* sub-retinal injection), and also allows for an internal control (i.e. untreated inferior retina). However, any measured therapeutic effect (i.e. on electroretinography) may only represent 50% of what might otherwise be achievable with vector delivery to both the superior and inferior retina. For proof-of-principle studies, evidence of a structural rescue effect that matches the location

of AAV delivery is preferable to an increase in the magnitude of the functional rescue effect.

An identical protocol that successfully demonstrated murine *Cdhr1* in wildtype *C57BL/6J* retinæ on western blot was persistently unsuccessful in detecting human CDHR1 following sub-retinal delivery of either AAV8.CDHR1 vector in *Cdhr1*^{-/-} mice. Successful western blotting of human CDHR1 has not been published, suggesting that the antibodies may depend on tertiary and quaternary CDHR1 protein structure in the human homolog which is disrupted during tissue processing for western blotting. Moreover, the CDHR1 ectodomain is cleaved *in vivo*, and may be easily lost into the solute; it is for this reason that the c-terminal fragment is more abundant on western blotting (Chapter 3). A further explanation is that a relatively low abundance of CDHR1 protein following AAV delivery may fall below the detection limit of the assay, once sample processing has been performed. Indirect evidence of full-length CDHR1 expression with each AAV8.CDHR1 vector was provided through successful immunostaining using both N-terminal and C-terminal anti-CDHR1 primary antibodies.

4.6 Conclusions

In this chapter, successful sub-cloning of the full-length CDHR1 transgene into four rationally designed expression plasmids was performed, followed by packaging of two lead constructs into AAV8 wildtype capsids with appropriate purity and titre. Sub-retinal injection of both AAV8.CDHR1 vectors in *Cdhr1*^{-/-} mice demonstrated exclusive expression and localisation in rod and cone photoreceptors with antibodies against the N- and C-terminus of human CDHR1, suggesting full-length protein expression. Retinal thickness measurements on OCT imaging appeared to detect a difference between vectors and retinal locations, suggesting a methodology that could support the high-throughput required to test multiple vector doses for both AAV8.CDHR1 constructs as part of the vector dosing study presented in Chapter 5.

5. AAV8.CDHR1 vector dosing study

5.1 Introduction

The *CDHR1 in vivo* expression study demonstrated likely overexpression of CDHR1 protein with both AAV8.CDHR1 vectors at a dose of 1.5×10^9 in *Cdhr1*^{-/-} mice (Chapter 4). Whilst diffuse, speckled immunostaining of murine *Cdhr1* at the base of the outer segments is seen in *C57BL/6J* mice (Chapter 3),^{47, 51, 52} perinuclear and inner segment accumulation of CDHR1 was evident following vector delivery at this dose. OCT imaging demonstrated superior and inferior retinal thinning with the enhanced vector when compared to the unenhanced vector at a dose of 1.5×10^9 vg, suggesting important differences between the constructs and the possibility of retinal toxicity secondary to overexpression. However, retinal thinning could not be determined with the unenhanced vector at 1.5×10^9 vg due to the constraints of experimental design.

Studies directly comparing the level of expression of genes expressed in photoreceptors in the mammalian retina were not available at the time of this study. However, two single-cell RNAseq papers have now been published which enable some comparison of the relative expression of genes amongst retinal cells.^{178, 179} Moreover, both studies identify *CDHR1* expression in bipolar, Müller-glia, retinal ganglion cells, microglia, amacrine cells and astrocytes. Expression in horizontal cells was identified in one study but not in the other. The role of CDHR1

in non-photoreceptor retinal cell types is unknown. Consequently, at the time of the study, the rational prediction of the dose of sub-retinal AAV could not be undertaken based on the known effective doses of similar gene therapy vectors that have been evaluated in murine models (i.e. AAV8.coRPGR¹⁰⁴). Moreover, numerous factors may affect transgene expression, including differences in vector design (such as the presence of *cis*-acting enhancing elements, promoters or transgene codon optimisation), the location of expression within the photoreceptor (i.e. outer segment protein expression may be limited by the cilium, and membrane-bound proteins theoretically more likely to elicit inflammation), the specific murine model, rate of degeneration, the method and/or timing of vector delivery.

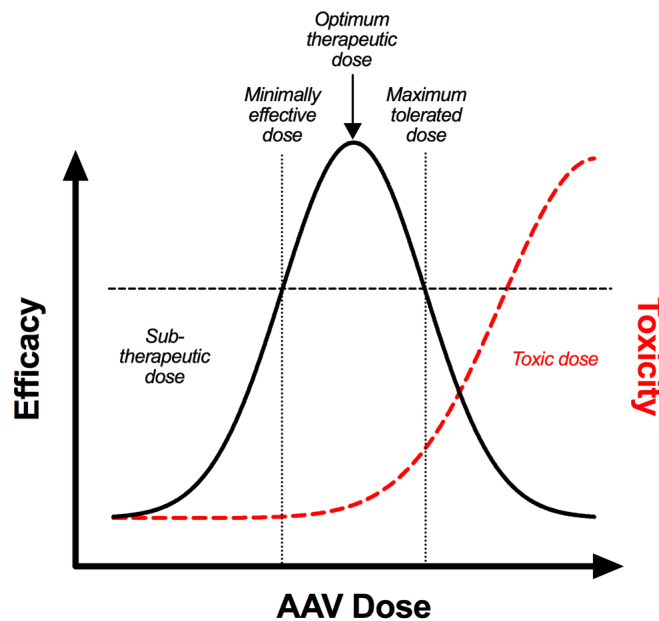


Figure 5.1 Theoretical dose-response relationships as they relate to toxicity and efficacy following sub-retinal delivery of AAV vector

The horizontal dashed line represents the threshold of efficacy, below which no beneficial effect is seen. The optimum therapeutic dose yields maximum efficacy. At higher doses, efficacy is adversely affected through a variety of mechanisms.

[Continued] The maximum tolerated dose is defined as the highest dose that does not produce measurable, or clinically significant, toxicity using the chosen outcome measure compared to control eyes. The minimally effective dose is defined as the smallest dose at which a therapeutic benefit is seen. The therapeutic dosing interval is the difference between these limits. Note that these dosing thresholds may vary depending on the selected outcome measure that determines efficacy and toxicity.

Since a single dose (1.5×10^9 vg) of AAV8.CDHR1 was likely to be in the toxic range for the enhanced vector, a dose-reduction (rather than dose-escalation) study design was appropriate to determine the maximum tolerated dose, the optimum therapeutic dose, and ideally, the minimally effective therapeutic dose which would permit the therapeutic dosing interval to be determined. These dose-response definitions as they relate to toxicity and efficacy following subretinal delivery of AAV are summarised in Figure 5.1.

Numerous retinal gene therapy vectors have been developed using murine models. However, not all vectors were assessed for their dose-response characteristics *in vivo* (Table 5.1). This approach risks a type II error, in which a given vector is considered ineffective due to the delivery of a non-optimal dose (either sub-therapeutic or toxic). A formal dosing study is necessary before it can be concluded that a given vector is ineffective. The therapeutic dose of any vector will depend on the numerous factors which influence transgene expression in photoreceptors, as described above. However, a formal dosing study provides strong evidence of the therapeutic and toxic characteristics of a given vector, which guides the selection of both vector and dose. Moreover, the selection of an optimum dose will

support downstream proof-of-principle pre-clinical studies that aim to determine the efficacy of gene therapy, and validate relevant outcome measures. These data may guide dose selection in onward phase 1 clinical studies following allometric scaling.

Gene ^(Ref)	AAV serotype	Promoter	Dose(s)	Formal dosing study	Target cell
<i>RPGR</i> ¹⁰⁴	AAV8	GRK1	1.5 x 10 ⁹	No	Photoreceptor
<i>CHM</i> ¹⁰³	AAV2	CBA	2 x 10 ⁸ – 2 x 10 ⁹	Yes: 1 log unit	RPE
<i>CHM</i> ¹⁸⁰	AAV8	CBA	1 x 10 ⁹	Not <i>in vivo</i>	RPE
<i>RPE65</i> ¹³²	AAV5	CBA	Not specified	No	RPE
<i>RPE65</i> ¹⁸¹	AAV2	CBA	1 x 10 ⁸ – 1 x 10 ¹⁰	Yes: 2 log units	RPE
<i>RDH12</i> ¹⁸²	AAV5	GRK1	1.3 x 10 ⁷ – 1.5 x 10 ⁹	Yes: <2 log units	Photoreceptor
<i>RPGRIP</i> ¹⁸³	AAV2	Mouse opsin	1 x 10 ⁹	No	Photoreceptor
<i>RP2</i> ¹⁵⁰	AAV8	GRK1	5 x 10 ⁷ – 1 x 10 ⁹	Yes: ~1.5 log units	Photoreceptor
<i>PDE6B</i> ¹⁸⁴	AAV9	Human rhodopsin	Not specified	No	Photoreceptor
<i>CNGA3</i> ¹⁸⁵	AAV5	CBA	Not specified	No	Photoreceptor
<i>CNGB3</i> ¹⁸⁶	AAV2 ^{tYF}	PR1.7	1 x 10 ⁹ – 4.2 x 10 ⁹	Yes: <0.5 log units	Cone photoreceptors

Table 5.1 Doses of AAV vector evaluated using sub-retinal delivery in murine models

Note that around half of studies do not undertake, or present results from, dose-response studies *in vivo*. AAV capsids are unmodified unless stated. rAAV – recombinant AAV

5.2 Aims

- The primary aim of this study was to determine the maximum tolerated and optimum therapeutic dose of both AAV8.CDHR1 vectors in *Cdhr1*^{-/-} and *C57BL/6J* mice in order to guide dose selection for a longitudinal, gene therapy efficacy and safety experiment (Chapter 6).
- The secondary aims were to:
 - Identify the lead vector construct for longitudinal evaluation
 - Determine the size of any rescue effect seen on electroretinography in order further qualify dose selection and to adequately power (*a priori*) a longitudinal, prospective study (Chapter 6).

5.3 Materials and methods

5.3.1 Vector dosing study design

An open-label, prospective, controlled dose reduction study of both AAV8.CDHR1 vectors was undertaken in *Cdhr1*^{-/-} and *C57BL/6J* mice. The overall experimental design is summarised in Figure 5.2. The justification for each aspect of the experimental design of this study is specified below.

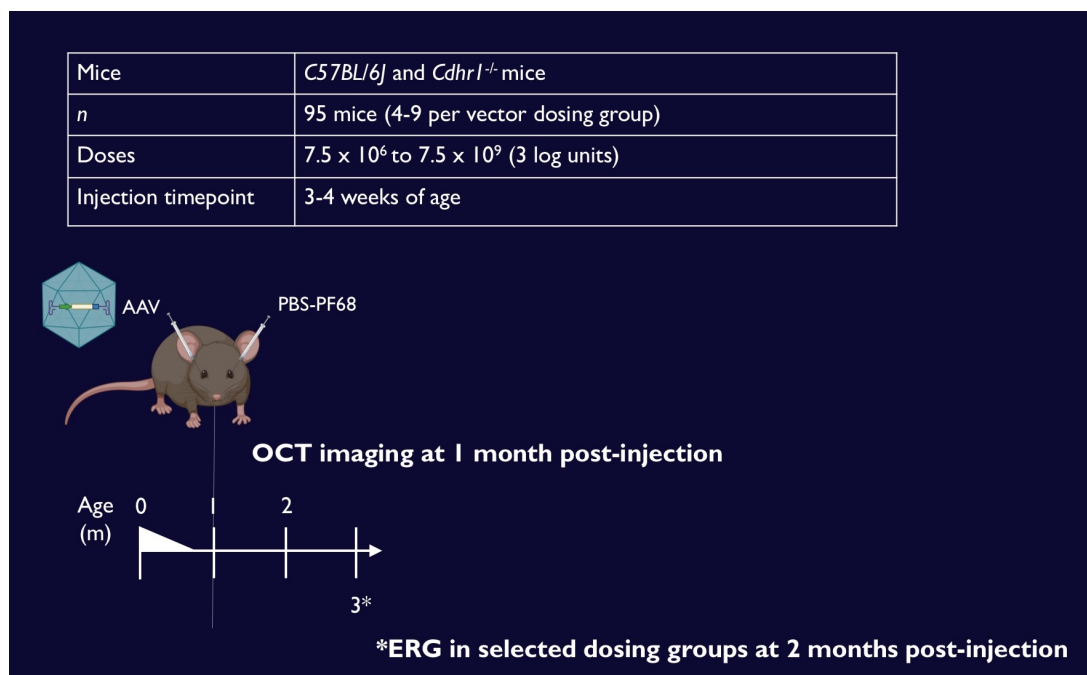


Figure 5.2 AAV8.CDHR1 vector dosing study design

5.3.1.1 Mouse strains

Evaluation of vector dosing was assessed in the *Cdhr1*^{-/-} mouse model that had been validated through data presented in Chapter 3. Evidence of safety in *C57BL/6J* mice at a chosen therapeutic dose, or across a therapeutic range, was considered important in order to support the safety of overexpression at any selected therapeutic dose - relevant for regulatory approval prior to clinical trial. The data generated from both strains would guide selection of the optimum dose for formal evaluation (Chapter 6). The *C57BL/6J* mouse strain was selected for the safety study, since the *Cdhr1*^{-/-} mouse strain had been backcrossed to the *C57BL/6J* background, minimising any strain-specific differences in OCT and/or ERG measurements between groups.

5.3.1.2 *Selection of control intervention*

Introduction of vector into the sub-retinal space necessitates temporary neurosensory retinal detachment, which may last around 24 hours following delivery of 1µl of saline.¹⁸⁷ Since the retinal tissues are separated from the underlying choroidal circulation, transient, relative retinal ischaemia may result, photoreceptor outer segments may temporarily shortened,¹⁸⁸ coupled with physical damage to the underlying RPE and Bruch's membrane which are necessarily breached using an *ab externo* surgical approach. Localised outer retinal thinning may be expected following hemi-retinal, surgically induced retinal detachment, which may depend on the duration of detachment, the properties of the vector and excipients, and the volume delivered.

However, specific functional benefits of surgically-induced retinal detachment (i.e. improved ERG responses following sub-retinal PBS injection) have been described.¹⁰⁴ This may be explained by removal of cellular debris from the outer retina in models of retinal degeneration in which such mechanisms are deficient – for example the Royal College of Surgeons Rat who exhibit a naturally occurring mutation in the *Mertk* gene in which debris accumulates between the outer segments and the RPE. This may result in an apparent therapeutic signal that may appear when the control eyes are untreated, since the retinal detachment itself is beneficial, independent of the test vector.¹⁸⁹ Since outer segments are abnormal in *Cdhr1*^{-/-} mice,⁴⁷ surgically induced retinal detachment might

conceivably be beneficial through a similar mechanism. It was therefore considered preferable to control for the effects of surgically induced retinal detachment using an identical volume of vehicle control in paired eyes. Increased variability in control eyes following an active control intervention may reduce the power of individual experiments, although further experimental mice could be added to dosing groups if necessary.

Creation of a null, or non-coding vector packaged identically in AAV8 wildtype capsids would permit further insights into the mechanism of dose-dependent toxicity by controlling for immune responses to the AAV8 capsid, and enabling transgene related effects to be determined. However, sub-retinally delivered null AAV8 vectors may produce retinal toxicity through as yet unknown mechanisms (unpublished lab data) but perhaps related to immune responses to particular nucleotide sequences within the null construct (i.e. unmethylated CpG dinucleotides). A toxic effect in control eyes may risk a type 1 error in demonstrating an apparent beneficial effect of AAV8.CDHR1 in treated eyes, through a relative absence of toxicity. Moreover, immune responses to AAV may not necessarily be symmetrical, adding further variability between eyes and reducing the statistical power of the experiment.

For these reasons, an identical volume of PBS-PF68 (0.001%), the AAV diluent, was used as the experimental control delivered by sub-retinal injection to fellow

eyes. This controlled for the effect of surgically induced retinal detachment and any unlikely toxic or therapeutic effect of the excipient only.

5.3.1.3 Dosing interval

In order to determine the maximum tolerated dose of AAV8.CDHR1 vectors in both *C57BL/6J* and *Cdhr1^{-/-}* mice, a dose reduction study was performed starting at a dose of 1.5×10^9 for the enhanced vector, and 7.5×10^9 for the unenhanced vector – as permitted by the achieved viral titres. 0.5 log unit dose decrements were delivered at 3-4 weeks of age (i.e. post-weaning), in the expectation that this dosing interval would be sufficient to separate groups whilst permitting an evaluation of a wide dosing range (i.e. 2-3 log units) as may be necessary to identify the maximum tolerated dose. Four to five animals were included in each group during the first round of dose evaluation. AAV8.CDHR1 dosing groups that demonstrated clear evidence of toxicity following analysis of outer retinal thickness on OCT measurements did not require further assessment. However, additional mice were injected in dosing groups in which toxicity was statistically uncertain. The OCT data for expanded dosing groups were analysed together since the protocols for intervention and evaluation were standardised.

5.3.1.4 Outcome measures

Primary outcome measure: Retinal thickness measurements on OCT imaging

Following the demonstration of successful, and exclusive, expression of CDHR1 in both rod and cone photoreceptors in *Cdhr1*^{-/-} mice, strategies to identify the maximum tolerated dose were considered. Quantification of CDHR1 mRNA or protein following retinal transduction, with comparison to wildtype CDHR1 expression is complicated by hemi-retinal vector delivery: physiological expression of CDHR1 in the superior retina would not yield equivalent measured CDHR1 expression from wildtype whole retinal lysate, since CDHR1 is expressed throughout the retina in wildtype animals. Moreover, quantification of expression (protein or mRNA) are less meaningful measures, since they can only provide indirect insights into the maximum tolerated dose of vector, and must be considered alongside other outcomes measures of retinal structure and function. For example, low CDHR1 expression may occur as a result of a sub-therapeutic vector dose, or due to photoreceptor toxicity and cell death. Furthermore, the time required for the optimisation and quantification of mRNA and/or protein assays following gene therapy would limit the number of mice that could be evaluated, reducing the power of the study and the dosing range that could be examined. Downstream *in vivo* assessments are not possible following tissue retrieval.

The principle of using retinal thickness as an outcome measure is based on the susceptibility of photoreceptors to the toxic effects of substances introduced into

the subretinal space, partly due to the vulnerability of the photoreceptor outer segment membrane discs to the pH, lipophilicity and other properties of solutes. Moreover, overexpression or inflammation in response to AAV delivery would also have a similar effect. Retinal thickness measurements on OCT imaging are highly reproducible outcome measures (Chapter 3) which would support a relatively high-throughput experimental plan. Furthermore, hemi-retinal delivery of AAV8.CDHR1 vector permits internal controls in both treated and control eyes by retinal location which further qualifies evidence of therapeutic benefit or toxicity. Moreover, this experimental design permits downstream evaluation of retinal function by electroretinography in order to qualify the doses found to be non-toxic on OCT imaging, an approach not possible using histological analysis or protein/mRNA assays as the primary outcome measure. Photoreceptor layer thickness (OPL-RPE) measurements would be determined at both superior and inferior retinal loci, as described in Chapter 3.

The interval between intervention and assessment of outer retinal thickness required sufficient time for recovery of mice from anaesthesia as specified in the Project Licence (i.e. one week), and for subretinal fluid to be resorbed to allow outer retinal measurements to be taken (i.e. 24 hours).¹⁸⁷ Furthermore, sufficient time was necessary to allow for any manifestations of toxicity that may result from inflammatory reactions to the viral capsid or the expressed transgene; either innate responses, through pattern recognition receptors (i.e. TLR9), or T-cell

responses or anti AAV neutralising antibody responses.¹⁹⁰ In consideration of these factors, a 4-week interval was considered appropriate between the intervention and OCT imaging.

Secondary outcome measures: Response amplitudes on electroretinography

OCT imaging, whilst likely to detect the maximum tolerated dose, would be unlikely in isolation to distinguish the therapeutic benefit of a range of non-toxic doses at early timepoints. Assessment of retinal function using ERG in animals identified to have received non-toxic doses of AAV8.CDHR1 vector (i.e. without retinal thinning on OCT imaging beyond the effect of surgically-induced retinal detachment) would increase the likelihood of identifying the optimum therapeutic dose for longitudinal evaluation in Chapter 6. ERG was undertaken to confirm the absence of toxicity in selected doses in the *C57BL/6J* group. ERG assessments were undertaken 2 months post-injection to determine whether rod and cone function had improved in AAV- versus PBS-injected eyes, since the responses may be dose-dependent. Furthermore, pilot ERG studies were used to perform an *a priori* power calculation to adequately power a longitudinal study evaluating the efficacy and safety of retinal gene therapy using identical techniques (Chapter 6).

5.3.1.5 Experimental numbers

The reproducibility of retinal thickness measurements on OCT imaging enabled a statistical difference to be detected using four animals (Chapter 4). In the absence

of an *a priori* power calculation, a cohort of 4-5 mice were evaluated at each dose, with a similar number of mice evaluated as part of a second cohort where data were inconclusive. This design limited the number of mice used whilst fulfilling the experimental aim. Tables 5.2 & 5.3 identify the numbers of experimental mice ($n=95$ overall) of each genotype in the relevant dosing groups, by vector.

AAV dose	<i>Cdhr1</i>^{-/-} ($n=26$)		<i>C57BL/6J</i> ($n=18$)	
	<i>1st cohort</i>	<i>2nd cohort</i>	<i>1st cohort</i>	<i>2nd cohort</i>
7.5 x 10 ⁹ vg	-	-	4	-
1.5 x 10 ⁹ vg	4	3	4	-
7.5 x 10 ⁸ vg	5	4	3	-
1.5 x 10 ⁸ vg	5	-	4	-
7.5 x 10 ⁷ vg	5	-	3	-

Table 5.2 Numbers of mice per dosing group in the unenhanced AAV8.GRK1.CDHR1.pA vector study

A total of 44 mice were used.

AAV dose	<i>Cdhr1</i>^{-/-} ($n=25$)		<i>C57BL/6J</i> ($n=26$)	
	<i>1st cohort</i>	<i>2nd cohort</i>	<i>1st cohort</i>	<i>2nd cohort</i>
1.5 x 10 ⁹ vg	-	-	4	-
7.5 x 10 ⁸ vg	4	3	3	-
1.5 x 10 ⁸ vg	4	5	4	-
7.5 x 10 ⁷ vg	5	-	5	-
1.5 x 10 ⁷ vg	4	-	5	-
7.5 x 10 ⁶ vg	-	-	5	-

Table 5.3 Numbers of mice per dosing group in the enhanced AAV8.GRK1.In.CDHR1.WPRE.pA vector study

A total of 51 mice were used.

5.4 Results

5.4.1 The maximum tolerated dose of AAV8.GRK1.CDHR1.pA is 1.5×10^8

OCT imaging 1-month post-injection with AAV8.GRK1.CDHR1.pA in both *C57BL/6J* and *Cdhr1*^{-/-} mice did not result in outer retinal thinning at a dose of 1.5×10^8 vg per eye compared to PBS-injected control eyes (Figure 5.3 & 5.4). This dose was designated as the maximum tolerated dose for the unenhanced AAV8.GRK1.CDHR1.pA vector.

5.4.1.1 *Retinal thickness measurements on OCT imaging in Cdhr1^{-/-} mice 1 month after sub-retinal injection with AAV8.GRK1.CDHR1.pA*

Outer retinal thickness measurements on OCT imaging in *Cdhr1*^{-/-} mice 1 month after sub-retinal injection of AAV8.GRK1.CDHR1.pA demonstrated dose-dependent superior retinal thinning in AAV treated eyes ($P=0.0008$ overall; two-way ANOVA for effect of dose). A dose of 1.5×10^9 vg resulted in superior retinal thinning when compared to a dose of 7.5×10^8 vg ($P=0.0025$). This observation was consistent with presumed overexpression on immunohistochemical analysis at 1.5×10^9 vg (Chapter 4). Furthermore, at 1.5×10^9 vg, mild inferior retinal thinning was present in some AAV-treated eyes, although this was not statistically significant ($P=0.051$). AAV doses of 7.5×10^8 , 1.5×10^8 and 7.5×10^7 vg did not produce superior or inferior retinal thinning. Normalised data comparing retinal thickness measurements at identical loci in paired AAV- and PBS-injected eyes confirm these findings (Figure 5.3).

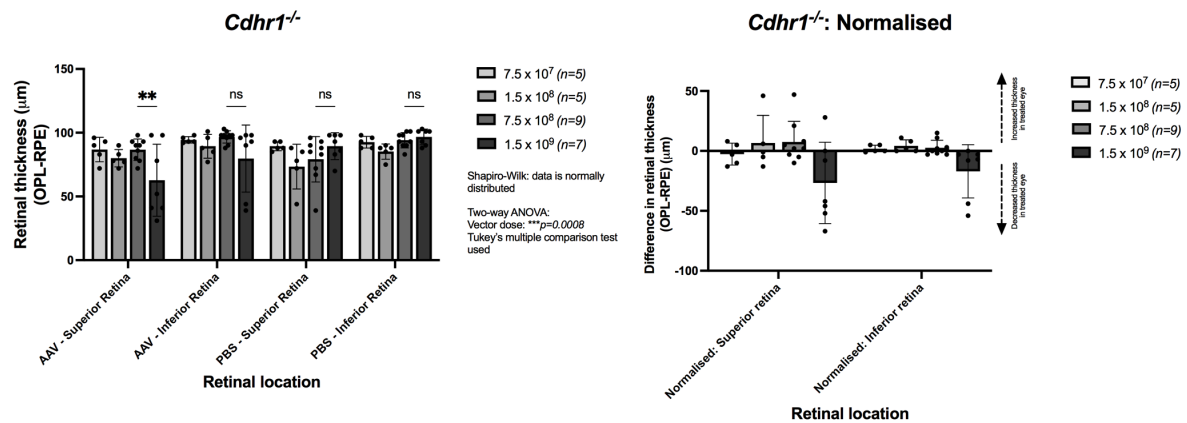


Figure 5.3 Retinal thickness measurements on OCT imaging 1 month post-injection with AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice.

The left panel presents raw data, and right panel presents normalised data from OCT imaging. Doses of 7.5×10^8 vg or less were non-toxic in *Cdhr1*^{-/-} mice.

5.4.1.2 Retinal thickness measurements on OCT imaging in C57BL/6J mice 1 month after sub-retinal injection with AAV8.GRK1.CDHR1.pA

In C57BL/6J mice, a clear dose-response relationship was observed between AAV dose and outer retinal thickness measurements ($P < 0.0001$ overall; two-way ANOVA for effect of dose) (Figure 5.4). Overall, a greater degree of superior retinal thinning was identified at equivalent doses of AAV8.GRK1.CDHR1.pA when compared to *Cdhr1*^{-/-} mice when normalised to inferior retinal locations. A dose of 1.5×10^8 vg did not produce retinal thinning in AAV-injected eyes which was observed with increasing magnitude at higher doses (e.g. $P = 0.047$ vs 7.5×10^8 , $P = 0.018$ for 1.5×10^9 , and $P < 0.0001$ for 7.5×10^9). Inferior retinal toxicity was observed only at a dose of 7.5×10^9 in AAV-injected eyes ($P < 0.0001$ vs. all other dosing groups).

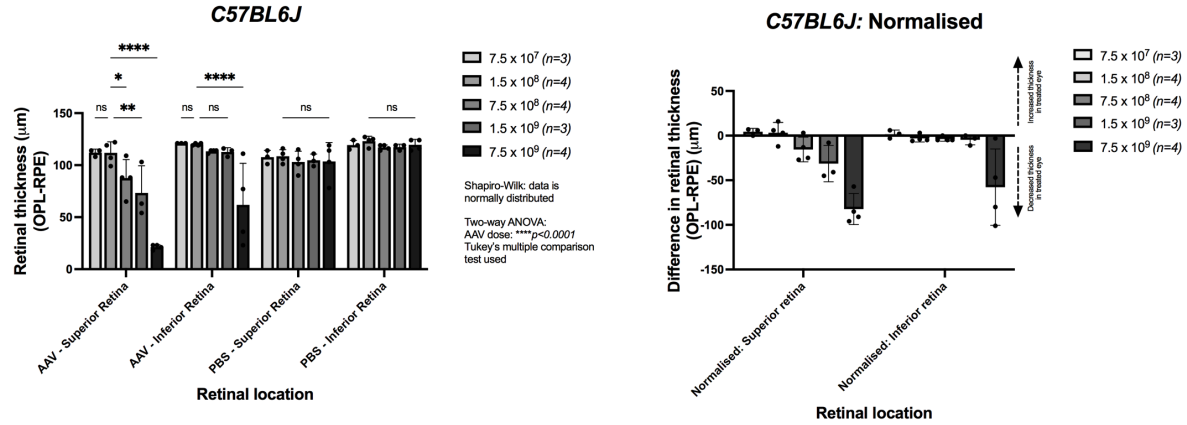


Figure 5.4 Retinal thickness measurements on OCT imaging 1 month post-injection of AAV8.GRK1.CDHR1.pA in C57BL/6J mice. The left panel presents raw data, and right panel presents normalised data from OCT imaging. Doses of 1.5×10^8 vg or less were non-toxic in C57BL/6J mice.

5.4.2 Electrophysiology demonstrates a functional improvement in rod and cone photoreceptor responses in a pilot cohort of *Cdhr1*^{-/-} mice injected with AAV8.GRK1.CDHR1.pA at a dose of 1.5×10^8

5.4.2.1 Dark-adapted ERG

Dark-adapted electroretinography was undertaken in *Cdhr1*^{-/-} mice in the 1.5×10^9 vg to 7.5×10^7 vg cohorts 2-months post-injection. In the 1.5×10^8 vg group, an improvement in A-wave responses ($P=0.0002$; $n=5$) and B-wave responses ($P=0.001$) were observed in AAV- versus paired PBS-injected eyes at 2-months post-injection. In the 7.5×10^8 vg group, a small benefit was identified in AAV-injected eyes, although this was not significant overall at this timepoint (A-wave – $P=0.13$; B-wave – $P=0.81$; $n=4$ eyes; Figure 5.5). Comparative responses at different AAV doses are presented in Figure 5.6, and normalised responses in

Figure 5.7. These data support the conclusion of an optimum therapeutic dose of 1.5×10^8 .

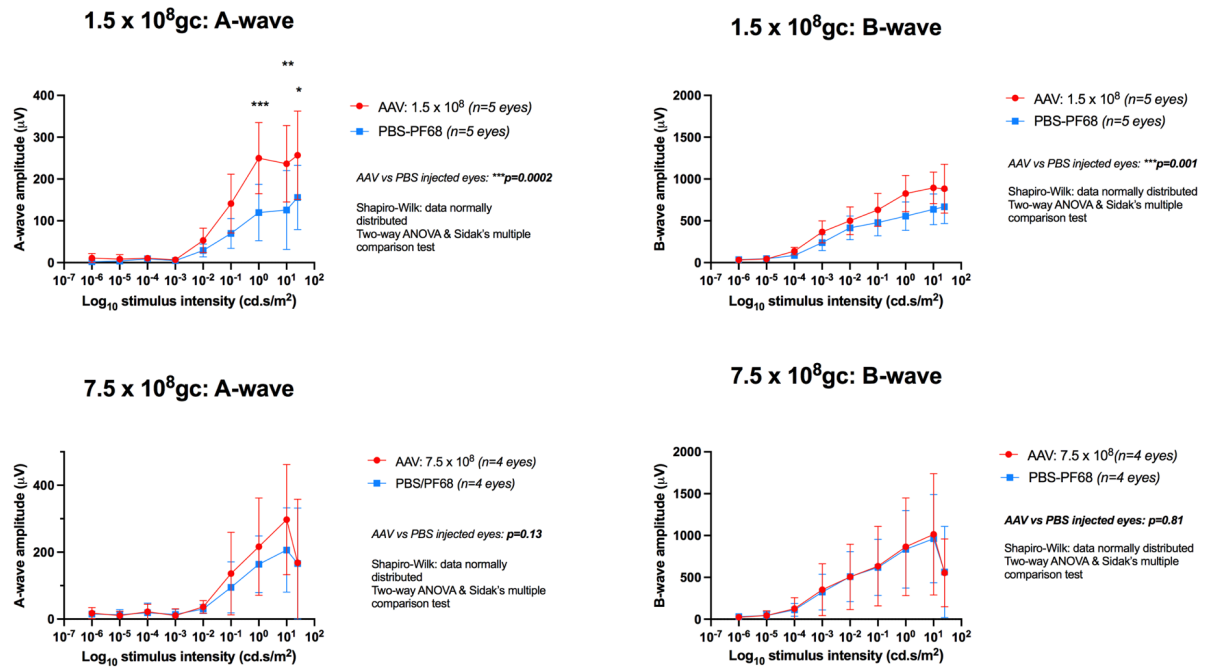


Figure 5.5 Dark-adapted ERG responses 2-months post-injection with AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice at two doses.

A significant rescue effect is identified at a dose of 1.5×10^8 on both A- ($P=0.0002$) and B-wave responses ($P=0.001$; $n=5$). A non-significant benefit is seen at a dose of 7.5×10^8 ($P=0.13$). Plots represent mean $\pm$ 95% C.I.

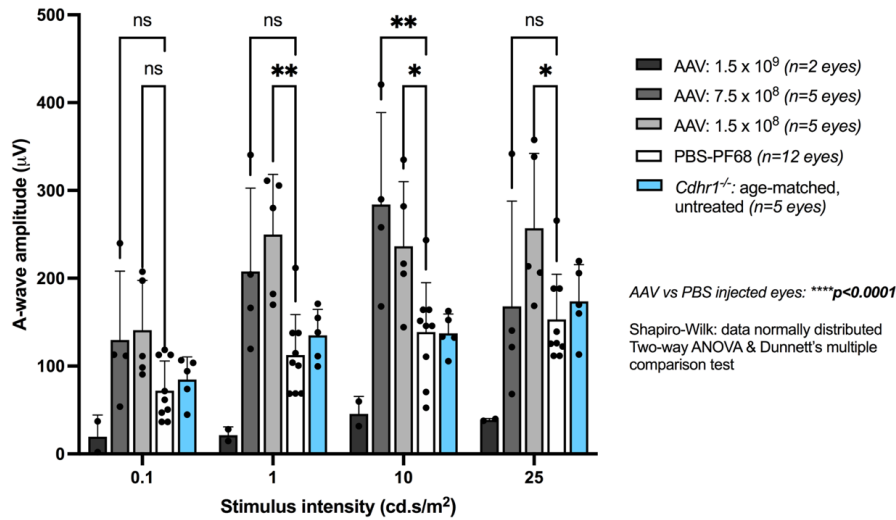


Figure 5.6 Dark-adapted ERG responses 2-months post-injection with AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice at different doses.

Whilst a dose of 1.5×10^9 showed reduced responses, doses of 1.5×10^8 and 7.5×10^8 yielded improved A-wave responses when compared to PBS-injected (pooled) and un-injected control eyes (Chapter 3). The similarity between the two control groups supports the lack of functional deficit or improvement following surgically induced retinal detachment in PBS-injected eyes in *Cdhr1*^{-/-} mice.

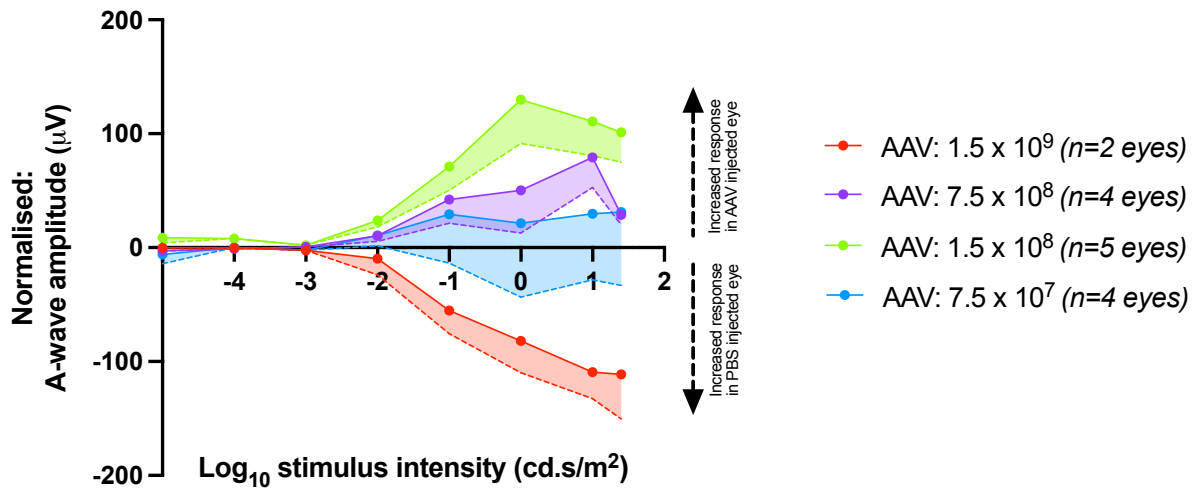


Figure 5.7 A comparison of normalised dark-adapted ERG responses according to dose of AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice.

Data represents mean and SEM of responses in AAV-injected eyes when normalised to paired PBS-injected control eyes. Improvements in A-wave amplitudes in AAV-injected eyes are greatest at a dose of 1.5×10^8 vg in this pilot experiment, with 7.5×10^8 vg and 7.5×10^7 vg demonstrating smaller responses.

5.4.2.2 Light-adapted ERG

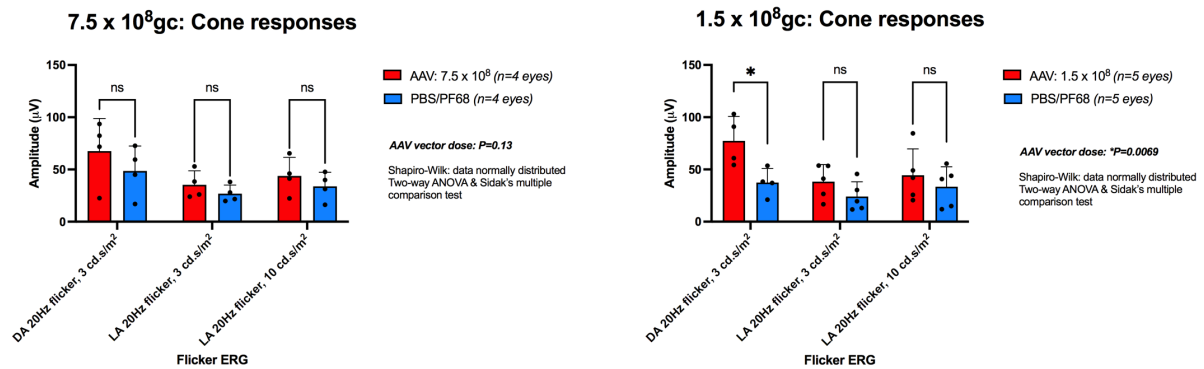


Figure 5.8 Flicker ERG responses 2-months post-injection with AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice.

A significant overall benefit is seen in flicker responses following AAV injection at a dose of 1.5×10^8 vg ($P=0.0069$), but not at 7.5×10^8 vg ($P=0.13$) in this small experimental cohort. A larger effect is seen in the dark-adapted flicker response.

Light-adapted ERG assessments showed improved responses in AAV- over PBS-injected control eyes at a dose of 1.5×10^8 vg per eye ($P=0.0069$ overall; two-way ANOVA for effect of treatment; $n=5$) (Figure 5.8 & 5.9). Improved responses were seen at a dose of 7.5×10^8 vg per eye; however, this was not statistically significant at this timepoint in a small cohort ($P=0.13$; two-way ANOVA for effect of treatment; $n=4$). A greater benefit was seen in dark-adapted versus light-adapted flicker responses which may indicate a rod contribution to this response. Normalised data (not shown) demonstrated greater response amplitudes on flicker ERG in all AAV-injected eyes at both doses compared to PBS-injected paired eyes.

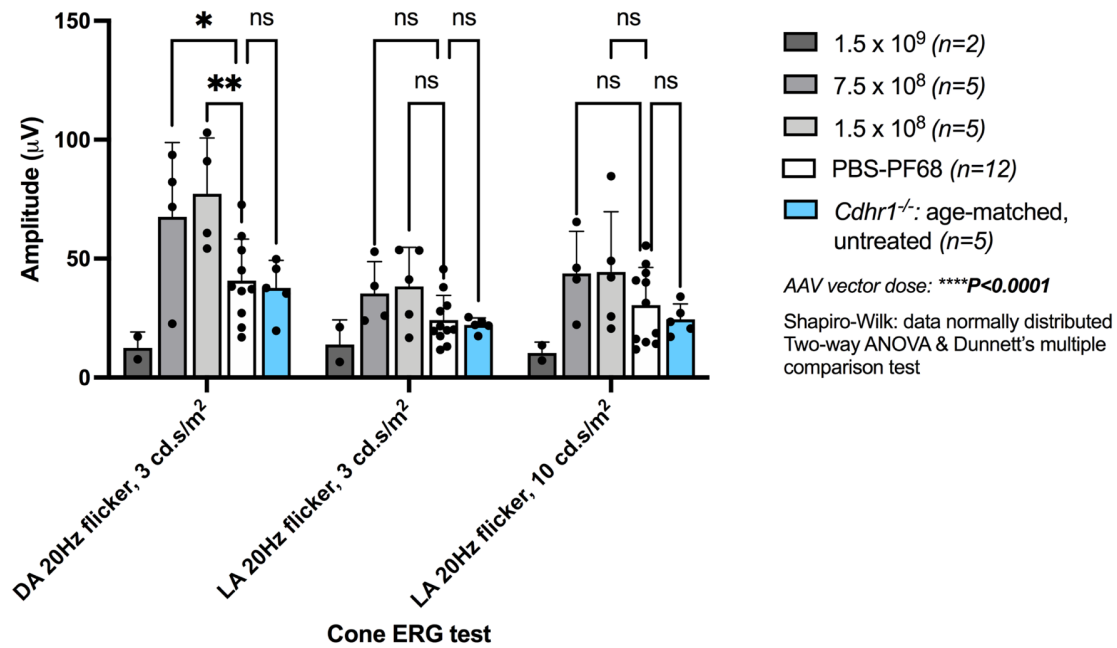


Figure 5.9 Light-adapted flicker ERG responses 2-months post-injection with AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice at different doses.

Doses of 1.5 x 10⁸ vg and 7.5 x 10⁸ vg yielded improved flicker responses when compared to PBS-injected (pooled) and un-injected control eyes (from natural history data in Chapter 3). The similarity between the control groups supports the lack of functional deficit or improvement in cone function following surgically induced retinal detachment in PBS-injected eyes.

5.4.3 Electroretinography did not detect a toxic effect of AAV8.GRK1.CDHR1.pA at a dose of 7.5x10⁸ in *C57BL/6J* mice.

Pilot ERG studies undertaken 2-months post-injection did not detect a difference in retinal function between AAV- and PBS-injected control eyes at a dose of 7.5 x 10⁸ vg in *C57BL/6J* mice (Figure 5.8), despite slight superior retinal thinning seen on OCT imaging in the same cohort at 1-month post-injection. Doses lower than 7.5 x 10⁸ vg were not evaluated by ERG, since toxicity was unlikely.

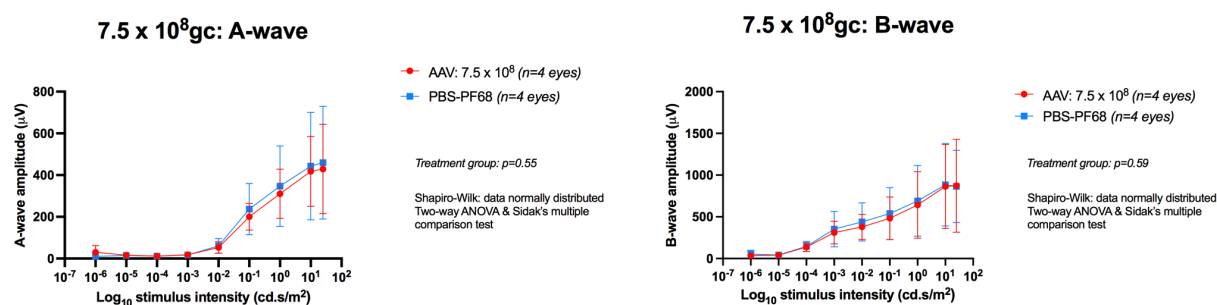


Figure 5.10 Dark-adapted, single-flash ERG responses 2-months post-injection with AAV8.GRK1.CDHR1.pA in *C57BL/6J* mice

There was no difference between ERG assessments in AAV- and PBS-injected control eyes at a dose of 7.5×10^8 vg on A-wave ($P=0.55$) or B-wave ($P=0.59$) response amplitudes in *C57BL/6J* mice. Plots represent mean $\pm$ 95% C.I.

5.4.4 The maximum tolerated dose of AAV8.GRK1.In.CDHR1.WPRE.pA is less than 1.5×10^7

OCT imaging 1-month post-injection with AAV8.GRK1.In.CDHR1.WPRE.pA did not identify a dose between 1.5×10^7 to 7.5×10^8 which did not produce some outer retinal thinning in *Cdhr1*^{-/-} mice. Time constraints prevented the testing of additional doses in order to identify the maximum tolerated dose in *Cdhr1*^{-/-} mice. In *C57BL/6J* mice, doses of 7.5×10^6 and 1.5×10^7 did not produce retinal thinning on OCT imaging.

5.4.4.1 Retinal thickness on OCT imaging in *Cdhr1*^{-/-} mice 1-month post-injection

A clear dose-response relationship was identified between outer retinal thickness measurements on OCT imaging and subretinal dose of AAV8.GRK1.In.CDHR1.WPRE.pA that was evident in both superior ($P=0.014$)

and inferior retinal locations (Figure 5.11). Superior retinal thinning was evident in the lowest dose tested; 1.5×10^7 vg. However, due to limitations of time, evaluation of doses below 1.5×10^7 vg was not undertaken for reasons further elaborated in the discussion. ERG assessments were not undertaken on this cohort.

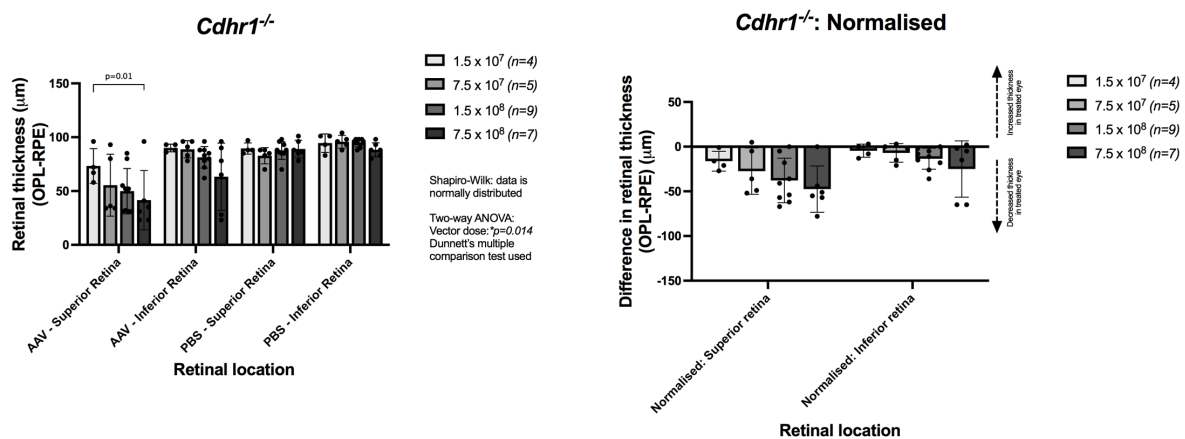


Figure 5.11 Retinal thickness measurements on OCT imaging 1 month post-injection of AAV8.GRK1.In.CDHR1.WPRE.pA in *Cdhr1*^{-/-} mice
The left panel presents raw data, and right panel presents normalised data. The maximum tolerated dose was not identified in *Cdhr1*^{-/-} mice since some retinal thinning was evident at the lowest dose tested.

5.4.4.2 Retinal thickness on OCT imaging in C57BL/6J mice 1-month post-injection

In *C57BL/6J* mice, a clear dose-response relationship was identified between outer retinal thinning and increasing doses of AAV8.GRK1.In.CDHR1.WPRE.pA. This was evident at both superior ($P < 0.0001$; two-way ANOVA for effect of vector dose and retinal location) and inferior retinal locations. Doses of 7.5×10^6 and 1.5×10^7 did not produce retinal thinning.

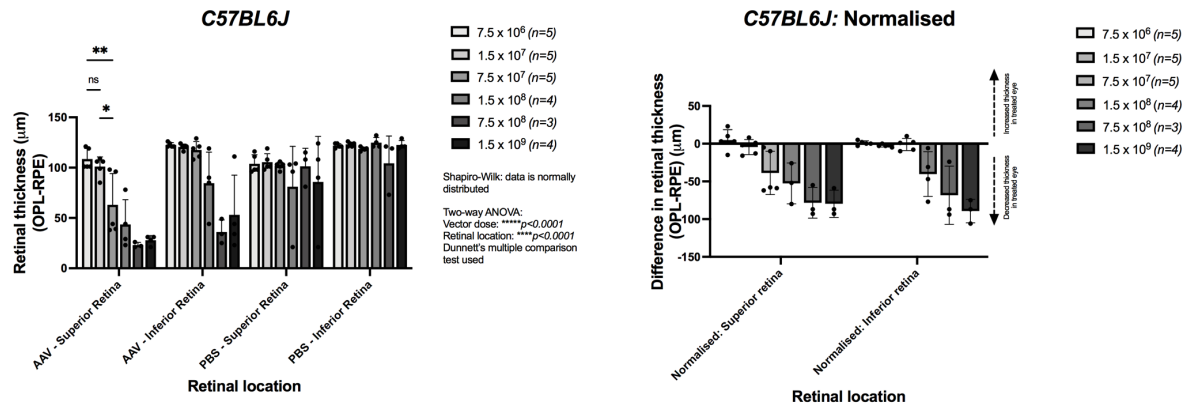


Figure 5.12 Retinal thickness measurements on OCT imaging 1 month post- injection of AAV8.GRK1.In.CDHR1.WPRE.pA in *C57BL/6J* mice

The left panel presents raw data, and right panel presents normalised data. A dose of 1.5×10^7 or less appeared to be safe in *C57BL/6J* mice according to these data.

5.5 Discussion

In this chapter, a novel experimental approach is demonstrated of using retinal thickness measurements on OCT imaging to perform relatively high-throughput screening of sub-retinally delivered vector doses across a ~ 3 log-unit dosing range. A similar experimental approach has been used in the evaluation of dose characteristics of AAV retinal gene therapy in a murine model of *Rpe65* mutant *rd12* mice using ERG as the main outcome measure over a 2 log-unit dosing range.¹⁸¹ A clear dose-response relationship is shown between the dose of both AAV8.CDHR1 vectors and retinal thickness measurements on OCT imaging 1-month post-injection in both *Cdhr1*^{-/-} and *C57BL/6J* strains, further confirming a biological effect of both AAV.CDHR1 vectors. However, OCT imaging provides additional information since the technique of hemi-retinal sub-retinal delivery of AAV vector enables the use of retinal location as an internal control. In this case,

inferior retinal thinning was evident at higher doses in which superior retinal thinning was also seen, supporting the designation of toxicity with lower numbers of experimental animals. This approach is not possible using ERG, since full-field responses are measured. This methodology should be considered for similar equivalent pre-clinical vector dosing studies which aim to identify the maximum tolerated, minimum effective and/or optimum therapeutic dose for a given vector. It should be noted, however, that dose-dependent transduction beyond the surgical bleb margins have been demonstrated in non-human primates.¹⁴¹ Furthermore, at doses of 1.5×10^9 , some CDHR1 expression was seen at inferior retinal locations. However, the selection of peripheral retinal loci for thickness measurements minimises the effect of transduction beyond the bleb.

Dose-dependent thinning of the inferior retina, seen most clearly in the evaluation of the enhanced AAV8.CDHR1 vector in *C57BL/6J* mice, may represent either a generalised effect of dose-dependent inflammation (i.e. to AAV capsid or nucleotide sequences), or an effect of overexpression of the CDHR1 transgene. Data acquired with the unenhanced AAV8.CDHR1 vector showed retinal thinning in wildtype mice at a 0.5 log-unit lower dose when compared to the *Cdhr1*^{-/-} mice. Assuming there are no independent differences between strains (i.e. inflammatory or immunogenicity responses to the same stimuli), this observation is most likely due to the effect of overexpression of the CDHR1 transgene at particularly high vector doses. This is logical since it can be assumed that overexpression of an outer

segment protein within photoreceptors in wildtype retinas competes with other outer segment proteins at the cilium resulting in dysfunction, toxicity and cell death that manifests as mild retinal thinning.

The maximum tolerated dose across both *Cdhr1*^{-/-} and *C57BL/6J* strains was identified as 1.5×10^8 vg for the unenhanced vector (AAV8.GRK1.CDHR1.pA). At this dose, a statistically significant rescue effect on both rod and cone function was identified 2-months post-injection in a pilot cohort of 5 mice. Although a dose of 7.5×10^8 vg did not produce retinal thinning in *Cdhr1*^{-/-} mice – and indeed demonstrated a potential rescue effect – mild retinal thinning was observed in some *C57BL/6J* mice at the same dose. Moreover, ERG demonstrated more favourable responses following sub-retinal injection of a dose of 1.5×10^8 vg, suggesting this dose was closest the optimum therapeutic dose for this vector, with a dose of 7.5×10^8 vg closest to the maximum tolerated dose in *Cdhr1*^{-/-} mice.

Demonstration of efficacy and safety in both *Cdhr1*^{-/-} and *C57BL/6J* strains at the same dose was considered essential in order to support clinical trials in individuals with hypomorphic *CDHR1* variants who express mutant CDHR1 (Chapter 7), and in whom overexpression is more likely following AAV.CDHR1 gene therapy. Furthermore, regulatory approval for clinical trials would be supported by evidence of safety in *C57BL/6J* mice at equivalent doses that were found to be beneficial in *Cdhr1*^{-/-} mice. A dose of 1.5×10^8 vg did not produce retinal

thinning in either strain, as was designated as the maximum tolerated dose for the unenhanced vector (Figure 5.13).

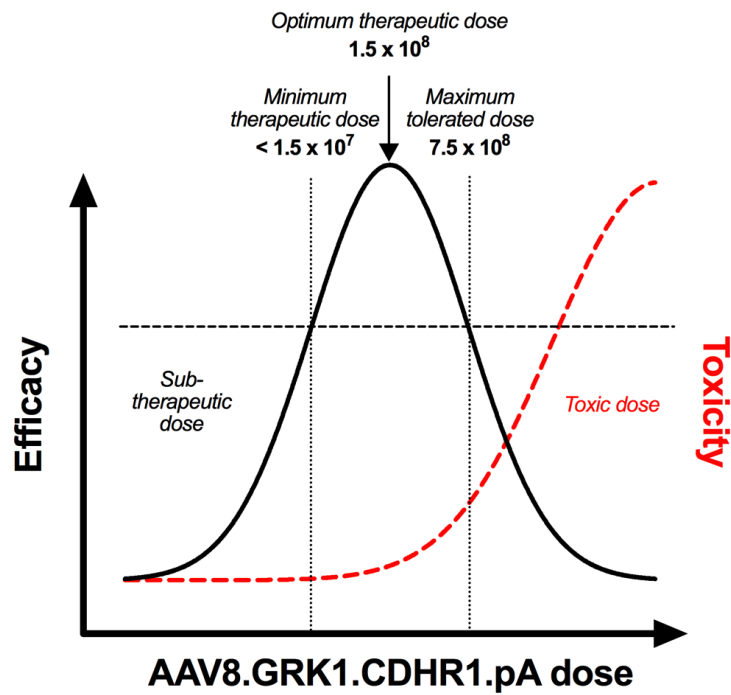


Figure 5.13 Dose-response relationships as they relate to toxicity and efficacy following sub-retinal injection of AAV8.GRK1.CDHR1.pA in *Cdhrl*^{-/-} mice

The dose-response relationships represented in this schematic chart relate to OCT imaging data and pilot ERG data of the unenhanced vector in *Cdhrl*^{-/-} mice.

It should be noted that OCT imaging may not be sufficiently sensitive to detect mild toxic effects when used as the primary outcome measure for retinal toxicity. Molecular markers at the level of the retina using qPCR for transcripts associated photoreceptor apoptosis¹⁹¹ or immunohistochemistry (such as TUNEL staining) may be used to identify retinal toxicity. However, murine models of retinal degeneration that demonstrate TUNEL staining also exhibit reduced retinal

thickness on OCT imaging.^{192, 193} Moreover, such molecular approaches exclude longitudinal analysis, and are more resource intensive, reducing the number of doses and animals that could be examined. In addition, OCT imaging is highly reproducible as an outcome measure, with low variability between subjects and timepoints, increasing the effective power to detect small effects in a relatively limited experimental cohort.

The enhanced CDHR1 expression vector (AAV8.GRK1.In.CDHR1.WPRE.pA), continued to demonstrate evidence of toxicity at a dose of 1.5×10^7 vg per eye in *Cdhr1*^{-/-} mice. However, in wildtype mice, doses of 1.5×10^7 vg and 7.5×10^6 vg did not produce retinal thinning when normalised to PBS-injected control eyes. Taken together, the observations of retinal thinning are not explainable by CDHR1 overexpression alone since toxicity was more pronounced in the *Cdhr1*^{-/-} strain at equivalent doses. Dose-dependent retinal toxicity (1×10^9 vg per eye) was identified following delivery of AAV8.scRP2 *in vivo* in the rd2 mouse using a vector containing the intronic sequence from CMV sub-cloned downstream from the GRK1 promoter.¹⁵⁰ The authors could not identify the mechanism of toxicity, although similarly considered that the viral capsid and transgene were unlikely to be responsible since similar doses were non-toxic in wildtype mice. However, the decreased tolerance for an additional toxic insult in degenerate photoreceptors in *Cdhr1*^{-/-} eyes may be lower compared to wildtype. Data presented in this chapter further support this suggestion since equivalent vector doses using the

two AAV8.CDHR1 constructs produce different degrees of retinal thinning, which cannot therefore be attributed solely to viral capsid related inflammation. It should be noted that full:empty AAV capsid ratios and endotoxin quantification were not evaluated in AAV preparations.

The evaluation of further lower doses of AAV8.GRK1.In.CDHR1.WPRE.pA in *Cdhr1*^{-/-} mice was considered. A dose of 7.5x10⁶ vg represents a theoretical MOI of 2.28 for each murine photoreceptor assuming subretinal delivery to the superior retina. Modelling using a Poisson distribution considered this ratio too low, and would likely result in a large proportion of photoreceptors remaining untransduced. Furthermore, time and resources were insufficient for the longitudinal assessment an additional vector, since two doses were to be evaluated in a longitudinal study in both *Cdhr1*^{-/-} and *C57BL/6J* mouse strains (Chapter 6). For these reasons, no further assessments were undertaken using the AAV8.GRK1.In.CDHR1.WPRE.pA vector.

5.6 Conclusions

In this chapter, a novel *in vivo* bioassay is presented in which OCT measures of outer retinal thickness were used to conduct a vector dosing study across a 3-log unit range in two mouse strains. A dose of 1.5×10^8 vg per eye was identified as the optimum therapeutic dose of the lead vector, AAV8.GRK1.CDHR1.pA, in the absence of retinal thinning following sub-retinal delivery in both *Cdhr1*^{-/-} and *C57BL/6J* strains. Furthermore, a pilot ERG study undertaken on a small cohort of mice identified a statistically significant therapeutic signal in *Cdhr1*^{-/-} mice at a dose of 1.5×10^8 vg per eye, as early as 2-months post-injection. The effect sizes identified on dark- and light-adapted ERG would enable an *a priori* power calculation for the longitudinal, prospective analysis of the safety and efficacy of *CDHR1*-gene therapy in both *Cdhr1*^{-/-} and *C57BL/6J* strains (Chapter 6). Moreover, the therapeutic dose of AAV8.GRK1.CDHR1.pA was shown to be non-toxic in wildtype mice using OCT imaging, indicating that overexpression at the chosen dose is well tolerated on measures of retinal structure.

6. Evaluation of *CDHR1* gene therapy

6.1 Introduction

Evaluation of retinal gene therapy in relevant and validated pre-clinical models of retinal degeneration has been instrumental in their development and translation into clinical trials and subsequent regulatory approval; for example, Luxturna® (voretigene neparvovec) for *RPE65*-associated retinal degeneration. Tables 3.1 & 5.1 identify key murine models used in the pre-clinical development of vectors currently in clinical trial. However, naturally occurring or generated zebrafish, chicken, cat, dog, sheep, pig, and non-human primate models of retinal degeneration have also proved helpful, overcoming some of the key biological differences between the human and murine retina.¹⁹⁴ In Chapter 3, structural, functional and behavioural data were presented that validated the *Cdhr1*^{-/-} murine model for the investigation of human *CDHR1*-associated cone-rod dystrophy. Chapter 4 describes the rational design and validation of two AAV8.CDHR1 vectors. A formal dose-toxicity study of AAV8.CDHR1 vectors in *Cdhr1*^{-/-} and *C57BL/6J* mouse strains is presented in Chapter 5, identifying the maximum tolerated dose and likely optimum therapeutic dose of AAV8.GRK1.CDHR1.pA – the lead, unenhanced vector.

This chapter presents experimental data evaluating the safety and efficacy of *CDHR1* retinal gene therapy in *Cdhr1^{-/-}* and *C57BL/6J* strains. Chapter 5 presents a detailed toxicity study of the unenhanced vector, identifying 1.5×10^8 vg as the optimum therapeutic dose from the 0.5 log unit dosing intervals that were evaluated. A subretinal dose of 1.5×10^8 vg exhibited a stronger rescue effect on dark- and light-adapted ERG 2-months post-injection in a small pilot study when compared to a dose of 7.5×10^8 vg. Moreover, retinal thinning was not observed in *C57BL/6J* mice at a dose of 1.5×10^8 vg. Based on previous observations (Figure 4.8 & 4.9), it was considered that a dose of 7.5×10^8 vg has a functional benefit that is limited by overexpression (Figure 5.13). Consequently, a dose of 1.5×10^8 was selected as the “high dose” for longitudinal evaluation.

Dose reduction studies identified a more limited therapeutic effect of doses below 1.5×10^8 vg when evaluated 2-months post-injection in small pilot cohorts (Figure 5.7). Specifically, dark-adapted ERG responses did not differ significantly between doses of 1.5×10^7 vg and 7.5×10^7 vg, both of which demonstrated a smaller benefit compared with a dose of 7.5×10^8 vg. However, the longer term functional therapeutic effect based on dose was unknown. In addition, small variations may have a large effect on outcome measures of electrophysiological testing in small cohorts. A dose of 1.5×10^7 vg was selected as the lower dose for longitudinal evaluation - one log unit below the high dose value - to ensure a high probability of a clear distinction between the groups on both ERG and OCT assessments. The

dosing groups for the longitudinal rescue experiments using the AAV8.GRK1.CDHR1.pA CDHR1 vector were selected as follows:

“High dose” – 1.5×10^8 vg per eye

“Low dose” – 1.5×10^7 vg per eye

6.2 Aims

The primary aim of this chapter was to evaluate the efficacy and safety of *CDHR1* retinal gene therapy using the AAV8.GRK1.CDHR1.pA vector in *Cdhr1*^{-/-} and *C57BL/6J* strains.

The primary outcome measures of efficacy in *Cdhr1*^{-/-} mice were evaluated through a comparison of AAV- versus PBS-injected paired control eyes as follows:

- Dark- and light-adapted ERG: rescue of rod and cone function, on response amplitudes and A-wave implicit times
- OCT imaging: Preservation of photoreceptor layer thickness measurements

In addition, exploratory outcome measures included:

- OCT evidence of morphological changes in outer segment length, and/or preservation of outer retinal structures.
- Preservation of photopic and/or scotopic optomotor reflex responses.
- Ultrastructural analysis of photoreceptor morphology.

Dark- and light-adapted ERG responses were compared with PBS-injected control eyes since full field ERG responses could not compare retinal locations. However, OCT data presented in Chapter 5 validated the use of retinal thickness measurements according to retinal location as an internal control that could be used to further qualify safety and efficacy. The effect of gene therapy on retinal thickness measures were evaluated through a comparison of superior (targeted) and inferior (non-targeted) retinal locations in AAV-injected eyes and between corresponding retinal loci in paired AAV- and PBS-injected eyes. Morphological changes on OCT imaging were also assessed by retinal location.

The primary outcome measures of safety in *C57BL/6J* mice were:

- Dark- and light-adapted ERG: the absence of a significant reduction in rod and cone function compared to PBS-injected control eyes.
- OCT imaging: The absence of photoreceptor layer thinning compared to paired PBS-injected control eyes.
- Optomotor testing: The absence of significant differences in photopic and scotopic optomotor reflexes between AAV- and PBS-injected control eyes.

The absence of a statistical difference in these outcome measures would be considered sufficient to demonstrate safety of overexpression of CDHR1 on wildtype photoreceptors following gene therapy.

The secondary aims were to:

- Identify a dose-response relationship between high and low vector doses on retinal function and structure.
- To determine the durability of any therapeutic benefit which would provide indirect evidence of stable CDHR1 transgene expression following gene therapy *in vivo*.
- To determine the effect of CDHR1 gene therapy on RPE structure as evaluated by autofluorescence imaging (SW-AF and NIR-AF).

6.3 Materials and methods

6.3.1 Animal strains

Cdhr1^{-/-} mice were backcrossed to *C57BL/6J* mice over at least 5 generations to limit the influence of unknown genetic variants, and to reduce any strain-specific variations in electroretinography and OCT imaging parameters with the *C57BL/6J* wildtype group. The *C57BL/6N* strain was not considered due to a frequent *Crb1* mutation (*rd8*) that could confound the effect of *Cdhr1* targeting.¹²⁸ *Cdhr1* is separated by 3 million base pairs from *Opn4* on murine chromosome 14, with a 3% probability of recombination over a single generation. The *Opn4* gene encodes melanopsin, expressed in intrinsically photosensitive retinal ganglion cells. Sequencing of the *Opn4* gene was not considered necessary since melanopsin offers a minor contribution to the full-field ERG, and structural and functional

effects on the OMR responses were not detected in *Opn4*^{-/-} mice in previous studies.¹⁹⁵ Moreover, experimental design involved the use of paired control eyes.

Following confirmation of genotype by PCR (Chapter 3), multiple breeding pairs were established to achieve large numbers of pups of a similar age for subretinal injection. Both male and female pups were included; the inclusion of littermates was considered advantageous to reduce the probability of genetic differences within breeding cohorts. *C57BL/6J* mice were acquired directly from Charles River at the required age; all were female to reduce the likelihood of attrition.

6.3.2 *A priori* power calculation

The AAV8.CDHR1 dosing study (Chapter 5) was designed to permit an *a priori* power calculation for this experiment based on ERG responses following subretinal injection of AAV8.GRK1.CDHR1.pA at 1.5×10^8 vg per eye (Table 6.1). Twenty-six *Cdhr1*^{-/-} mice were required to detect a difference between treated and control eyes with 95% power. A power calculation was not undertaken for the low dose *Cdhr1*^{-/-} or high and low dose *C57BL/6J* groups since large differences were not identified in pilot studies (Chapter 5). A group of mice of approximately the same size ($n=24$) was selected for the “low dose” *Cdhr1*^{-/-} group (1.5×10^7 vg). Similarly, the high dose and low dose *C57BL/6J* groups included 25 and 26 mice, respectively. Additional mice were injected in each of the four groups to account for ~10% attrition rate.

Light intensity (cd.s/m ²)	Group	Mean (μV)	SD (μV)	Effect size	<i>n</i> (95% power)	<i>n</i> (99% power)
1	AAV:1.5 x 10 ⁸	249.80	68.48	1.677	18	24
	<i>PBS control</i>	134.98	29.60	-		
10	AAV:1.5 x 10 ⁸	236.54	73.60	1.348	26	38
	<i>PBS control</i>	137.31	21.91	-		
25	AAV:1.5 x 10 ⁸	256.98	85.11	0.979	48	68
	<i>PBS control</i>	173.66	41.84	-		

Table 6.1 A priori power calculation for longitudinal study of AAV8.CDHR1 gene therapy in *Cdhr1*^{-/-} mice

Values are based on dark-adapted single-flash ERG data in mice 2 months after sub-retinal injection as part of a pilot study presented in Chapter 5; *n*=5. Power calculations were based on Cohen's effect size, whereby the SD of both groups was accounted for in the calculation.

Table 6.2 details the number of mice injected in each cohort and the numbers of mice that were culled across the experiment. Low dose cohorts were culled after the 6-month timepoint. High-dose cohorts were kept until the 18-month timepoint at which time final assessments were undertaken.

6.3.3 Experimental design

The experimental design was of an open-label, prospective, longitudinal, controlled study using objective, structural (OCT imaging), functional (dark- and light-adapted ERG), and behavioural (optomotor reflex; OMR) outcome measures.

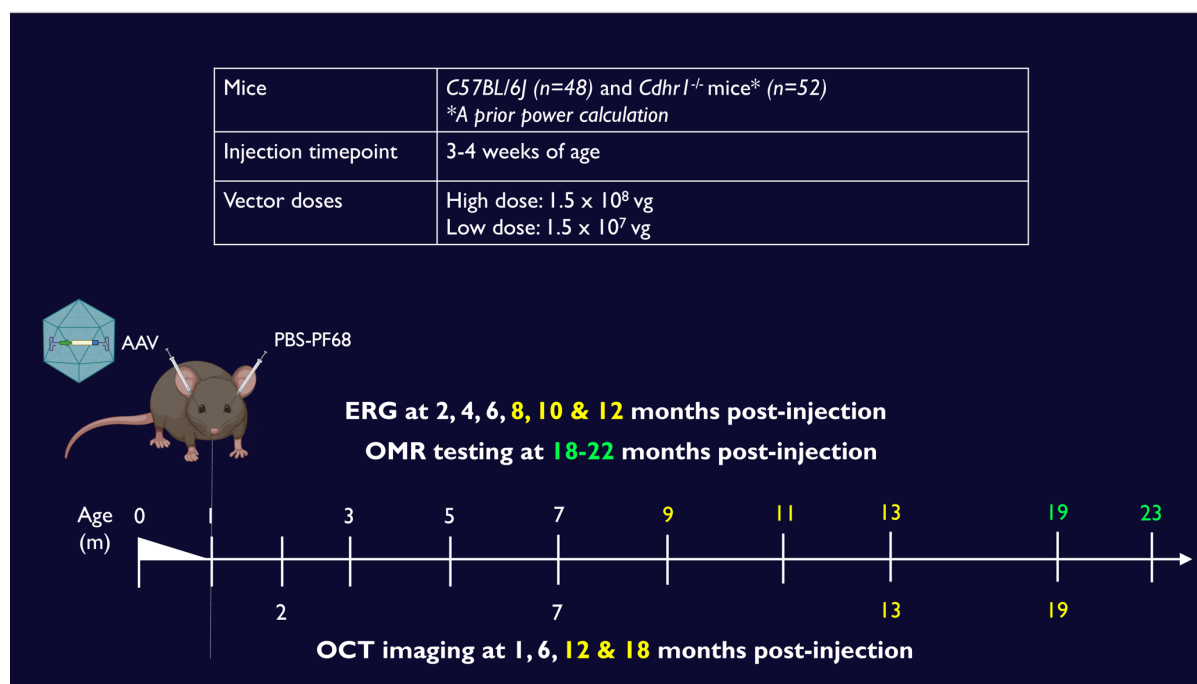


Figure 6.1 Design of longitudinal AAV8.GRK1.CDHR1.pA gene therapy efficacy and safety study

Yellow font (OCT and ERG) and green font (OMR) indicate additional experiments undertaken in high dose groups only. White font indicates experimental procedures undertaken in all dosing groups. 100 experimental mice were used. Tissue recovery was undertaken in high-dose *Cdhr1*^{-/-} eyes at 21-months post-injection for histological analysis.

All mice underwent subretinal injection between 3 weeks and 1 day and 3 weeks and 6 days postnatal age (i.e. post-weaning), to prevent the requirement for surgical division of the eyelids and to ensure experimental methods remained consistent with those outlined in Chapter 5 from which *a priori* power calculations were derived. Moreover, retinal degeneration, as determined by detailed phenotyping of the *Cdhr1*^{-/-} mouse (Chapter 3), was relatively slow with photoreceptor layer retinal thickness measurements around 80% of wildtype controls at 1 month; there was no justification for earlier intervention.

1.5µl of AAV8.GRK1.CDHR1.pA vector in 0.001% PBS-PF68 was delivered by *ab externo* sub-retinal injection at a dose of either 1.5×10^8 vg or 1.5×10^7 vg in both the *Cdhr1^{-/-}* and *C57BL/6J* groups. The sub-retinal injection was delivered superiorly in all cases, as described in Chapter 2. Subretinal haemorrhage of any size, significant ocular surface bleeding, and animals with pre-existing anatomical or developmental abnormalities underwent Schedule 1 sacrifice and were not included. All other animals were recovered and eyes inspected daily in the early post-operative period. No further exclusions based on structural or functional findings were permitted following recovery after sub-retinal injection. However, dropout due to poor recovery or husbandry concerns is detailed in Table 6.2.

Group	Mice (n)	Follow-up	Drop out (n)	Reasons for exclusion
<i>Cdhr1^{-/-}</i> High dose	28	21 months: <i>n</i> =28 at 6 months <i>n</i> =24 at 12 months <i>n</i> =20 at 18 months	8	Husbandry concerns: 6 Culled for poor recovery: 2
<i>Cdhr1^{-/-}</i> Low dose	24	6 months: <i>n</i> =24 at 2 months <i>n</i> =23 at 4 months <i>n</i> =23 at 6 months	1	Non-recovery following 2-month ERG assessment: 1
<i>C57BL/6J</i> High dose	23	22 months: <i>n</i> =21 at 6 months <i>n</i> =19 at 12 months <i>n</i> =12 at 18 months	9	Husbandry concerns: 7 Culled for poor recovery: 2
<i>C57BL/6J</i> Low dose	25	6 months: <i>n</i> =25 at 2 months <i>n</i> =24 at 4 months <i>n</i> =24 at 6 months	1	Non-recovery following ERG at 2 months: 1

Table 6.2 Numbers of injected mice per cohort and exclusions

Following inclusion of mice in each cohort after successful subretinal injection, no investigator-led exclusions were made, except on the basis of animal welfare concerns. High dose – 1.5×10^8 vg; Low dose – 1.5×10^7 vg

6.3.3.1 *Selection of controls*

Three strategies for experimental controls were incorporated into the study design; age-matched *C57BL/6J* mice (between genotypes to control for CDHR1 overexpression), paired eyes with active vehicle control intervention (between eyes to control for the effect of retinal detachment), and within eye internal control (between retinal locations to control for the effects of localised AAV vector delivery). Experimental procedures were undertaken according to standardised protocols for all groups; the integrity of the data presented in this chapter was considered crucial for regulatory approval (e.g. MHRA) prior to clinical trial.

Paired control eyes underwent sub-retinal injection with an identical volume of PBS in 0.001% PF68, thereby controlling for the effect of the retinal detachment and excipient but not AAV vector or transgene. The control substance was prepared at the same time as the test vector and stored under the same conditions. The increased variability from paired sub-retinal injections was offset by the advantage in controlling for the effect of surgically-induced retinal detachment. A single superior injection was favoured over both superior and inferior injections to create an internal control within each eye in which any toxic or therapeutic effect could be expected in a superior-inferior gradient on retinal imaging. Demonstrating proof-of-principle of efficacy was considered more beneficial than the overall therapeutic effect size on ERG relative to wildtype animals which may be achieved with more extensive AAV delivery.

6.3.4 Retinal imaging

All mice underwent retinal imaging including near infra-red reflectance, short-wavelength and near infra-red autofluorescence and OCT imaging of both AAV- and PBS-injected eyes at 1- and 6-months post-injection. Mice in the high-dose groups underwent additional retinal imaging at 12- and 18-months post-injection. Retinal imaging protocols, retinal loci and definitions of retinal thickness measurements were identical to those outlined in Chapter 3. Photoreceptor OS length and measurement of the radial extent of OS regeneration in high dose *Cdhr1*^{-/-} groups were assessed at all timepoints by a masked observer, blinded to the treatment allocation.

6.3.5 Electroretinography

Dark- and light-adapted ERG assessments were undertaken in all groups at 2-, 4- and 6-months post-injection, with additional ERG assessments at 8-, 10-, 12-months post-injection in the high-dose groups (Figure 6.1), as described in Chapter 3. In addition, A-wave implicit times were measured for all groups on dark-adapted ERG at light intensities of 0.01 cd.s/m² and greater and on light-adapted single-flash responses at both 3 and 10 cd.s/m².

6.3.6 Optomotor reflex behavioural evaluation

Mice in the high dose *Cdhr1*^{-/-} and *C57BL/6J* strains underwent photopic and scotopic OMR testing under standardised conditions at 18-22 months post-

injection (Section 2.5.5). OMR responses (head tracking) were counted across 3 x 30 second intervals (i.e. three technical replicates) at two speeds of rotation in both clockwise and anti-clockwise direction to isolate responses from left (PBS-injected) and right (AAV-injected) eyes, respectively.⁹⁶ Video recordings were reviewed by a masked observer, blinded to genotype and treatment allocation, who counted smooth pursuit head tracks in the direction of rotation of the OKN drum.

6.3.7 Tissue recovery and transmission electron microscopy

Eyes were recovered following schedule 1 sacrifice by 100µl of intraperitoneal Pentoject® (Pentobarbital sodium 20% w/v), a sufficient dose for mice up to 60g in weight. A 10-0 nylon suture was used to mark the superior aspect of the eye prior to enucleation. Mouse eyes were fixed in 2% glutaraldehyde, 2% paraformaldehyde in 0.1M cacodylate buffer at pH 7.4 for 1 hour at room temperature before removal of the cornea and lens and fixing for a further hour. Further steps were performed by Dr. Thomas Burgoyne: The eyes were incubated in 1.5% potassium ferricyanide and 1% osmium tetroxide for 1 hour at 4°C before *en bloc* staining with undiluted UA-Zero (Agar Scientific) for 2 hours at room temperature. The eyes were dehydrated in an ethanol series (70%, 90% and 100% ethanol) followed by propylene oxide before leaving in 1:1 propylene oxide:epon overnight at room temperature. The following day the epon was replaced twice before embedding the eyes overnight at 60°C. ~100nm thick sections were cut and viewed on a JEOL

1400+ transmission electron microscope fitted with a Gatan Orius SC100B charge-coupled device camera.

6.4 Results

6.4.1 High-dose AAV8.GRK1.CDHR1.pA slows photoreceptor cell death in *Cdhr1*^{-/-} mice to 18-months post-injection

In *Cdhr1*^{-/-} mice, OCT imaging identified a slowing of photoreceptor layer thinning that was sustained to 18-months post-injection following high-dose AAV8.GRK1.CDHR1.pA (1.5 x10⁸ vg) (Figure 6.2 & 6.4). At equivalent superior retinal loci, mean photoreceptor layer thickness measurements were 76.6µm in AAV- versus 49.7µm in PBS-injected eyes (two-way ANOVA: $P<0.0001$ for effect of treatment; $n=28$) at 6-months post-injection. At 18-months, the difference had increased to 64.5µm versus 23.5µm ($P<0.0001$; $n=20$), with further thinning in PBS-injected eyes limited by a floor effect.

A mild, but significant, therapeutic effect was seen inferiorly in AAV- versus PBS-treated eyes at 6-months ($P=0.012$; $n=28$) and 12-months ($P=0.003$; $n=26$), although was not significant at 18-months post-injection ($P=0.125$; $n=20$).

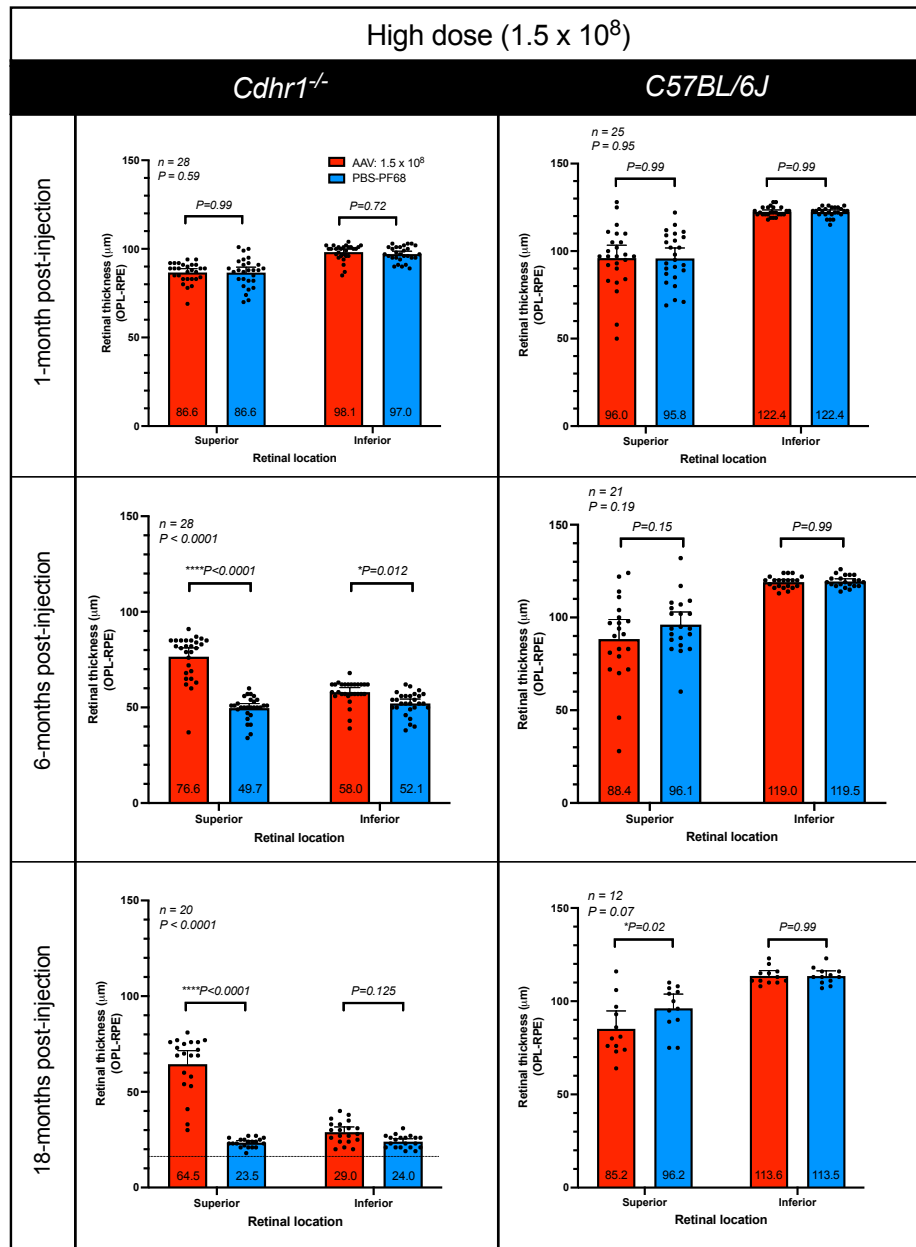


Figure 6.2 Photoreceptor layer thickness measurements on OCT imaging following high-dose AAV8.GRK1.CDHR1.pA to 18 months post-injection in *Cdhr1*^{-/-} and *C57BL/6J* mice

AAV-injected *Cdhr1*^{-/-} eyes demonstrate a slowing of photoreceptor degeneration as demonstrated by preserved photoreceptor layer thickness measurements at 6-months post-injection ($P < 0.0001$), and at all later timepoints. The rescue effect is marked superiorly where the bleb is delivered, and mild inferiorly at 6- and 12-months post-injection. In *C57BL/6J* mice, there is no significant retinal thinning in AAV-injected eyes, except in the superior retina at 18-months. Plots represent mean + 95% C.I. Shapiro-Wilk indicated parametric datasets; P values are derived from two-way ANOVA (effect of treatment) with Šídák's multiple comparison test.

However, retinal structure was preserved in the superior half of the inferior retina (Figure 6.3), when compared to the peripheral inferior retinal loci where OCT measurements were derived. This is a likely consequence of a superior-inferior gradient of transduction at 1.5×10^8 vg, beyond the limits of the initial bleb.

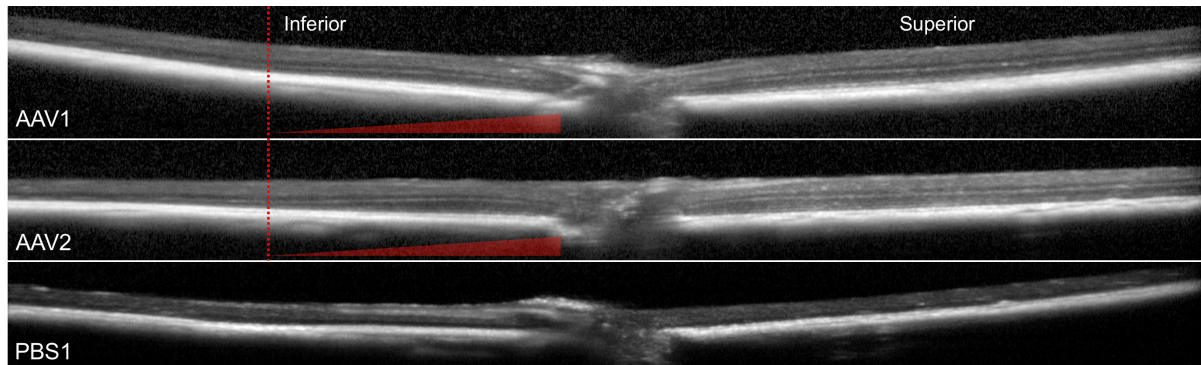


Figure 6.3 Structural superior-to-inferior therapeutic gradient following high-dose AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice.

Representative OCT images from two mice 18-months post-injection showing a superior to inferior gradient of structural preservation that is lost (dashed red line) in the peripheral inferior retina where retinal structure appears similar to a PBS-injected control eye at the same timepoint.

In the low dose cohort, a mild slowing of photoreceptor layer thinning was observed in the superior retina of AAV- versus PBS-injected eyes ($P=0.028$; $n=20$) at 6-months post-injection. The therapeutic effect was significant overall ($P=0.021$), although the effect size was small and restricted to the superior retina (Figure 6.4). There was no identifiable therapeutic effect on the inferior retina in AAV-injected eyes at a dose of 1.5×10^7 vg at either timepoint. Raw OCT images illustrate the structural rescue effect of high dose AAV8.GRK1.CDHR1.pA (1.5×10^8 vg) on the preservation of the photoreceptor layer in AAV- versus PBS-injected

control eyes (Figures 6.3 & 6.5-6.7). This effect is sustained to at least 18-months after intervention, the latest timepoint measured.

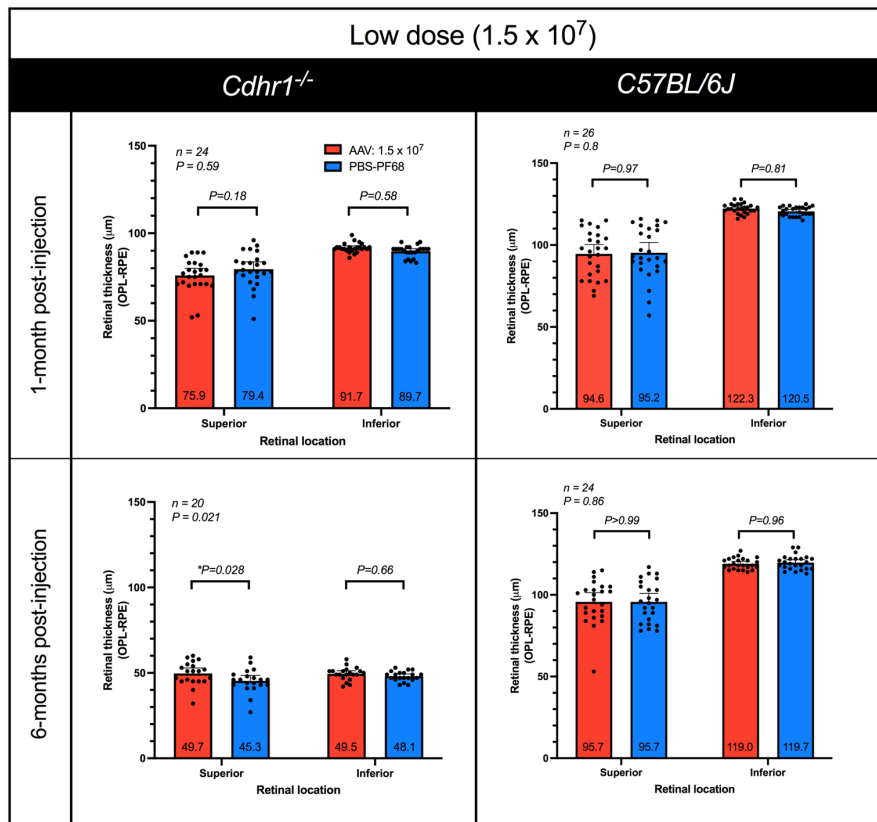


Figure 6.4 Photoreceptor layer thickness measurements on OCT imaging following low-dose AAV8.GRK1.CDHR1.pA to 6 months post-injection in *Cdhr1*^{-/-} and *C57BL/6J* mice

AAV-injected eyes demonstrated a modest rescue effect superiorly at 6-months post-injection compared to PBS-injected eyes at 6-months ($P=0.028$) in *Cdhr1*^{-/-} mice. In *C57BL/6J* mice, treatment groups are matched at all timepoints and retinal locations. Plots represent mean + 95% C.I. Shapiro-Wilk indicated parametric datasets; P values are derived from two-way ANOVA for effect of treatment with Šídák's multiple comparison test.

6.4.2 High-dose AAV.GRK1.CDHR1.pA regenerates photoreceptor outer segments in *Cdhr1*^{-/-} mice, sustained to 21-months post-injection

In Chapter 3, longitudinal OCT imaging data are presented which identify shortened photoreceptor OS in *Cdhr1*^{-/-} mice at 1-month of age (Figure 3.6), which

are no longer discernible by 6-months of age. Following high dose AAV8.GRK1.CDHR1.pA injection (1.5×10^8 vg), preservation and regeneration of the OCT layer representing the photoreceptor OS was identified in AAV-injected eyes to 18-months post-injection on OCT imaging (Figures 6.3, 6.5-6.7). These effects were not identified in the low-dose cohort, or PBS-injected eyes.

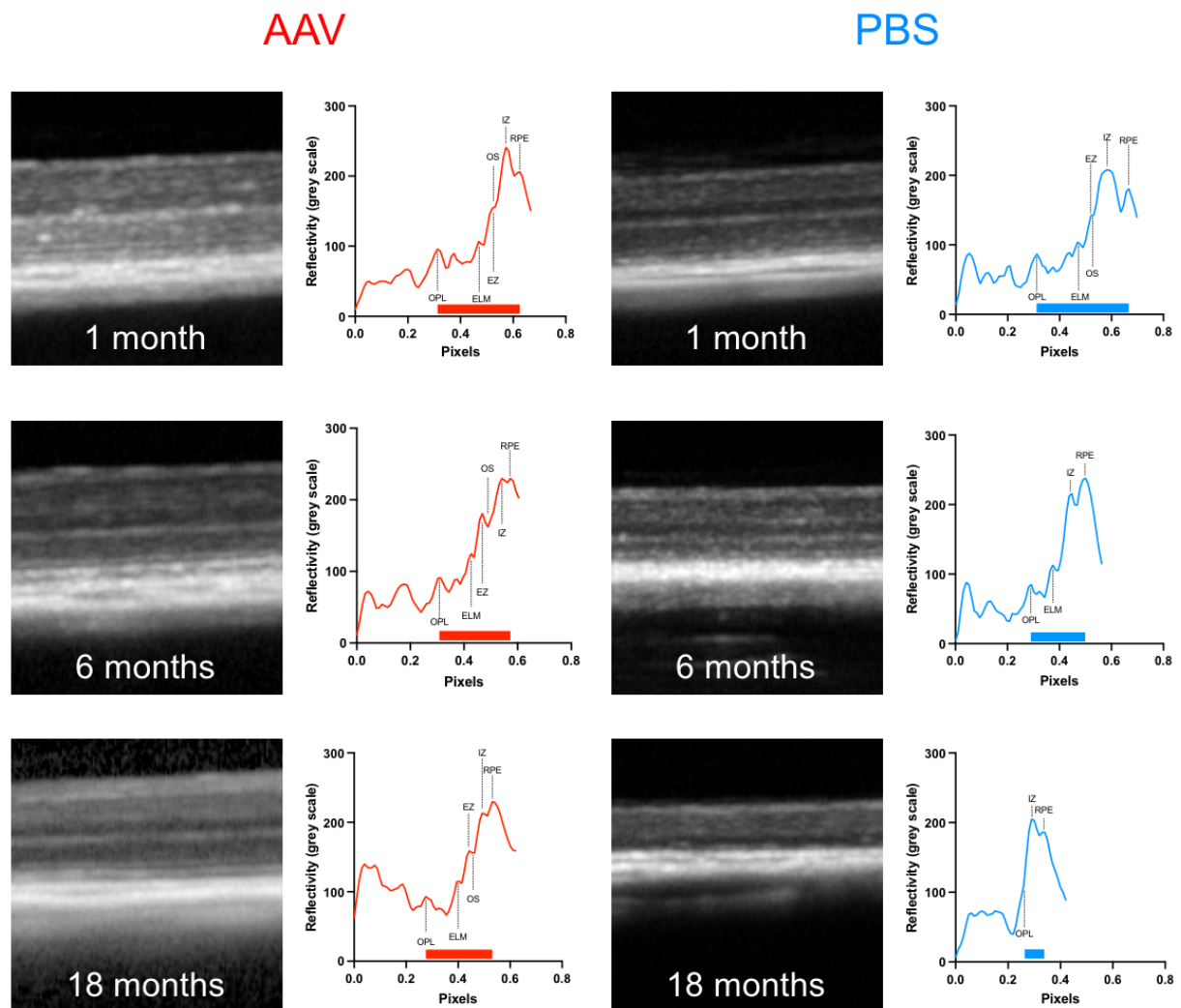


Figure 6.5 Morphological outer retinal features on OCT imaging following high-dose AAV8.GRK1.CDHR1.pA to 18 months post-injection Raw OCT images from a single *Cdhr1*^{-/-} mouse show preservation of the photoreceptor layer in the superior retina following AAV-injection to 18-months post-injection, with severe outer retinal degeneration in the paired PBS-injected eye at the same loci. Reflectivity profiles highlight congenitally shortened OS at 1-month in both treatment groups which lengthened and become defined at 6- and

[Continued...] 18-months post injection in AAV-, but not PBS-injected eyes. Increased reflectivity of the ellipsoid zone was seen in AAV-injected eyes from 6-months and later timepoints, which appeared iso-reflective to the ELM at 1-month in both groups. In PBS-injected eyes, the EZ and OS were absent at 6-months, and near total outer retinal degeneration with loss of ONL is seen at 18-months. The ELM was preserved to 18-months post-injection in AAV-injected eyes, and absent from 12-months in PBS-injected eyes. Coloured bars on reflectivity profiles indicate photoreceptor layer thickness measurements which diverge in treatment groups. Reflectivity profiles were generated from the presented raw OCT images in the axis of the OCT A-scan using ImageJ.

High-dose AAV8.GRK1.CDHR1.pA was found to lengthen the layer representing photoreceptor OS on OCT imaging in *Cdhr1*^{-/-} mice as early as 1-month post-injection, which increased in radial extent across the superior retina to 18-months post-injection ($P < 0.0001$ at all timepoints vs. PBS-injected eyes; Figure 6.7). OCT evidence of OS lengthening of the superior half of the inferior retina was observed (shown in Figure 6.3) in some AAV-injected eyes yielding a significant overall benefit compared with PBS-injected control eyes, although the effect size was smaller (data not shown).

Transmission electron microscopy confirmed OS regeneration following high dose AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} eyes to 21-months post-injection (Figure 6.8). OS measured approximately 30µm, similar to wildtype,¹⁹⁶ with regular alignment, orientation and disc spacing.⁵² Incisures in rod outer segments were identifiable with normal morphology of nascent outer segment discs along the periciliary ridge of the inner segment.^{197, 198} Photoreceptor and outer segments were not identifiable in paired PBS-injected control eyes (data not shown).

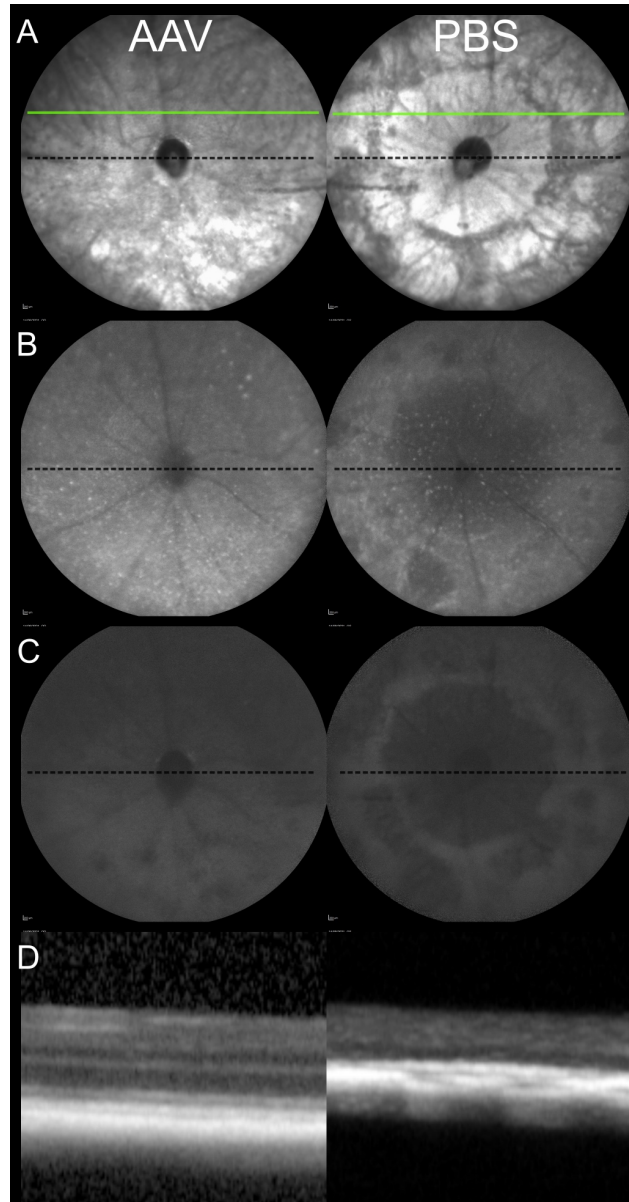


Figure 6.6 Multimodal retinal imaging findings in a representative *Cdhrl*^{-/-} mouse 18-months post-injection with AAV.GRK1.CDHR1.pA

IR reflectance (A), SW-AF (B), NIR-AF (C), and OCT images (D) are presented for paired AAV- (right) and PBS-injected eyes (left). Dashed lines represent the approximate limits of the sub-retinal bleb. In AAV-injected eyes, *en face* retinal imaging techniques demonstrate an absence of features of retinal degeneration in the superior retina, although mild degenerative features are seen inferiorly (A-C). In PBS-injected eyes, severe generalised retinal degeneration is seen with a distinct peripapillary distribution with degeneration, but not loss, of the RPE. OCT images from equivalent retinal loci (as indicated by green line in A) show a clear rescue effect in AAV-injected eyes with preservation of ONL, EZ and OS regeneration. PBS-injected eyes exhibit severe outer retinal degeneration.

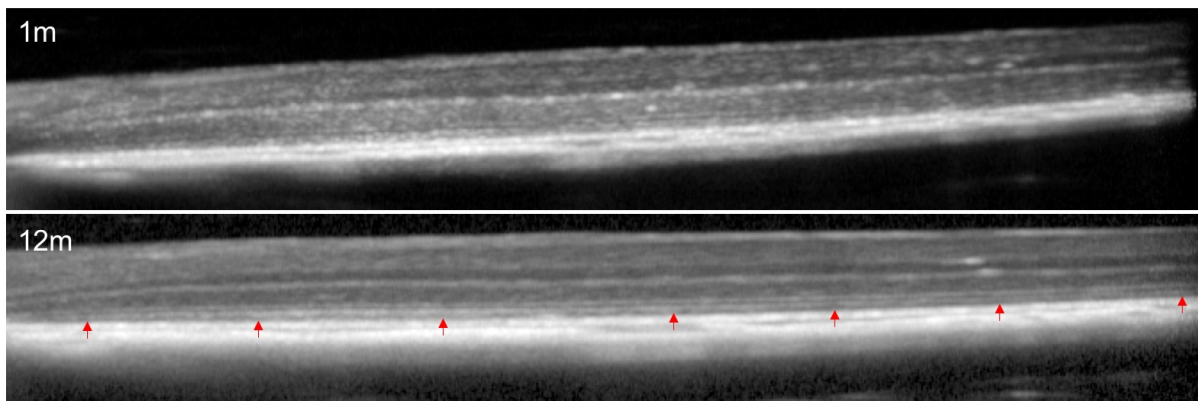
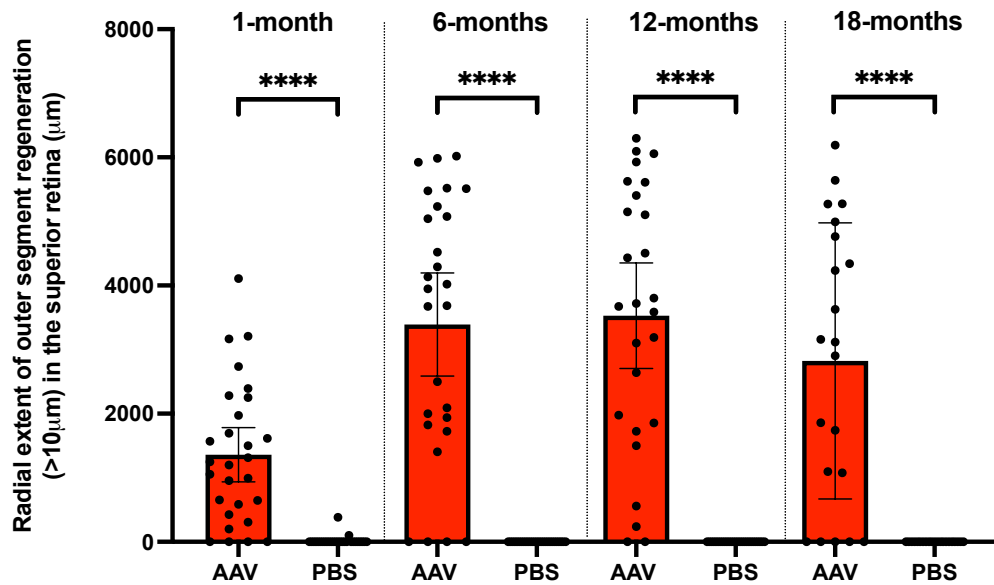


Figure 6.7 Timing and extent of outer segment regeneration on OCT imaging following high dose AAV.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice
Upper: Measurement of the radial extent of OS regeneration by an observer blinded to the treatment allocation demonstrated evidence of OS regeneration at 1-month post-injection, which increased in extent to 12-months and was sustained to 18-months post-injection ($P < 0.0001$ at all timepoints). Plots represent mean and 95% CI. OS regeneration was not identified in PBS-injected eyes. *Lower:* OCT images of the superior retina of the same *Cdhr1*^{-/-} mouse showing regeneration of congenitally shortened OS at 12-months post AAV-injection. The reflectivity of the ellipsoid band appears greater where OS length is greater (see Discussion).

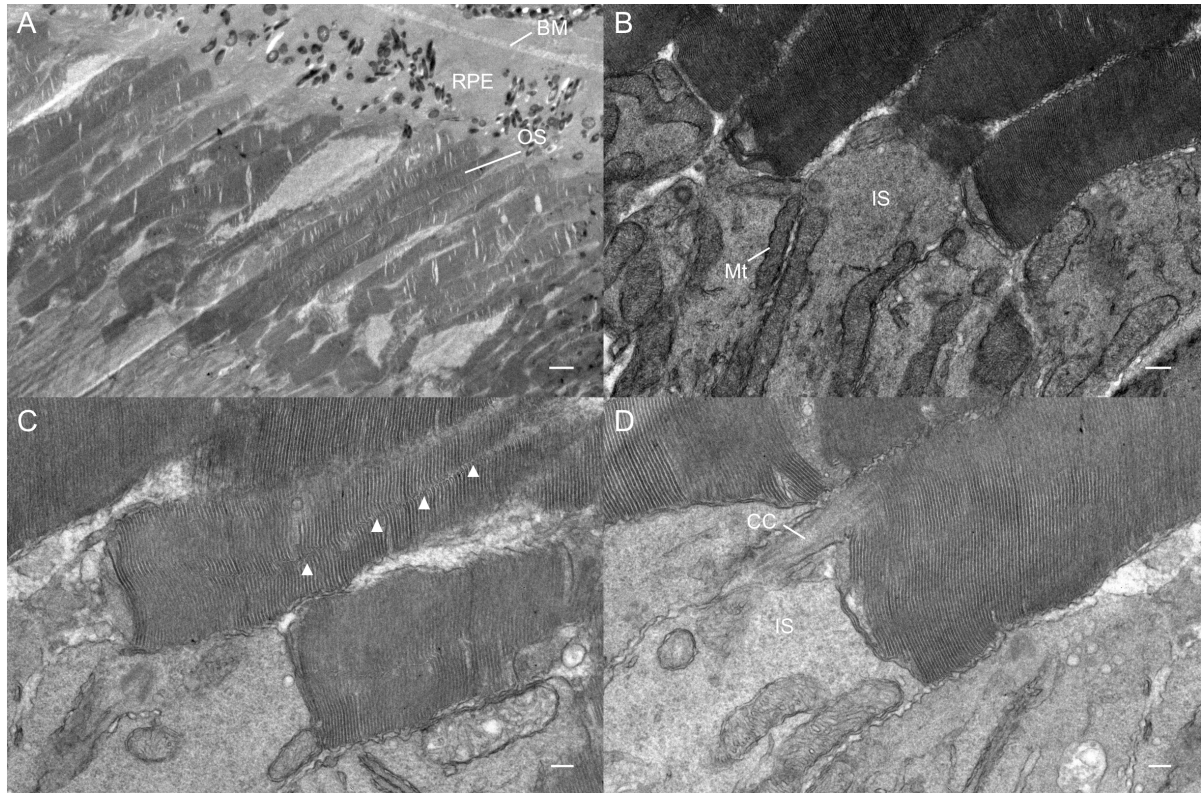


Figure 6.8 Restoration of outer segment length, alignment and morphology on transmission electron microscopy 21-months after high dose AAV.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice

(A) Full-length (~30µm), aligned and correctly orientated photoreceptor OS are identified in the superior retina following *CDHR1* gene therapy. **(B)** Elongated, vertically orientated mitochondria are identified in the IS with normal morphology compared to wildtype mice (data not shown). **(C)** Incisures (arrowheads) in the rod photoreceptor OS are demonstrated, supporting the restoration of the higher-order organisation following *CDHR1* gene therapy. **(D)** Normal morphology of nascent photoreceptor discs with close alignment to the periciliary ridge of the inner segment. Regular spacing of the OS discs throughout the length of the proximal OS is apparent adjacent to the connecting cilium. In the PBS-injected fellow eye of this mouse, photoreceptors and outer segments were not identifiable. Tissue recovery and fixation performed by the author; embedding, sectioning and TEM image capture performed by Dr. Thomas Burgoyne. Scale bars represent 2µm, 400nm, 20nm and 20nm, respectively. BM – Bruch's membrane; Mt – mitochondrion; CC – connecting cilium.

6.4.3 High-dose AAV.GRK1.CDHR1.pA increases the reflectivity of the ellipsoid zone and preserves the external limiting membrane in *Cdhr1*^{-/-} mice to 18-months post-injection

In *Cdhr1*^{-/-} mice, the EZ is no longer discernible on OCT imaging after 6-months of age (Chapter 3). Moreover, when present, the EZ appears less reflective compared to age-matched wildtype controls, appearing similar in width and reflectivity to the ELM (Figure 3.6, 6.5 & 6.7). High-dose AAV8.GRK1.CDHR1.pA preserved the EZ to 18-months post-injection in *Cdhr1*^{-/-} mice, associated with an increase in EZ reflectivity and broadening of the EZ band over time (Figure 6.5-6.7). In the inferior peripheral retina, the EZ is lost with continued retinal degeneration at later timepoints (Figure 6.3). Transmission electron microscopy confirmed the number and morphology of mitochondria in the inner segments of AAV-treated *Cdhr1*^{-/-} eyes were as per wildtype controls – the anatomical correlate for the EZ on OCT imaging (Figure 6.8). Moreover, the ELM, which is normally lost by 12-months of age in *Cdhr1*^{-/-} mice is preserved to at least 18-months following high-dose AAV8.GRK1.CDHR1.pA in AAV-injected eyes (Figure 6.5-6.7). The ELM does not appear to increase in intensity at later timepoints.

6.4.4 High-dose AAV.GRK1.CDHR1.pA prevents RPE degeneration to 18-months post-injection in *Cdhr1*^{-/-} mice

In PBS-injected *Cdhr1*^{-/-} mice, progressive, patchy RPE atrophy was demonstrated, most evident on NIR-AF imaging at 18-months post-injection

(Figure 6.6 & 6.9). High dose AAV8.GRK1.CDHR1.pA prevented RPE degeneration on NIR-AF imaging compared to PBS-injected eyes ($P<0.0001$) at 18-months post-injection. Moreover, this effect was greater in treated superior retinal locations, although a benefit was also identified inferiorly in AAV-injected eyes ($P<0.0001$ for both retinal locations versus PBS-injected eyes; Figure 6.18). Contiguous areas of RPE atrophy were not present at the 6-month timepoint in PBS-injected eyes, and was therefore not assessed in the low dose groups.

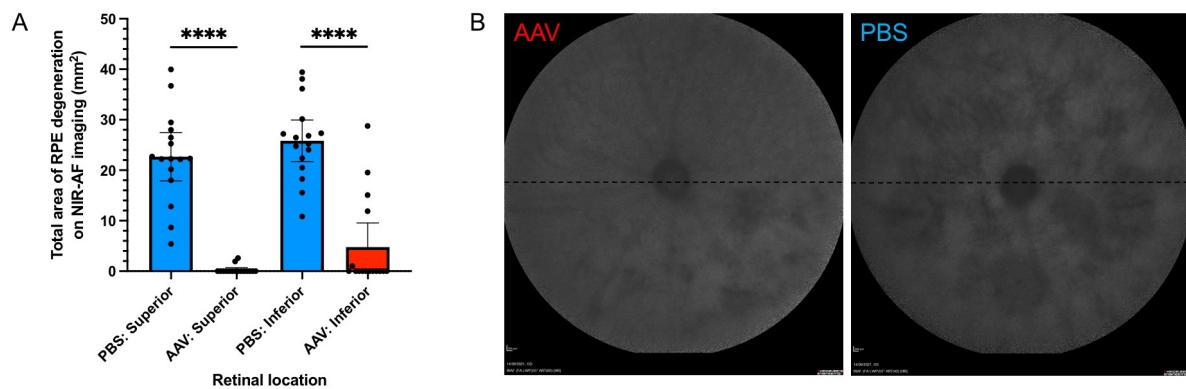


Figure 6.9 Area of RPE degeneration by retinal location in *Cdhrl*^{-/-} mice 18-months post-injection with high-dose AAV8.GRK1.CDHR1.pA

(A) AAV gene therapy slows down RPE degeneration as seen on NIR-AF imaging; $P<0.0001$ overall compared to PBS-injected eyes. This effect is most marked in the superior retina of AAV-treated eyes ($P<0.0001$), although the beneficial effect is also seen inferiorly ($P<0.0001$). Severe, generalised RPE degeneration is seen in PBS-injected eyes **(B)**. Overall, inferior retinal locations exhibited more RPE degeneration than superior ones, despite superior surgically-induced retinal detachments. The area of degeneration was calculated using Heidelberg imaging software following standardised 55° NIR-AF imaging centred on the optic disc. Shapiro Wilk: non-parametric datasets. Wilcoxon paired signed rank test used.

6.4.5 OCT imaging excludes outer retinal thinning to 18-months post sub-retinal injection of AAV8.GRK1.CDHR1.pA in *C57BL/6J* mice

Photoreceptor layer thickness measurements were no different between AAV- and PBS-injected eyes in *C57BL/6J* mice to 18-months post-injection: in the high-dose group at 6-months ($P=0.19$; $n=21$), and 18-months ($P=0.07$; $n=12$), and in the low-dose group at 6-months ($P=0.86$; $n=24$), as shown in Figures 6.2 and 6.4. However, Šídák's multiple comparison test identified a small difference between superior retinal locations at 18-months ($P=0.02$) with a small effect size (see Discussion). Other than variable focal outer retinal disruption at the injection site in both AAV- and PBS-injected control eyes, no obvious morphological alterations were identified in the high- or low-dose *C57BL/6J* groups.

6.4.6 Electroretinography demonstrates rescue of cone and rod photoreceptor function in *Cdhr1*^{-/-} mice to 12 months post-injection

6.4.6.1 *Dark-adapted ERG*

Consistent with observations from pilot experiments presented in Chapter 5, a significant difference in A-wave amplitudes on the dark-adapted luminance series was observed between AAV- and PBS-injected eyes in *Cdhr1*^{-/-} mice as early as 2-months post-injection of AAV8.GRK1.CDHR1.pA at 1.5×10^8 vg ($P<0.0001$; $n=28$) (Figure 6.10). The difference in A-wave amplitudes (i.e. effect size) between AAV- and PBS-injected eyes increased at each successive timepoint to 12-months post-injection (Figure 6.10).

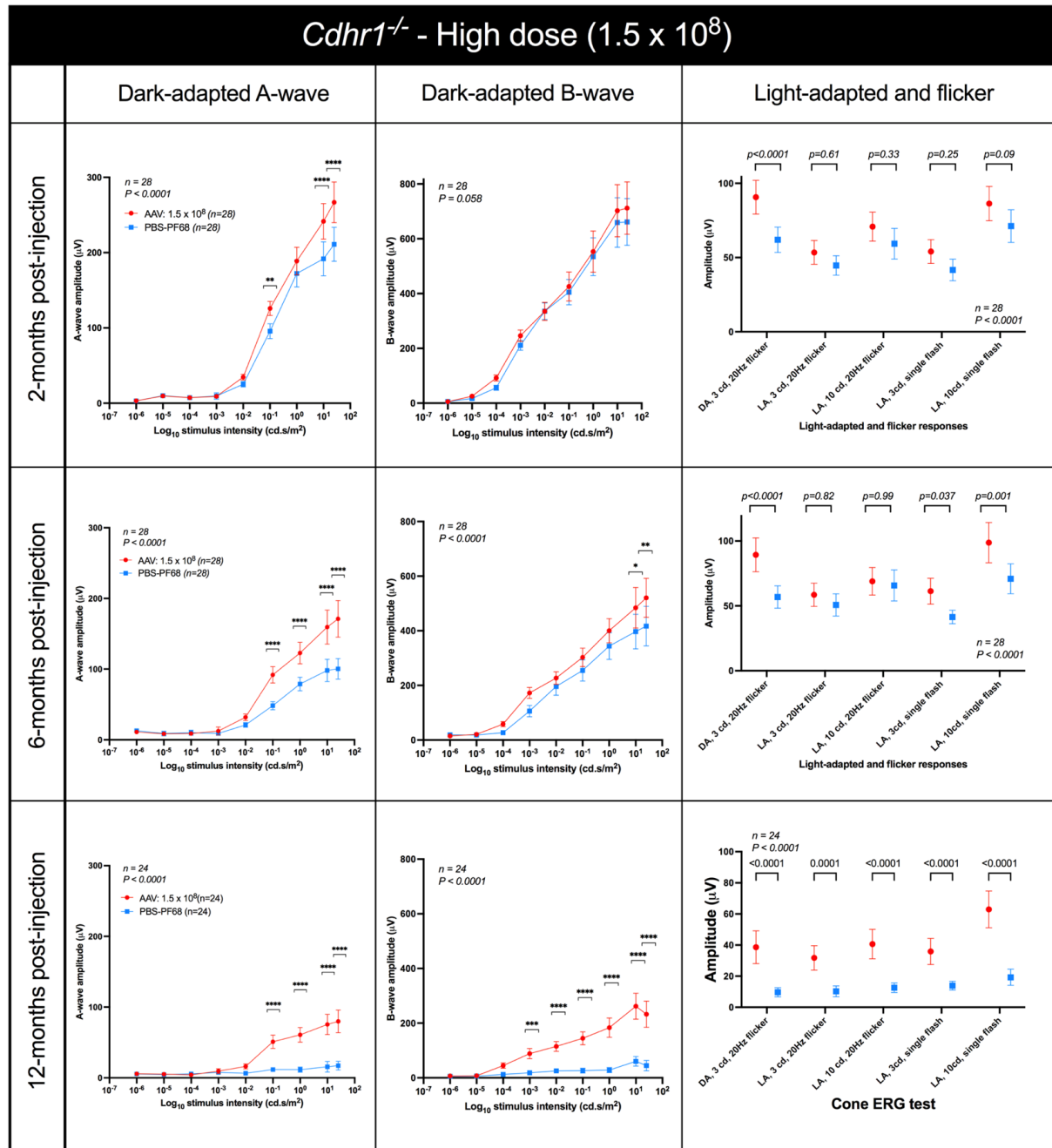


Figure 6.10 Dark- and light-adapted ERG following high-dose AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice to 12 months post-injection
 Rescue of rod and cone photoreceptor function in AAV-treated eyes is demonstrated as early as 2-months post-injection, with the therapeutic effect size increasing at each successive timepoint. Plots represent mean + 95% C.I. Shapiro-Wilk indicated parametric datasets; P values were derived from two-way ANOVA for effect of treatment with Šídák's multiple comparison test for further comparisons.

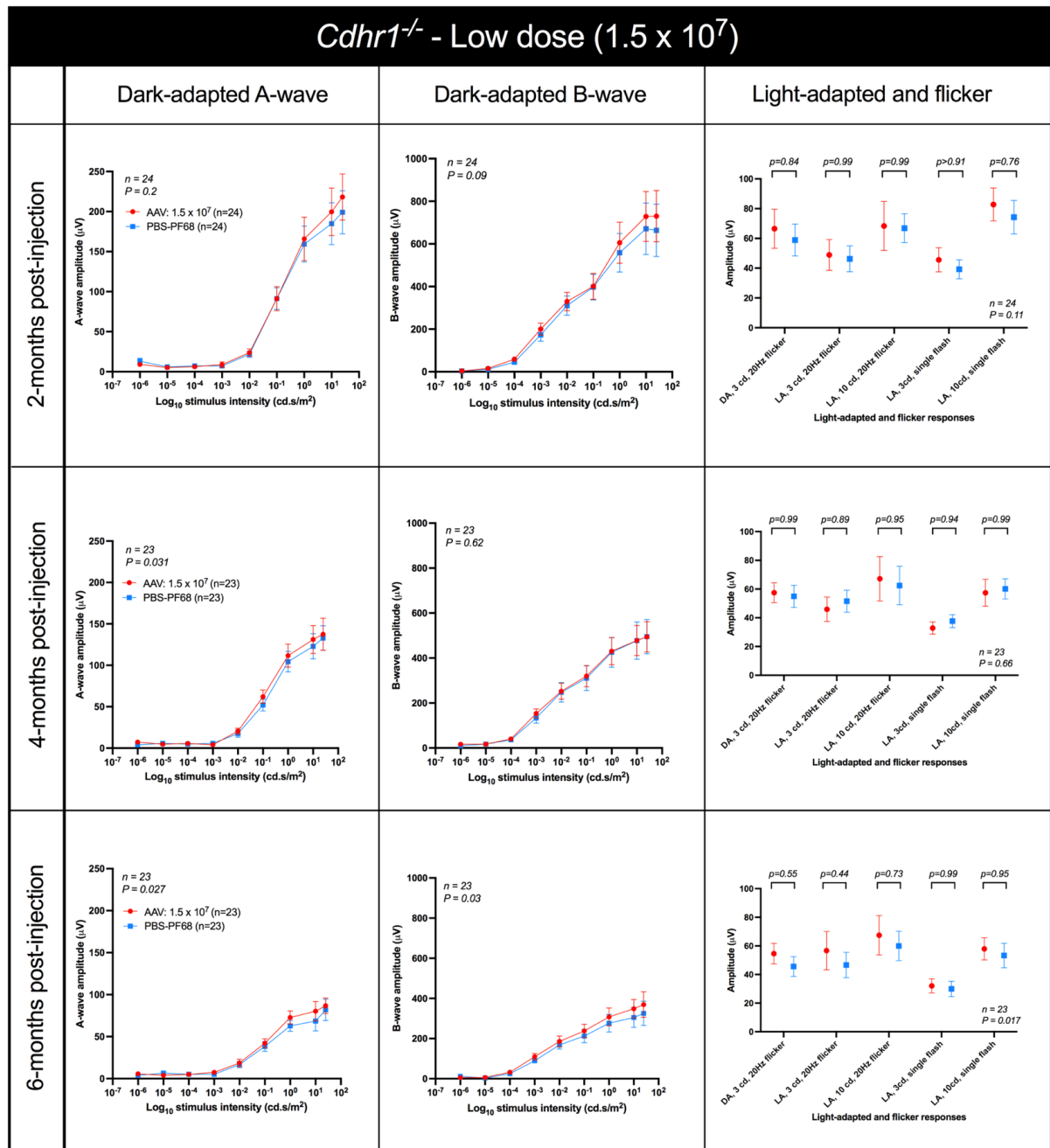


Figure 6.11 Dark- and light-adapted ERG following low-dose AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice to 6 months post-injection

A statistical benefit of AAV- over PBS-injected eyes was demonstrated in A-wave responses from 4-months post-injection, and B-wave responses from 6-months post-injection. Light-adapted single-flash and flicker responses were improved overall, although not significantly different for individual tests. Plots represent mean + 95% C.I. Shapiro-Wilk indicated parametric datasets; P values were derived from two-way ANOVA (for effect of treatment) with Šidák's multiple comparison test used for post-hoc analysis of light-adapted responses.

The low dose group (1.5×10^7 vg) demonstrated marginally greater A-wave response amplitudes in AAV- versus PBS-injected eyes that was evident from 4-months post-injection ($P<0.031$; $n=23$), although the effect size was much smaller and unlikely to be clinically significant (Figure 6.11).

In the high dose group, B-wave amplitudes were not significantly different at 2-months post-injection between AAV- and PBS-injected eyes ($P=0.058$), although became significantly greater in AAV-injected eyes from 4-months ($P=0.015$; $n=28$ – data not shown), with a greater effect size seen at each successive timepoint ($P<0.0001$ from 6-months; Figure 6.10). In the low dose group, a very small benefit was evident in the B-wave response amplitudes at 6-months post injection ($P=0.03$; $n=23$), although not at earlier timepoints. This is unlikely to be clinically significant.

Raw ERG traces recorded simultaneously from paired *Cdhr1*^{-/-} eyes 12-months post-injection illustrate the benefit of *CDHR1* gene therapy (Figure 6.12). A-wave responses, representing rod and combined rod-cone photoreceptor responses, are present in AAV-injected eyes only at this timepoint. Moreover, B-wave responses (ON-bipolar cells) are significantly larger and oscillatory potentials (amacrine cells) are only discernible in AAV-treated eyes.

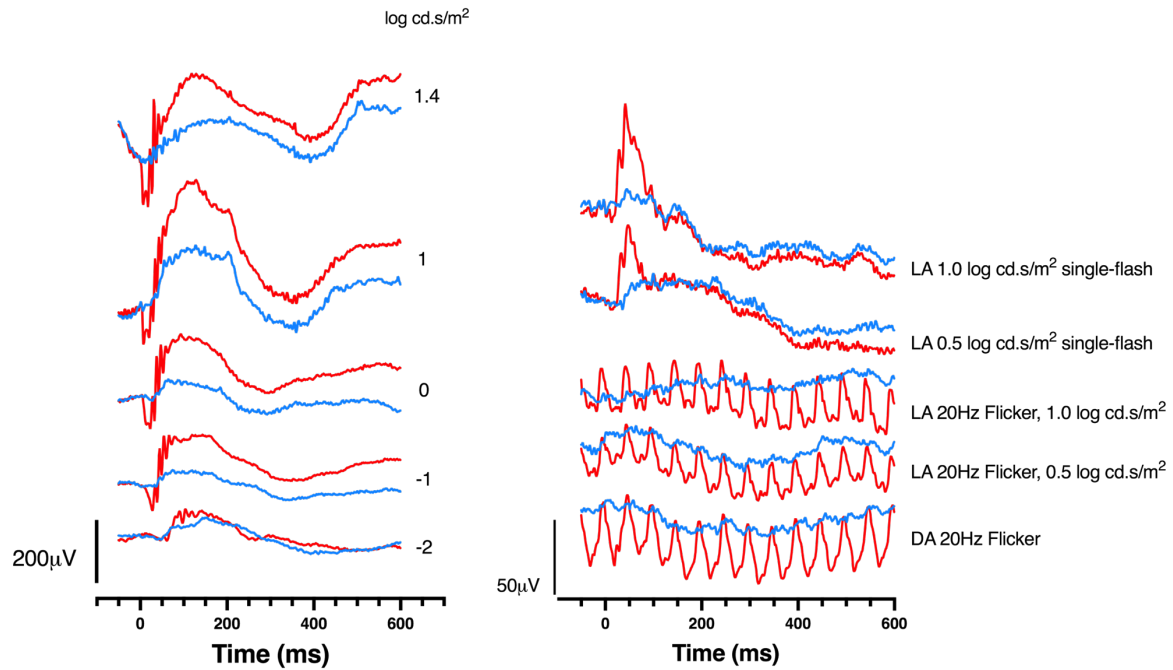


Figure 6.12 Raw dark- and light-adapted ERG traces showing rescue of photoreceptor function in *Cdhr1*^{-/-} mice at 12 months post-injection of high-dose AAV8.GRK1.CDHR1.pA

Raw ERG traces recorded simultaneously from the same mouse 12-month post-injection show preserved responses in the AAV- (red, right eye) versus the PBS-injected eye (blue, left eye). Note the presence of the A-wave on dark-adapted ERG in AAV-treated eyes only, indicating preserved rod and cone photoreceptor function, which are absent in the PBS-injected eye. B-wave responses are also larger and oscillatory potentials discernible in AAV-injected eyes only.

6.4.6.2 Light-adapted ERG

Following high-dose AAV8.GRK1.CDHR1.pA, a significant benefit was seen in dark-adapted flicker responses at 2-month post-injection compared to PBS-injected control eyes ($P < 0.0001$; $n = 28$). At this early timepoint, other light-adapted single flash and flicker response amplitudes were better preserved in AAV- versus PBS-injected eyes, although not significant individually (Figure 6.10). At 4-months post-injection, light-adapted single-flash response amplitudes were better

preserved in AAV-injected eyes ($P=0.0021$; $n=28$ – data not shown), with increasing effect size at each successive timepoint. Light-adapted flicker response amplitudes were significantly larger in AAV-injected eyes from 8 months post-injection at both 3 cd.s/m² ($P=0.037$; $n=26$) and 10 cd.s/m² ($P=0.0003$), which became more significant at later timepoints ($P<0.0001$ at 12-months for both light-adapted flicker responses; $n=24$) (Figure 6.10).

In the low dose group, although mean response amplitudes were greater in AAV-injected eyes at each timepoint, and significant overall at 6-months ($P=0.017$; $n=23$), multiple comparison testing failed to detect a significant benefit in any individual light-adapted or flicker ERG test (Figure 6.11).

Raw light-adapted ERG traces recorded simultaneously 12-months post-injection in a single *Cdhr1*^{-/-} mouse illustrate the benefits of high-dose AAV-injection on cone photoreceptor function that is sustained to at least 12-months post-injection (Figure 6.12). Light-adapted single-flash and flicker responses are observed in the AAV-injected eye and near-absent in the fellow PBS-injected eye.

6.4.7 Shortened A-wave implicit times in *Cdhr1*^{-/-} mice following high-dose AAV8.GRK1.CDHR1.pA at 12-months post-injection

6.4.7.1 Dark-adapted A-wave implicit times

Dark-adapted A-wave implicit times were significantly shorter in AAV- versus PBS-injected *Cdhr1*^{-/-} eyes (mean, 6.0 vs. 10.2ms at 25 cd.s/m²) at 2-months post-injection in the high-dose group ($P<0.0001$ at all light intensities; $n=28$), a difference which increased to 12-months post-injection when A-waves were not easily discernible in PBS-injected eyes (Figures 6.15 & 6.16). Moreover, A-wave implicit times in AAV-injected *Cdhr1*^{-/-} eyes were not significantly different from AAV-injected *C57BL/6J* eyes at the highest light intensities at 2-months post-injection ($P>0.92$), although this difference became significant at later timepoints.

Conversely, A-wave implicit times were significantly longer in *Cdhr1*^{-/-} versus *C57BL/6J* eyes ($P<0.0001$ overall, and at the 4 highest light intensities) at 2-months following PBS injection. In the low dose group, A-wave implicit times were not significantly different between AAV- and PBS-injected eyes to 6-months post-injection in *Cdhr1*^{-/-} mice ($P=0.43$; $n=22$) (Figure 6.15).

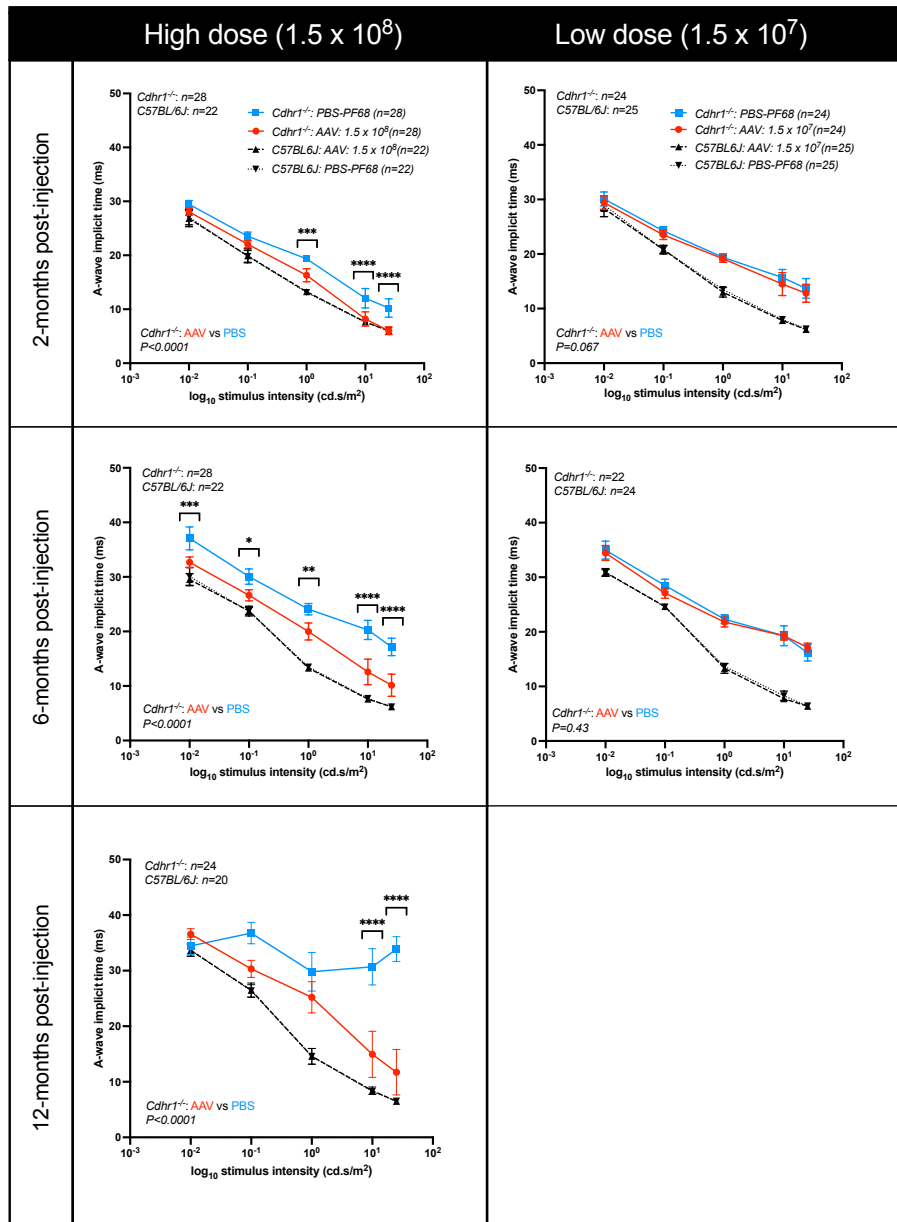


Figure 6.13 Dark-adapted A-wave implicit times following subretinal injection of AAV8.GRK1.CDHR1.pa to 12 months post-injection

Data are plotted from both AAV- and PBS-injected *Cdhr1*^{-/-} and *C57BL/6J* mice, at the five highest light intensities on the dark-adapted luminance series. In the high dose group, AAV-injected *Cdhr1*^{-/-} eyes demonstrated shorter A-wave implicit times compared to PBS-injected eyes ($P < 0.0001$ at all timepoints). This effect was sustained to 12-months. A benefit was not seen in the low dose group. In *C57BL/6J* mice, A-wave implicit times were no different in AAV- and PBS-injected eyes in either dosing group. Plots represent mean + 95% C.I. Shapiro-Wilk indicated parametric datasets; P values are derived from two-way ANOVA for effect of treatment with Šídák's multiple comparison test.

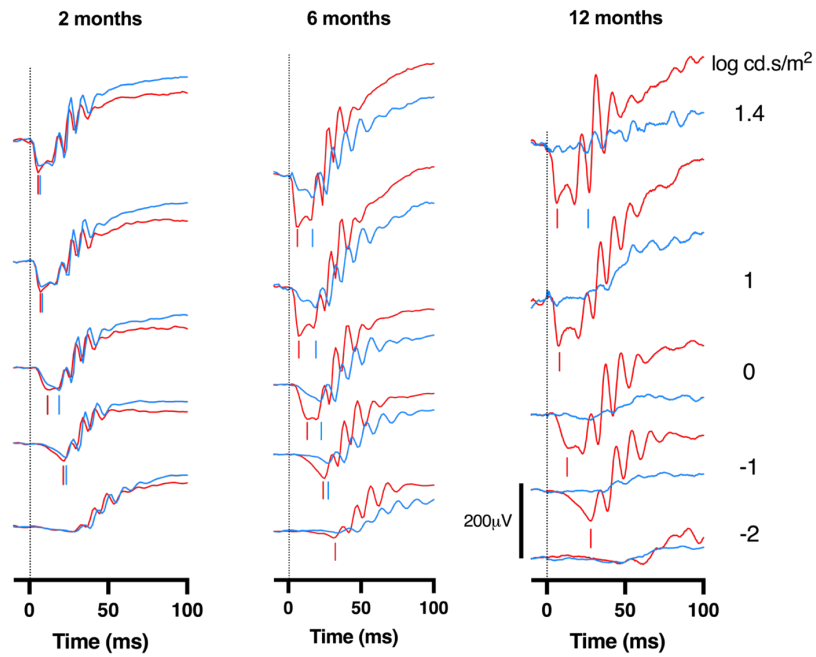


Figure 6.14 Raw dark-adapted ERG traces illustrating A-wave implicit times after high dose AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice

Longitudinal ERG traces are presented for the same *Cdhr1*^{-/-} mouse over 12 months. Vertical bars mark A-wave peaks for AAV-(red) and PBS-(blue) injected eyes. Note that at 2-months post-injection, A-wave implicit times are shorter in AAV-injected eyes, a difference that increases at the 6-month timepoint. At 12-months, A-waves are not clearly discernible in all PBS-injected eyes.

6.4.7.2 *Light-adapted single-flash A-wave implicit times*

Sub-retinal injection of AAV8.GRK1.CDHR1.pA at a dose of 1.5×10^8 shortened A-wave implicit times on light-adapted, single flash ERG versus PBS-injected control eyes at 2-months post-injection ($P=0.03$; $n=28$) in *Cdhr1*^{-/-} mice (Figure 6.17 & 6.18). The difference in light-adapted A-wave implicit times between treated and control eyes increased further at 6-months ($P<0.0001$; $n=28$) and 12-months ($P<0.0001$; $n=24$). The rescue effect was highly significant at two light intensities at the latest timepoint, although was more prominent at 10cd.s/m² where implicit times at 12-months were not significantly different from AAV-

injected *C57BL/6J* eyes ($P=0.23$; two-way ANOVA for the effect of treatment with Šídák's multiple comparison test for all statistics presented in this section).

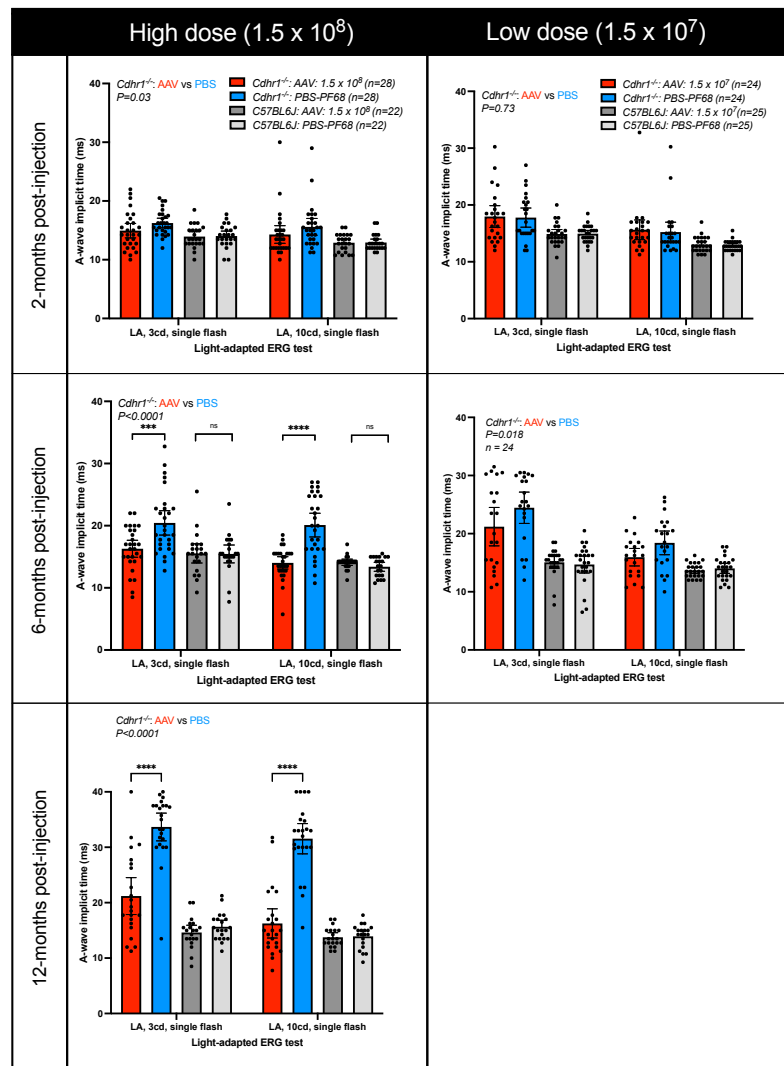


Figure 6.15 Cone photoreceptor response kinetics following subretinal injection of AAV8.GRK1.CDHR1.pA to 12 months post-injection

High-dose AAV- shortens A-wave implicit times on light-adapted single flash ERG in *Cdhr1*^{-/-} mice from 2-months post-injection ($P=0.03$) with increasing effect at later timepoints ($P<0.0001$). AAV-injected responses in *Cdhr1*^{-/-} eyes were no different to those in *C57BL/6J* at 6-months ($P=0.58$), although differed at 12-months ($P<0.0001$). In the low-dose group, responses were shorter in AAV-injected eyes at 6-months ($P=0.018$), but not at 2-months post-injection ($P=0.73$). AAV-injection did not affect A-wave implicit times in *C57BL/6J* mice at any dose or timepoint. Plots represent mean + 95% C.I. Shapiro-Wilk indicated parametric datasets; P values are derived from two-way ANOVA for the effect of treatment with Šídák's multiple comparison test.

In the low dose group, A-wave implicit times on light-adapted single flash ERG were no different in AAV and PBS-injected eyes at 2 months ($P=0.73$, $n=24$). However, A-wave implicit times were shorter in AAV-injected eyes at 6 months post-injection overall ($P=0.018$; $n=22$) (Figure 6.17), although were not significant for individual light intensities.

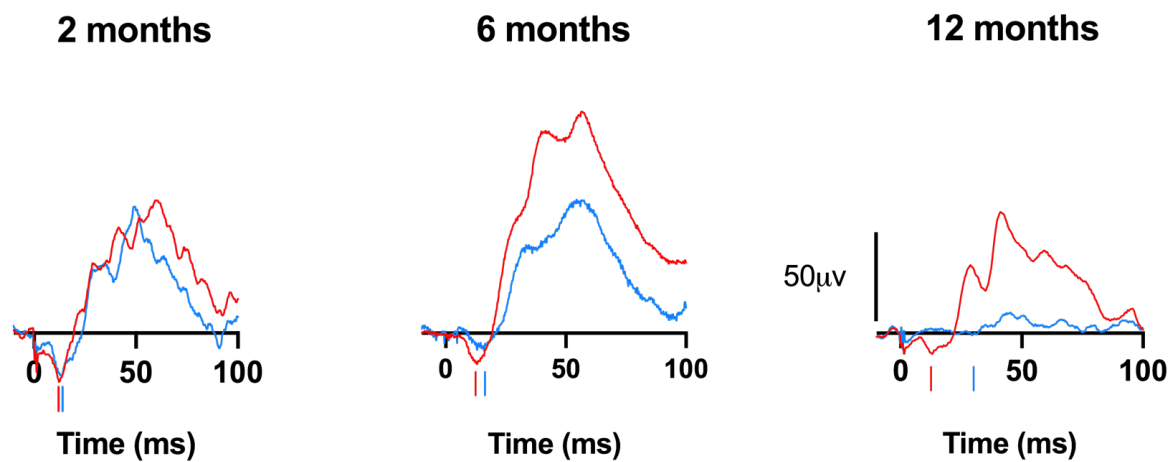


Figure 6.16 Raw light-adapted ERG traces illustrating A-wave implicit times after high dose AAV8.GRK1.CDHR1.pA in *Cdhr1*^{-/-} mice

Longitudinal ERG traces are presented for the same *Cdhr1*^{-/-} mouse over 12 months. Increases in response amplitudes at 6 months, consistent with natural history data (Chapter 3). Vertical bars indicate A-wave peaks for AAV-(red) and PBS-(blue) injected eyes. At 2-months post-injection, A-wave implicit times are shorter in AAV-injected eyes, a difference that increases at the 6-month timepoint. At 12-months, A-waves are not clearly discernible in all PBS-injected eyes.

6.4.8 Electrophysiology excludes cone and rod dysfunction in *C57BL/6J* mice to 12 months post-injection of AAV8.GRK1.CDHR1.pA

6.4.8.1 Dark-adapted ERG

In the high-dose group, there was no difference in A-wave amplitudes ($P=0.052$; $n=19$) and B-wave amplitudes ($P=0.55$) in *C57BL/6J* mice 12-months post-

injection, with no statistical difference between amplitudes at any light intensity on multiple comparison testing. The two groups were well-matched at all earlier timepoints (Figure 6.17). Similarly, in the low dose group, there was no difference between AAV- and PBS-injected eyes in A- ($P=0.49$; $n=24$) or B-wave ($P=0.95$) amplitudes at 6-month post-injection (Figure 6.18), the latest timepoint tested. A-wave implicit times, were no different between AAV- and PBS-injected eyes to 6-months (low dose) and 12-months post-injection (high dose) in *C57BL/6J* mice on dark-adapted ERG ($P>0.99$ at all light intensities) (Figure 6.13).

6.4.8.2 *Light-adapted ERG*

There was no difference in light-adapted single-flash and flicker response amplitudes between high dose AAV- and PBS-injected eyes to 12-months post injection in *C57BL/6J* mice ($P>0.99$ overall; $n=19$), with no differences in individual ERG tests of the cone system on multiple comparison testing ($P>0.97$ for all tests) (Figure 6.17). In the low dose cohort, there was no difference in responses between AAV- and PBS-injected eyes to 6-months post-injection ($P=0.59$ overall; $n=24$) (Figure 6.18), with no differences on individual tests ($P>0.95$ for all).

A-wave implicit times on light-adapted, single-flash ERG at both 3 and 10 cd.s/m², were no different between AAV- and PBS-injected eyes in *C57BL/6J* mice in both low- ($P=0.96$) and high-dose ($P=0.26$) groups at 6- and 12-months post-injection, respectively (Figure 6.15).

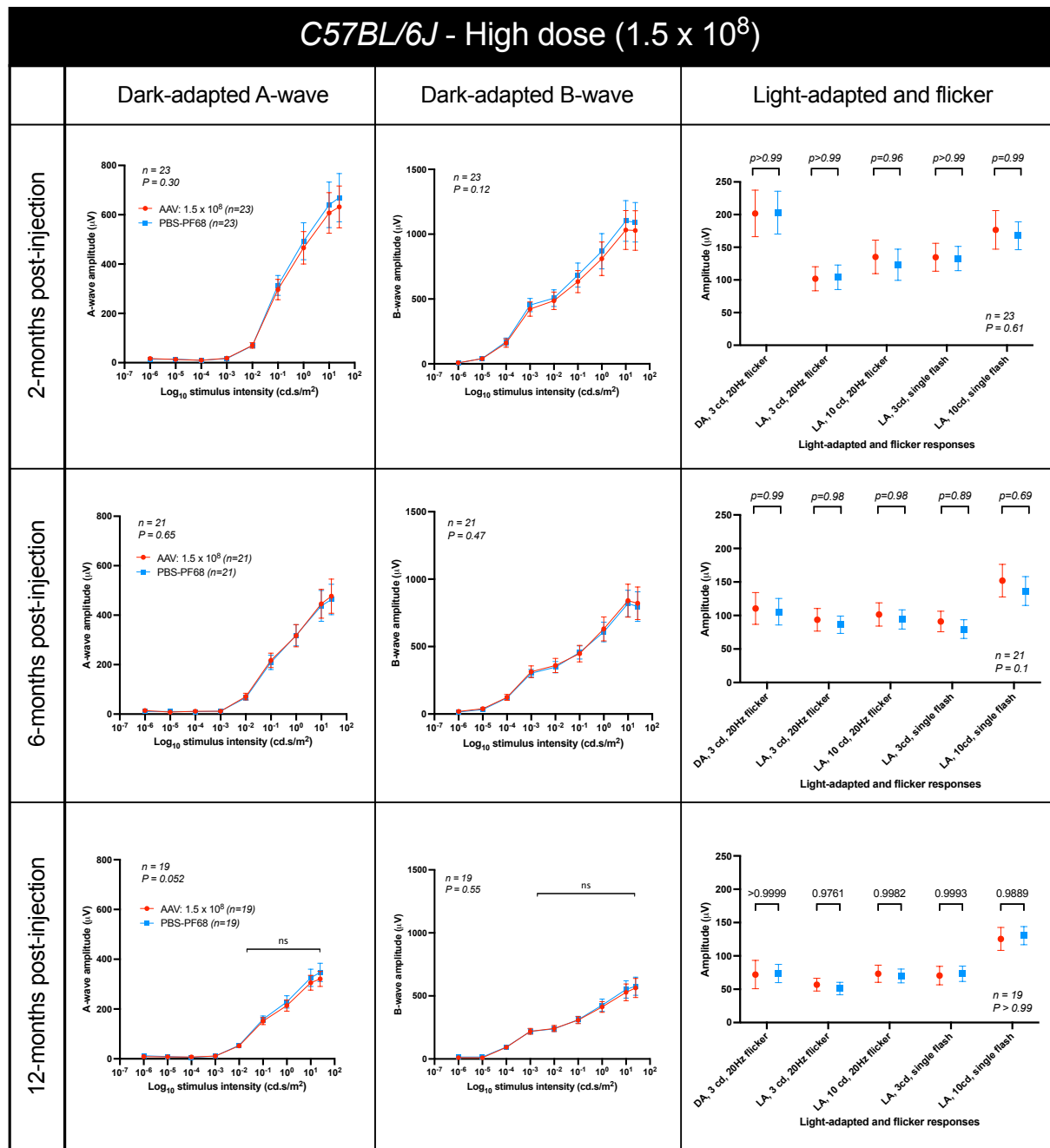


Figure 6.17 Dark- and light-adapted ERG following high-dose AAV8.GRK1.CDHR1.pA in C57BL/6J mice to 12 months post-injection

There is no difference in dark- and light-adapted ERG between AAV- and PBS-injected eyes in C57BL/6J mice to 12-months post-injection. Plots represent mean + 95% C.I. Shapiro-Wilk indicated parametric datasets; P values are derived from two-way ANOVA for effect of treatment, stated overall between treatment groups at all light intensities. Šídák's multiple comparison test is used to compare individual light-adapted and flicker tests between treatment groups.

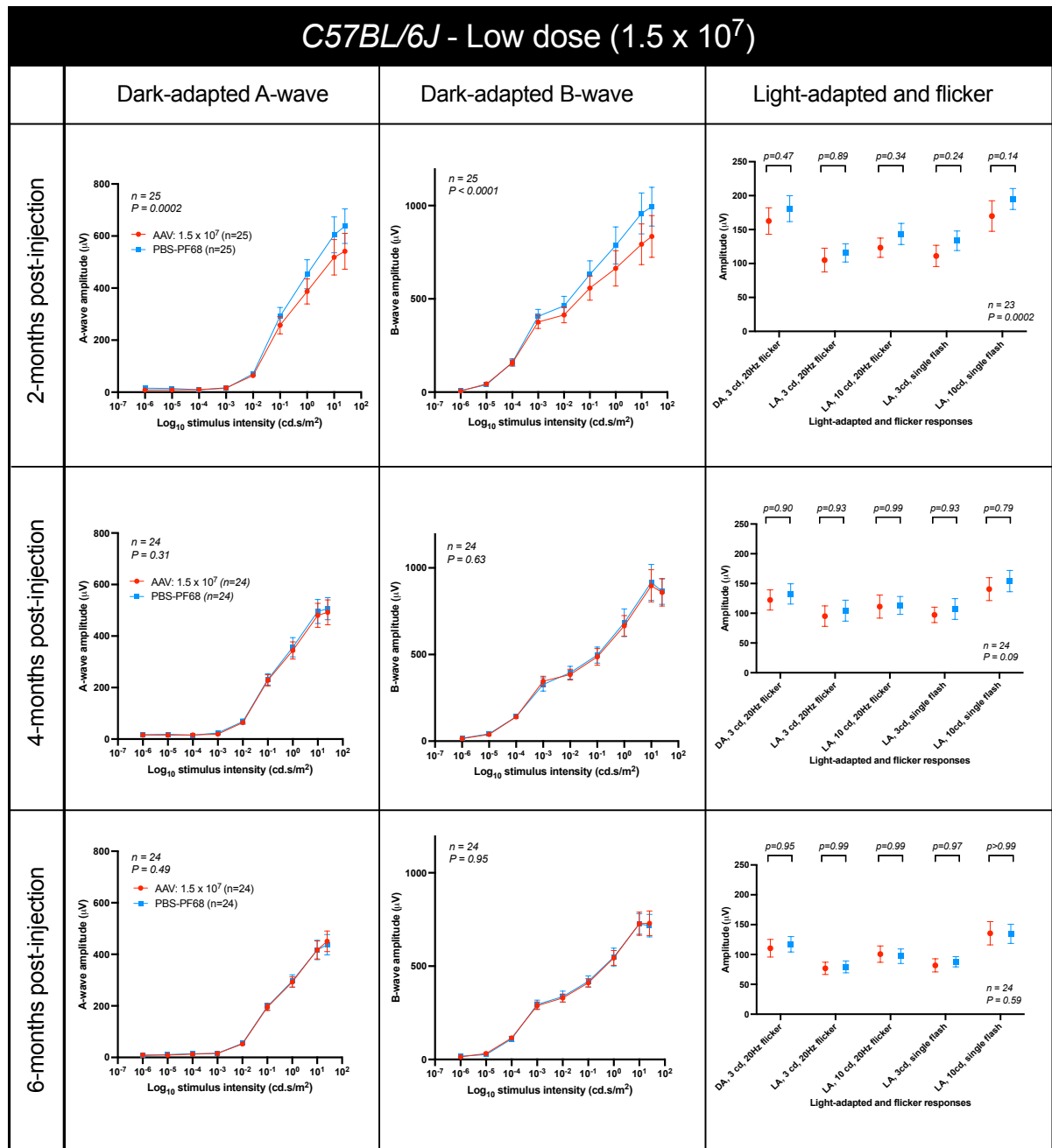


Figure 6.18 Dark- and light-adapted ERG following low-dose AAV8.GRK1.CDHR1.pA in C57BL/6J mice to 6 months post-injection
 Dark- and light-adapted ERG does not detect differences between AAV- and PBS-injected eyes to 6-months post-injection with low-dose AAV8.GRK1.CDHR1.pA. Plots represent mean + 95% C.I. Shapiro-Wilk indicated parametric datasets; P values are derived from two-way ANOVA for effect of treatment with Šídák's multiple comparison test. The recorded difference between groups at 2-months was due to a faulty electrode which were subsequently alternated between AAV- and PBS-injected eyes at later timepoints, and all other ERG assessments.

6.4.9 High-dose AAV8.GRK1.CDHR1.pA rescues photopic and scotopic optomotor reflex responses in *Cdhr1*^{-/-} mice to 21-months post-injection

In Chapter 3, natural history data are presented identifying the extinction of photopic and scotopic OMR in *Cdhr1*^{-/-} mice at 20-24 months of age, whilst responses remained identifiable in age-matched *C57BL/6J* controls. Optomotor responses were preserved in AAV-injected *Cdhr1*^{-/-} eyes ($n=20$) under photopic conditions (1000 lux) at 19-months, and scotopic conditions (0.01 lux) at 21-months post-injection at two speeds of rotation of the OKN drum; $P<0.0001$ for AAV- versus PBS-injected eyes for all speeds and at both levels of illuminance (Figure 6.19).⁹⁶ The best responders demonstrated optomotor responses that were comparable to *C57BL/6J* mice tested under the same conditions (Figure 6.20).

6.4.10 High-dose AAV8.GRK1.CDHR1.pA does not affect photopic and scotopic optomotor reflex responses in *C57BL/6J* mice to 22-months post-injection

Optomotor testing under photopic (20-months) and scotopic (22-months post-injection) conditions did not identify a difference between AAV- and PBS-injected eyes in *C57BL/6J* mice at either 15s or 30s per rotation of the OKN drum (Figure 6.20); $P=0.25$ – photopic; $P=0.12$ – scotopic OMR testing.

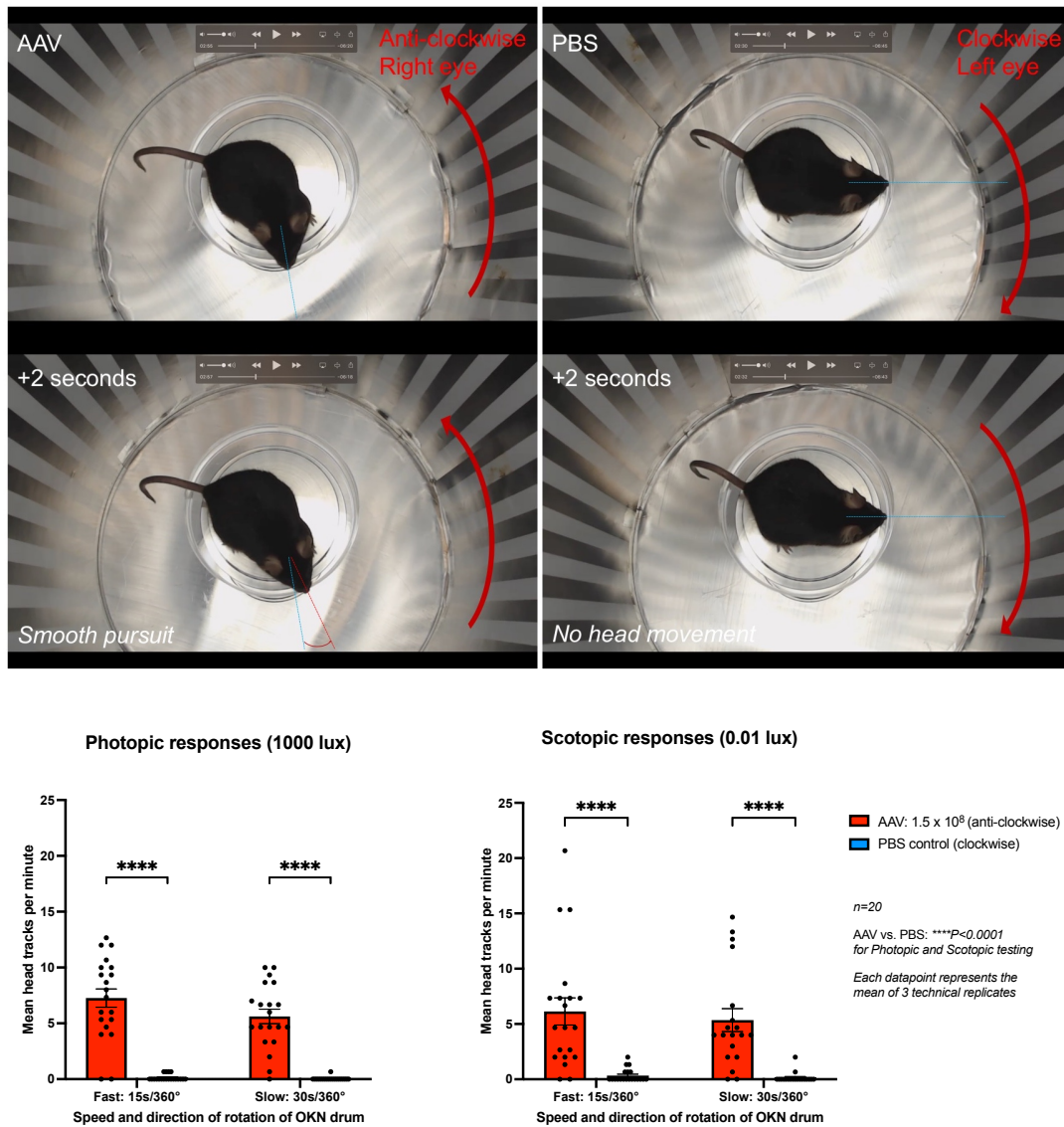


Figure 6.19 Rescue of photopic and scotopic optomotor reflexes to 21-months post-injection of high-dose AAV.GRK1.CDHR1.pA in *Cdhrl*^{-/-} mice *Upper left panel* shows responses from the AAV-injected right eye isolated by anticlockwise rotation of the OKN drum under photopic conditions. A smooth pursuit head movement (optomotor response) in the direction of rotation of the drum is maintained for 2 seconds, indicating visualisation and fixation of the vertical grating. *Upper right panel* shows an absence of optomotor response from the PBS-injected control eye. *Lower left panel* shows significantly greater OMR responses in AAV- versus PBS-injected control eyes at both fast (15s/rotation; $P<0.0001$) and slow (30s/rotation; $P<0.0001$) OKN drum speeds under photopic conditions (1000 lux) at 19-months post-injection, with beneficial effects also demonstrated under scotopic conditions (0.01 lux) at 21-months post-injection at both rotation speeds ($P<0.0001$), shown in the *lower right panel*. Statistical tests presented are two-way ANOVA for the effect of treatment.

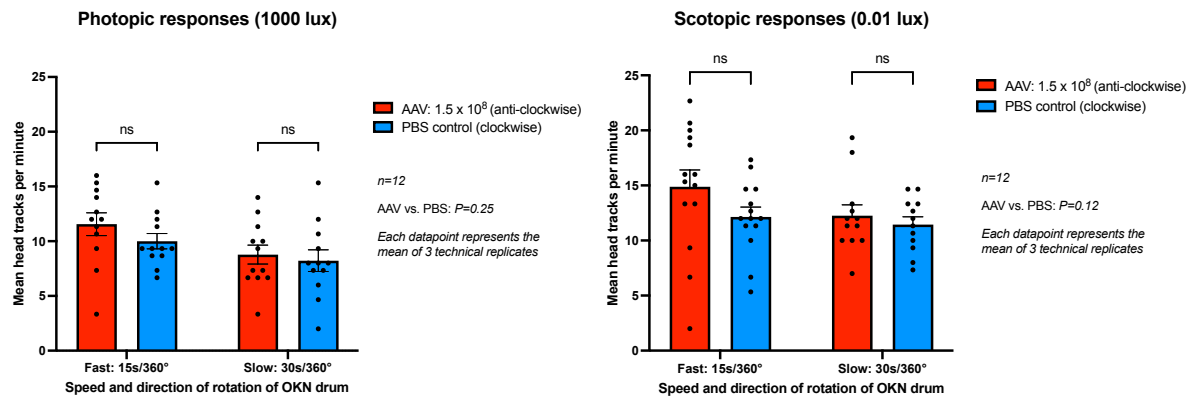


Figure 6.20 Preservation of photopic and scotopic optomotor reflexes to 22-months post-injection following high-dose AAV8.GRK1.CDHR1.pA in *C57BL/6J* mice

Photopic (1000 lux) and scotopic (0.01 lux) optomotor responses were no different between AAV- and PBS-injected eyes in *C57BL/6J* mice at 20- and 22-months post-injection, respectively ($P=0.25$ for photopic, $P=0.12$ for scotopic testing on two-way ANOVA for the effect of treatment). Moreover, no differences were observed between groups at two speeds of rotation of the OKN drum at either level of illuminance, at a fixed spatial frequency of 0.1 cycles per degree. Plots present mean of three technical replicates. Error bars represent SEM.

6.4.11 Correlation of structural and functional rescue following *CDHR1* gene therapy

In *Cdhr1*^{-/-} mice, AAV-injection modified the relationship between structural and functional measures seen in PBS-injected and untreated control eyes, further supporting a slowing of photoreceptor degeneration ($R^2>0.98$ for all groups, Figure 6.21).

In *C57BL/6J* mice, high dose AAV8.GRK1.CDHR1.pA injection did not affect the structure-function correlation observed in PBS-injected and untreated control eyes, further supporting other observations of safety of *CDHR1* gene therapy.

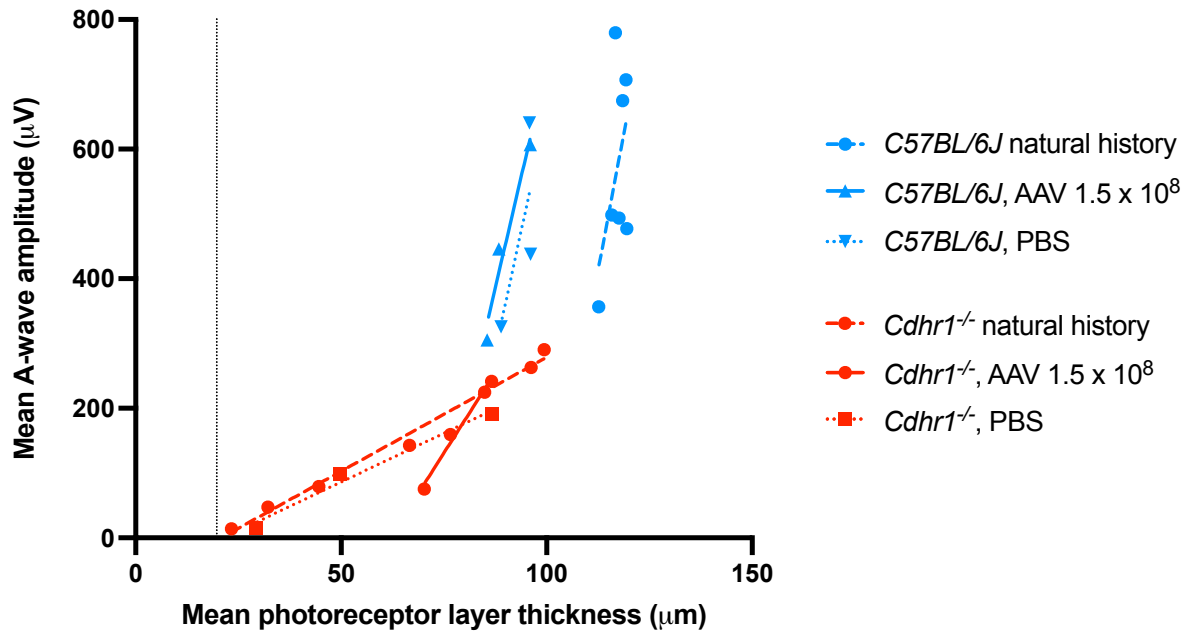


Figure 6.21 Structure-function correlation in *Cdhr1*^{-/-} and *C57BL/6J* mice following *CDHR1* gene therapy

Plots represent mean A-wave amplitudes on dark-adapted, single-flash ERG at 10 cd.s/m² taken at 2-, 6- and 12-months post-injection and mean superior photoreceptor layer thickness measurements on OCT imaging at 1-, 6- and 12-months post-injection. Simple linear regression lines are plotted for natural history data (dashed lines, Chapter 3), AAV-injected eyes (solid lines), and PBS-injected eyes (dotted lines). In *C57BL/6J* mice, all three groups demonstrate a similar structure-function relationship. In *Cdhr1*^{-/-} mice, structural and functional degeneration is slowed in AAV-injected eyes compared to natural history data and PBS-injected eyes, as exemplified by differences in the slopes. The dashed vertical line represents the floor of retinal thickness measurements.

6.4.12 Matched rod and cone photoreceptor rescue following *CDHR1* gene therapy

Functional rescue of rod and cone photoreceptor responses were well matched in AAV-injected eyes relative to age- and treatment-matched *C57BL/6J* mice (Figure 6.22), differences also apparent in PBS-injected eyes.

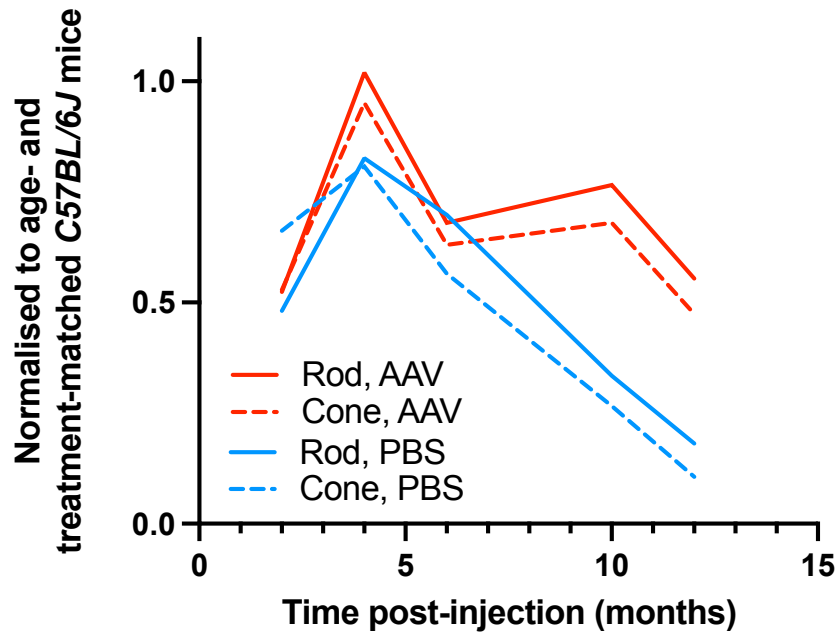


Figure 6.22 Functional rod and cone photoreceptor rescue in *Cdhr1*^{-/-} mice relative to *C57BL/6J* following high dose AAV8.GRK1.CDHR1.pA

Rescue of rod and cone responses are well matched in *Cdhr1*^{-/-} mice to 12-months post-injection of high-dose AAV8.GRK1.CDHR1.pA. Note that normalised cone responses also fall below those of rods in PBS-injected *Cdhr1*^{-/-} eyes relative to *C57BL/6J*. All responses are normalised to age-matched and treatment-matched *C57BL/6J* mice. Peak normalised responses at 5 months of age are consistent with *Cdhr1*^{-/-} natural history data (Chapter 3). Cone responses are derived from light-adapted flicker responses at 10 cd.s/m² and rod responses from dark-adapted single-flash B-wave responses at 0.01 cd.s/m².

6.5 Discussion

CDHR1-associated retinal degeneration is an as yet untreatable cause of blindness. Following validation of the *Cdhr1*^{-/-} mouse model (Chapter 3), development and *in vivo* validation of two vector constructs expressing the *CDHR1* cDNA (Chapter 4), and identification of the optimum therapeutic dose of the lead vector construct to within 0.5 log units (Chapter 5), data presented in this chapter demonstrate proof-of-principle of the safety and efficacy of the first therapy for

this disease in a relevant pre-clinical model. A discussion on the translational relevance of these data towards phase 1 clinical trial is presented in Chapter 8, following the presentation of data relating to the human phenotypes in Chapter 7.

6.5.1 Safety of *CDHR1* gene therapy

Dark- and light-adapted ERG did not detect a difference in A- and B-wave response amplitudes (Figures 6.17 & 6.18), or A-wave implicit times (Figures 6.13 & 6.15) following sub-retinal injection with AAV8.GRK1.CDHR1.pA in *C57BL/6J* mice at either high- or low-dose compared with PBS-injected control eyes. Moreover, behavioural testing at 20-22-months post-injection did not detect a difference in photopic and scotopic optomotor responses between AAV- and PBS-injected eyes in *C57BL/6J* mice.

Consistent with these functional data, longitudinal OCT imaging did not detect a difference in overall photoreceptor layer thickness measurements between AAV- and PBS-injected eyes to 18-months (high dose) and 6-months post-injection (low dose). However, a difference was detected on multiple comparison testing of paired superior retinal locations at 18-months post-injection ($P=0.02$), although the effect size was small. Large numbers of *C57BL/6J* mice ($n=48$), and a long duration of follow-up (6-months for low dose, and 22-months for high-dose cohorts) were chosen in the experimental design to ensure sufficient statistical power and follow-up to detect small toxic effects of CDHR1 overexpression on retinal structural and

function. The supplementation of therapeutic doses of *CDHR1* gene therapy in an individual with two wildtype *CDHR1* alleles is not a clinically relevant scenario, and diminishes the significance of the observed mild superior retinal thinning in *C57BL/6J* mice at the 18-month timepoint. This follow-up is longer than is typically presented for safety data in pre-clinical studies of retinal gene therapy^{104, 199} and should be sufficient to support regulatory approval.

As discussed in Chapter 5, *CDHR1* is expressed at the base of the outer segment, and is thus trafficked across the connecting cilium with other outer segment proteins. Although CDHR1 is not directly involved in protein trafficking across the cilium,⁴⁷ overexpression of an outer segment protein may overload ciliary transport mechanisms in rod and cone photoreceptors, impairing the transport of key OS proteins, such as opsins (rhodopsin, S- and M-cone opsins etc.) or other proteins critical for phototransduction (CNG channels, PDE etc.). Delivery of any dose of AAV8.GRK1.CDHR1.pA will lead to a relative overexpression of CDHR1 in *C57BL/6J* mice. However, the data presented in this chapter support the safety of CDHR1 overexpression following sub-retinal delivery of AAV8.GRK1.CDHR1.pA at a dose of 1.5×10^8 vg and 1.5×10^7 vg on both rod and cone photoreceptors of *C57BL/6J* mice. Moreover, a dominant negative effect of human CDHR1 following vector transduction on native murine *Cdhr1*, or immune responses to the AAV-encoded human CDHR1 orthologue are made less likely by the safety data presented in this Chapter. The translational relevance of these

safety data to gene therapy for human *CDHR1*-associated late-onset macular dystrophy caused by hypomorphic variants are discussed further in Chapter 8.

6.5.2 Efficacy of *CDHR1* gene therapy

A single sub-retinal injection of 1.5×10^8 vg of AAV.GRK1.CDHR1.pA delivered at 3-4 weeks of age preserved rod- and cone-photoreceptor response amplitudes, reduced rod- and cone photoreceptor response implicit times, slowed photoreceptor cell death, preserved outer retinal structures (e.g. ELM and EZ on OCT imaging) and regenerated full-length, morphologically normal photoreceptor outer segments at 22-months post-injection in AAV-injected *Cdhr1*^{-/-} eyes. A clear beneficial effect on visual behaviour through photopic and scotopic optomotor testing was demonstrated in AAV-injected eyes at 19-21 months post-injection. The treatment benefit persisted to at least 21-months post-injection, the most durable response following AAV-gene therapy demonstrated in any pre-clinical murine model of retinal degeneration to date.

PBS-injected *Cdhr1*^{-/-} control eyes exhibited severe outer retinal degeneration without identifiable OS, and with minimal or absent functional responses at the equivalent timepoints. Evidence of a true therapeutic effect was further supported by the dose-dependent and location-dependent nature of the observed therapeutic effects (i.e. using the inferior retina as an internal control) and incremental therapeutic effects on both longitudinal structural and functional analyses, with

a greater therapeutic effect size demonstrated across four timepoints on OCT imaging and six timepoints on dark- and light-adapted ERG.

CDHR1 functions in the development of nascent photoreceptor outer segment discs⁵² and the higher order organisation of the outer segment.⁵³ It is logical that restoration of the CDHR1 transgene to rod and cone photoreceptors may result in progressive regeneration of photoreceptor outer segments as demonstrated on OCT imaging and confirmed on electron microscopy in this study. Moreover, this beneficial morphological correction appears to begin as early as 1-month, increases to 12-months, and persists to at least 21-months post-injection. Late OS regeneration (i.e. seen at 6-months onwards) may reflect the time required for photoreceptor transduction, a process which has been shown to further increase between 6- and 12-months post-injection in the murine retina.¹⁷⁴ Moreover, the process of outer segment renewal occurs at a rate of approximately 2.3µm per day with the outer segment measuring approximately ~20-25µm in *C57BL/6J* mice.²⁰⁰

However, the potential for OS lengthening in any individual *Cdhr1*^{-/-} photoreceptor may be dependent on the OS length of surrounding photoreceptors (i.e. dependent on uniformity of transduction within the retina in space and time). Indeed, on OCT imaging, OS regeneration was identified within contiguous retinal regions at 6-months post-injection which further lengthened and broadened in radial extent to 12-months post-injection (Figure 6.7). Successful transduction of

photoreceptors within a region may be necessary to observe OS regeneration on OCT imaging, following which adjacent transduced retina may regenerate their OS. This hypothesis may explain the increasing radial extent of OS regeneration at the 12- versus 6-month timepoint following AAV injection (Figure 6.7).

An alternative explanation is that non-transduced photoreceptors interfere with normalisation of retinal structures seen on OCT imaging, as supported by continued retinal thinning in the superior retina over 18-months, albeit at a much slower rate than seen in PBS-injected eyes. Ultrastructural analysis at 21-months post-injection confirms the regeneration of full-length, aligned and organised OS in treated *Cdhr1*^{-/-} photoreceptors, by which time non-transduced photoreceptors could be assumed to have undergone cell death. Assuming all adequately transduced photoreceptors were preserved to 18-months post-injection, transduction efficiency based on OCT imaging data could be estimated at approximately 75%, based on data from Figure 6.2. This observation may suggest that further dose refinement (i.e. <0.5 log units) might further enhance the beneficial structural, functional and behavioural treatment effects observed.

Cone-rod dystrophies are typified by a generalised reduction in the intensity of the ellipsoid zone.²⁰¹ The increasing reflectivity of the EZ following AAV delivery in *Cdhr1*^{-/-} mice appears to parallel OS regeneration seen on OCT imaging: increasing at later timepoints, in contiguous superior retinal regions, and

continues to progress to 12-months post-injection. Moreover, ultrastructural analysis at 21-months post-injection identified mitochondria inner segment of AAV-treated eyes that appeared indistinguishable from those observed in wildtype mice. Current evidence suggests that the hyperreflective EZ identified on OCT imaging is formed through the alignment of mitochondrial rich regions within photoreceptor inner segments.¹¹³ The finding of broadening of, and an increase in the reflectivity of the EZ suggests reorganisation and realignment of photoreceptor inner and outer segments. Furthermore, increased reflectivity of the EZ appears to occur in retinal locations in which OS regeneration is marked (Figure 6.7). Increased numbers of mitochondria within the inner segments in these locations may be required to support an increase in cellular metabolism of rod and cone photoreceptors following OS regeneration following gene therapy.

Previously, increased production of glial fibrillary acidic protein was demonstrated in *Cdhr1*^{-/-} mice, suggesting activation of Müller-glia as likely non-specific finding in retinal degeneration.⁴⁷ Persistence of the ELM, formed from the footplates of Müller-glia, suggests preservation of these cells which may also be a non-specific effect of slowing of photoreceptor degeneration. However, formal histological confirmation is required, comparing Müller-glia activation in AAV-treated eyes. The absence of RPE atrophy demonstrated strongly in AAV-treated retinal locations is likely to be a non-specific effect of the prevention of photoreceptor cell death.

Outer segment regeneration is likely to form a key mechanism by which CDHR1 gene therapy rescues rod and cone photoreceptor function. Shortened, misaligned and dysmorphic outer segments were previously demonstrated in *Cdhr1*^{-/-} mice at 1-month of age, associated with a ~50% reduction in rhodopsin⁴⁷; both findings may explain the severe functional deficits at this timepoint, outlined in Chapter 3, which appear out of proportion to photoreceptor layer thickness measurements (~80% of age-matched wildtype mice at 1-month). Moreover, shortened photoreceptor OS have been associated with retinal dysfunction in various forms of retinal degeneration that affect the cilium, such as *RPGR*²⁰², *PROM1*^{54, 203, 204} and *PRPH2*.²⁰⁵⁻²⁰⁷ Data presented in this chapter validate the *in vivo* quantification of OS length on OCT imaging as a biomarker for OS regeneration, through confirmation on ultrastructural analysis. OS lengthening and regeneration may represent a valuable outcome measure for molecular therapies aimed at restoring function of genes involved in OS disc morphogenesis (*CDHR1*, *PRPH2*, *PROM1*), and indeed genes critical for normal function of the connecting cilium (e.g. *CEP290*) in pre-clinical and clinical studies. Moreover, quantification of rhodopsin and S- and M-opsins within the outer segments of AAV- versus PBS-injected *Cdhr1*^{-/-} eyes may further clarify the mechanism of functional rescue.⁴⁷

The slowing of photoreceptor cell death may provide a second mechanism to explain functional rescue – increased response amplitudes and reduced A-wave implicit times of *Cdhr1*^{-/-} eyes following AAV-injection. However, at 1-month post-

injection, OCT imaging identified early OS regeneration without a slowing of photoreceptor cell death at that timepoint. This suggests that early OS regeneration may explain shortened A-wave implicit times on both dark- and light-adapted ERG in AAV-injected *Cdhr1*^{-/-} eyes at 2-months post-injection in the high-dose group. Electron microscopy undertaken at 2-months post-injection may confirm this. Rescue of B-wave implicit times was evident from 4-months injection in the high-dose AAV group. This likely represents an effect of reduced photoreceptor cell death in AAV-treated eyes, rather than any direct beneficial effect on ON-bipolar cells. The rescue effect on B-wave function was incremental at successive timepoints.

A limitation of the mouse model in the context of an experimental design involving superior sub-retinal injections is that cones are asymmetrically distributed in the murine retina as outlined in Chapter 1, with M-cones more numerous in the dorsal (superior) retina and S-cones in the ventral (inferior) retina forming an opposing, overlapping gradient. Thus, any rescue effect of CDHR1 gene therapy may preferentially act on M-cones. Coloured stimuli were not used in ERG testing protocols to determine whether the rescue effect on light-adapted and flicker responses segregated with cone populations. However, the finding of cone-rescue on light-adapted ERG and photopic optomotor testing were apparent despite the assumption that the majority of S-cones in the ventral retina were untransduced following intervention. In humans, CDHR1 is expressed equally in S-, L- and M

cones within the macula, where subretinal AAV8.CDHR1 would be delivered.¹⁷⁹ Overall, it is unlikely that the dorsal-ventral gradient of S- and M-cones in the murine retina would adversely affect the translational impact of these data.

Optomotor behavioural responses support paired-ERG data in demonstrating a functional rescue effect of *CDHR1* gene therapy in *Cdhr1*^{-/-} and safety in *C57BL/6J* mice. OMR behavioural testing is able to separate responses from left and right eyes based on the direction of rotation of the OKN drum in murine subjects without ocular occlusion,⁹⁶ head fixation or anaesthesia, whilst remaining comparable psychometrically to optokinetic testing.²⁰⁸ Some controversy exists as to whether scotopic optomotor responses exist in murine subjects.^{94, 95} Wildtype mice were found to maintain 41% of response levels at 0.005 lux compared to 30 lux, although *Cnga3*^{-/-} mice lacking cones performed better under scotopic conditions.⁹⁴ Functional testing using OMR is limited by between subject variability, and the impact of other sensory distractors (scent, noise etc.). Although variations exist in OMR between murine models, in this study assessment of paired eyes reduces the significance of variability between genotypes and subjects. Moreover, the outcomes of OMR testing reflect those seen on dark- and light-adapted ERG in both strains. The demonstration of a behavioural rescue effect, as shown in pre-clinical studies of *RPE65* gene therapy,¹³² is likely to be of value in regulatory approval since it substantiates a clinically significant therapeutic benefit on visual function from *CDHR1* gene therapy.

6.6 Conclusions

The data presented in this chapter demonstrate proof-of-principle of the first therapy for *CDHR1*-associated retinal degeneration, and indeed of any disease phenotype caused by dysfunction of a cadherin *in vivo*. AAV8.GRK1.CDHR1.pA delivered by sub-retinal injection at a dose of 1.5×10^8 vg preserved rod- and cone-photoreceptor response amplitudes and reduced light- and dark-adapted photoreceptor response implicit times on electroretinography to at least 12-months post-injection in *Cdhr1*^{-/-} mice when compared to PBS-injected control eyes. A slowing of photoreceptor cell death, complete regeneration of rod photoreceptor outer segment length, alignment and morphology on electron microscopy, increased reflectivity of the ellipsoid zone and preservation of the ELM on OCT imaging were seen only in AAV-injected eyes, with the treatment effect sustained to at least 21-months post-injection. A clear benefit of high-dose AAV8.GRK1.CDHR1.pA on visual behaviour was demonstrated on photopic and scotopic optomotor testing, whilst responses were absent in PBS-injected eyes at 19-21 months post-injection – the most durable therapeutic response of any pre-clinical murine study of AAV retinal gene therapy. Longitudinal structural, functional and behavioural testing, using identical experimental protocols, did not detect any significant toxic effects of AAV8.GRK1.CDHR1.pA at 1.5×10^8 vg in *C57BL/6J* mice to 22-months post-injection when compared to PBS-injected control eyes, except for very mild thinning on OCT imaging in matched superior retinal locations measured at 18-months post-injection. A dose of 1.5×10^7 vg was

neither significantly therapeutic in *Cdhr1*^{-/-} mice, nor toxic in *C57BL/6J* mice to 6-months post-injection.

7. Human *CDHR1*-associated retinal degeneration

7.1 Introduction

Since the original descriptions of *CDHR1*-associated retinal degeneration in 2010,^{49, 50} clinical reports have been published describing sequence variants and phenotypes of individuals with biallelic *CDHR1* variants (section 1.3). However, these data are insufficient to support the commercialisation, funding, ethical approval and study design of a phase 1 clinical trial of *CDHR1* gene therapy.

Although cone-rod and macular dystrophy appear to be the most common disorders associated with biallelic *CDHR1* variants, these phenotypes have not been described in detail. For example, age of symptom onset, nature of presenting symptoms, natural history of visual decline, disease symmetry, multimodal retinal imaging findings and morphology have not been described systematically according to retinal phenotype. Similarly, genotype-phenotype associations in *CDHR1*-associated retinal degeneration have not been clearly defined for most variants. These data are required to support onward clinical trials for case identification, and verification, particularly for variants of uncertain significance (VUS) as defined by American College of Medical Genetics (ACMG)⁸⁹ criteria. Natural history data in particular are essential for the ethical approval of clinical trials and to determine the optimum timing for intervention. Moreover, detailed

phenotyping may identify clinically relevant, objective, and reproducible endpoints for clinical trial design.

The aim of this Chapter was to characterise *CDHR1*-associated retinal degeneration in order to support the translation of *CDHR1* gene therapy into clinical trial by addressing the shortfalls in clinical data highlighted above. Commercialisation of clinical trial programmes are dependent on reliable epidemiological data, essential to support the viability of clinical programmes due to the high cost of manufacturing GMP grade AAV vector. A further key objective was to calculate the global genetic prevalence of *CDHR1*-associated retinal degeneration, using a methodology reported elsewhere²³ using clinically validated pathological sequence variants identified in this work. Furthermore, an additional aim was to estimate the genetic prevalence of *CDHR1*-associated retinal degeneration by phenotype, following characterisation of genotype-phenotype associations. This would aid the design of subsequent clinical trials.

7.2 Aims

The aim of this chapter was to further characterise the clinical phenotypes of human *CDHR1*-associated retinal degeneration. The specific objectives were:

- To identify and characterise the clinical phenotype(s) associated with biallelic variants in *CDHR1*.

- To expand the description of pathogenic sequence variants in *CDHR1*.
- To describe genotype-phenotype associations.
- To characterise the natural history of disease by phenotype
- To quantify retinal thinning in *CDHR1*-associated cone-rod and macular dystrophy by age and location within the macula
- To model the rate of growth of macular atrophy in *CDHR1*-associated macular dystrophy
- To estimate the global genetic prevalence of *CDHR1*-associated retinal degeneration, and to determine the prevalence according to phenotype.

7.3 Materials and methods

7.3.1 International study collaboration

Experts in retinal genetics (ophthalmologists or geneticists) were contacted in order to identify individuals with retinal degeneration attributed to biallelic variants in *CDHR1* who had undergone clinical assessment in their centre. Collaborators were invited to contribute anonymised, retrospective clinical data that had been attained in the routine clinical care of the identified patients. For case inclusion, the results of molecular genetic testing and review of clinical information and multimodal retinal imaging studies was considered with supporting data. Centralised ethical approval for an anonymised patient database to support this study was granted by the Oxford University Hospitals institutional

review board, and further supported by ethical approvals at contributing centres as appropriate.

7.3.2 Clinical phenotyping

A standardised spreadsheet was used to collect relevant clinical information: demographics, family history, symptoms, age of onset and diagnosis, longitudinal visual acuity data across all visits at that centre, refraction, ocular and systemic co-morbidities, the method and results of molecular genetic testing (including variants in other genes), segregation analysis, and available investigations (colour fundus photographs, OCT, FAF, visual field testing, ERG, colour vision testing etc.). Digital copies of original investigations were sent to the investigators, including digital retinal images with Heidelberg E2E files for primary analysis of OCT and FAF images, where available.

7.3.3 Molecular genetic analysis

Molecular genetic confirmation of *CDHR1* variants was used to validate case inclusion. The nature of molecular genetic testing varied between subjects; index cases typically underwent whole genome (WGS), whole exome (WES) or targeted next-generation sequencing (NGS) with variants confirmed by Sanger sequencing. The targeted next-generation sequencing panels, and read depths are likely to have varied between contributing centres. Clinical data were reviewed with the results of molecular genetic testing to validate the identified variants. For novel

variants, ACMG⁸⁹ recommended *in silico* prediction tools for splicing (ASSP,¹⁴⁴ MaxENT¹⁴⁵ NNSPLICE, Human Splice Finder) and missense variants (SIFT, Polyphen, Provean, Mutation Taster) were examined together with evolutionary sequence conservation, segregation data and allele frequency (GnomAD).

7.3.4 Clinical phenotypes

A description of clinical features was undertaken according to genotype: individuals with two null variants *in trans* (stop gained, start lost, frameshift or those affecting the canonical splice donor (GT) or acceptor (AG) sites) were designated as CRD for the purposes of phenotypic description. Individuals with degeneration of the macula in the absence of biallelic null variants were designated as MD. In this group, peripheral retinal function on full-field ERG was found to be in the normal range or reduced compared to age-matched controls.⁵⁵ However, those with extinguished ERGs were excluded from this group. RP was defined as rod-predominant degeneration with relative preservation of the fovea in early disease. Atypical phenotypes were those that did not match these designated groups. A description of each phenotype involved the analysis of data relating to the age of onset, symptoms at presentation, best corrected visual acuity (logMAR) and symmetry, refraction (spherical equivalent), ocular and systemic comorbidities, retinal imaging studies (colour images, widefield colour images and FAF, OCT & FAF imaging), and the results of visual field testing and electroretinography where available.

7.3.5 Natural history of *CDHR1*-associated retinal degeneration

Visual acuity is a key primary outcome measure in clinical trials. Retrospective visual acuity data were collected for each patient; those specified in Snellen or Decimal format were converted into logMAR for analysis. The definition of severe visual impairment used for the analysis was logMAR <1.0 (Snellen 6/60 equivalent) in the better seeing eye (World Health Organisation (WHO) definition²⁰⁹). Individuals with “counting fingers”, “hand movements” and “perception of light” or worse were designated as logMAR 2.0, 2.5 and 3.0, respectively, which were approximated from previously published studies.²¹⁰

Data for individuals with CRD and MD phenotypes were analysed separately. Raw visual acuity data were compared in both eyes to determine symmetry by phenotype. In order to illustrate the natural history of visual loss by phenotype, the best corrected visual acuity in the better seeing eye was plotted against age at all timepoints for both CRD and MD groups. Kaplan-Meier analysis was used to identify the proportion of individuals with CRD and MD who had severe visual impairment by the WHO definition (i.e. logMAR of less than 1.0 in the better seeing eye) by age for both phenotype groups. Analysis of full-field ERG assessments was undertaken for both CRD and MD groups following classification of scotopic B-wave (rod) responses at 0.01 cd.s/m² and photopic flicker (cone) responses as “normal”, “reduced” or “extinguished” according to the testing laboratory. The age distribution according to ERG category was further compared

using a two-tailed t-test for each phenotype. Visual field and colour vision results were considered too variable for direct quantitative comparison.

7.3.6 Quantitative analysis of multimodal retinal imaging studies in *CDHR1*-associated retinal degeneration

For primary analysis of retinal thickness measurements, 50 Heidelberg E2E files were available for individuals with CRD, and 29 for individuals with MD, enabling a primary quantitative analysis of retinal thickness measurements.

7.3.6.1 *Macular thinning on OCT imaging*

A primary analysis of total retinal thickness measurements (Bruch's membrane to ILM) using Heidelberg Spectralis OCT imaging was undertaken in both CRD and MD, as previously described.²¹¹ Briefly, mean total retinal thickness measurements were recorded from 9 ETDRS subfields: the central subfield (1mm around the fovea), and 8 additional subfields at 1-3 (inner ring) and 3-6mm (outer ring) eccentricity to the fovea divided into four cardinal quadrants (Figure 7.13).²¹¹ Image stacks were individually inspected, automated segmentation lines were corrected where necessary and centration of the ETDRS grid on the fovea was confirmed. Retinal thickness measurements were compared by genotype, age and location within the ETDRS subfields, with reference to published normative data acquired from 34 adult controls using the Heidelberg Spectralis imaging system.²¹¹

7.3.6.2 *Macular atrophy on fundus autofluorescence imaging*

Identifying the rate of progression of macular atrophy provides information regarding the natural history of the disease, and are likely to form outcome measures for clinical trial. Areas of RPE loss within the macula were measured (mm²) manually on 30° SW-AF imaging using the internal Heidelberg Spectralis software in patients with macular dystrophy. Follow-up images were overlaid on the baseline image to correct for differences in focus between sessions. Since baseline images varied in dioptric focus between subjects, the area of loss of RPE was expressed as a percentage of the overall area within the image.

7.3.7 Genetic prevalence of *CDHR1*-associated retinal degeneration

For recessive disorders, disease alleles exist in equilibrium within a population, according to the Hardy-Weinberg principle, where p is the allele frequency (AF) of the wildtype allele and q , the AF of mutant allele:

$$p^2 + 2pq + q^2 = 1$$

7.3.7.1 *Overall genetic prevalence*

The global genetic prevalence of *CDHR1*-associated retinal degeneration was estimated using a methodology used by Hanany *et al.*²³ The population frequency of validated disease-associated variants in *CDHR1* were identified for six

characterised global genetic populations from the Genome Aggregation Database (GnomAD; <https://gnomad.broadinstitute.org>): European (non-Finnish), European (Finnish), South Asian, East Asian, Latino/Admixed American, African/African-American. The overall frequency of individuals with biallelic pathological variants (i.e. affected individuals) and heterozygous individuals (i.e. unaffected carriers) was calculated for each population based on all identified pathological *CDHR1* variants, including clinically verified novel variants from this study. An estimation of the global prevalence of *CDHR1*-associated retinal degeneration was calculated through a multiplication of population frequency with current world population estimates (World Bank; <https://data.worldbank.org>).

7.3.7.2 *Genetic prevalence by disease phenotype*

A further analysis was undertaken by separating known hypomorphic *CDHR1* variants from null variants in order to calculate the frequency of macular dystrophy (biallelic hypomorphic, or a hypomorphic disease-associated variant on at least one allele) and cone-rod dystrophy (biallelic null, or known severe missense variant) for each population, according to an expanded Hardy-Weinberg equation with three alleles equilibrium, where p is the AF of wildtype allele, q – AF of null allele, r – AF of hypomorphic allele:

$$p^2 + 2pq + q^2 + 2qr + r^2 + 2rp = 1$$

Hardy-Weinberg identifier	Alleles	Predicted clinical phenotype
p^2	WT / WT	Normal
$2pq$	WT / null	Carrier: null
q^2	null / null	CRD
$2pr$	WT / hypomorphic	Carrier: hypomorphic
$2qr$	Null / hypomorphic	MD
r^2	Hypomorphic / hypomorphic	MD

Table 7.1 Allele categories and assumed clinical phenotypes for genetic prevalence calculations

Further specific calculations were undertaken for the c.783G>A_{CDHR1} variant.

7.4 Results

7.4.1 International *CDHR1* study collaboration and case inclusion

Seventy-nine retinal specialists or geneticists contributed retrospective, anonymised clinical data for 157 individuals with retinal degeneration attributed to biallelic variants in *CDHR1*. A list of contributors is specified in Appendix A.4, and the global distribution of the contributing centres is illustrated in Figure 7.1. Of 157 individuals with retinal degeneration attributable to biallelic *CDHR1* variants, 7 were excluded due to incomplete genetic data, and 4 excluded due to inadequate clinical data. 146 patients were included in the study.



Figure 7.1 Global distribution of contributors to the *CDHR1*- associated retinal degeneration study

7.4.2 Retinal phenotype in *CDHR1*-associated retinal degeneration

Of 146 reported individuals, sufficient clinical data were available to assign a phenotype to 135 individuals: 100 with cone-rod dystrophy, 32 with macular dystrophy, 2 with a rod-predominant dystrophy, and 1 with an atypical phenotype. 8 individuals had a null variant (frameshift, stop gained, truncating, start lost) *in trans* with a hypomorphic variant. No individuals exhibited clinical syndromes of early-onset retinal dystrophy (i.e. onset before 2 years of age) or Leber Congenital Amaurosis (i.e. congenital nystagmus, oculodigital sign, and amaurosis). There were no cases in which deficits of rod or cone function were present in the absence of retinal degeneration (i.e. CSNB or achromatopsia).

	Cone-rod dystrophy (n=100)	Macular dystrophy (n=32)
Sex	Male - 43 Female - 57	Male - 22 Female - 10
Age of onset	Median – 20 years (n=90) (range: 3 - 45)	Median – 44 years (n=24) (range: 20 - 68)
Symptoms at onset	Night vision problems – 31.5% Blurry central vision – 24.5% Reading difficulties – 10.5% Glare – 9.8% Peripheral field defects – 9.1% Dyschromatopsia – 7% Others – 6.3% Central scotoma – 1.4%	Reading difficulties – 46.7% Night vision problems – 26.7% Blurry central vision – 16.7% Central scotoma – 3.3% Asymptomatic – 6.7%
Refraction Mean S.E. (D)	-3.34D (n=47) (SD: 3.84D)	-1.79D (n=20) (SD: 3.13D)

Table 7.2 Clinical characteristics of 132 individuals with *CDHR1*-associated retinal degeneration

7.4.2.1 *CDHR1*-associated cone-rod dystrophy

There were with 43 males and 57 females in the CRD cohort. The retinal phenotype of *CDHR1*-associated CRD was characterised by the onset of symptoms attributable to either cone dysfunction (colour vision disturbance, glare/photophobia, central visual disturbance, difficulty reading) and/or rod dysfunction (night vision or visual field problems). Glare and peripheral visual field deficits were noted at presentation in CRD patients only. The median age at symptom onset was 20 years. Onset before the age of 10 was reported in 10 cases. Most individuals were myopic with a mean spherical equivalent of -3.34D, and a high degree of refractive symmetry. Figure 7.2 presents a graphical representation of age of onset, primary symptom at presentation, disease symmetry and refraction in CRD versus MD patients.

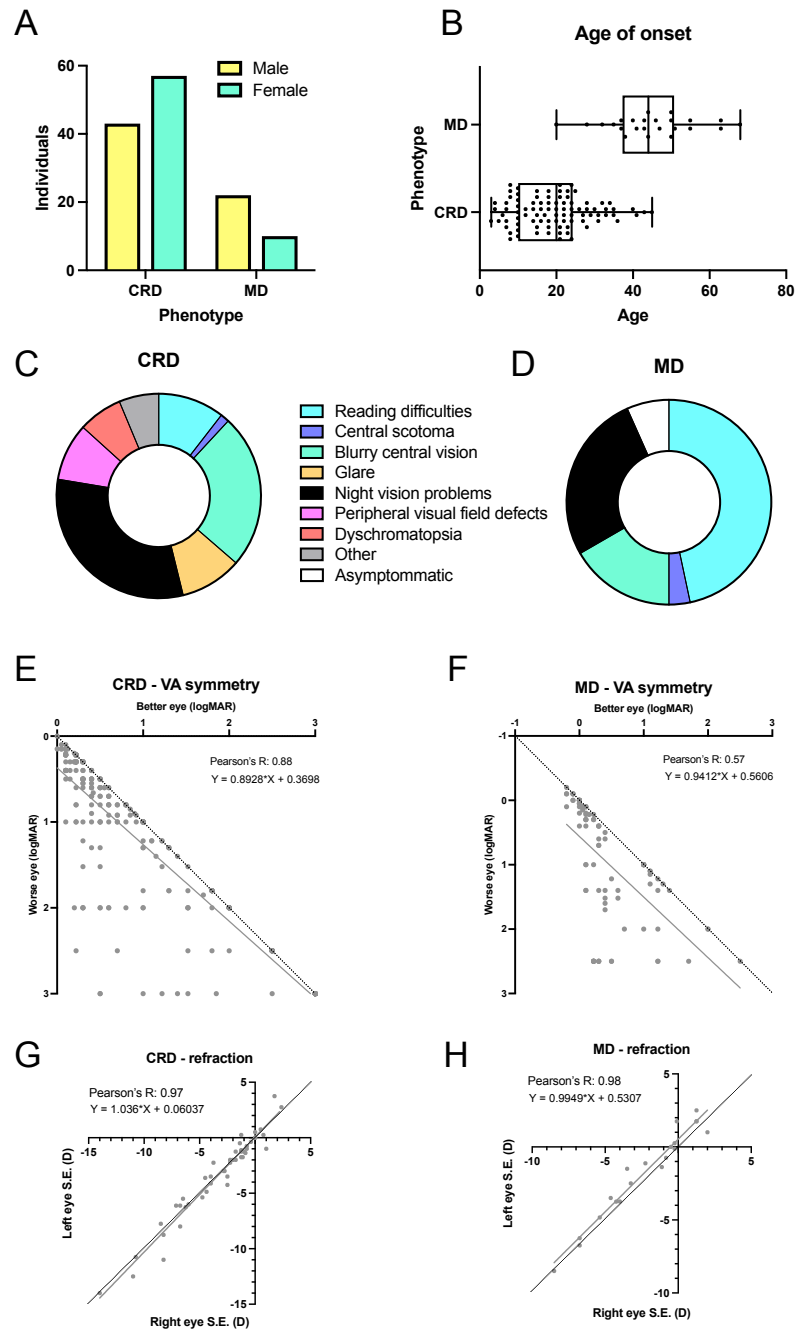


Figure 7.2 Clinical features of *CDHR1*-associated retinal degeneration
 Cone-rod dystrophy patients are described in the left column, and macular dystrophy on the right. Clinical symptoms are presented in the charts at the top; almost half of MD patients present with reading difficulties. Symmetry of VA is greater in CRD patients (Pearson's rank: 0.88; $P < 0.0001$) than MD patients (Pearson's rank: 0.57; $P < 0.0001$) due to late foveal sparing; simple linear regression lines are plotted. Lower plots present refractive data as spherical equivalent (S.E.); most patients in both CRD and MD groups are myopic.

All patients with CRD experienced a progressive decline in visual acuity. The median age of severe visual impairment, defined by the WHO as logMAR <1.0 in the better seeing eye was 40 years of age (95% C.I. 36.2 – 44.5 years) ($n=95$, Figure 7.10). All individuals with CRD were classified as having severe visual impairment by this definition by the age of 65. Colour vision, where tested, was typically limited to identification of the Ishihara test plate by the age of ~20 years.

Fundoscopy or colour imaging

In early disease, fundus examination frequently reveals peripapillary atrophy (Figure 7.8), an abnormal foveal reflex and irregularities of the ILM reflex where present in younger patients (Figure 7.4). Later, foveal atrophy develops with subsequent RPE loss (Figure 7.3 & 7.5). Mid-peripheral retinal thinning may be evident although pigment migration may be absent in early disease. Scalloped areas of RPE loss in the periphery were frequent (Figure 7.8). Waxy disc pallor and generalised arteriolar attenuation may be seen. In late disease, extensive pigment migration was typical with coalescence of scalloped areas of RPE loss (Figure 7.4). Epiretinal membrane and CMO may occur as with RP, although CMO was seen rarely in this cohort.

Fundus autofluorescence imaging

In early disease, an enlargement of the foveal hypoautofluorescent signal was seen (Figure 7.3), which expanded further with disease progression. There was variable

loss of macular pigment. Generalised hyperautofluorescence was identifiable within the macula or around the posterior pole in early disease; where clearly defined, this area of hyperautofluorescence appeared to identify the leading edge of centripetal outer retinal degeneration on OCT imaging and appeared to coincide with the limits of the ELM.

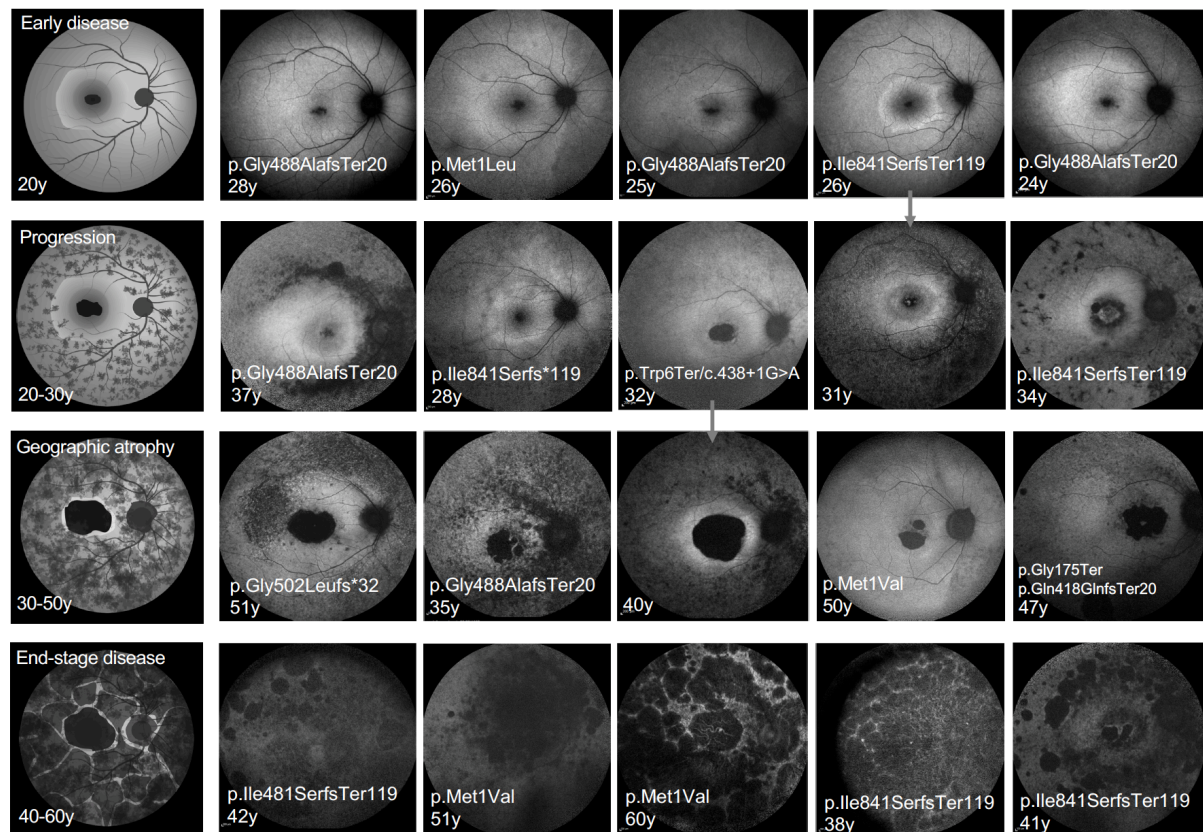


Figure 7.3 Fundus autofluorescence imaging in *CDHR1*-associated cone-rod dystrophy

In early disease (~20 years), an ovoid horizontal hypofluorescent area is seen at the fovea, with variable loss of macular pigment. Surrounding hyperautofluorescence is often present. With increasing age (20-30 years) loss of RPE at the fovea is seen with progressive retinal degeneration in the mid-periphery encroaching on the macula. Geographic atrophy is seen from 30-50 years of age with confluent loss of RPE centred on the fovea. In end-stage disease (40-60 years), scalloped areas of mid-peripheral RPE loss merge with atrophic areas in the macula leaving a reticulated appearance formed from residual RPE.

Patchy hypoautofluorescence developed around the fovea before RPE loss occurred at one or more foci within this area. Concentric enlargement of areas of RPE loss occurred which became confluent with other foci. In end-stage disease, coalescence of macular RPE loss merged with scalloped RPE loss in the mid-periphery to leave a coarse reticulated pattern formed of residual islands of RPE (Figure 7.3).

Optical coherence tomography

Severe, generalised thinning of the outer nuclear layer was seen in early disease with shortening of the layer representing the photoreceptor outer segments. The EZ was absent at the periphery of 30° OCT scans and attenuated centrally with reduced reflectivity compared to age-matched controls.²¹² Deposits of high reflectivity may be seen overlying the RPE. The ELM was preserved at later timepoints compared to the EZ, and extended more peripherally. In the absence of focal atrophy, the EZ was present at the fovea, with an optical gap²¹³ demonstrable in most patients overlying the sub-foveal RPE. With progressive disease, centripetal ONL thinning encroached on the central macula. Poor visual acuity was recorded in some cases despite the absence of RPE atrophy at the fovea. Outer retinal atrophy developed with subsequent RPE death and choroidal hypertransmission. In some cases, the foveal RPE was preserved as an island within a large atrophic area, preserving some recordable visual acuity. In end-stage disease, the outer retina and RPE was lost with choroidal atrophy.

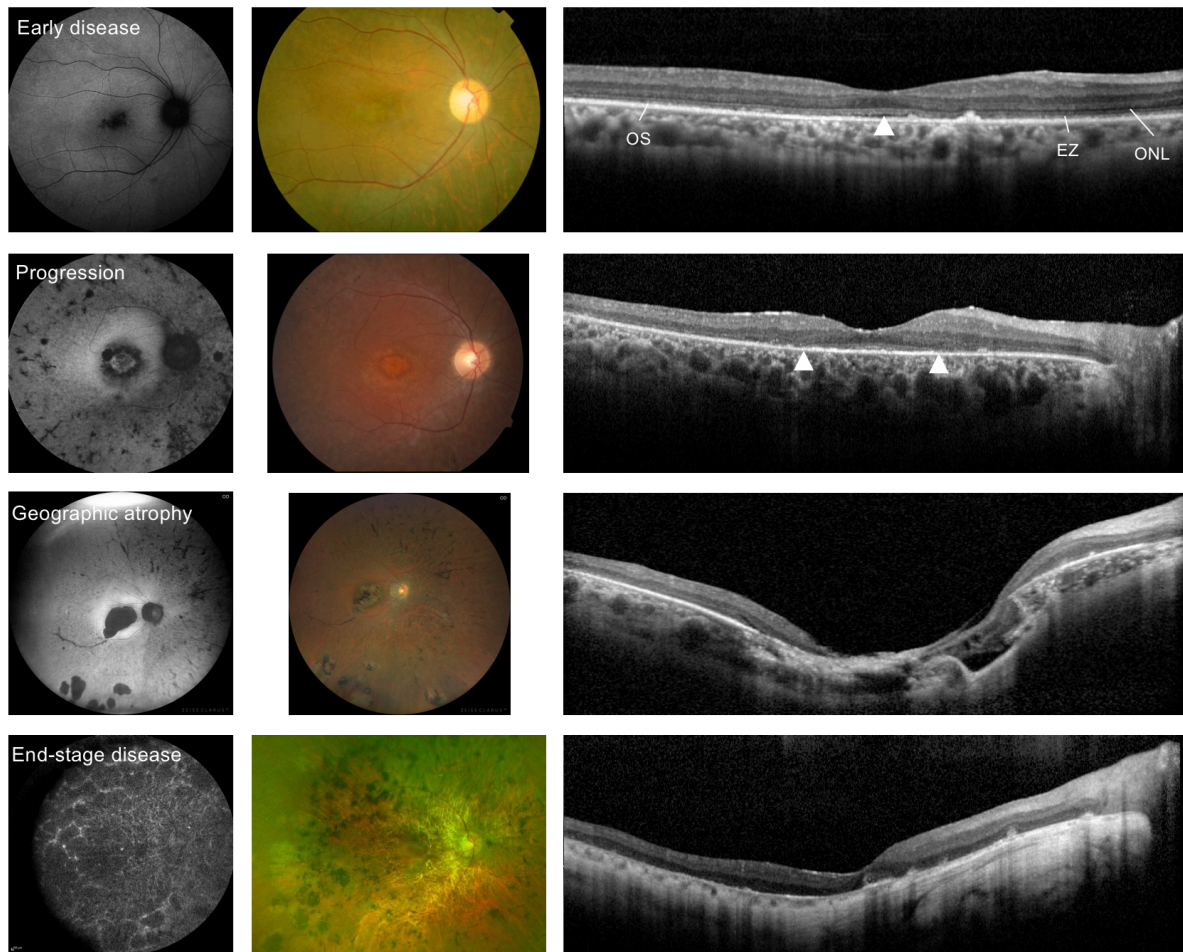


Figure 7.4 OCT imaging characteristics in *CDHR1*-associated cone-rod dystrophy

In early disease, generalised outer nuclear layer (ONL) thinning is seen in association with shortening of the photoreceptor outer segments and reduced reflectivity of the EZ. An optically empty area is present under the EZ at the fovea (arrowhead). With disease progression, the peripheral ONL is lost with focal RPE loss in the parafovea, although the EZ and ELM may be preserved at the fovea (between arrowheads), before foveal involvement/geographic atrophy with choroidal hypertransmission. In late disease, the outer retina and RPE is lost with choroidal atrophy.

In two individuals, a posterior staphyloma developed around the fovea (i.e. type II staphyloma) affecting all retinal layers, RPE and choroid with documented

progression around the age of 30-40 (Figure 7.9). In one individual with CRD, subfoveal elevation of the RPE was seen, not present in affected relatives (Figure 7.9).

ERG

Full-field ERG was characterised by marked rod and cone dysfunction in early disease with macular dysfunction (i.e. severely reduced P50 on pattern ERG). Patients varied in terms of relative severity of loss of rod and cone responses with most exhibiting predominant cone dysfunction, although two individuals exhibited recordable cone but absent rod responses. However, non-recordable full-field ERGs were seen in most patients, including as early as 19-years of age (Figure 7.11). No individuals with CRD, including 7 children, exhibited a normal ERG.

Visual field

Central scotomas with generalised, progressive visual field constriction was seen, the severity of which partly depended on age. Typically, by the fourth decade, kinetic visual field testing identified visual field constriction to $<10^\circ$ with the Ve isopter, although may be near normal in the first two decades. Residual temporal islands of visual field may be retained (Figure 7.5). Scotopic microperimetry demonstrated severe deficits in macular sensitivity (Figure 7.10).

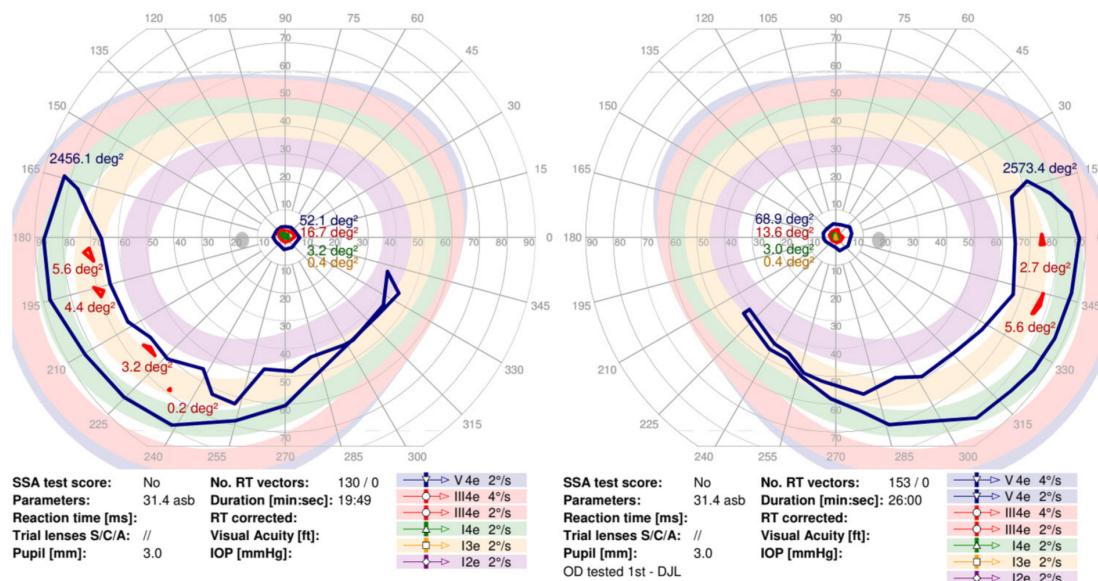


Figure 7.5 Kinetic visual field testing in *CDHR1*-associated cone-rod dystrophy

Severe generalised visual field constriction to $<10^\circ$ with a residual temporal island of vision is demonstrated in this 29-year-old patient homozygous for c.1485+2T>C.

7.4.2.2 *CDHR1*-associated macular dystrophy

In the MD cohort, there were 22 males and 10 females. *CDHR1*-associated macular dystrophy was most commonly characterised by the onset of symptoms relating to macular dysfunction (reading difficulties, central scotoma, blurred central vision) in middle age. The median age at first symptom was 44 years. However, MD was detected incidentally as a finding on clinical examination in some individuals who did not have visual symptoms. In some cases, peripheral retinal degeneration may be present, depending on the specific *CDHR1* variants, age at examination and/or possible disease modifiers (Figure 7.9).

Fundoscopy or colour imaging

In early disease, loss of the foveal reflex may be evident. Yellow flecks are often present in the parafovea which may be diffuse or well defined. These lesions resolve leaving punctate areas of atrophy with new flecks appearing more eccentrically (Figure 7.7). Peripapillary atrophy is present in most cases in early disease, and progresses over time (Figure 7.8). Areas of atrophy develop most commonly within the parafovea, expand and may coalesce with other atrophic areas with total loss of the RPE exposing choroidal vessels. This may preserve the foveal centre in some cases (Figure 7.6 & 7.7). In others, a persistent tongue of retinal tissue may protrude vertically from above or below, or from the papillomacular bundle, preserving visual acuity. Alternatively, an island of foveal tissue may persist before it is lost. In some patients, mid-peripheral retinal degeneration encircling the optic disc may be seen, in addition to scalloped areas of RPE loss in the far periphery (Figure 7.9).

Fundus autofluorescence imaging

In early disease, punctate hyperautofluorescent flecks may be seen in the parafovea, which appear more eccentrically leaving behind hypoautofluorescent areas, which gradually become atrophic. Atrophic areas expand until RPE loss, characterised by total loss of AF signal, develops in the parafovea. The fovea appears to be relatively spared with disease progression until late, when round or ovoid areas of total RPE loss may resemble central areolar choroidal dystrophy

(CACD) which expands concentrically over time. Widefield SW-AF imaging most commonly identifies an unaffected peripheral retina. However, the area of speckled autofluorescent signal may extend beyond the macula as disease progresses. In some cases, speckled autofluorescent signal and scalloped areas of retinal degeneration/RPE loss may be seen in the far periphery (Figure 7.9).

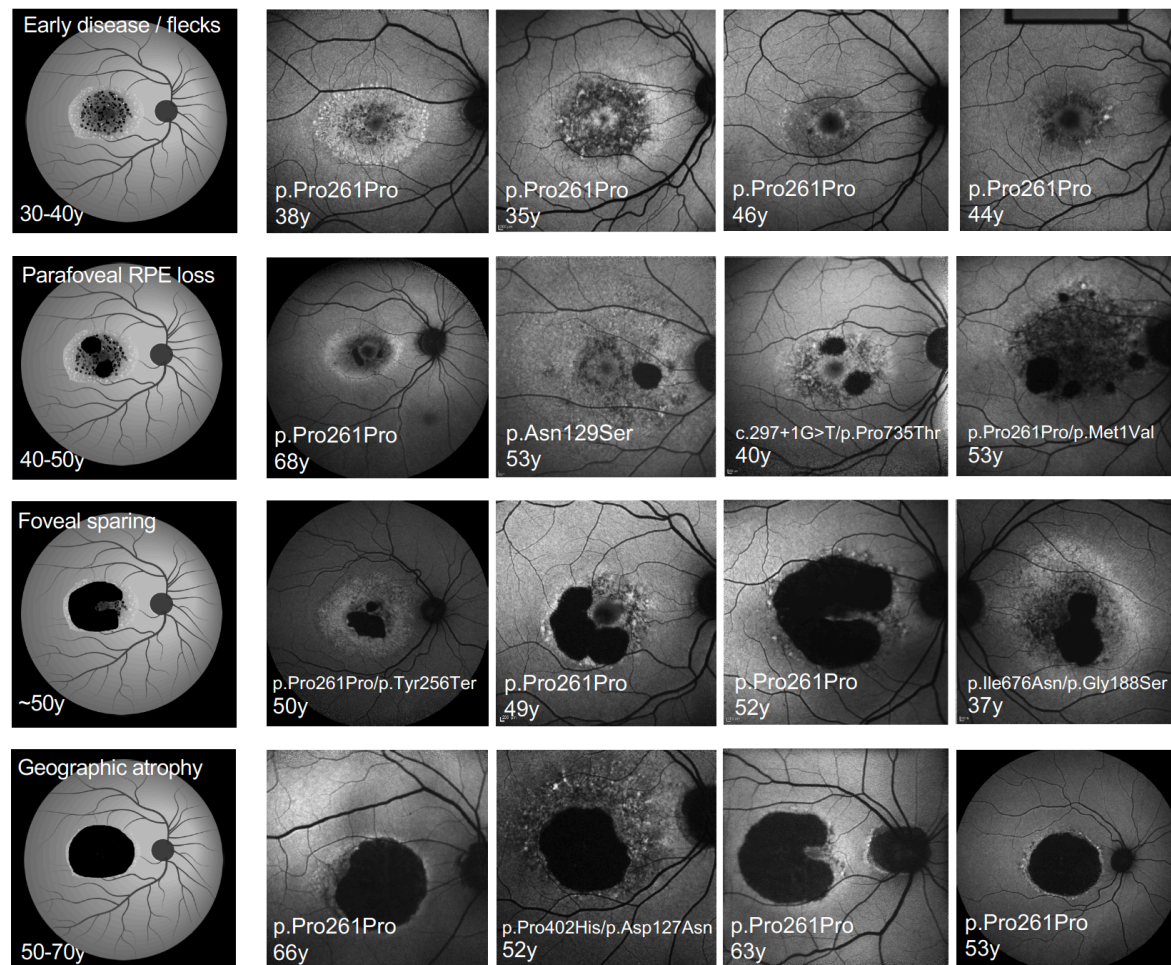


Figure 7.6 Fundus autofluorescence imaging in *CDHR1*-associated macular dystrophy

Punctate hyperautofluorescent flecks centered on the fovea may be seen in early/pre-symptomatic disease (30-40 years), spreading centrifugally leaving speckled hypoautofluorescence at the fovea. Areas of parafoveal RPE atrophy develop (40-50 years), and enlarge with initial foveal sparing in most cases (~50 years), before the fovea is lost and the area of RPE atrophy enlarges to a concentric area centered on the fovea with a hyperautofluorescent rim (50-70 years).

Optical coherence tomography

Yellow flecks appear as hyperreflective foci on OCT imaging between the ELM and RPE, partly obscuring the EZ. In affected parafoveal areas, hyperreflective foci are seen in the ONL which becomes thinner over time, with the EZ and ELM less discernible, and subsequent loss of the RPE. The fovea is spared until late, accounting for the greater degree of visual acuity asymmetry between eyes. In end stage disease, the fovea is lost and complete outer retinal atrophy develops, characterised by RPE loss and choroidal hypertransmission (Figure 7.7). In most cases, the retina beyond the area of atrophy appears normal with preservation of the laminar retina architecture.

ERG & Visual field testing

Individuals with MD exhibited normal or abnormal full-field ERG responses. However, individuals with abnormal ERGs (outside the reference range for the testing laboratory) were significantly older (Figure 7.11) than those with normal ERGs. No individuals within the MD group exhibited extinguished responses. Pattern ERG typically revealed macular dysfunction as a reduced or absent P50 response. Visual field testing typically demonstrated a central scotoma, which became absolute following RPE loss. In patients with peripheral retinal degeneration (Figure 7.9), peripheral retinal function may be affected on visual field and ERG testing. Microperimetry may detect reduced retinal sensitivity in the parafovea (Figure 7.10).

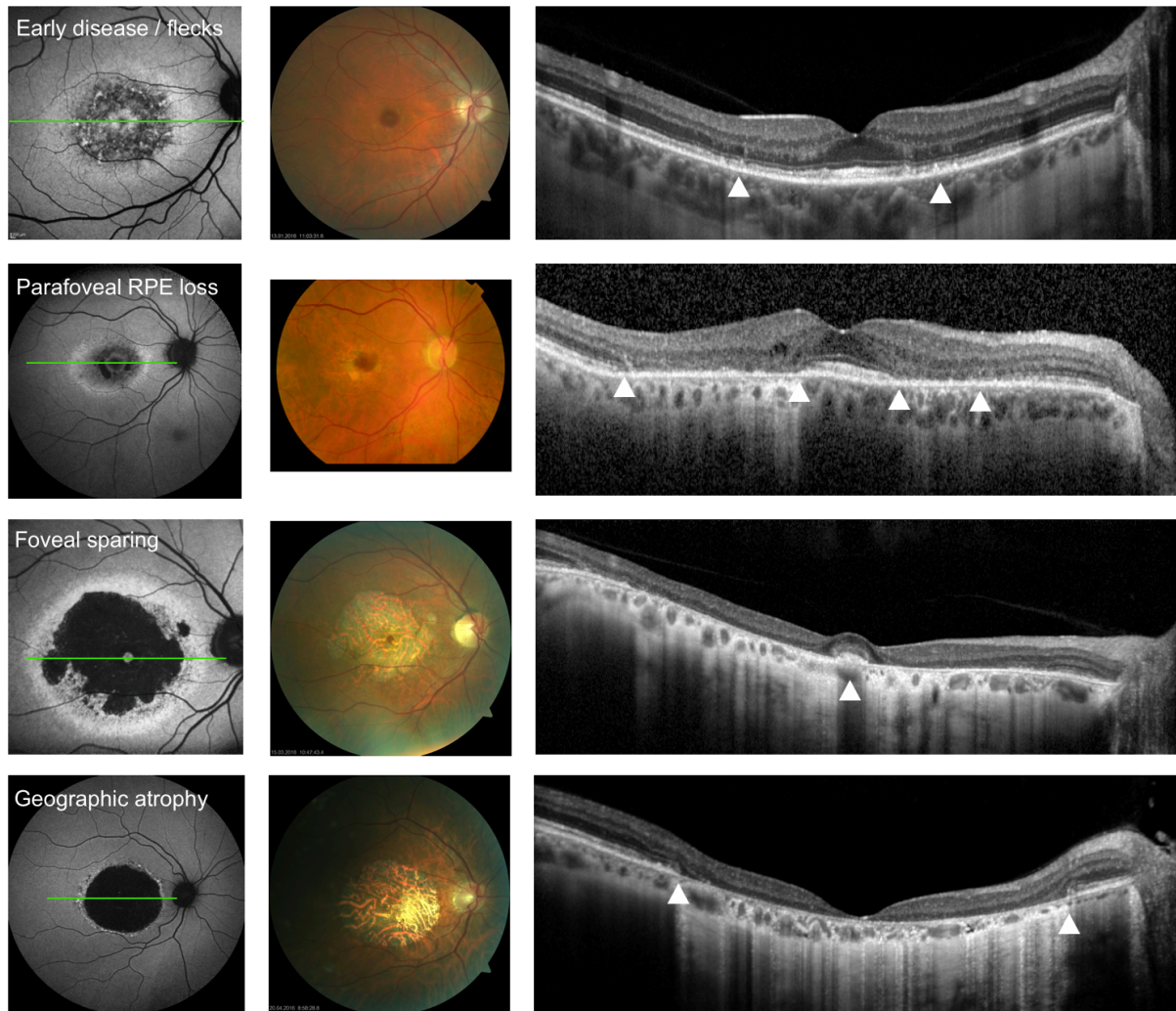


Figure 7.7 OCT imaging characteristics in *CDHR1*-associated macular dystrophy

In early disease, subtle parafoveal flecks may be present on clinical examination and identifiable on OCT imaging as hyperreflective foci in the outer retina (arrowhead). RPE atrophy develops in the parafovea, associated with loss of the overlying ONL (between arrowheads). RPE atrophy progresses with foveal sparing characterised by residual foveal RPE (in this case supporting a visual acuity of logMAR 0.4). Geographic atrophy is characterised by choroidal hypertransmission secondary to loss of the RPE with preservation of the peripheral retina.

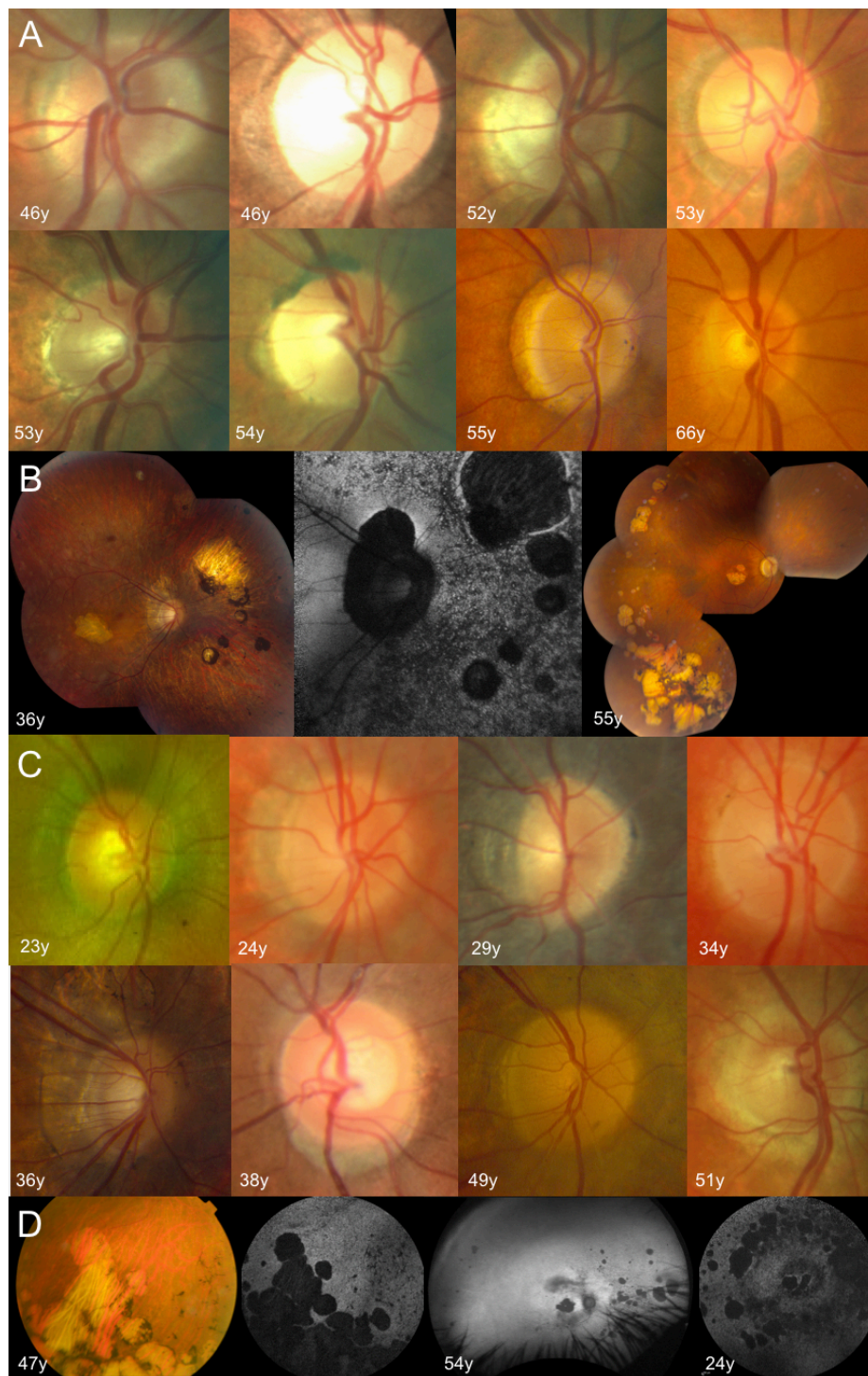


Figure 7.8 Atrophic features in *CDHR1*-associated retinal degeneration
(A) Peripapillary atrophy is evident in *CDHR1*-associated macular dystrophy, which may help to distinguish it from Stargardt's. **(B)** Scalloped peripheral retinal degeneration may be present on clinical examination. **(C)** Peripapillary atrophy in individuals with *CDHR1*-associated cone-rod dystrophy **(D)** Progressive, scalloped peripheral retinal degeneration may become extensive in late disease.

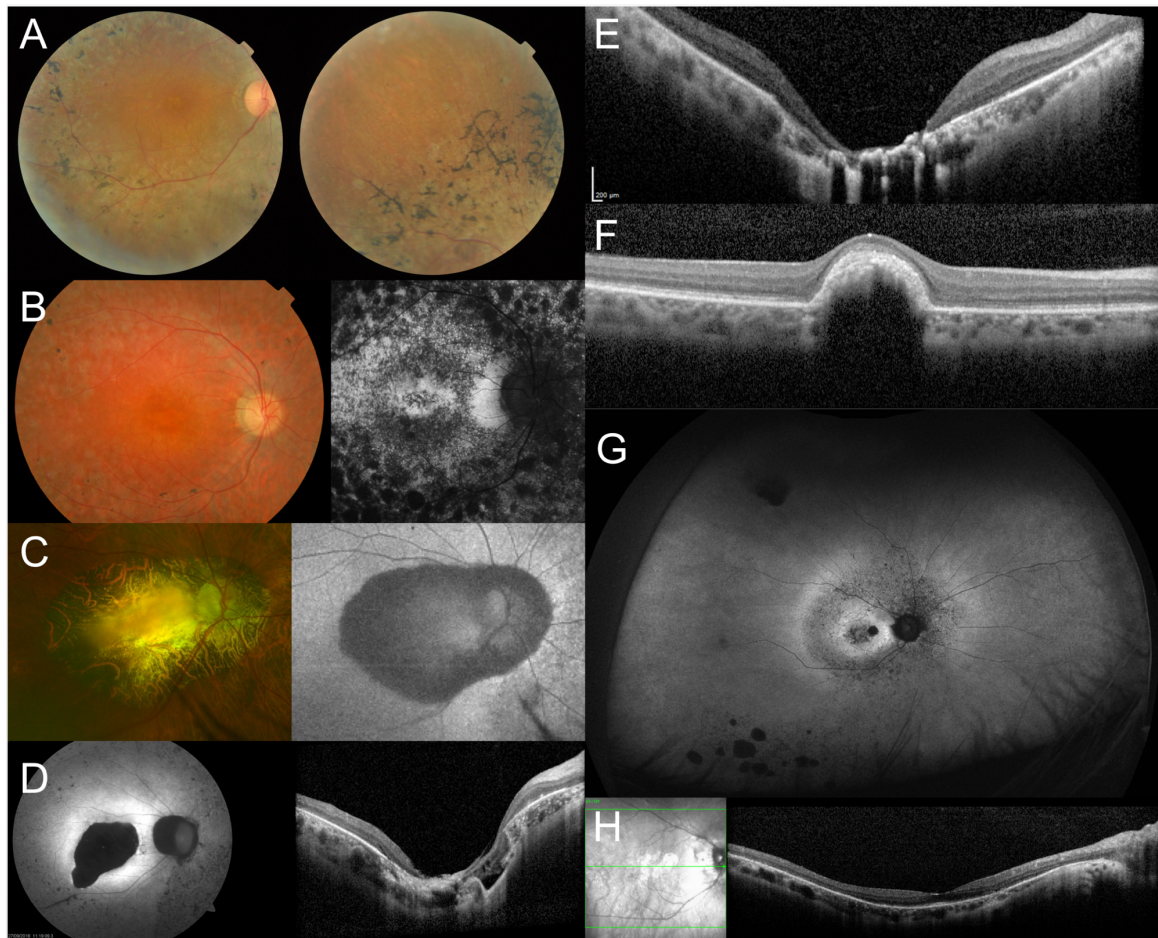


Figure 7.9 Atypical retinal features in *CDHR1*-associated retinal degeneration

Retinitis pigmentosa in a 44-year-old homozygous for p.Val733Phe showing peripheral pigment migration and relative sparing of the fovea (**A**), and a 24-year old homozygous for p.Ile841SerfsTer119 (**B**). (**C**) RPE loss encircling the disc and macula in an 84-year old homozygous for p.Glu201Lys. Unilateral posterior staphylomas are seen in two individuals with CRD: (**D**) a 36-year old homozygous for p.Gly113AlafsTer2 and (**E**) a 41-year old compound heterozygote with documented progression over 10 years on OCT. (**F**) Sub-foveal elevation in a 16-year-old homozygous for p.Gly488AlafsTer20. (**G**) A c.783G>A homozygote with mid-peripheral retinal degeneration on SW-FAF imaging associated with retinal thinning at the limits of the macula OCT (**H**), aged 44.

7.4.4.3 *CDHR1*-associated retinitis pigmentosa

Two individuals exhibited a retinal phenotype of classic rod-cone dystrophy, or RP as previously described,⁵⁸ characterised by mid-peripheral pigment migration and

centripetal outer degeneration without significant macular involvement for the stage of disease (Figure 7.9).

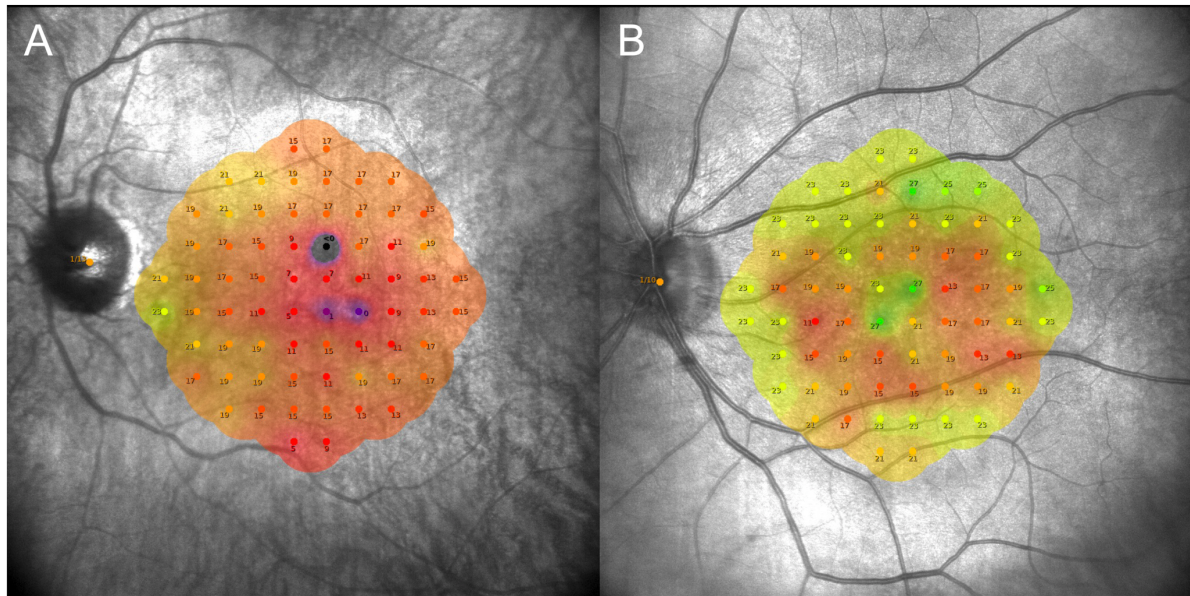


Figure 7.10 Microperimetry in *CDHR1*-associated retinal degeneration
(A) Microperimetry 24-year old individual with CRD (homozygous for p.Gly488AlafsTer20) demonstrates severe generalised reduction in macular sensitivity in early disease **(B)** A 43-year-old individual with MD (c.783G>A homozygote) demonstrates reduction in sensitivity in the parafovea, coinciding with the distribution of outer retinal degeneration on OCT imaging.

7.4.4.4 *Atypical phenotypes*

One individual exhibited a retinal phenotype that was atypical to those described above in the context of biallelic *CDHR1* variants, and in the absence of an alternative genetic or clinical explanation. This 84-year-old individual exhibited bilateral total RPE atrophy involving the macula and peripapillary regions, harbouring homozygous previously described²¹⁴ pathogenic *CDHR1* variant (c.601G>A, p.Glu201Lys; Figure 7.9). OCT imaging showed normal retinal structure beyond the atrophy. However, ERG showed abnormal rod and cone

function with late-onset nyctalopia (in the 8th decade). This case may represent a severe MD phenotype with extensive atrophy resulting from the confluence of peripapillary and macular atrophy. The patient was not symptomatic until 63 years of age, although data at presentation are not available.

7.4.3 Natural history of *CDHR1*-associated retinal degeneration: visual function

7.4.3.1 Visual acuity

Individuals with MD ($n=31$) retained vision in the better seeing eye to a later age when compared to the CRD cohort ($n=95$; $P<0.0001$; Figure 7.11). In CRD, a logMAR BCVA of better than 2.0 (counting fingers) in the better seeing eye was not identified over 55 years of age. Kaplan-Meier survival analysis identified a significant difference between rates of visual decline in CRD and MD groups ($P<0.0001$), with median age of severe visual impairment by WHO criteria of 40 years (95% CI: 36.3 to 44.4 years) in the CRD group and 71 years (95% CI: 50 to 80.5 years) in the MD group. All individuals with CRD were severely visually impaired by the age of retirement, although only 33% of the MD cohort were at the same timepoint.

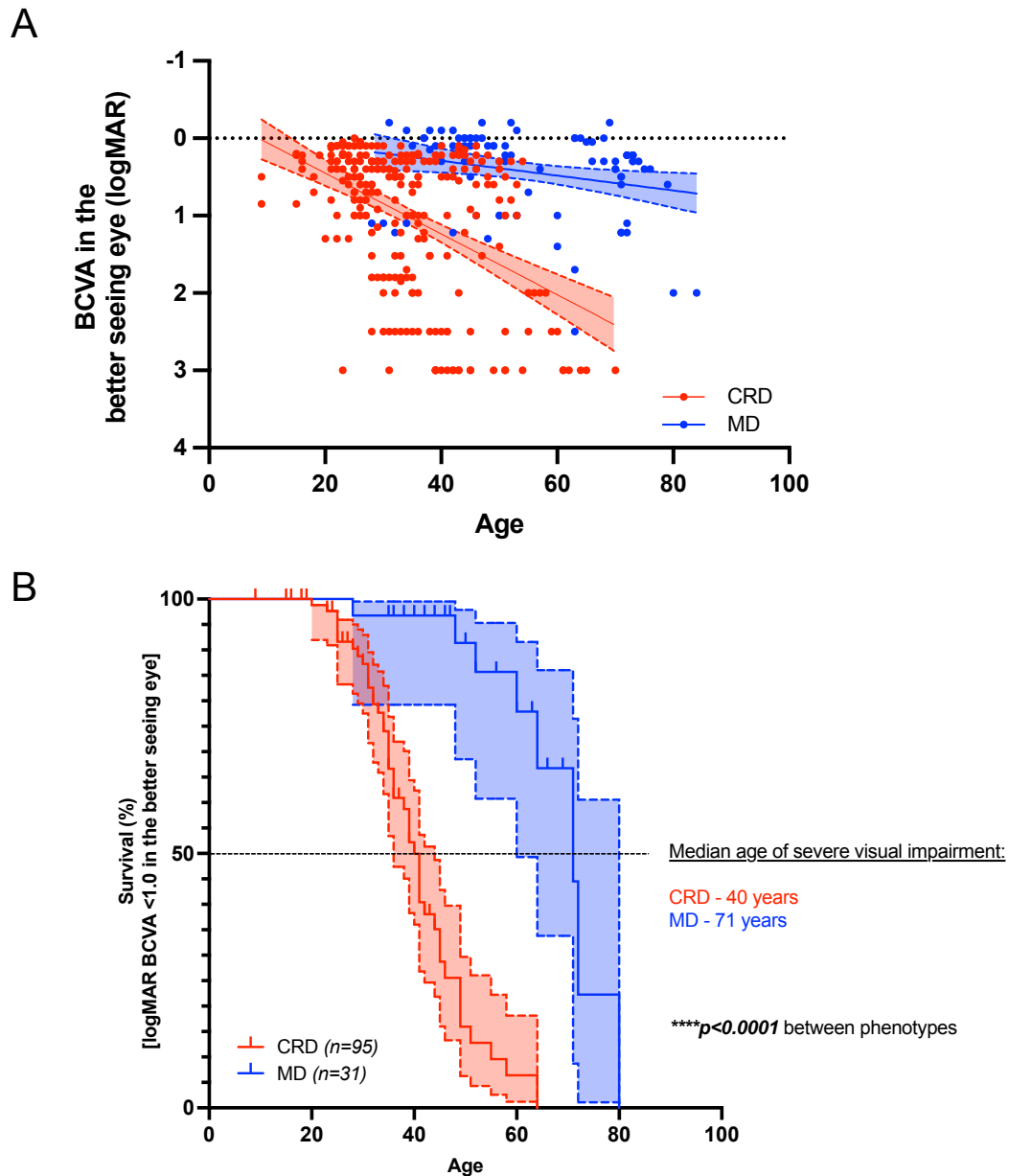


Figure 7.11 Natural history of visual acuity decline in *CDHR1*-associated retinal degeneration

(A) A significantly better visual acuity is demonstrated in the better seeing eye of individuals with MD versus CRD with age (non-parametric dataset; two-tailed Mann-Whitney $P < 0.0001$; $n = 353$ data points). A linear regression line is plotted + 95% C.I. (B) Kaplan-Meier survival analysis shows that individuals with CRD developed severe visual impairment (defined as logMAR 1.0 or worse in the better seeing eye) at a significantly earlier age (median: 40 years), compared to MD (median: 71 years) with comparison of survival curves using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests both demonstrating a highly significant difference ($P < 0.0001$). Shaded areas indicate 95% CI for both MD and CRD groups.

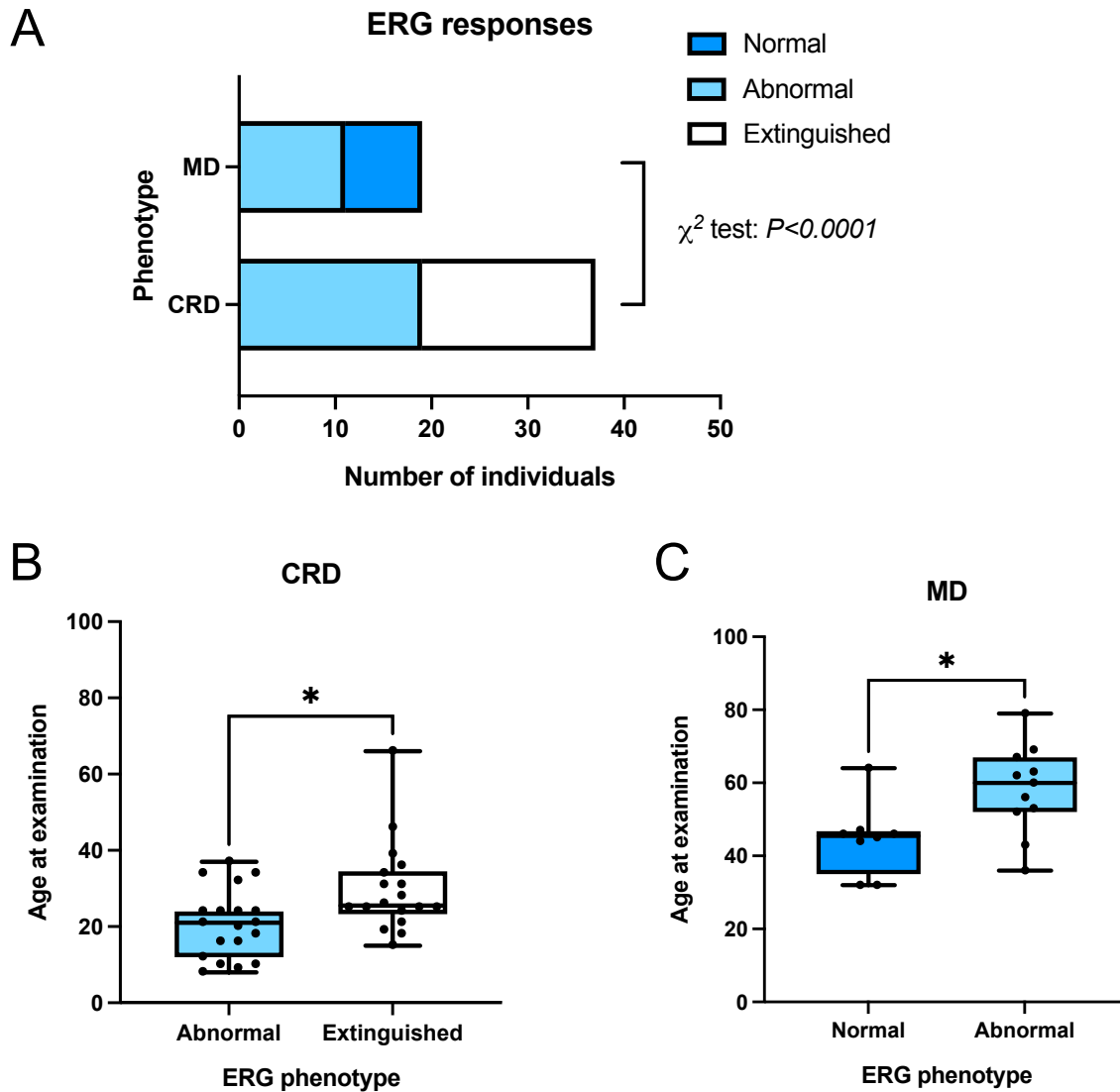


Figure 7.12 ERG responses in *CDHR1*-associated retinal degeneration

(A) Full-field ERG responses (scotopic single-flash responses at 0.01 cd.s/m², and photopic 30Hz flicker responses) were categorized as normal, reduced, or extinguished according to phenotype. No individuals with CRD ($n=37$) exhibited normal ERGs, and no individuals with MD ($n=19$) exhibited extinguished ERGs: categories differed significantly between CRD and MD groups (Chi-square contingency testing (27.94, 4df) $P < 0.0001$). (B) In the CRD group, individuals with extinguished ERGs were significantly older than those with abnormal ERGs (Shapiro-Wilk: parametric datasets; two-tailed, unpaired t-test with Welch's correction $P = 0.0152$) (C) In the MD group, individuals with abnormal ERGs were significantly older than those with normal ERGs (Shapiro-Wilk: parametric datasets; two-tailed, unpaired t-test with Welch's correction $P = 0.0157$).

7.4.3.2 *Electroretinography*

Individuals with CRD ($n=37$) demonstrated abnormal or extinguished scotopic- and photopic full-field ERG responses (Figure 7.12). No individuals with CRD demonstrated normal responses, including 4 individuals who were examined at the age of 10 or younger. Conversely, no individuals with MD ($n=19$) demonstrated extinguished ERGs, with the group exhibiting normal or abnormal ERG tests. In both CRD and MD, age was a significant predictor of ERG response categories ($P=0.016$ for both groups), indicating a deterioration of visual function with time. In compound heterozygotes with a null allele *in trans* with a hypomorphic allele ($n=7$), there were no extinguished ERGs although subgroup analysis was not possible due to a small sample size.

7.4.4 Natural history of *CDHR1*-associated retinal degeneration: quantitative multimodal retinal imaging analysis

7.4.4.1 *Retinal thickness measurements on OCT imaging*

Quantitative analysis of macular OCT imaging data revealed that all individuals with CRD, regardless of age, had central subfield retinal thickness measurements (mean 181.6 μm) less than 2 SDs below a control population of adults (mean 277.4 μm ; $P<0.0001$, two-way ANOVA for effect of genotype). Moreover, all 8 other ETDRS subfields were significantly thinner compared to controls ($P<0.0001$ for all subfields, two-way ANOVA for effect of genotype; Figure 7.14). The variation in retinal thickness measurements were greater in the inner (3 mm) ring (SD range:

29.9 to 44.0), compared to the outer (6 mm) ring (SD range: 21.0 to 29.2), with a greater mean reduction (94.1 versus 71.4 μm). Relative to adult controls, the reduction in the central 1mm was 69% and 67%, 1-3mm was 75% and 66% and 3-6mm was 78% and 77% for CRD and MD groups, respectively (Figure 7.14). Superior and inferior retinal thickness measurements within the central 3mm ($P=0.997$) and 6mm ($P=0.62$; two-way ANOVA for effect of retinal location) were not significantly different in individuals with CRD.

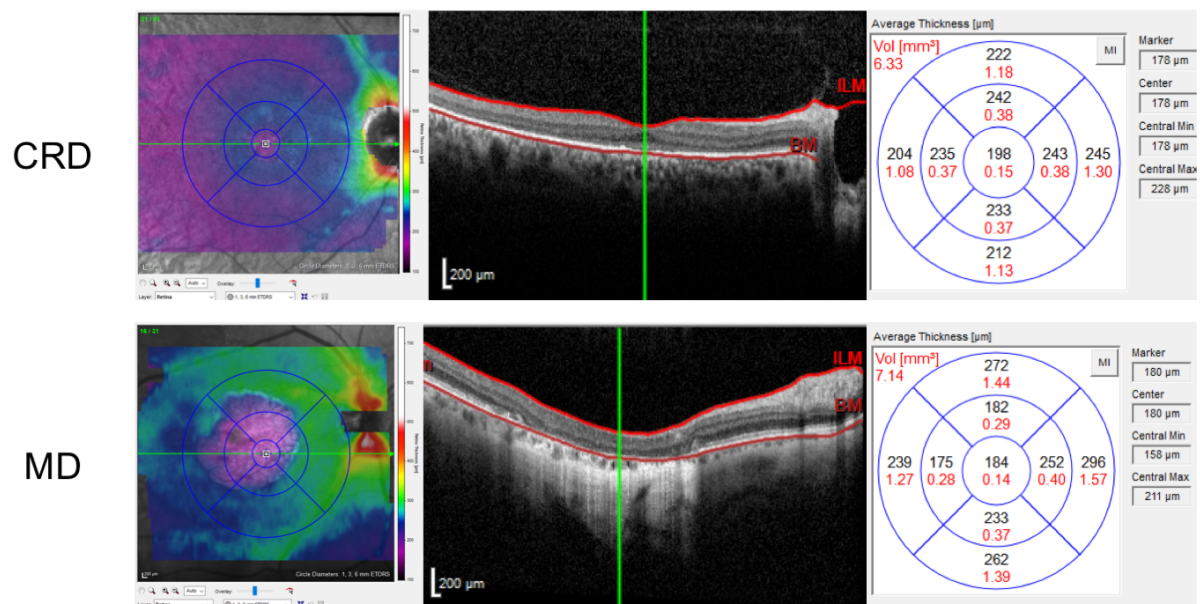


Figure 7.13 Retinal thickness measurements derived from OCT imaging
The ETDRS grid was centered on the fovea, defining nine subfields at 1-, 3- and 6-mm eccentricity in the cardinal quadrants. Mean retinal thickness was recorded for each subfield. Individuals with CRD exhibited generalised thinning across all subfields, although more variable in the central 3mm. Individuals with MD exhibited atrophy in the central 3mm with relative preservation from 3-6mm.

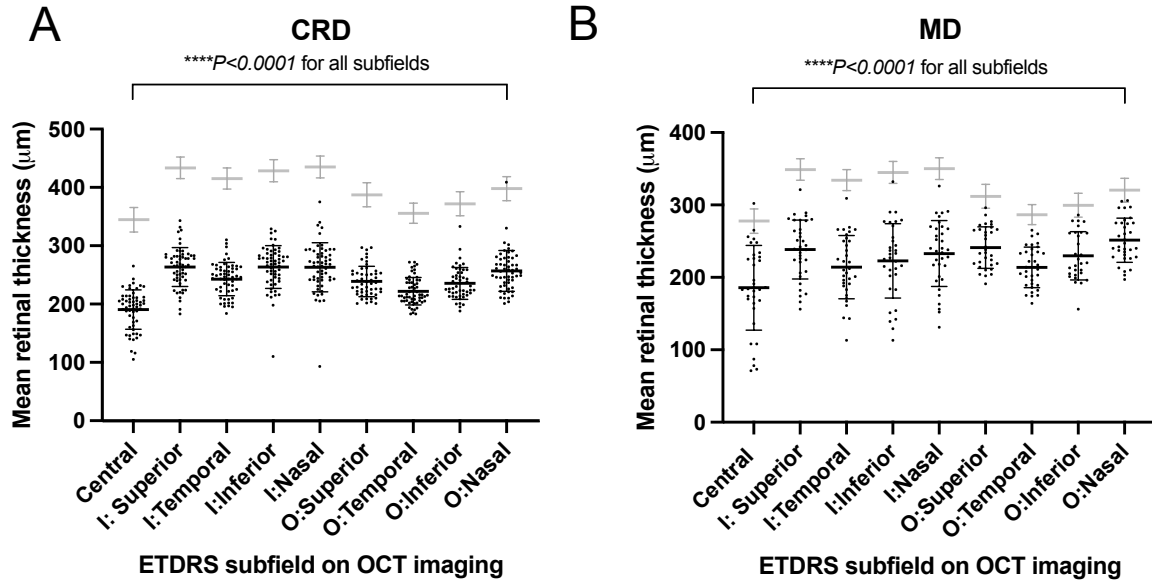


Figure 7.14 Retinal thickness measurements on OCT imaging in *CDHR1*-associated retinal degeneration

(A) Individuals with CRD and (B) MD demonstrate retinal thinning across all 9 ETDRS subfields compared to controls ($P<0.0001$ for effect of genotype in all subfields). Across both phenotypes, a greater variability was seen in the central and inner (I) 1-3mm subfields, compared to outer (O) 3-6mm subfields. In the CRD group, outliers were those patients ($n=2$) with foveal staphylomas. Grey bars indicate normative Spectralis OCT data (mean $\pm$ 1SD) from a published adult cohort. Shapiro-Wilk: all datasets were parametric; two-way ANOVA with Šidák's multiple comparison testing for the effect of retinal location. I – inner, 1-3mm subfield; O – outer, 3-6mm subfield.

In the MD group, significant retinal thinning was also observed in all nine ETDRS subfields ($P<0.0001$ for all subfields; two-way ANOVA for effect of genotype), compared to age-matched controls (Figure 7.14). The variability was even greater than CRD patients in the central subfield and inner (1-3mm) subfields (SD range: 52.4 to 66.5 μ m) than in the 3-6mm subfield (SD range: 20.7 to 35.7 μ m) with a greater mean average reduction (123 versus 56.5 μ m), indicating that atrophic changes were greatest within 3mm of the fovea. Superior and inferior retinal

thickness measurements were not significantly different in the 1-3mm ($P=0.08$) and 3-6-mm subfields ($P=0.09$; two-way ANOVA for effect of retinal location with Šídák's multiple comparison testing).

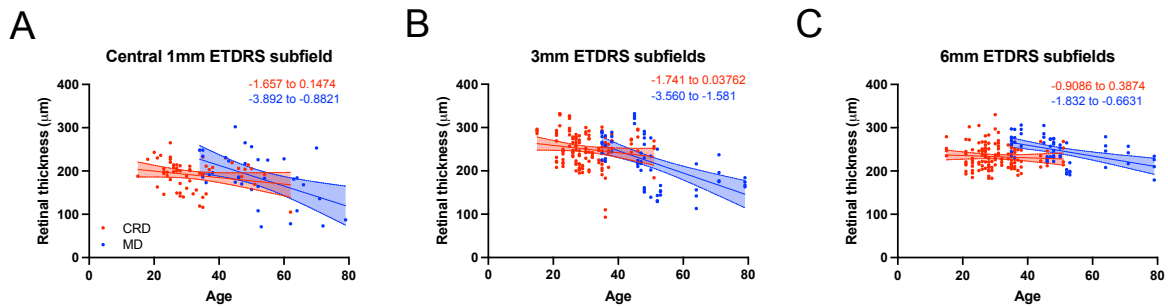


Figure 7.15 Longitudinal retinal thickness on OCT imaging in *CDHR1*-associated CRD and MD patients

In both CRD and MD groups, the rate of retinal thinning is greater in the central 1mm and 1-3mm subfields, which appear similar, than the 3-6mm subfields which are relatively spared in both phenotypes. Across all subfields, individuals with CRD demonstrate a slower rate of retinal thinning, in part due to lower baseline values, a younger cohort and a floor effect ($\sim 100\mu\text{m}$). Values indicate 95% CI for the slopes of linear regression; note the quicker rate of retinal thinning in all ETDRS subfields in the MD group. Data are derived from right eyes.

Longitudinal comparison of retinal thickness measurements identified retinal thinning across both MD and CRD phenotypes in all ETDRS subfields (Figure 7.14). The rate of retinal thinning appeared greater in MD versus CRD patients in all subfields (95% CI of linear regression lines shown in Figure 7.14) due in part to the more advanced age of the MD cohort, thinner baseline values in the CRD group and a floor effect ($\sim 100\mu\text{m}$) in end-stage outer retinal degeneration. In both phenotypes, the rate of retinal thinning was slowest in the 3-6mm subfield.

7.4.4.2 RPE atrophy on FAF imaging

Individuals with MD exhibited an exponential growth of RPE atrophy on SW-AF imaging (Figure 7.16). The age at which RPE atrophy had developed to 50% of its maximum area was 47.6 years (95% CI: 46.9 to 48.2 years). Beyond this limit, a ceiling effect prevented further relevant centrifugal expansion of RPE atrophy.

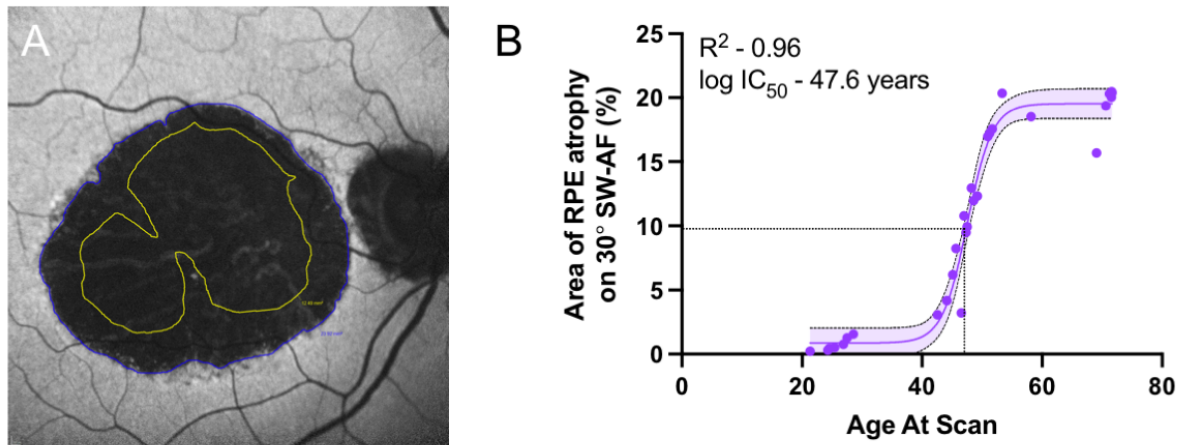


Figure 7.16 Modelling the rate of growth of RPE atrophy in *CDHR1*-associated macular dystrophy

(A) SW-AF image of a c.783G>A homozygote with baseline tracing at 67-years (yellow) overlaid on latest image (blue) to adjust for dioptric focus (B) Areas of absolute RPE atrophy on 30° SW-AF imaging were measured longitudinally in MD patients ($n=9$) using data from right eyes only. A sigmoidal function was fitted to the data (R^2 : 0.96). The 95% CI for the $\log IC_{50}$ was 46.9 to 48.2 years: within this range, the area of RPE atrophy was 50% of maximum.

7.4.5 Genetics of *CDHR1*-associated retinal degeneration

7.4.5.1 Pathological sequence variants in *CDHR1*

A total of 63 different *CDHR1* variants were present in the cohort of 146 patients. These clinically validated pathological sequence variants in *CDHR1* include 21 null variants (start lost, stop gain, frameshift), 16 splice/intronic variants, 23 missense variants, 2 in-frame deletions and 1 synonymous variant. Missense

variants appear more commonly in extracellular cadherin domains 1st, 2nd, 4th and 5th, with the 3rd and 6th cadherin domains relatively spared. Missense variants associated with MD are concentrated towards the c-terminal 1st and 2nd cadherin domains (Figure 7.17). All clinically validated pathological *CDHR1* sequence variants identified in the cohort and other published reports, are presented in the Appendix listed from 5' to 3' and illustrated against a schematic of the human *CDHR1* protein in Figure 7.17.

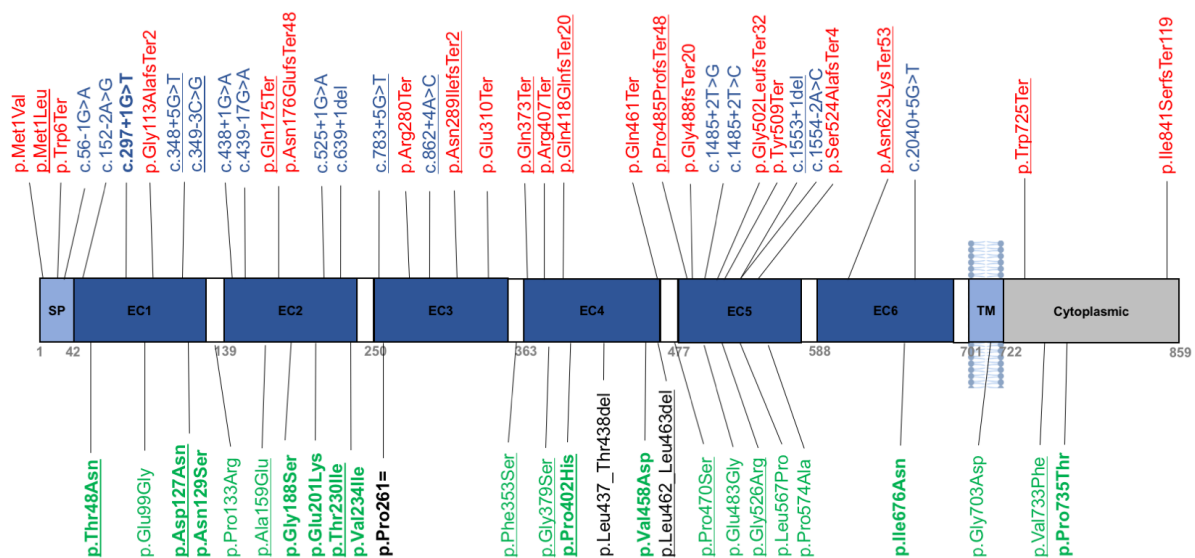


Figure 7.17 Clinically validated pathological *CDHR1* sequence variants
Twenty-five novel (underlined) and previously published pathological *CDHR1* sequence variants are illustrated against the *CDHR1* protein domains. Red – start lost, stop gained, and truncating variants; Blue – intronic/splice variants; Green – missense variants; Black – synonymous variants or in-frame deletions. Bold font indicates variants associated with macular dystrophy.

7.4.5.2 Novel sequence variants in *CDHR1*

Twenty-five novel disease-associated variants in *CDHR1* identified in this cohort were novel, annotated with ACMG criteria for pathogenicity in Table 7.3. The novel variants were generally rare ($MAF < 3.4 \times 10^{-4}$) or absent from genome aggregation databases. One novel variant affected the start codon (p.Met1Leu) resulting in an alternate start codon 38bp downstream, although the missense variant itself is not predicted to be damaging.

<i>CDHR1</i> variant	Protein consequence	Genomic co-ordinate dbSNP	MAF	Phenotype	Alleles	ACMG
c.1A>T	p.Met1Leu Start loss	10-85954517 rs794726954	8.6×10^{-6}	CRD	6	Pathogenic
c.143C>A	p.Thr48Asn	10-85955337 rs201698218	3.8×10^{-4}	MD	3	Likely pathogenic
c.348+5G>T	p.?	10-85957597	NP	-	1	Uncertain significance
c.349-3C>G	p.?	10-85958785	NP	-	1	Likely pathogenic
c.379G>A	p.Asp127Asn	10-85958818 rs1189565760	6.4×10^{-6}	-	1	Uncertain significance
c.476C>A	p.Ala159Glu	10-85960394	NP	-	1	Uncertain significance
c.523C>T	p.Gln175Ter	10-85960441 rs781781440	4.2×10^{-6}	-	2	Pathogenic
c.689C>T	p.Thr230Ile	10-85962785 rs141173656	3.4×10^{-4}	MD	3	Likely pathogenic
c.783+5G>T	p.?	10-85962884	NP	-	1	Likely pathogenic
c.862+4A>C	p.?	10-85964365 rs1352559639	8.0×10^{-6}	-	1	Uncertain significance
c.866_872delins TTTGAAATTA ATGAC	p.Asn289IlefsTer2	10-85965586	NP	-	1	Pathogenic
c.1058T>C	p.Phe353Ser	10-85968024 rs771316043	1.2×10^{-5}	RP	2	Uncertain significance
c.1117C>T	p.Gln373Ter	10-85968082	NP	CRD	3	Pathogenic
c.1135G>A	p.Gly379Ser	10-85968101 rs572616777	2.0×10^{-5}	CRD	2	Uncertain significance
c.1205C>A	p.Pro402His	10-85968522	NP	-	1	Uncertain significance
c.1219C>T	p.Arg407Ter	10-85968536 rs373978786	7.1×10^{-6}	-	1	Pathogenic

<i>CDHR1</i> variant	Protein consequence	Genomic co-ordinate dbSNP	MAF	Phenotype	Alleles	ACMG
c.1254del	p.Gln418GlnfsTer20	10-85968571	NP	-	2	Pathogenic
c.1383_1388del	p.Leu462_Leu463del	10-85970819	NP	CRD	2	Likely pathogenic
c.1408C>T	p.Pro470Ser	10-85970844 rs779854349	8.0 x 10 ⁻⁶	-	1	Uncertain significance
c.1458_1462del	p.Pro485ProfsTer48	10-85970889	NP	CRD	2	Pathogenic
c.1553+1del	p.?	10-85971472	NP	-	1	Pathogenic
c.1576G>A	p.Gly526Arg	10-85971950	NP	CRD	2	Uncertain significance
c.1868dup	p.Asn623LysfsTer53	10-85972932	NP	-	3	Pathogenic
c.2175G>A	p.Trp725Ter	10-85973972	4.0 x 10 ⁻⁶	CRD	2	Pathogenic
c.2197G>T	p.Val733Phe	10-85973994	NP	RP	2	Uncertain significance

Table 7.3 ACMG classification of novel *CDHR1* variants

Twenty-five novel sequence variants in *CDHR1* are classified as per ACMG criteria.⁸⁹ Alleles counts are from a total of 292 alleles of 146 individuals within the cohort. MAF – mean allele frequency (GnomAD); NP – not present. Phenotype is annotated in homozygous cases.

In silico assessments of 10 missense variants and five splice variants are presented in Table 7.4 with their evolutionary conservation presented in the Appendix.

7.4.5.3 Genotype-phenotype associations in *CDHR1*-associated CRD

Individuals with biallelic truncating variants in *CDHR1* exhibited clinical features of CRD (Figure 7.17). However, several missense variants (p.Gln379Ser, p.Glu483Gly, p.Gly526Arg, p.Pro574Ala) and two in-frame deletions were noted to produce CRD in the homozygous state and hence may result in a null-like effect. Moreover, all splice variants, with the exception of c.297+1G>T, were associated with CRD when homozygous, or *in trans* with a null allele.

Amino acid change predictions							
DNA	Protein	MAF	SIFT [0-1]	Provean	Polyphen-2 [0-1]	Mutation Taster [0-1]	Notes
c.1A>T	p.Met1Leu Start loss	8.6 x 10 ⁻⁶	Deleterious – low confidence	Neutral -0.5	Benign 0.001	Disease causing 1.0	Start codon 38 bp downstream
c.143C>A	p.Thr48Asn	3.8 x 10 ⁻⁴	Deleterious	Deleterious -3.8	Probably damaging 1.0	Disease causing 1.0	Highly conserved
c.379G>A	p.Asp127Asn	6.4 x 10 ⁻⁶	Deleterious	Deleterious -3.8	Probably damaging 1.0	Disease causing 1.0	Highly conserved
c.476C>A	p.Ala159Glu	-		Deleterious -3.8	Probably damaging 0.99	Disease causing 1.0	Highly conserved
c.689C>T	p.Thr230Ile	3.4 x 10 ⁻⁴	Deleterious	Deleterious -2.5	Possibly damaging 0.85	Polymorphism 0.6	Gain of SA
c.1058T>C	p.Phe353Ser	1.2 x 10 ⁻⁵	Deleterious	Deleterious -6.8	Probably damaging 1.0	Disease causing 1.0	Highly conserved Gain of SD
c.1135G>A	p.Gly379Ser	2.0 x 10 ⁻⁵	Deleterious	Deleterious -3.3	Probably damaging 1.0	Disease causing 1.0	Highly conserved Gain of SD
c.1205C>A	p.Pro402His	-		Deleterious -3.3	Probably damaging 0.99	Polymorphism 0.72	Highly conserved Gain of SD
c.1408C>T	p.Pro470Ser	8.0 x 10 ⁻⁶	Deleterious	Deleterious -7.0	Probably damaging 1.0	Disease causing 1.0	Highly conserved
c.1576G>A	p.Gly526Arg	-		Deleterious -6.8	Probably damaging 1.0	Disease causing 1.0	Highly conserved
c.2197G>T	p.Val733Phe	-		Neutral -2.0	Possibly damaging 0.87	Disease causing 0.91	Highly conserved
Splice variant predictions							
DNA	Predicted effect	MAF	MaxEnt [0-12]	ASSP [0-12]		NNSPLICE [0-1]	
c.348+5G>T	Broken donor site	-	10.1 → 3.0	12.1 → 4.9		0.99 → 0	
c.349-3C>G	Broken acceptor site	-	-34.7 → -26.45	11.0 → 3.0		-	
c.783+5G>T	Broken donor site	-	7.9 → -0.9	11.2 → 0		0.26 → 0	
c.862+4A>C	Broken donor site	8.0x10 ⁻⁶	10.1 → 8.3	12.9 → 9.1		0.99 → 0.96	
c.1553+1del	Broken donor site	-	7.52 → -28.5	10.8 → 0		0.95 → 0	

Table 7.4 *In silico* analysis of novel *CDHR1* sequence variants

ACMG recommended *in silico* prediction tools were used for the predictions of missense variants (SIFT, Provean, Polyphen-2, Mutation Taster), and splice variants (MaxEnt, HSF, ASSP, NNSPLICE). All missense variants occur at highly conserved residues (see Appendix), with the exception of Thr230Ile. Three splice variants are predicted to abolish splicing at that position *in silico*, with two others reducing the efficiency of splicing. SD - splice donor; SA - splice acceptor.

7.4.5.4 *Genotype-phenotype associations in CDHR1-associated MD*

32 individuals in the cohort had clinical features of macular dystrophy. Variants associated with MD were predominantly missense variants, clustered within the first two cadherin domains (Figure 7.17). None of these individuals had biallelic truncating variants or variants affecting the canonical splice sites, with the exception of c.297+1G>T, associated previously with MD when *in trans* with a mild variant on the second allele.⁵⁵ Individuals (*n*=11) with a hypomorphic *variant in trans* with a null allele had an intermediate phenotype with macular predominant disease associated with variable peripheral retinal degeneration.

7.4.6 Global genetic prevalence of *CDHR1*-associated retinal degeneration

115 disease-associated variants in *CDHR1* were identified. Start lost, stop gained, frame-shifting mutations, and those affecting the canonical splice donor or acceptor sites were identified from population databases by Hanany *et al* (66 variants).²³ An additional 49 validated *CDHR1* variants were added from previously published reports, or unpublished novel variants from this study (25

variants). 32 variants were not present in GnomAD, presumably due to a very low population frequency or in relatively under-represented ethnic populations.

7.4.6.1 Overall genetic prevalence of *CDHR1*-associated retinal degeneration

The allele frequency (AF) of 115 disease-associated *CDHR1* variants were extracted from six major ethnic world populations from the GnomAD genome aggregation database.

Region	Population	Affected individuals	Heterozygous carriers
UK	66,650,000	3,180	914,456
North America	574,000,000	27,390	7,875,434
Europe	735,802,007	35,111	10,095,402
Finland	5,516,176	200	66,075
Africa	1,258,311,831	18,433	9,595,219
Latin America	646,059,776	1,880	2,200,133
South Asia	1,870,824,410	8,367	7,895,919
East Asia	1,646,834,954	119,451	27,812,246
WORLDWIDE	6,737,349,154	210,832	65,540,429
<i>Worldwide frequency</i>		<i>1 in 31,956</i>	<i>1 in 103</i>
Europe & North America	1,309,802,007	62,502	17,970,836
<i>Frequency in Europe & North America</i>		<i>1 in 20,956</i>	<i>1 in 73</i>

Table 7.5 Global genetic prevalence of *CDHR1*-associated retinal degeneration

Population data are derived from The World Bank (current as of 2020). Affected individuals harbour two pathological *CDHR1* alleles *in trans*; heterozygous carriers harbour any single pathological *CDHR1* allele.

The sum of the combined AFs of all pathological *CDHR1* variants in each major world population was determined, and the Hardy-Weinberg equation was applied to determine the number of affected individuals and carrier frequency (data summarised in Table 7.5). The worldwide genetic prevalence of *CDHR1*-associated retinal degeneration, regardless of phenotype, is approximately 210,000 individuals (1 in every ~32,000 individuals) with >65 million carriers worldwide (1 in ~103) who harbour at least one disease-associated *CDHR1* variant. In Europe and North America, the frequency of affected individuals is 1 in ~21,000 with a carrier rate of 1 in ~73.

7.4.6.2 *Genetic prevalence of CDHR1-associated retinal degeneration by phenotype*

CDHR1 variants ($n=115$) were classified into null/presumptive null ($n=93$) or hypomorphic ($n=22$) groups. Start lost, stop gain, frameshifting and truncating variants, or where missense or splice variants had been shown to result in CRD were included in the null group. Variants associated with MD were designated as hypomorphic. The Hardy-Weinberg equation was used to approximate the equilibrium of three allele categories within each population (wildtype, hypomorphic, and null), as described in the methods. Individuals with two null variants *in trans* were categorised as CRD, and those with one or more hypomorphic alleles *in trans* with a second disease-associated *CDHR1* variant, as MD (Table 7.1). 3 rare variants associated with RP were not present on GnomAD.

Region	Population	Macular dystrophy	Cone-rod dystrophy
UK	66,500,000	3,120	53
North America	574,000,000	26,930	460
Europe	735,802,007	34,521	590
Finland	5,516,176	200	0
Africa	1,258,311,831	12,429	6,004
Latin America	646,059,776	1,374	506
South Asia	1,870,824,410	6,098	2,269
East Asia	1,646,834,954	118,366	1,085
WORLDWIDE	6,737,349,154	199,918	10,968
<i>Worldwide frequency</i>		<i>1 in 33,701</i>	<i>1 in 614,281</i>
Europe & North America	1,315,318,183	61,651	1,051
<i>Frequency in Europe & North America</i>		<i>1 in 21,335</i>	<i>1 in 1,251,921</i>

Table 7.6 Genetic prevalence of *CDHR1*-associated cone-rod and macular dystrophies

Individuals with two null/functional null alleles were designated as CRD, and those with one or more hypomorphic alleles as MD.

Genetic prevalence estimates suggest that macular dystrophy is the most common clinical manifestation of *CDHR1*-associated retinal degeneration, accounting for 94.8% of cases, whilst cone-rod dystrophy accounts for approximately 5.2% (Table 7.6). Worldwide, the approximate frequency of *CDHR1*-associated MD is 1 in every ~34,000 individuals, with CRD occurring approximately in 1 in every ~614,000 individuals. However, in Europe & North America, the frequency of MD is higher at 1 in ~21,000 individuals with >60,000 affected individuals; CRD is expected to affect ~1,000 individuals across these regions. Of the >65 million carriers of a

pathological *CDHR1* variant, ~15.7 million carry a null *CDHR1* allele (1 in ~429), and ~49.8 million carry a pathological hypomorphic allele (1 in ~135) (Table 7.7).

Population	Carriers of a null allele	Carriers of a hypomorphic allele
UK	118,289	794,109
North America	1,021,021	6,854,413
Europe	1,308,832	8,786,569
Finland	1,475	64,600
Africa	5,476,282	4,118,937
Latin America	1,141,332	1,058,801
South Asia	4,111,702	3,784,216
East Asia	2,650,850	25,161,397
WORLDWIDE	15,711,495	49,828,934
<i>Worldwide carrier frequency</i>	<i>1 in 429</i>	<i>1 in 135</i>

Table 7.7 Global carrier frequencies of null and hypomorphic *CDHR1* alleles

7.4.6.3 Genetic prevalence of the *CDHR1* c.783G>A variant

Estimation of the genetic prevalence of *CDHR1*-associated retinal degeneration associated with homozygosity, or compound heterozygosity of the common c.783G>A_{*CDHR1*} variant was undertaken with three alleles in equilibrium: *p* – wildtype; *q* – c.783G>A and *r* – all other pathogenic *CDHR1* alleles. Worldwide, an estimated 32,000 individuals are homozygous for the c.783G>A_{*CDHR1*} variant with an additional 30,000 individuals compound heterozygous with the c.783G>A_{*CDHR1*} variant on one allele with another pathologic allele *in trans*. Of the

~62,000 affected individuals worldwide, 93% are of European ancestry (principally in Europe and North America) affecting an average of 1 in ~46,000 individuals from these populations (Table 7.8).

Region	Population	c.783G>A, homozygotes	c.783G>A, compound heterozygotes
UK	66,500,000	2,308	1,307
North America	574,000,000	13,799	11,285
Europe	735,802,007	17,688	14,466
Finland	5,516,176	191	9
Africa	1,258,311,831	50	1,827
Latin America	646,059,776	51	519
South Asia	1,870,824,410	18	739
East Asia	1,646,834,954	4	1,398
WORLDWIDE	6,737,349,154	31,802	30,243
<i>Worldwide frequency</i>		<i>1 in 211,851</i>	<i>1 in 222,777</i>
Europe & North America	1,315,318,183	31,678	25,759
<i>Frequency in Europe & North America</i>		<i>1 in 41,521</i>	<i>1 in 51,063</i>

Table 7.8 Genetic prevalence of the c.783G>A *CDHR1* variant

7.5 Discussion

In this Chapter, the largest series of patients ($n=146$) with retinal degeneration secondary to biallelic variants in *CDHR1* is described. Individuals with biallelic null variants, and severe missense or splice variants develop cone-rod dystrophy with median age of 20 years at symptom onset. The primary symptom at presentation may relate to either cone or rod dysfunction. This group have severe deficits in both rod and cone function with abnormal or extinguished scotopic and photopic ERGs. The median age of severe visual impairment in *CDHR1*-associated CRD patients is 41 years (95% CI: 36.3 to 44.5 years) with no individual sustaining vision $>\log\text{MAR}$ 2.0 after the age of 55 years in the better seeing eye.

The multimodal retinal imaging characteristics of CRD are described in detail, with four typical stages on FAF imaging: early expansion of foveal hypoautofluorescence with loss of macular pigment, RPE atrophy, fovea-involving geographic atrophy and end-stage disease with pan-retinal degeneration of the outer retina and RPE. OCT imaging demonstrates early thinning of the ONL, reduced reflectivity of the EZ, and shortening of the photoreceptor OS consistent with data from the *Cdhr1*^{-/-} mouse in Chapter 3. Furthermore, functional deficits appear similarly out of proportion to structural appearances, as demonstrated on ERG and microperimetry, with visual acuity sometimes affected more greatly than may be anticipated from OCT appearances of the outer retina overlying the fovea.

OCT retinal thickness measurements in the CRD cohort are less than two SD below healthy adult controls in all macular ETDRS subfields; these observations mirror murine data from Chapter 3. Loss of the RPE appears to begin in the parafovea; quantitative OCT imaging data confirms a greater variability in the central 3mm zone versus the 3-6mm zone when compared to controls. Peripapillary atrophy is a very common phenotypic feature which may aid in the clinical distinction between *CDHR1*- and *ABCA4* retinopathies - the most common cause of macular dystrophy where peripapillary sparing is characteristic.²³ Peripapillary and mid-peripheral coalescing RPE degeneration are also demonstrable in the *Cdhr1*^{-/-} mouse model at late timepoints (>18 months) on autofluorescence imaging (Figure 6.6 & 6.8). Disease progression as determined by photoreceptor layer thickness measurements on OCT imaging appears approximately linear in the *Cdhr1*^{-/-} mouse model (Chapter 3). In CRD patients, there appears to be more variability which may be due to the macular derivation of presented OCT data that are not directly translatable from murine subjects. Without more detailed normative OCT imaging data based on age, it is difficult to form direct, quantitative comparisons of longitudinal structural changes between species based on a scaling of respective lifespans. Similarly, a relative lack of ERG data from human CRD patients limit functional comparisons. However, there is some variability in CRD in the relative severity of rod and cone involvement in CRD that in general appears greater than that observed in the *Cdhr1*^{-/-} mouse.

Individuals with one or more hypomorphic *CDHR1* variants develop macular dystrophy. Variants are typically missense, although a single splice variant (c.297+1G>T) has been described in association with MD.¹⁴⁹ It is possible that this may be explained by in-frame exon skipping (exon 3) caused by abolition of this canonical splice donor site. However, splice assays are required to confirm this, and to support the pathogenicity of five novel splice variants in *CDHR1* described in this Chapter. These observations suggest that macular photoreceptors appear to be more susceptible to structural abnormalities conferred by hypomorphic *CDHR1* variants. The cause for this remains unknown although a clustering of MD-associated missense variants in the c-terminal 1st and 2nd cadherin ectodomains suggest a particularly important role within the macula, perhaps interacting with an as yet unidentified binding partner. Individuals with biallelic hypomorphic variants appear in general to avoid peripheral retinal degeneration, although this was seen together with an abnormal full field ERG in a c.783G>A*CDHR1* homozygote individual suggesting a role for as yet unidentified disease modifiers. Individuals with a hypomorphic allele *in trans* with a null allele are more likely to isolated develop isolated macular dystrophy (in 10 of 14 cases), or macular dystrophy with variable peripheral retinal degeneration (4 of 14 cases); these individuals were assumed to develop MD for the purposes of genetic prevalence estimates. However, it has been demonstrated that other forms of monogenic retinal degeneration can evolve from a MD into a more widespread peripheral retinal degeneration over time.²¹⁵ The clinical phenotype may depend

on age at examination, the combination of variants in any individual, and as yet undetermined disease modifiers.

Individuals with *CDHR1*-associated macular dystrophy develop symptoms at a median age of 44 years, although they may be asymptomatic at disease detection. The most common presenting symptom is difficulty reading or abnormalities with central vision (66.6%) although 27% presented with night vision problems, suggesting peripheral retinal dysfunction in some individuals in early disease. A greater degree of foveal sparing in many cases explains the lower degree of symmetry of corrected visual acuity in MD (Pearson's rank: 0.57; $P < 0.0001$) versus CRD patients (Pearson's rank: 0.88; $P < 0.0001$). The median age at severe visual impairment was estimated at 71 years of age (95% CI 60.5 to 80.6 years). The multimodal retinal imaging findings are described in detail with early flecks (particularly in pre-symptomatic individuals), with subsequent parafoveal RPE atrophy, foveal sparing central atrophy and finally RPE and outer retinal atrophy in late disease with concentric enlargement which may be classified clinically as central areolar choroidal dystrophy (CACD). Accordingly, *CDHR1* should be considered in a patient with CACD in whom a dominant family history is not present.⁵⁶ Quantitative OCT analysis demonstrates progressive parafoveal outer retinal degeneration affecting the central 3mm, sparing the 3-6mm subfields until late.

Overall, a genotype-phenotype association exists in *CDHR1*-associated retinal degeneration in which the retinal phenotype may be generally predicted from the combination of variants. This is likely to further support an onward clinical programme, and aid in the interpretation of new disease-associated variants. Protein modelling may further qualify the interpretation of missense variants associated with CRD (i.e. functional nulls), versus those associated with MD (hypomorphic). The absence of experimental structures for *CDHR1* makes this more challenging. However, residues of known functional importance (i.e. calcium binding sites) may provide further evidence for the severe functional effects of some *CDHR1* missense variants, validated through clinical correlation. For example, it should be noted that the 3rd cadherin ectodomain is the least conserved⁵¹, and accordingly, does not harbour any disease-associated missense variants for any phenotype discovered to date. Furthermore, the observation of RP with specific hypomorphic variants may suggest alternative interactions between *CDHR1* and its binding partners in cones versus rods, which differ structurally in the continuity of OS discs with the plasma membrane. At least two reports identify RP patients where hypomorphic *CDHR1* alleles may contribute to a milder disease phenotype.^{57, 58}

Genetic prevalence estimates using allele frequency data of clinically validated *CDHR1* variants predict more than 200,000 affected individuals worldwide, with a global frequency of approximately 1 in 32,000 individuals. Approximately 65

million individuals harbour a pathological variant in the gene (~1 in 100 individuals); 50 million are hypomorphic and 15 million are null *CDHR1* alleles. Moreover, genotype-phenotype data generated during this study and further validated using other published reports enabled an approximate prevalence of disease by phenotype: with ~95% of affected individuals predicted to manifest macular predominant disease, and around 5% to exhibit cone-rod dystrophy. This calculation assumed that compound heterozygotes (i.e. harbouring null and hypomorphic variants *in trans*) develop macular dystrophy which may be true in approximately 70% of cases (Appendix). However, some peripheral retinal involvement may be present in this group. In this Chapter, 100 of 146 individuals (68.4%) manifested CRD, a difference which may be explained by likely misclassification of individuals with *CDHR1*-associated MD as dry age-related macular degeneration in the general population, which is further discussed in Chapter 8. Moreover, the previous estimate of genetic prevalence excluded many disease-associated variants, based on the variant filtering strategy, reliance on population databases alone and omission of many novel variants identified in this thesis.²³ In addition, it should be noted that population genetic data from continental Africa, and South Asia are inadequately developed; the allele frequencies of disease-associated variants in *CDHR1* in those continents is poorly understood many of which may be assumed to be unique, occurring independently to those identified in Europeans.

Of the estimated 62,700 individuals affected across Europe and North America (~1 in 21,000 across all phenotypes), there are approximately 31,700 c.783G>A homozygotes and 25,700 compound heterozygotes who harbour the c.783G>A variant on one allele. Taken together, the c.783G>A variant exists on at least one allele in 57,400 individuals in Europe and North America; 91.9% of all individuals predicted to harbour biallelic *CDHR1* variants. The c.783G>A_{CDHR1} variant is consequently a prime therapeutic target for bespoke gene editing approaches.

The methodology used to generate disease prevalence estimates is subject to the general limitations and assumptions of the Hardy-Weinberg equilibrium (e.g. no selection, genetic drift, mutation migration, or survival benefit from heterozygosity).²¹⁶ The calculations are based on the prevalence of biallelic *CDHR1* variants that are presumed to be fully penetrant (i.e. disease-causing in all individuals). Disease may manifest at a variable age, as illustrated in Figure 7.2, and some will be too young to manifest the disease. The Hardy-Weinberg equilibrium assumes random mating, which is rarely the case in human populations. For example, the equilibrium predicts 4 individuals across Europe homozygous for the p.Ile841SerfsTer119 *CDHR1* variant (AF: 7.19E-05). However, in this study, 25 individuals with CRD secondary to biallelic p.Ile841SerfsTer119 variants are identified, which is a probable manifestation of both a founder effect and inbreeding within sub-populations. The calculations assume that there is no selection pressure caused by *CDHR1* variants (i.e. no

survival benefit in heterozygous individuals). Furthermore, it ignores the genetic diversity of populations within continents. However, despite these limitations, the methodology has been shown to be a reliable estimate of carrier frequency for inherited retinal degenerations.^{23, 217} Moreover, curation and clinical validation of genetic variants allowed for a more precise estimation of genetic prevalence; analogously the true prevalence and carrier frequency of other monogenic inherited retinal degenerations may be greater than previously estimated. For *CDHR1*-associated retinal degeneration, this methodology increased the genetic prevalence estimate from 10,000 to 210,000 affected individuals worldwide.⁴⁵ This estimate may grow further as new pathological alleles are reported.

Three other cadherins expressed in the retina (*CDH23*, *PCDH15* and *CDH3*) which are known to underlie monogenic retinal degeneration exhibit systemic clinical manifestations due to expression of the relevant proteins outside the retina (USH1D, USH1F and hypotrichosis with juvenile macular dystrophy). This study did not identify any consistent systemic associated disease associated with *CDHR1* variants. However, expression of CDHR1 in the olfactory bulb²¹⁸ warrants formal objective investigation of olfactory function in CRD patients, as has been demonstrated in *CNGB1*-associated RP.²¹⁹

7.6 Conclusion

The global genetic prevalence of *CDHR1*-associated retinal degeneration is >200,000 individuals worldwide (1 in ~32,000 individuals), with ~10,000 harbouring biallelic null variants (cone-rod dystrophy phenotype) and ~200,000 with one or more hypomorphic variants (macular dystrophy phenotype). Currently, 115 disease-associated variants have been identified. Cone-rod dystrophy presents at a median age of 20 years with symptoms of rod or cone dysfunction with onset of severe visual impairment at a median age of 41 years. Macular dystrophy presents at a median age of 44 years, most often with reading difficulties and central visual problems, with median age of severe visual impairment at 71 years. The multimodal retinal imaging characteristics of both *CDHR1*-associated CRD and MD are described in detail in this chapter. Individuals with CRD demonstrate progressive, generalised thinning of the macula on OCT imaging, which is more variable in the central 3mm. Those with MD exhibit a more rapid thinning within the central 3mm once disease has started at a later age. The expansion of RPE atrophy in MD follows a sigmoid function with a log phase between the age of 40 and 50 years, followed by a plateau as the degeneration reaches its limit within the macula. These data will support the ethical approval, funding and clinical trial design of *CDHR1* gene therapy.

8. General Discussion

8.1 *CDHR1*-associated retinal degeneration as a target for gene therapy

For a retinal gene therapy investigational medical product (IMP) to progress to phase 1 clinical trial, a number of prerequisites should be satisfied (summarised in Figure 8.1) which incorporate the FDA recommendations on “Human Gene Therapy for Retinal Disorders”, 2018.⁸⁸

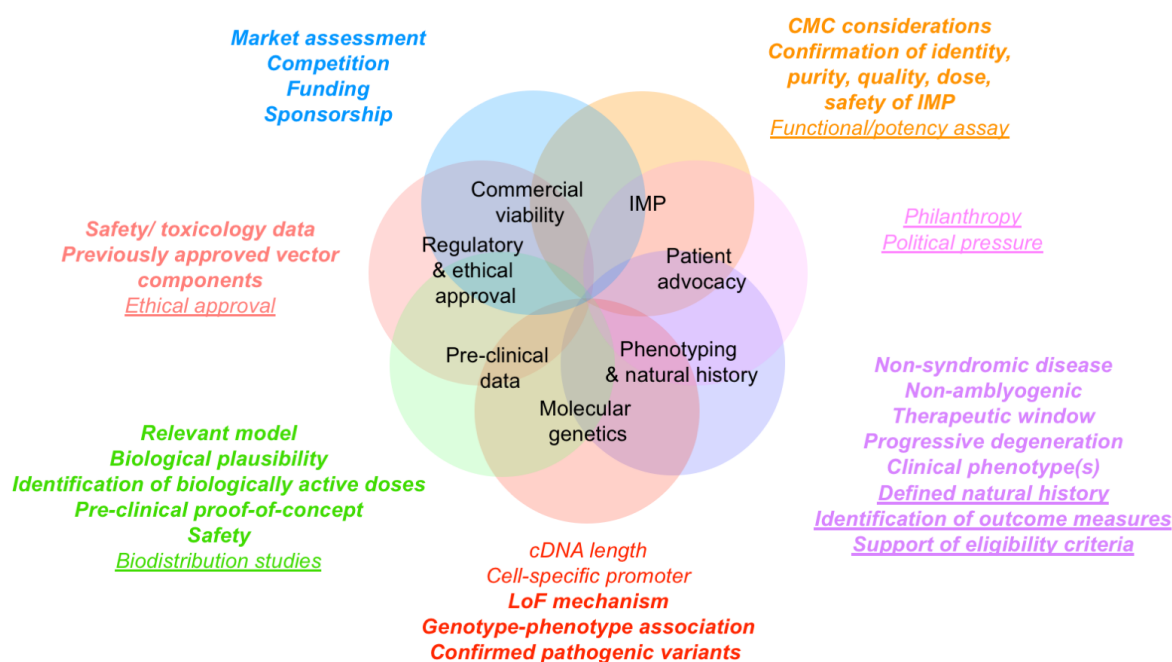


Figure 8.1 Elements supporting the *CDHR1* retinal gene therapy programme

Bold typeface highlights areas supported by data presented in this thesis and linked commercial agreements. Underlined are essential or desirable elements that require further development prior to clinical trial. CMC – chemistry, manufacturing, control; LoF – loss of function; cDNA – coding sequence; IMP – investigational medical product.

For *CDHR1*-associated retinal degeneration, the data presented in this thesis greatly support the case for phase 1 clinical trial by addressing shortfalls of data in key areas (highlighted in Figure 8.1). The *Cdhr1*^{-/-} mouse was validated as a disease model²²⁰ (Chapter 3), before demonstration of the photoreceptor cell specificity of *CDHR1* transgene expression of the lead vector (Chapter 4). The safety and efficacy of the optimised dose (Chapter 5) of AAV8.GRK1.CDHR1.pA (henceforth referred to as the investigational medical product: IMP) is demonstrated in Chapter 6 with demonstration of structural, functional and behavioural rescue in the *Cdhr1*^{-/-} mouse, sustained to at least 21-months post-injection. These data satisfy the FDA criteria of biological plausibility,⁸⁸ in the absence of a direct functional assay (discussed in 8.4.2). Biological active doses of the IMP are formally established in Chapter 5 and further refined in Chapter 6.

Data presented in Chapter 7 systematically describe the clinical phenotypes associated with biallelic *CDHR1* variants, expand the spectrum of disease-associated variants, and identify a genotype-phenotype correlation. Observations appear to confirm a loss-of-function disease mechanism for both CRD and MD, in addition to the non-syndromic nature of *CDHR1*-associated retinal degeneration. The onset of symptoms after the age of visual maturity (~7 years) suggests that amblyopia is unlikely to limit visual outcomes, as it may do in other gene therapy programmes for severe/early onset disease (i.e. *RPE65*, *CNGA3*, *CNGB3*)²²¹. The clinical data presented define the natural history of *CDHR1*-associated CRD and

MD in which a therapeutic window exists for both diseases (described further in section 8.5.2). In CRD, severe visual impairment occurred in all individuals by 65 years of age compared to ~35% of patients with X-linked retinoschisis (XLRS) using the same definitions.²²² The MD cohort were comparable to the XLRS cohort with ~33% exhibiting severe visual impairment by this age. These data greatly support the ethical and regulatory approval for any subsequent clinical trial. However, a prospective natural history study, guided by data in Chapter 7, may further define the short-term natural history of disease, identify suitable outcome measures, and refine eligibility criteria for clinical trials (discussed below).

Since government research organisations (i.e. MRC) generally do not fund clinical trials of AAV gene therapy due to the high cost of production of GMP grade clinical vector, commercial funding and sponsorship is essential. The genetic prevalence estimates presented in Chapter 7 were important in securing commercial sponsorship and licencing of the *CDHR1* clinical trial programme, with an estimated >200,000 affected individuals worldwide.

Timelines for retinal gene therapy programmes from gene discovery to proof-of-concept pre-clinical studies, through to clinical trial and approval are listed for 8 genes in Table 8.1. Severe forms of retinal degeneration with clear phenotypes and inheritance patterns (i.e. X-linked) predominate. Although *RPE65* gene therapy took 16 years from proof-of-concept to marketing approval, the commercial,

manufacturing, regulatory processes and infrastructure are now better developed and rationalised. Ethical and regulatory approvals are required, although expected, prior to phase 1 clinical trial, for reasons outlined in this section.

Gene (Year of discovery)	Pre-clinical proof-of- concept	Phase 1 clinical trial	Phase 3 clinical trial	Approval
<i>RPE65</i> (1997) ^{73, 74}	2001 ⁷⁶	6 years (2007) ²²³	11 years (2012) ¹³⁰	16 years (2017)
<i>CHM</i> (1990) ²²⁴	2013 ¹⁰³	1 year (2014) ¹⁶⁸	6 years (2019)	-
<i>RPGR</i> (1996) ²²⁵	2017 ¹⁰⁴	<1 year (2017) ¹²⁹	-	
<i>RS1</i> (1997) ²²⁶	2004 ²²⁷	9 years (2015) ¹⁷⁵	-	
<i>CNGA3</i> (1998) ²²⁸	2010 ¹⁰⁶	5 years (2015) ²²⁹	-	
<i>CNGB3</i> (2000) ²²⁸	2010 ²³⁰	5 years (2015) ^{np}	-	
<i>MERTK</i> (2000) ²³¹	2001 ²³²	10 years (2011) ²³³	-	
<i>PDE6B</i> (1993) ²³⁴	1996 ⁷¹	21 years (2017) ^{np}	-	

Table 8.1 Timeline of progress in AAV retinal gene therapy programmes

Genes are listed in order of the development of the clinical trials programmes. Where more than one programme exists for a gene, the first to phase 1 clinical trial is presented. Phase 1 clinical trials may commence within a year of publication of pre-clinical data; this may relate to the protection of intellectual property.

Patient advocacy is a further factor which may provide financial and political support for single gene clinical trial programmes (i.e. *RDH12*; <http://www.rdh12.org>) which could be further developed by patients and relatives of individuals with *CDHR1*-associated visual loss.

8.2 Pre-clinical safety and efficacy of *CDHR1* gene therapy

In Chapters 4-6, data are presented outlining the safety and efficacy of *CDHR1* gene therapy in a pre-clinical model of the disease that had been validated in Chapter 3.²²⁰ Table 8.1 lists the relevant pre-clinical proof-of-principle studies of AAV gene therapy vectors for retinal degeneration. In this study, the therapeutic effect sizes were larger on dark-adapted ERG and present at earlier timepoints (2 months) when compared to other equivalent pre-clinical studies (i.e. *RPGR*-related XLRP²³¹). Moreover, the therapeutic benefit was demonstrated to 21-months post-injection; pre-clinical studies typically limit reporting of outcomes to 6 months post-injection (*CHM*¹⁰³, *RPGR*¹⁰⁴). The exceptional durability of therapeutic effect will support onward regulatory approval.

Preservation of outer nuclear layer thickness occurring in combination with lengthening of photoreceptor OS and increased intensity of the EZ on OCT imaging have not been demonstrated for any other pre-clinical AAV gene therapy. These structural findings provide a clear basis for the consecutive functional and behavioural benefits observed in treated versus control eyes. Conventional electron microscopy confirmed regeneration of full-length photoreceptor outer segments. Immunoelectron microscopy is awaited to confirm the ultrastructural localisation of the *CDHR1* transgene in rod and cone photoreceptors outer segments. Improvements in outer segment morphology on electron microscopy have been shown following *RPGRIP1*⁸³ and *PRPH2*⁷² gene therapy. However, *in*

in vivo OCT imaging permitted an assessment of longitudinal morphological changes at the same retinal location from 1 to 18-months post-injection, with early OS regeneration at 1-month which further developed over 12-months, persisting to 18-months post-injection in most subjects. These observations suggest the possibility of an early structural (OCT imaging) and functional signal (i.e. mesopic microperimetry) in human clinical trials at 1-month post-injection. In humans, a slower rate of retinal degeneration over an equivalent time period may result in further beneficial functional effects over time as the outer segments continue to regenerate, without significant retinal degeneration. A comparison of the rate of ONL thinning between eyes may therefore not be significant, or indeed necessary. This reduces the experimental importance of the untreated fellow eye which is discouraged by the FDA due to disease asymmetry and difficulties with masking.⁸⁸ Whilst photoreceptor cell death was not entirely prevented in treated areas in the murine model, further dose refinement (i.e. <0.5 log units between groups) may have overcome this.

Complementing the strong therapeutic signals on structural and functional testing, a clear benefit on visual behaviour was demonstrated in *Cdhr1*^{-/-} mice, to 21-months after delivery of the IMP, using optomotor testing. In the *RPE65* programme, the modified Morris water maze test demonstrated improvements in rod-mediated visual behaviour following gene therapy in *Rpe65*^{-/-} mice,¹³² and the novel multi-luminance mobility test (MLMT) to show improvements in scotopic

visual function in the onward phase 3 clinical trial, supporting regulatory approval.^{88, 130} Analogously, the demonstration of a benefit in photopic and scotopic visual behaviour in pre-clinical studies of *CDHR1* gene therapy further strengthens the case for clinical trial. However, due to the slow nature of *CDHR1*-associated retinal degeneration, the demonstration of a functional visual benefit is not expected in the short-term. However, it should be noted that form vision is eventually lost in *CDHR1*-associated CRD, at which point visual function would fall below that required to complete the MLMT without treatment.

The safety data presented in Chapter 6 excludes retinal toxicity of the therapeutic dose of the IMP in *C57BL/6J* mice on cone and rod function (ERG) and retinal structure (OCT imaging) to 12-months post-injection, and on photopic optomotor testing to 18-months post-injection. A small degree of retinal thinning was seen at 18-months post-injection in AAV injected eyes on multiple comparison testing at superior retinal locations. However, this experimental design does not reproduce any plausible scenario in humans (i.e. to replace *CDHR1* in healthy individuals). Moreover, assessment to 22-months post-injection exceeds the 6-month duration typically reported in pre-clinical gene therapy safety studies.^{103, 104} We anticipate that these comprehensive and robust safety data, generated through standardised techniques, should be acceptable to regulators for phase 1 clinical trial. Furthermore, these data support *CDHR1* gene therapy in patients with MD due to hypomorphic variants (discussed further below).

8.3 Towards a phase 1/2 clinical trial of *CDHR1* gene therapy

Figure 8.1 highlights areas where further work is required to support the clinical trial programme. First in human trials of IMP require regulatory approval with assessment of the pre-clinical data with particular regard to the relevance of disease models²²⁰ (Chapter 3), toxicology (Chapter 5), estimation of dose in humans (8.5.1) in addition to key elements of clinical trial design (discussed further below). Since the constituent elements of the IMP have been used safely in other clinical trials (e.g. wildtype AAV8 capsids, GRK1 promoter), regulatory approval will be simplified,¹²⁹ and satisfy the FDA's condition of "reasonable safety" of the route of administration. Further experiments using larger animal models - naturally occurring canine models of *CDHR1*-associated retinal degeneration have not yet been described⁹⁸ - and non-human primate work should not be necessary. Applications for regulatory and ethical approval will be supported greatly by the pre-clinical safety data and clinical natural history data presented in this thesis.

However, a prospective natural history study to identify and validate outcome measures for clinical trial and the development of a functional/potency assay to assess the biological activity of clinical grade GMP AAV vector prior to clinical use would greatly support the programme.

8.3.1 Prospective *CDHR1* natural history study

For *CDHR1*-associated CRD and MD, a prospective natural history study is warranted to support all phases of onward clinical trials of *CDHR1* gene therapy. Natural history studies are encouraged by the FDA for all clinical development programmes.⁸⁸ A key study objective would be to identify structural and functional outcome measures that are able to detect change over a relatively short period as candidate endpoints for clinical trials (discussed in section 8.5).²³⁵ Tests that are recognised by regulatory bodies are of particular value. Moreover, these natural history data are likely to guide case selection, refine clinical trial protocols, enable power calculations for phase 3 studies, determine test-retest variability in the cohort, and form a reference dataset for future comparison.

The clinical data presented in Chapter 7 will guide the design of the prospective natural history study with proposed outcome measures outlined in Table 8.2. For example, full-field ERG is unlikely to detect differences in clinical trials where vector is delivered locally within the macula by subretinal injection. However, chromatic full-field stimulus threshold (FST) testing²³⁶⁻²³⁸ or mesopic²³⁹ and scotopic²⁴⁰ microperimetry may detect more subtle changes in rod and cone function within the macula which were not assessable in *Cdhr1*^{-/-} mice and for which data were not available in Chapter 7. Microperimetry should focus on change in the central 3mm in both CRD and MD cohorts, both of which demonstrated greater retinal thinning within these regions. Quantitative analysis

of retinal thickness measurements in ETDRS subfields on OCT imaging, and RPE atrophy on FAF (Chapter 7) would determine if quantitative changes can be detected over a short interval and consequently represent discriminating endpoints. Linear modelling of lesion growth rates on FAF imaging has been shown of value in describing the natural history of geographic atrophy secondary to dry AMD, and forms a key endpoint in gene therapy trials for AMD.²⁴¹ Similarly, these may represent key endpoints in trials of retinal gene therapy.

OCT imaging data with longitudinal ONL and OS thickness measurements, ELM and EZ reflectivity and length, and macular volume within the ETDRS sectors would be of particular interest.^{134, 242} Preservation of ONL thickness is an important outcome measure, although the effect may only be detected years later as progressive retinal degeneration occurs in the fellow eye. Colour vision testing²⁴³ would naturally be of interest, given the role of *CDHR1* in the development of cone outer segments; this has not been systematically evaluated. In addition to BCVA data presented in Chapter 6, low luminance visual acuity^{244, 245} and contrast sensitivity testing²⁴⁶⁻²⁴⁸ have been demonstrated as useful markers of central macular function in other forms of retinal degeneration.

The slow nature of retinal degeneration in CRD and MD would necessitate a comparison of outcome measures over a 2 or 3-year study with at least three

timepoints. Additional timepoints may be necessary to determine the test-retest variability of individual tests in each phenotype.

Test	Cone-rod dystrophy	Macular dystrophy
<i>Clinical assessment</i>		
BCVA (ETDRS chart)	✓	✓
Low luminance visual acuity	✓	✓
Contrast sensitivity	✓	✓
Colour vision testing	✓	✓
Clinical assessment and fundus examination	✓	✓
<i>Retinal imaging</i>		
Fundus autofluorescence:		
• SW-AF (area of absolute loss of AF)	✓	✓
• NIR-AF	✓	✓
Optical coherence tomography:		
• ONL thickness	✓	✓
• EZ reflectivity and length	✓	✓
• ELM reflectivity and length	✓	✓
• Outer segment length	✓	✓
• Macular volume, ETDRS gird	✓	✓
<i>Functional testing</i>		
Goldmann kinetic perimetry (V4e isopter)	✓	
Microperimetry		
• Mesopic	✓	✓
• Scotopic	✓	✓
Full-field stimulus threshold (FST)	✓	✓
<i>Patient reported visual function</i>		
Visual function (NEI-VFQ-25)	✓	✓

Table 8.2 Proposed outcome measures for a prospective *CDHR1* natural history study

Correlation between structural and functional outcome measures, particularly within the macula would be of interest. National Eye Institute's Visual Function Questionnaire; NEI-VFQ25.

8.3.2 Development of a functional assay for AAV8.CDHR1 GMP vector

Phase 3 clinical trials of gene therapy products ideally require a biological, quantitative *in vitro* assay to confirm the activity and potency of GMP clinical grade AAV vector production batches prior to surgical delivery, as specified by the FDA.^{86, 88} The development of a functional assay for the *RPE65* gene therapy programme was more straightforward since the gene encodes a well characterised isomerase catalysing the conversion of all-*trans* retinyl esters into 11-*cis* retinal, which may be exploited for this purpose.^{249, 250} For structural proteins, the identification of a functional assay is more challenging since their activity is more difficult to define and quantify. Moreover, for genes driven by a photoreceptor-specific promoter, an ideal cell line has not been identified for *in vitro* testing; Y79 or WERI cells do not highly express GRK1-driven transgenes.¹⁵³ Primary photoreceptors cultured *ex vivo* cannot be used for transduction. These hurdles are common to any gene therapy programme involving structural photoreceptor proteins (i.e. *CDHR1*, *RPGR*, *PROM1*, *PRPH2*).

The mode of action of the AAV8.CDHR1 IMP is not well characterized. The pursuit of a functional assay for the *CDHR1* programme may benefit from the more detailed characterisation the protein interactions of *CDHR1* within the retina. The identity of the inner segment binding partner and the enzyme catalysing the cleavage of the *CDHR1* ectodomains, for example, may yield an opportunity for the development of a functional assay. Direct confirmation of ADAM10 as the

catalyst for the cleavage of the CDHR1 ectodomain is required. A functional *in vitro* assay will most likely necessitate the development of a non-photoreceptor cell line in which an endogenous promoter (i.e. cone-rod homeobox (CRX), or other) enables the efficient expression of GRK1-driven transgenes.

Table 8.3 specifies assays that are optimised, or require further development for the CDHR1 programme as specified by the FDA.^{86, 88} A direct, biological quantitative effect may be demonstrable on ERG 2-months post-injection and on OCT imaging (i.e. OS regeneration). However, the FDA discourage *in vivo* potency assays and more than 20 mice may be required. Indirect (non-biological) *in vivo* confirmation of potency of GMP-grade IMP can be confirmed in *Cdhr1*^{-/-} mice with cellular localisation and protein expression using immunohistochemistry.

Assay		CDHR1 programme
Biological	<i>In vitro</i> (organ, tissue, cells)	<u>Novel cell line expressing GRK1-driven transgenes</u>
	<i>In vivo</i>	ERG & OCT
	<i>Ex vivo</i>	-
Non-Biological	Immunochemical	Immunohistochemistry
	Biochemical	<u>Western blot, MS, qPCR</u>
	Molecular attributes	<u>Protein interactions, post-translational modifications</u>
Multiple assays	Combination of above	Combination of above

Table 8.3 FDA-specified analytic methods to measure vector potency

The aim of an assay, or combination of assays, is to determine the quality, consistency and stability of AAV production lots. At least one assay must be quantitative. Bold type: assays characterised in this thesis. Underlined font: assays that require further development. MS – mass spectrometry; qPCR – quantitative PCR.

Three non-biological assays may be developed further to support the programme: *in vivo* expression of CDHR1 may be quantified using RT-PCR, and confirmation of full length CDHR1 may be achieved through mass spectrometry (allowing sequencing) or western blotting (both non-biological). All require *in vivo* delivery in *Cdhr1*^{-/-} mice. Attempts at western blot against the human CDHR1 isoform were unsuccessful using all commercially available primary anti-CDHR1 antibodies. Confirming interactions with known and unknown binding partners (PROM1, unidentified inner segment binding partner, and unconfirmed catalyst), and other post-translational modifications beyond cleavage of the ectodomain may provide further indirect evidence of biological activity (i.e. molecular attributes). For the CDHR1 programme, it is likely that a multiple assay matrix will be required to determine the potency of GMP-grade AAV vector, awaiting a suitable cell line.

8.4 Phase 1/2 clinical trial of *CDHR1* gene therapy

This section considers the design of a proposed phase 1/2 clinical trial exploring dose-escalation, patient selection, and outcomes measures. The proposals are made in reference to FDA guidance.⁸⁶⁻⁸⁸ Dose-escalation may be a non-randomized, open-label study with a dose-expansion phase randomised and masked.

8.4.1 Proposed dose-escalation for phase 1/2 clinical trial for CRD

Although allometric scaling has not been formally quantified between the murine and human eye in the context of retinal gene therapy, a proposed dosing range for a phase 1/2 clinical trial can be approximated from pre-clinical retinal gene therapy studies and phase 1 clinical trials for other programmes.

Transgenes expressed in rod and cone photoreceptors driven by the GRK1 promoter are most relevant (e.g. *RPGR*). In published pre-clinical studies of *RPGR* gene therapy, doses of $2\text{--}4 \times 10^9$ vg are generally therapeutic in relevant murine models. However, pre-clinical dose-response data are not reported; lower or higher doses may have otherwise shown benefit.^{104, 251, 252} The therapeutic dose of AAV8.CDHR1 is ~1 log unit lower than that shown to be beneficial in *RPGR* gene therapy studies in murine models (Table 8.4).

	Pre-clinical		Clinical: Phase 1/2	
Vector	Murine	Canine	Dosing range	Optimum dose
AAV8.coRPGR ^{104, 129} (<i>NightStaRx/Biogen</i>)	2×10^9	-	2×10^9 – 4×10^{11}	5×10^{10}
AAV5.RPGR (<i>MeiraGTx</i>)	Not reported	-	Not reported	Not reported
rAAV2tYF-GRK1- hRPGRco ^{253, 254} (<i>AGTC</i>)	4×10^8 – 4×10^9	1.2×10^{10} – 3.0×10^{12} 6×10^{11}	Not reported	Not reported
AAV8.CDHR1	1.5×10^8	-	Proposed range: 1×10^8 – 5×10^{10}	

Table 8.4 Dosing of subretinal gene therapy targeting photoreceptors
Comparisons are made for AAV vector constructs driven by the GRK1 promoter. Doses are expressed in total quantity of vector delivered (vg). Doses in bold are the optimum doses where formal dose-toxicity studies were undertaken.

In a phase 1/2 study of *RPGR* gene therapy, a vector dose of $\sim 5 \times 10^{10}$ vg showed greatest benefit.¹²⁹ For *CDHR1* gene therapy, data presented in this thesis suggest a starting dose of approximately 1×10^8 vg (Table 8.5)⁸⁸, with 0.5 log unit dose increments to 5×10^{10} vg as tolerated. This study design would be consistent with the FDA's recommendation of plausible benefit in all dosing groups.⁸⁸ Individuals with advanced CRD may be enrolled into the lower dose groups (1×10^8 and 5×10^8 vg), with those with early degeneration into the subsequent four dosing groups. Additional individuals may be treated at the most likely optimum therapeutic dose as required (dose confirmation; equivalent to experiments in Chapter 5). Assuming the safety and efficacy endpoints are met, a phase 3 trial may investigate the efficacy of the IMP in a larger cohort at the two most efficacious doses (equivalent to experiments in Chapter 6).

Cohort	<i>n</i>	Target dose (vg)	Patient characteristics
1	3	1×10^8	Advanced degeneration
2	3	5×10^8	Advanced degeneration
3	3	1×10^9	Early disease
4	3	5×10^9	Early disease
5	3	1×10^{10}	Early disease
6	3	5×10^{10}	Early disease

Table 8.5 Proposed dose-escalation for a Phase 1/2 clinical trial for *CDHR1*-associated CRD

Up to six cohorts of 3 individuals (a maximum of 18 in total) receive an increasing dose of AAV8.GRK1.CDHR1.pA between 1×10^8 and 5×10^{10} vg with 100 μ l blebs.

8.4.2 Proposed dose-escalation for phase 1/2 clinical trial for MD

A phase 1/2 clinical trial for individuals with *CDHR1*-associated MD may be combined with the CRD trial, with two-stages, allowing safety data from the CRD cohort to refine the dose-escalation range for the MD cohort (Figure 8.2). The FDA, in general, support the inclusion of a broader patient population if early trials show safety.⁸⁸ However, it remains to be determined whether safety data from the phase 1/2 clinical trial of CRD together with pre-clinical safety data (Chapters 5&6) are sufficient for regulatory approval for the MD cohort. Individuals heterozygous for variants associated with *CDHR1*-related MD do not exhibit disease, excluding a dominant negative disease mechanism. Moreover, subretinal delivery of a therapeutic dose of the IMP in *C57BL/6J* mice with two wildtype *Cdhr1* alleles is safe, indicating that overexpression of CDHR1 is tolerated in mammalian photoreceptors (Chapter 6). It should be noted that Luxturna® (*RPE65* gene therapy) has been applied to individuals with hypomorphic missense variants²⁵⁵ without any reported differences in safety and efficacy.

A lower dose of the IMP may be required to achieve a therapeutic effect in the context of presumed *CDHR1* variants associated with MD since it is assumed that mutant transcripts are partially functional. The dosing range for a phase 1/2 clinical trial may be refined by combining phase 1/2 data from CRD patients with data presented in Chapter 5 suggesting that retinal toxicity may occur at a ~0.5 log unit lower dose in *C57BL/6J* mice when compared to *Cdhr1*^{-/-} mice. Taken

together, these observations, support a phase 1/2 clinical trial of gene replacement for *CDHR1*-related MD.

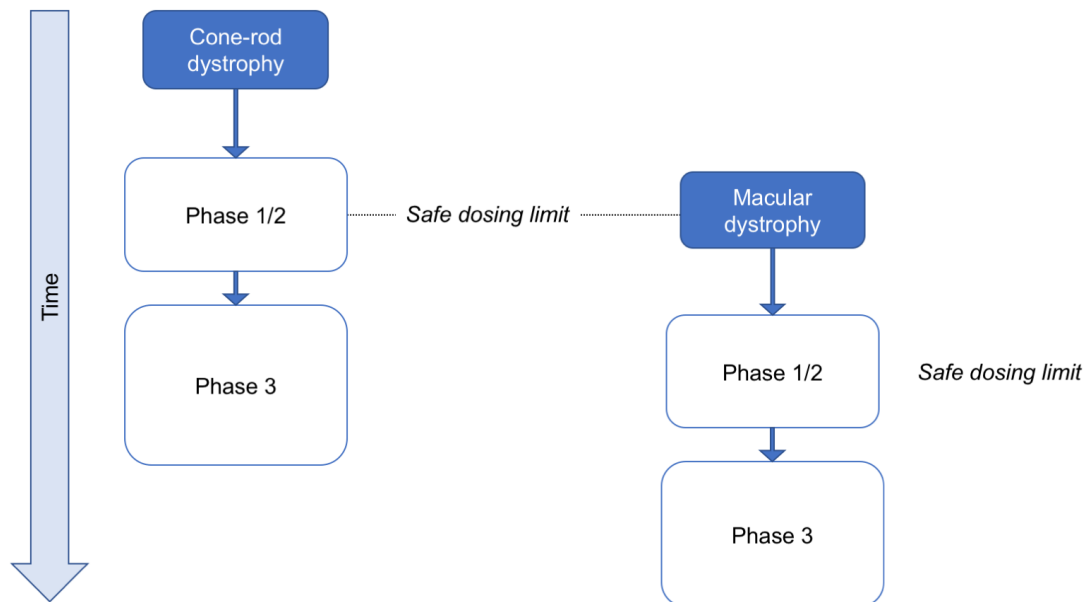


Figure 8.2 Proposed design of phase 1/2 clinical trial of *CDHR1* gene therapy

A staggered trial design is proposed where the safe dosing range in the CRD cohort is used to refine the dose-escalation range for the MD cohort.

8.4.3 Patient selection for phase 1/2 clinical trial of *CDHR1* gene therapy

In Chapter 6, *CDHR1* gene therapy slowed photoreceptor cell death with structural and functional recovery of surviving, dysfunctional rod and cone photoreceptors. Data presented in Chapter 7 show progressive retinal thinning associated with a reduction in visual acuity, suggesting early intervention when a greater number of target cells are available for transduction. Natural history data presented in this thesis will support the selection of patients for clinical trial.

Table 8.6 outlines proposed general inclusion criteria, typical for phase 1/2 retinal gene therapy clinical trials. Additional inclusion (BCVA or baseline microperimetry) and exclusion criteria (i.e. ocular comorbidities) relating to the study eye may be determined at a later date. The sections below identify ideal candidates with CRD and MD for phase 1/2 clinical trial. In general, 100µl of vector delivered in 2-3 blebs targeting the central 3mm with superior, inferior and temporal retinotomies, avoiding the papillomacular bundle, would be suited to both CRD and MD groups based on quantitative OCT data (Chapter 7).

Inclusion criteria:
<i>Clinical diagnosis of either CRD or MD attributed to biallelic variants in CDHR1</i>
<i>Age >18 years</i>
<i>Capacity to consent for study</i>
<i>Able to comply with treatment and follow-up</i>
Exclusion criteria:
<i>Pregnancy or lactation</i>
<i>Immunocompromise</i>
<i>Co-morbidity that increases risk of systemic immunosuppression (i.e. TB, HIV).</i>
<i>Amblyopia in either eye</i>
<i>Previous participation in interventional clinical trial in the past 12 weeks, or previous recipient of gene or cell-based therapy</i>
<i>Any other pathology that affects visual function or prevents the assessment of treatment effects</i>

Table 8.6 Proposed patient selection criteria for phase 1/2 clinical trial
Additional exclusion and inclusion criteria relating to the study eye (baseline BCVA, microperimetry sensitivity etc.) will be determined at a later date.

8.4.3.1 *Cone-rod dystrophy*

Individuals with CRD exhibit generalised retinal thinning which is most apparent in the central 3mm, overall and on longitudinal analysis. This observation appears consistent with severe, generalised depression of retinal sensitivity on microperimetry from a single individual (Figure 8.3). Improvements in retinal function following gene therapy will fall within the dynamic range of mesopic microperimetry (i.e. not limited by a floor effect). Gene therapy should target this region in addition to adjacent areas within the macula. Treatment at the earliest possible timepoint is recommended in order to preserve remaining rod and the cone photoreceptors. Figure 8.3 identifies an ideal candidate with CRD in whom ONL and EZ are evident within the macula on OCT imaging.

8.4.3.2 *Macular dystrophy*

In *CDHR1*-associated MD, thinning of the retina occurs in the central 3mm in early disease, correlating with loss of retinal sensitivity on scotopic microperimetry (Figure 8.3). This is followed by the development of RPE atrophy in the parafovea which, once established, increases in size according to a log function culminating in RPE and outer retinal atrophy with subsequent loss of the fovea. Since visual acuity is preserved until late in the disease, clinical trials should not exclude individuals with MD who retain an excellent BCVA. Since foveal cones are not lost until late, avoidance of foveal detachment may be advised.

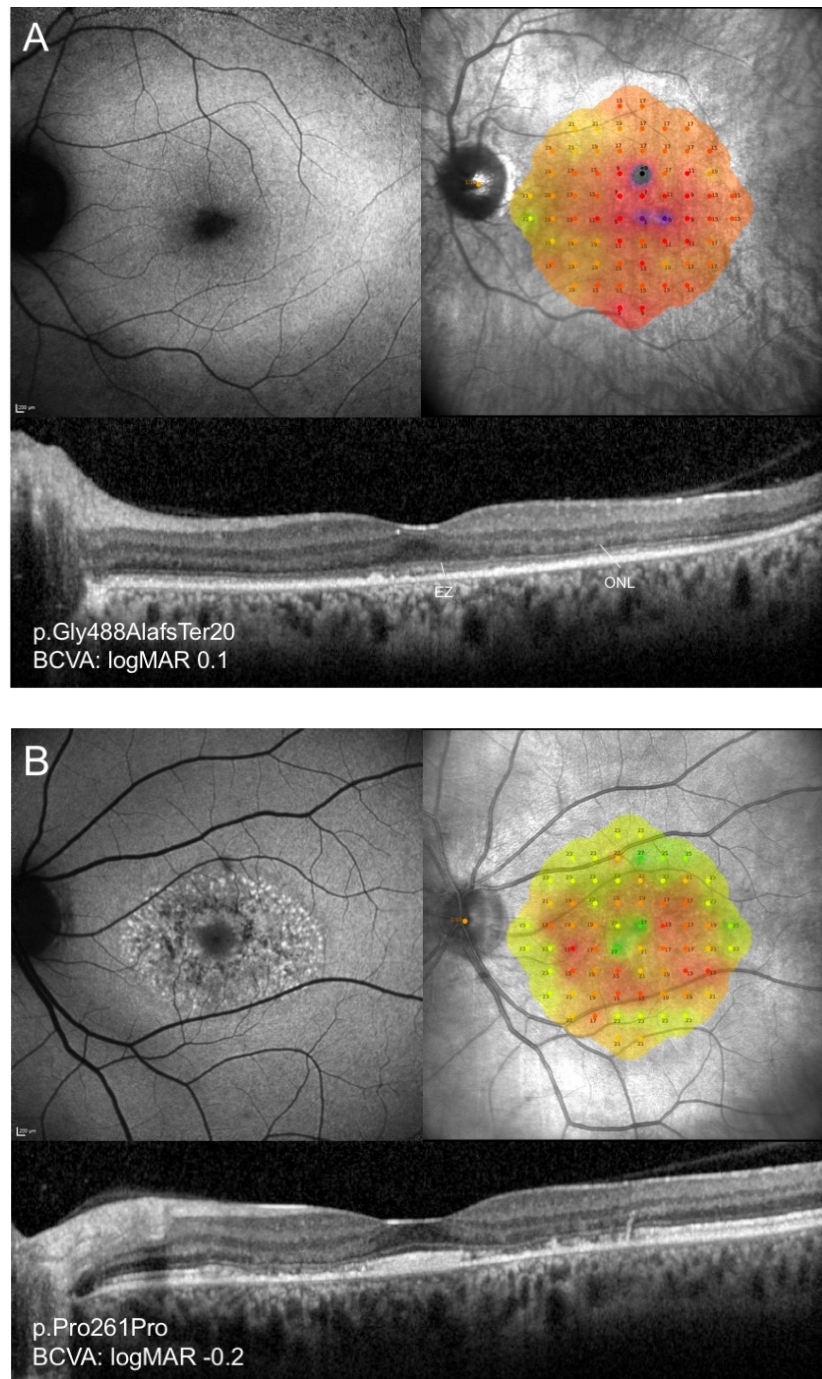


Figure 8.3 Ideal candidates for a phase 1/2 clinical trial of *CDHR1* gene therapy

(A) A 24-year-old female with *CDHR1*-associated CRD, homozygous for p.Gly488AlafsTer20. Generalised depression of retinal sensitivity is seen on microperimetry, most apparent centrally. **(B)** A 47-year-old female with *CDHR1*-associated MD, homozygous for the c.783G>A p.Pro261Pro variant. Retinal sensitivity is reduced between 1 and 3mm eccentricity, with preservation of foveal sensitivity and BCVA. These observations are consistent with OCT imaging data.

CDHR1-gene therapy may prevent photoreceptor cell death, and subsequent RPE atrophy which is presumed to occur through a secondary mechanism. Accordingly, treatment prior to the onset RPE death is recommended since the natural history of RPE loss is of progressive enlargement regardless of aetiology (i.e. degenerative, infectious, toxic). Intervention may slow down or arrest the expansion of RPE loss by preventing photoreceptor cell death. Transduction of the central 3mm appears an important goal of gene therapy surgery in order to prevent further retinal thinning and preserve and/or recover retinal sensitivity in this area (Figure 8.3). Areas of RPE loss, once developed, will form absolute scotomas on microperimetry.

8.4.4 Outcome measures for phase 1/2 clinical trial

Although establishing a safe dosing range is the primary aim of a phase 1 clinical trial, the FDA strongly recommend that study endpoints assess potential clinical benefit.⁸⁸ This is due to the relatively small number of eligible patients, enabling more efficient progression to phase 2 (identification of the optimum dose(s)) and phase 3 (determination of efficacy) clinical trials. The FDA suggest that marketing applications should include evidence of a clinically significant benefit, such as an improvement in visual function (i.e. visual acuity measured by ETDRS chart), reduction in symptoms, or structural evidence of reduced rate of photoreceptor loss on OCT or FAF imaging. Consequently, assessment of these endpoints is necessary in phase 1/2 clinical trials, as proposed in Table 8.7.

Outcome measures
<i>Possible primary outcome measures:</i>
Incidence of dose limiting toxicities (DLTs)
Proportion of patients with a DLT
Incidence of treatment emergent adverse events (TEAEs)
Proportion of patients with a TEAE
<i>Secondary outcome measures: changes from baseline in treated vs. control eyes</i>
Best-corrected visual acuity (ETDRS)
Retinal sensitivity on Microperimetry
Mesopic Microperimetry (dB)
Scotopic Microperimetry (dB)
SD-OCT imaging: - ONL thickness - OS length - EZ intensity and length - ELM intensity and length - Macular volume
Fundus autofluorescence (AF) imaging: - Change in area of RPE loss
*Low luminance visual acuity (LLVA) testing
*Contrast sensitivity testing
*Colour vision testing
*Visual Field testing: Kinetic perimetry
*Full-field stimulus threshold testing
*Reading speed

Table 8.7 Proposed outcome measures for Phase 1/2 clinical trial

Outcomes will be assessed over a 24-month period. DLT - dose-limiting toxicities; TEAE - Treatment emergent adverse effects; dB – decibels. *Possible exploratory endpoints.

In addition, measurement of systemic cellular and humoral immune responses to the AAV8 capsid and *CDHR1* transgene may be undertaken.⁸⁸ Systemic peri-operative administration of oral corticosteroids is suggested for all patients, as previously described,¹⁹⁰ with supplementary periocular steroids as necessary.¹²⁹

8.5 Patent filing, commercialisation and vector production

The cadherins are a superfamily of cell adhesion molecules, widely expressed in living organisms. The experimental data outlined in this thesis are the first demonstration of a molecular therapy that has successfully ameliorated a disease phenotype caused by perturbations of a cadherin *in vivo*.

A UK priority patent application to protect the intellectual property generated in this DPhil thesis was filed on behalf of the University of Oxford by JA Kemp LLP for the vectors described herein. Commercial agreements have been obtained to licence the IMP towards a phase 1 clinical trial for *CDHR1* programme, with the hope of marketing the IMP as a treatment for *CDHR1*-associated retinal degeneration in the future. Plasmids have been supplied under MTA, and the sponsor has produced 6mls high-grade AAV vector for validation achieving a titre of 9.2×10^{12} vg/ml with a full:empty capsid ratio of approximately 79% (data from commercial partner).

The international *CDHR1* study has identified >150 individuals to date with retinal degeneration who may be considered for the onward phase 1/2 clinical trial for patients with currently untreatable cone-rod and macular dystrophies. The global burden of *CDHR1*-associated retinal degeneration has been estimated. Although further natural history data may be required in parallel, the data presented in this thesis has significantly advanced the field in understanding

CDHR1-associated retinal degeneration, and present clear evidence of efficacy of the first molecular therapy aimed to target the disease *in vivo*. Ultimately, we hope that the data presented herein will translate swiftly into clinical trial for the benefit of all patients around with world with *CDHR1*-associated retinal degeneration.

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Appendix

A.1 Publications

At the time of submission, the following publications have resulted from research presented in this thesis:

Yusuf IH, McClements ME, MacLaren RE, Charbel Issa P. “Deep phenotyping of the *Cdhr1*^{-/-} mouse validates its use in pre-clinical studies for human *CDHR1*-associated retinal degeneration” *Exp Eye Res*, Jul;208:108603. doi: 10.1016/j.exer.2021.108603. Epub 2021 May 6. Online ahead of print. PMID: 33964272.

Yusuf IH, Garrett A, Charbel Issa P, MacLaren RE. “Retinal cadherins and the retinal cadherinopathies: Current concepts and future directions” *Progress in Retinal Eye Research*, 2022, Jan 20;101038. doi: 10.1016/j.preteyeres.2021.101038. PMID: 35066146

Charbel Issa P, Gliem M, **Yusuf, IH**, Birtel J, Muller PL, Mangold E, Bolz HJ. “A Specific Macula-Predominant Retinal Phenotype Is Associated With the CDHR1 Variant c.783G>A, a Silent Mutation Leading to In-Frame Exon Skipping” *IOVS*, 2019 Aug 1;60(10):3388-3397. doi: 10.1167/iovs.18-26415. PMID: 31387115.

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A.3 *CDHR1* variants associated with retinal degeneration

<i>CDHR1</i> variant	Protein consequence	Genomic co-ordinate dbSNP	Mean allele frequency	Pheno-type	Initial report
c.1A>G	p.Met1Val Start loss	10-85954517 rs794726954	0.00002024	CRD	Ba-Abbad <i>et al</i> , 2020 ⁵⁵
c.1A>T	p.Met1Leu Start loss	10-85954517 rs794726954	0.000008556	CRD	<u>Novel</u>
c.18G>A	p.Trp6Ter	10-85954534 rs1220602138	0.00001651	-	Charbel Issa <i>et al</i> , 2019 ⁵⁶
c.56-1G>A	Splice acceptor	10-85955249 rs751972593	0.00001198	CRD	Stingl <i>et al</i> , 2017 ⁵⁷
c.143C>A	p.Thr48Asn	10-85955337 rs201698218	0.0003826	MD	<u>Novel</u>
c.152-2A>G	Splice acceptor	10-85956259	NP	CRD	Bessette <i>et al</i> , 2017 ⁵⁸
c.296A>G	p.Glu99Gly	10-85956402	NP	CD	Stingl <i>et al</i> , 2017 ⁵⁷
c.297+1G>T	Splice donor	10-85956407 rs1464226905	0.000007953	MD	Ba-Abbad <i>et al</i> , 2020 ⁵⁵
c.338del	p.Gly113Alafs Ter2	10-85957580 rs747425652	0.000003979	CRD	Henderson <i>et al</i> , 2010 ⁴⁹
c.348+5G>T	Splice region	10-85957597	NP	-	<u>Novel</u>
c.349-3C>G	Splice region	10-85958785	NP	-	<u>Novel</u>
c.379G>A	p.Asp127Asn	10-85958818 rs1189565760	0.000006386	-	<u>Novel</u>
c.386A>G	p.Asn129Ser	10-85958825 rs1424495418	0.00001277	MD	Oishi <i>et al</i> , 2016 ⁵⁹
c.398C>G	p.Pro133Arg	10-85958837	NP	-	Tiwari <i>et al</i> , 2016 ⁶⁰
c.438+1G>A	Splice donor	10-85958874	NP	-	Charbel Issa <i>et al</i> , 2019 ⁵⁶
c.439-17G>A	Intron	10-85960340 rs771510913	0.000008672	-	Tiwari <i>et al</i> , 2016 ⁶⁰
c.476C>A	p.Ala159Glu	10-85960394	NP	-	<u>Novel</u>
c.523C>T	p.Gln175Ter	10-85960441 rs781781440	0.000004204	-	<u>Novel</u>

CDHR1 variant	Protein consequence	Genomic co-ordinate dbSNP	Mean allele frequency	Pheno-type	Initial report
c.524dup	p.Asn176GlufsTer48	10-85960441 rs781781440	0.00001114	CRD	Ostgergaar d <i>et al</i> , 2010 ⁵⁰
c.562G>A	p.Gly188Ser	10-85961599 rs748412274	0.00006820	MD	Ba-Abbad <i>et al</i> , 2020 ⁵⁵
c.601G>A	p.Glu201Lys	10-85961638 rs200646223	0.0001592	Atypical	Maeda <i>et al</i> , 2018 ²¹⁴
c.639+1del	Splice donor	10-85961675 rs745932099	0.000004173	-	Stingl <i>et al</i> , 2017 ⁵⁷
c.689C>T	p.Thr230Ile	10-85962785 rs141173656	0.0003465	MD	<u>Novel</u>
c.700G>A	p.Val234Ile	10-85962796 rs148356887	0.0002227	-	Maeda <i>et al</i> , 2018 ²¹⁴
c.783G>A	p.Pro261=	10-85962879 rs147346345	0.003052	MD	Glöckle <i>et al</i> , 2014 & Charbel Issa <i>et al</i> , 2019 ^{56, 61}
c.783+5G>T	Splice region	10-85962884	NP	-	<u>Novel</u>
c.838C>T	p.Arg280Ter	10-85964337 rs191858417	0.00001988	CRD	Boulanger-Scemama <i>et al</i> , 2015 ⁶²
c.862+4A>C	Splice region	10-85964365 rs1352559639	0.000007954	-	<u>Novel</u>
c.866_872delinsTTTGA AATTAATGAC	p.Asn289IlefsTer2	10-85965586	NP	-	<u>Novel</u>
c.928C>T	p.Glu310Ter	10-85965648	NP	-	Stingl <i>et al</i> , 2017 ⁵⁷
c.1058T>C	p.Phe353Ser	10-85968024 rs771316043	0.00001193	RP	<u>Novel</u>
c.1117C>T	p.Gln373Ter	10-85968082	NP	CRD	<u>Novel</u>
c.1135G>A	p.Gly379Ser	10-85968101 rs572616777	0.00001991	CRD	<u>Novel</u>
c.1205C>A	p.Pro402His	10-85968522	NP	-	<u>Novel</u>
c.1219C>T	p.Arg407Ter	10-85968536 rs373978786	0.000007071	-	<u>Novel</u>

CDHR1 variant	Protein consequence	Genomic co-ordinate dbSNP	Mean allele frequency	Pheno-type	Initial report
c.1254del	p.Gln418Glnfs Ter20	10-85968571	NP	-	<u>Novel</u>
c.1311_1316 del	p.Leu437_Thr 438del	10-85968625 rs1257781536	0.000003983	-	Stingl <i>et al</i> , 2017 ⁵⁷
c.1373T>A	p.Val458Asp	10-85970809 rs760942217	0.000003976	MD	Ba-Abbad <i>et al</i> , 2020 ⁵⁵
c.1381C>T	p.Gln461Ter	10-85970817	NP	CRD	Duncan <i>et al</i> , 2012 ⁶³
c.1383_1388 del	p.Leu462_Leu 463del	10-85970819	NP	CRD	<u>Novel</u>
c.1408C>T	p.Pro470Ser	10-85970844 rs779854349	0.000007953	-	<u>Novel</u>
c.1448A>G	p.Glu483Gly	10-85970879	NP	CRD	Stingl <i>et al</i> , 2017 ⁵⁷
c.1458_1462 del	p.Pro485Profs Ter48	10-85970889	NP	CRD	<u>Novel</u>
c.1463del	p.Gly488fsTer 20	10-85970894 rs756678484	0.00005966	CRD	Ba-Abbad <i>et al</i> , 2013 ⁶⁴
c.1485+2T>G	Splice donor site Linking of exons 12-14	10-85970923 rs767366723	0.000003980	CRD	Cohen <i>et al</i> , 2012 ⁶⁵
c.1485+2T>C	Splice donor site	10-85970923 rs767366723	0.000007959	CRD	Nikopoulos <i>et al</i> , 2015 ⁶⁶
c.1503_1507 del	p.Gly502Leufs Ter32	10-85971418 rs1266986282	0.00001061	CRD	Charbel Issa <i>et al</i> , 2019 ⁵⁶
c.1527T>G	p.Tyr509Ter	10-85971445 rs1477733493	0.000003976	CRD	Arno <i>et al</i> , 2016 ⁶⁷
c.1553+1del	Splice donor	10-85971472	NP	-	<u>Novel</u>
c.1554-2A>C	Splice acceptor Exon 15 skipping	10-85971933	NP	-	Riera <i>et al</i> , 2017 ⁶⁸
c.1570_1592 del	p.Ser524Alafs Ter4	10-85971944 rs751597954	0.000003976	CRD	Charbel Issa <i>et al</i> , 2019 ⁵⁶
c.1576G>A	p.Gly526Arg	10-85971950	NP	CRD	<u>Novel</u>
c.1700T>C	p.Leu567Pro	10-85972764	NP	RP	Stingl <i>et al</i> , 2017 ⁵⁷

<i>CDHR1</i> variant	Protein consequence	Genomic co- ordinate dbSNP	Mean allele frequency	Pheno- type	Initial report
c.1720C>G	p.Pro574Ala	10-85972784	NP	CRD	Nikopoulos <i>et al</i> , 2015 ⁶⁶
c.1868dup	p.Asn623Lysfs Ter53	10-85972932	NP	-	<u>Novel</u>
c.2027T>A	p.Ile676Asn	10-85973091 rs186486854	0.0003290	-	Maeda <i>et al</i> , 2018 ²¹⁴
c.2040 + 5 G>T	Splice donor site	10-85973109 rs1446751435	0.000003984	-	Stingl <i>et al</i> , 2017 ⁵⁷
c.2087_2090 del	p.Asp696Alafs Ter3	10-85973884 rs1589313739	NP	CRD	Beryozkin <i>et al</i> , 2015 ²⁵⁶
c.2108G>A	p.Gly703Asp	10-85973905	NP	-	Stingl <i>et al</i> , 2017 ⁵⁷
c.2175G>A	p.Trp725Ter	10-85973972	0.000003981	CRD	<u>Novel</u>
c.2197G>T	p.Val733Phe	10-85973994	NP	RP	<u>Novel</u>
c.2203C>A	p.Pro735Thr	10-85974000 rs780447091	0.000007975	-	Ba-Abbad <i>et al</i> , 2020 ⁵⁵
c.2522_2528 delTCTCTG A	p.Ile841SerfsT er119	10-85974312 rs1429453310	0.00003313	CRD	Arno <i>et al</i> , 2016 ⁶⁷

Variants previously reported in the literature with adequate phenotyping data are quoted. Variants reported in databases (i.e. ClinVar) but without clinical verification are not listed.

A.4 Sequence conservation of novel missense variants in *CDHR1*

p.Thr48Asn

species	match	gene	aa	alignment
Human			48	GNMALFSLPEDTPVGSHVYTLNGT
mutated	not conserved		48	GNMALFSLPEDN ^N PVGSHVYTLNG
Ptroglyodytes	all identical	ENSPTRG00000002693	48	GNMALFSLPED ^A PVGSHVYTLNG
Mmulatta	all identical	ENSMUG00000000658	48	GNMALFSLPED ^A PVGSHVYTLNG
Fcatus	all identical	ENSFCAG00000001649	29	GNMALFSLPED ^A PV
Mmusculus	all identical	ENSMUSG000000021803	48	GNMALFSLPED ^A PVGSHVYTLNG
Ggallus	all identical	ENSGALG00000002314	50	GNMALLSLSEDA ^A PVGSHVYTLNG
Trubripes	all identical	ENSTRUG00000011689	48	GNMALFSISEDA ^A AVGTHVYVLNG

p.Asp127Asn

species	match	gene	aa	alignment
Human			127	LVAEKVVILVTDANDEAPR F IQEP
mutated	all conserved		127	VVILVTD ^N ANDEAPR F IQE
Ptroglyodytes	all identical	ENSPTRG00000002693	127	VVILVTD ^D ANDEAPR F IQE
Mmulatta	all identical	ENSMUG00000000658	127	VVILVTD ^D ANDEAPR F IQE
Fcatus	all identical	ENSFCAG00000001649	108	VAEKVTILVTD ^D ANDEAPS F IQE
Mmusculus	all identical	ENSMUSG000000021803	127	VVILVTD ^D ANDEAPR F IQE
Ggallus	all identical	ENSGALG00000002314	129	TVSEKVRILVMD ^D ANDESPE F INT
Trubripes	all identical	ENSTRUG00000011689	127	D ^D ANDEAPK F QNL

p.Ala159Glu

species	match	gene	aa	alignment
Human			159	IPAGSIIIFKVHAVDRDTGSGGSVT
mutated	not conserved		159	IPAGSIIIFKVHE ^E VDRDTGSGGSV
Ptroglyodytes	all identical	ENSPTRG00000002693	159	IPAGSIIIFKVHA ^A VDRDTGSGGSV
Mmulatta	all identical	ENSMUG00000000658	159	IPAGSSIFKVHA ^A VDRDTGSGGSV
Fcatus	not conserved	ENSFCAG00000001649	140	XXXXXXXXXXXXXXXXXXXXXXXXXXXX
Mmusculus	all identical	ENSMUSG000000021803	159	IPAGSSIFKVQA ^A EDKDTGSGGSV
Ggallus	all identical	ENSGALG00000002314	161	SPSGSSIFKIEA ^A VDRDTGSGGSI
Trubripes	all identical	ENSTRUG00000011689	159	AAAESSIYRVQA ^A VDKDMGSGGSV
Drerio	no homologue			
Dmelanogaster	all identical	FBgn0261053	277	AKRGTELDYVIA ^A RKNPLFQK---
Celegans	not conserved	Y71D11A.1	1469	LGEGEAIGTISV----TGSTSPA
Xtropicalis	no homologue			

p.Thr230Ile

species	match	gene	aa	alignment
Human			230	LHGADVVFSA T TTVTNVNVEDVQDM
mutated	not conserved		230	LHGADVVFSA I TTVTNVNVEDVQ
Ptrogodytes	all identical	ENSPTRG00000002693	230	LHGADVVFSA T TTVTNVNVEDVQ
Mmulatta	all conserved	ENSMUG00000000658	230	LHGADVVGPC S TATQRPDSFL
Fcatus	not conserved	ENSFCAG00000001649	211	XXXXXXXXXXXX X XXXXX
Mmusculus	all identical	ENSMUSG000000021803	230	LRGADMVFSA T TTVTINVEDV
Ggallus	all identical	ENSGALG00000002314	233	LRGDNKVFSAT T TTVTNVNVEDVQD
Trubripes	not conserved	ENSTRUG000000011689	236	YKGRHQVLSST A TITINVIDAQ
Drerio	no homologue			
Dmelanogaster	not conserved	FBgn0261053	351	-PGQDSRNIAGMEI V IVVQDVQD
Celegans	not conserved	Y71D11A.1	1537	--KENPETAR A LIHVAIIRDVNE
Xtropicalis	no homologue			

p.Phe353Ser

species	match	gene	aa	alignment
Human			353	RIVDLN NHPPT F YGESGPQNR F EL
mutated	not conserved		353	RIVDLN NHPPT S YGESGPQ
Ptrogodytes	all identical	ENSPTRG000000002693	353	RIVDLN NHPPT F YGESGPQ
Mmulatta	all identical	ENSMUG00000000658	353	RIVDLN NHPPT F YGESGPQ
Fcatus	all identical	ENSFCAG000000001649	335	RIVDLN NHPPT F YG
Mmusculus	all identical	ENSMUSG000000021803	354	RIVDLN NHPPT F YGESGP
Ggallus	all identical	ENSGALG000000002314	356	RVVDLN NHPPT F YGESGPQNR F E
Trubripes	all identical	ENSTRUG000000011689	359	RVVDLN NHPPT F YGENGPQ
Drerio	no homologue			
Dmelanogaster	all identical	FBgn0261053	473	FLPDRT NSPPY F END-----HYV
Celegans	not conserved	Y71D11A.1	1656	EAVDRNGNHDST HVIV YIDGAPTS--
Xtropicalis	no homologue			

p.Phe379Ser

species	match	gene	aa	alignment
Human			379	NEHPPQGEILR G LKI TVNDS D QGA
mutated	not conserved		379	NEHPPQGEILR S LKI TVNDS D QG
Ptrogodytes	all identical	ENSPTRG000000002693	379	NEHPPQGEILR G LKI TVNDS D QG
Mmulatta	all identical	ENSMUG00000000658	379	NEHPPQGEILR G LKI TVNDS D QG
Fcatus	all identical	ENSFCAG000000001649	361	YEHPPQGEILR G LKI TVNDS D QG
Mmusculus	all identical	ENSMUSG000000021803	380	FEHPPQGEILR G LKI TVNDS D QG
Ggallus	all identical	ENSGALG000000002314	382	YEHPPGEILR G LKI TVNDS D QG
Trubripes	all identical	ENSTRUG000000011689	385	REHPPAGEILR G FKI TVNDS D QG

p.Pro402His

species	match	gene	aa	alignment
Human			402	ANAKFNLQLVGPRGIFRVVPQT VL
mutated	not conserved		402	ANAKFNLQLVG H RGIFRVVPQT V
Ptroglyodytes	all identical	ENSPTRG00000002693	402	ANAKFNLQLVG P RGIFRVVPQT V
Mmulatta	all identical	ENSMUG00000000658	402	ANAKFNLQLVG P RGIFRVVPQT V
Fcatus	all identical	ENSFCAG00000001649	384	ANAKFNLRLVG P GGIFRVVPLT V
Mmusculus	all identical	ENSMUSG000000021803	403	ANAKFNLRLVG P GGIFRVVPQT V
Ggallus	all identical	ENSGALG00000002314	405	ANAKFNLRLVG P GGIFRVVPLT V
Trubripes	all identical	ENSTRUG00000011689	408	ANAKFKLRLVG P SRVLRVVPQT V
Drerio	no homologue			
Dmelanogaster	not conserved	FBgn0261053	527	KNGVFSLTLLN N NGTFEISPNV A
Celegans	not conserved	Y71D11A.1	1686	TTQSFQLQTAS D DVIYRIVGGNDDGLFEL
Xtropicalis	no homologue			

p.Pro470Ser

species	match	gene	aa	alignment
Human			470	VIQLLDT NDNVPKFDSLYYVARI
mutated	not conserved		470	VIQLLDT NDNV S KFDSLYYVAR
Ptroglyodytes	all identical	ENSPTRG00000002693	470	VIQLLDT NDNV P KFDSLYYVAR
Mmulatta	all identical	ENSMUG00000000658	470	VIQLLDT NDNV P KFDSLYYVAR
Fcatus	all identical	ENSFCAG00000001649	452	VIQLLDT NDNV P KFTSH
Mmusculus	all identical	ENSMUSG000000021803	471	VIQLLDT NDNV P KFTSHYYIA
Ggallus	all identical	ENSGALG00000002314	473	AIRLLDT NDNV P KFSSDYIARI
Trubripes	all identical	ENSTRUG00000011689	476	VINLKDT NDNV P KFTSEYYIGR
Drerio	no homologue			
Dmelanogaster	all identical	FBgn0261053	598	TVYINDV NDNA P VFEQPAYSVEL
Celegans	all identical	Y71D11A.1	1754	TVNINPTISNAHA P KFTRD

p.Gly526Arg

species	match	gene	aa	alignment
Human			526	GADLFLIHPSTGLI YTPPWASLDA
mutated	not conserved		526	GADLFLIHPST R LI YTPPWASLD
Ptroglyodytes	all identical	ENSPTRG00000002693	526	GADLFLIHPST G LI YTPPWASLD
Mmulatta	all identical	ENSMUG00000000658	526	GADLFLIHPST G LI YTPPWASLD
Fcatus	all identical	ENSFCAG00000001649	508	GADPFLIHPST G IF YTPQARAS
Mmusculus	all identical	ENSMUSG000000021803	527	GSDLFLIHPST G LI YTPPWASLD
Ggallus	all identical	ENSGALG00000002314	529	GADLFLIHPST G II YTPPWAVLD
Trubripes	all identical	ENSTRUG00000011689	532	GSDLFHIHSS S GLI TTPPWTSLD
Drerio	no homologue			
Dmelanogaster	all identical	FBgn0261053	654	LNTSLNLDAET G LITVSTNKHGFD
Celegans	not conserved	Y71D11A.1	1804	GCADQLGIDNSG R VA YK--V
Xtropicalis	no homologue			

p.Val733Phe

species	match	gene	aa alignment
Human			733 A T F W R N K K S N K V L P M R R V L R K R P S
mutated	not conserved		733 A T F W R N K K S N K F L P M R R V L R K R P
Ptrogodytes	all identical	ENSPTRG00000002693	733 A T F W R N K K S N K V L P V R R V L R K R P
Mmulatta	all identical	ENSMUG00000000658	733 A T F W R N K K S N K V L P V R R V V R K R P
Fcatus	all identical	ENSFCAG00000001649	715 A T F W R N K K S N K V L P V R R V L R K R P
Mmusculus	all identical	ENSMUSG000000021803	734 A T F W R N K K S N K V L P V R R V L R R R P
Ggallus	all conserved	ENSGALG00000002314	735 N K R S N K I M P V R R I I K K R Q
Trubripes	all conserved	ENSTRUG000000011689	738 A I Y M R N A K S N R I A P V R R I I K R R P K

A.5 Founder mutations in *CDHR1*

Variant	Consequence	Population	MAF (%) in founder population	MAF (%) in other groups	Relative frequency
c.783G>A	p.Pro261Pro In-frame exon skipping	European/ European Finnish	0.49% 781/154,234 alleles	0.0638% 82/128,568 alleles	7.7
c.1463del	p.Gly488fs*20	North Indian/Pakistani (South Asian)	0.0457% 14/30,616 alleles	0.000453% 1/220,810 alleles	1008.8
c.2522_2528 del	p.Ile841Serfs*119	Eastern European/ Greek	0.00618% 7/113,220 alleles	0% 0/122,214 alleles	-
c.1381C>T	p.Gln461*	Arab	-	-	-
c.524dup	p.Asn176Glu fs*48	Faroese	0.00163% 2/122,862	0.000407% 1/245,904 alleles	4

A.6 Phenotype of *CDHR1* compound heterozygotes with truncating and hypomorphic alleles *in trans*

Phenotype	Reports	Variants		<i>n</i>
		Allele 1 - Hypomorphic	Allele 2 – Null	
Macular dystrophy	2 – Ba-Abbad 1 – Charbel Issa ⁵⁶ 1 – Pt. no 3 1 – Pt. no 20 1 – Pt. no 21 1 – Pt. no 33 1 – Pt. no 58 1 – Pt. no 117 1 – Pt. no 124	p.Pro261Pro p.Pro735Thr p.Pro261Pro p.Gly188Ser p.Pro261Pro p.Gly188Ser p.Pro261Pro p.Pro261Pro p.Pro261Pro p.Pro470Ser	p.Met1? c.297+1G>T p.Ser524AlafsTer4 p.Gly502LeufsTer32 p.Met1? p.Arg407Ter c.1552+1del p.Tyr256Ter c.1485+2T>G p.Trp6Ter	10
Macular dystrophy + peripheral retinal degeneration	1 – Charbel Issa 1 – Pt. no 9 1 – Pt. no 36 1 – Pt. no 45 1 – Pt. no 83	p.Pro261Pro p.Pro261Pro p.Pro261Pro p.Pro261Pro p.Pro261Pro	p.Ile841SerfsTer119 c.783+5G>T p.Gln373Ter p.Ile841SerfsTer119 p.Met1?	4

List of Primers

All primers that bind the reverse strand (RV) have been presented in their reverse complement of 5' to 3'.

Primers for *Cdhr1* genotyping:

1	CDHR1_WT_FWD1	5' - ATCTGGTCTGAGAGCCCCCAT - 3'
2	CDHR1_WT_RV1	5' – CAAACACAGACTGGGGGCGGTA – 3'
3	CDHR1_KO_FWD1	5' – GGAAGGTGCCACTCCCCTGTCC – 3'
4	CDHR1_KO_RV1	5' – CTTACCTGTTTG TATCGCTACATCC – 3'

Primers for amplification of the CDHR1 cds for GRK1.CDHR1.WPRE.pA:

5	CDHR1_HpaI_FWD	5' - ATTGTTAACGCCACCATGAGGCG - 3'
6	CDHR1_HindIII_RV	5' – CAAACACAGACTGGGGGCGGTA – 3'

Primers for sequencing GRK1.CDHR1.WPRE.pA plasmid:

7	GRK1.CDHR1.WPRE.pA_Seq1	5' - GCTACGTAGATAAGTAGCAT - 3'
8	GRK1.CDHR1.WPRE.pA_Seq2	5' – CGTAGCCATGCTCTAGGTAC – 3'
9	GRK1.CDHR1.WPRE.pA_Seq3	5' – CCATCATCAGCATTCTGAT – 3'
10	GRK1.CDHR1.WPRE.pA_Seq4	5' – CCATCTCCATCACTCAGAGC – 3'
11	GRK1.CDHR1.WPRE.pA_Seq5	5' – CACAGCTGTGGATCCAGATAC – 3'
12	GRK1.CDHR1.WPRE.pA_Seq6	5' – CCTTCCTGATACAGACCAAGG – 3'
13	GRK1.CDHR1.WPRE.pA_Seq7	5' – CGCTATGTGGATACGCTGC – 3'

14	GRK1.CDHR1.WPRE.pA_Seq8	5' – CCTCGACTGTGCCTTCTAGT – 3'
15	GRK1.CDHR1.WPRE.pA_Seq9	5' – GCTGACTAATTGAGATGCATG – 3'
16	GRK1.CDHR1.WPRE.pA_Seq10	5' – CTCATAGCTCACGCTGTAGG – 3'
17	GRK1.CDHR1.WPRE.pA_Seq11	5' – CCAATGCTTAATCAGTGAGG – 3'
18	GRK1.CDHR1.WPRE.pA_Seq12	5' – CCGTAAGATGCTTTTCTGTG – 3'
19	GRK1.CDHR1.WPRE.pA_Seq13	5' – CGGTCACAGCTTGTCTGTAA – 3'
20	GRK1.CDHR1.WPRE.pA_Seq14	5' – GCACCAGGCTAAATCCCAG – 3'

Primers for PCR amplification of intronic sequence:

21	CAGInt_FWD_1	5' – TCGCTGCGCGCTGCCTTCGC – 3'
22	CAGInt_RV_1	5' – AGCTTGATATCGAATTCCTGCAGC – 3'

Primers for amplification of the CDHR1 cds for GRK1.CDHR1.WPRE.pA:

23	CDHR1_EcoRV_FWD	5' - ATTGATATCGGCACCATGAGGCGC - 3'
24	CDHR1_HindIII_RV	5' – CCAAGCTTTTAGAAGTAAGCC – 3'

Primers for sequencing CAG.CDHR1.WPRE.pA plasmid:

25	CDHR1_RV_CAG_1	5' – GAAACATGGCTCTGTTTCAGC – 3'
26	CDHR1_RV_CAG_2	5' – CATCACCTGGTTGAAGAGC – 3'

Primers for AAV quantification by qPCR:

27	CDHR1_qPCR_FWD_1	5' – GCTCTCACGTATACAC – 3'
28	CDHR1_qPCR_RV_1	5' – TCCTGGTGACCGATGC – 3'
29	CDHR1_qPCR_FWD_2	5' – GCTGCCTTCCTGATAC – 3'
30	CDHR1_qPCR_RV_2	5' – ATCCGCATTGAGTGGC – 3'