

Optimisation of therapies for inherited retinal and mitochondrial diseases

A thesis submitted to the University of Dublin for the
degree of Doctor of Philosophy

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and that, except where noted, it is entirely my own work.

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September 2017

Summary

Inherited retinal degenerations (IRDs) are the most frequent cause of vision loss in people of working age. They have highly varied causes and pathophysiologies, and are typically incurable. Over 250 genes have been implicated in this wide range of diseases (RetNet; sph.uth.edu/retnet). However, the combination of rapid advancements in both technologies to determine the genetic nature of these diseases, and in the field of gene therapy, mean that the prospect of developing effective treatments for many of these diseases is in sight. Mitochondrial retinal diseases represent a subgroup of IRDs caused by mutations in genes governing mitochondrial function. These can be nuclear genes required for healthy mitochondria or genes encoded by the mitochondrial genome (such as *ND1-6* in Leber hereditary optic neuropathy; LHON). Studies have shown that retinal ganglion cells (RGCs) are seen to be dysfunctional in several mitochondrial diseases, and so the ability to effectively target these with relevant therapeutics is of significant importance. A chemically induced model of LHON utilising rotenone (a potent Complex I inhibitor) has allowed for the testing of therapies aimed at treating LHON.

In Chapter 2 of this work, a codon-optimised version of the yeast gene *Ndi1* (termed *ophNdi1*) was tested for its efficacy in protecting against RGC loss caused by rotenone-induced Complex I deficiency. *Ndi1* is a single-subunit, nuclear gene encoded by *S. cerevisiae* that has previously been shown to be effective in treating a rotenone-mediated Complex I deficiency model of LHON (Chadderton et al., 2013). Administered intravitreally in an AAV2/2 vector, under the control of a *CMV* promoter, *ophNdi1* was found to be effective at a viral dose administered as low as one sixth that of the original *CMV-Ndi1* viral vector. *ophNdi1* and *Ndi1* both demonstrated significant protection against rotenone in terms of RGC survival, optic nerve thickness, and also visual acuity threshold as measured by optokinetic response (OKR). The protective effect of *ophNdi1* was equal to or greater than that of *Ndi1*, even with a lower viral titre. Overall, this study has validated that codon-optimisation is a valid strategy for increasing the expression and effectiveness of a construct, and that *ophNdi1* has the potential to offer significant benefit in LHON.

Next, an approach was taken to generate potential novel promoters to direct AAV transgene expression preferentially towards RGCs. While several human clinical trials using AAV have targeted RGCs for diseases such as LHON, these have used non-cell type

specific promoters such as cytomegalovirus (*CMV*) or chicken- β -actin (*CBA*). The development of specific promoters for AAV would be valuable both for gene expression studies and, for safety, in restraining expression only to the target tissue. In Chapter 3, a pipeline was developed, the goal of which was to streamline the selection process for novel promoters using a combination of expression data and conservation of upstream sequence. Following from the use of this pipeline, one putative promoter sequence, *Nefh*, was tested in an AAV2/2 vector. It was found that *Nefh* offered highly preferential expression of EGFP in RGCs when injected intravitreally into adult mouse eyes, as compared to an AAV2/2-*CMV-EGFP* vector. Two minimal versions of the *Nefh* promoter sequence were designed, utilising only the highly conserved regions of the *Nefh* sequence. These were tested in AAV2/2 vectors, and while it was found that initial data suggests that *Nefh_short* drives some degree of expression at the RNA level, the *msMin_Nefh* promoter (driving EGFP expression) appears to lack both the expression level of *Nefh* and its specificity.

Finally, a preliminary study looking at copy number variants (CNVs) in photoreceptors was undertaken in Chapter 4. A small number of individual rod photoreceptors (n=20) were sequenced using low-coverage whole genome sequencing to determine the presence or absence of megabase-scale CNVs, as had been reported in other neuronal cells (Cai et al., 2014; McConnell et al., 2013).

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"Your talk," I said, "is surely the handiwork of wisdom because not one word of it do I understand."

-Flann O'Brien, *The Third Policeman*

I decided to include this quote, as *The Third Policeman* is my favourite discovery from my PhD. I think it sums up that fatalistic humour everyone reaches at some point, when you're on your thirtieth paper of the day and the words turn to mush, or when it's 11pm and the protocol you're following seems right but everything keeps going wrong.

This thesis has been the work of some late evenings, a growing dependence on caffeine, and my lab's worrying (but welcome!) dependence on chocolate. But more importantly, it's been the work of people – the people who have encouraged me, helped me, chastised me (when I needed it!) and kept me sane. I don't believe I could possibly list everyone who deserves to be mentioned here, but I'll try my best. First, acknowledgements must go to the Irish Research Council. IRC funding has allowed me to produce this work, has kept me fed and clothed, and has allowed me to pursue my goals. I also acknowledge Science Foundation Ireland (SFI), for supporting my research. I acknowledge also Daniel Maloney and Dr. Arpad Palfi for their permission in reproducing figures.

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You can never have too many friends, and I feel particularly fortunate in that regard. I'll leave the Smurfit with far more friends than I entered with. Paul, Darragh, Lara, Eric, Bennett, Daniel, we've been through it all since we were undergrads, and I'm glad to have gone through it with you guys. Aido, Jeff, Ciara, Eoin, Natalie and Ema, if getting to know you all was all I received from the PhD, I'd be happy. To my gaming group (Locky, Joe, Liam, Liam, and our great-grandfather Conor who got us started), you've been a constant source of sanity and insanity, and I'd be a sadder, more normal man without you. Colm, well, there aren't really any words, are there. Fourteen years and a surprisingly similar life path will do that to you. But we did it together, and I'm glad.

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Thesis table of abbreviations

AAV	Adeno-associated virus
ADA-SCID	Adenosine deaminase severe combined immunodeficiency syndrome
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAF	B allele frequency
BWA	Burrows-Wheeler alignment
CBA	Chicken β -actin
CBS	Circular binary segmentation
ChAT	Choline acetyltransferase
CMV	Cytomegalovirus
CNV	Copy number variation
CoQ	Coenzyme Q
CRISPR	Clustered regularly interspersed short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DOA	Dominant optic atrophy
EF	Enrichment factor
EGFP	Enhanced green fluorescent protein
EGFR-PTK	Epidermal growth factor receptor protein tyrosine kinase
EL	Expression level
EMA	European medicines agency
EPA	Environmental protection agency
ERG	Electroretinogram
ETS	Electron transport system

FACS	Fluorescent activated cell sorting
GABA	gamma-Aminobutyric acid
GCL	Ganglion cell layer
GEM	Genome multitool
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GS	Gene score
HA	Human influenza haemagglutinin tag
HEK293	Human embryonic kidney 293 cells
HMM	Hidden Markov model
HSPG	Heparan sulphate proteoglycan
hSYN	Human synpasin 1
iBRB	Inner blood retinal barrier
ICC	Immunocytochemistry
IHC	Immunohistochemistry
ILM	Inner limiting membrane
INL	Inner nuclear layer
IPL	Inner plexiform layer
IRD	Inherited retinal degeneration
ITR	Inverted terminal repeat
KSS	Kearns-Sayers syndrome
LASN	LTR-ADA-SV40 promoter-Neo selectable marker
LCA	Leber congenital amaurosis
LHON	Leber hereditary optic neuropathy
LIANTI	Linear amplification via transposon insertion
MAD	Median absolute deviation
MALBAC	Multiple annealing and looping-based amplification cycles
MAR	Median angle of refraction
MCS	Multiple cloning site
MDA	Multiple displacement amplification
MELAS	Mitochondrial encephalomyopathy,

MERRF	lactic acidosis, and stroke-like episodes Myoclonic epilepsy with ragged red fibres
MLS	Mitochondrial localisation signal
mRGC	Melanopsin-positive retinal ganglion cell
NAD ⁺ /NADH	Nicotinamide adenine dinucleotide
NARP	Neuropathy, ataxia and retinitis pigmentosa
ND1-6	NADH-ubiquinone oxidoreductase chain (1-6)
Ndi1	NADH-ubiquinone oxidoreductase 1
NEFH	Neurofilament, heavy polypeptide
NEFL	Neurofilament, light polypeptide
NEFM	Neurofilament, medium polypeptide
NGS	Next-generation sequencing
NHP	Non-human primate
NSE	Neuron-specific enolase
OKR	Optokinetic response
ONL	Outer nuclear layer
ophNdi1	Human optimised Ndi1
OPL	Outer plexiform layer
OR	Outer retina
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
qPCR	Quantitative polymerase chain reaction
RGC	Retinal ganglion cell
RNA	Ribonucleic acid
RNFL	Retinal nerve fibre layer
ROS	Reactive oxygen species
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RT-PCR	Reverse transcriptase PCR
scAAV	Self-complementary AAV
SCS	Single-cell sequencing

SNCG	Synuclein gamma
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase 1
SPARCL1	SPARC-like protein 1
SPF	Specific pathogen-free
SVD	Singular value decomposition
TCD	Trinity College Dublin
tRNA	Transfer RNA
VP1-3	Viral protein 1
VSNL1	Visinin-like 1
WGS	Whole genome sequencing

Chapter 1: Introduction

Significant progress has been made in gene therapy in recent years. Over two decades after the first approved clinical trial using a viral gene therapy, several therapies have now been approved for commercial use – Glybera for lipoprotein lipase deficiency, Strimvelis for ADA-SCID, and T-VEC, an oncolytic used in melanoma. In particular, the retina has been the focus of many gene therapy studies. The retina is a confined but readily accessible target, and retinal neurons are non-dividing – thus, a gene therapy can in theory provide long-term benefit. As can be seen in the following sections, the retina, and retinal diseases, are a rich target for gene-based therapeutic strategies.

1.1 The retina

The retina is a thin layer of neural tissue (0.1-0.3mm), broken up into several distinct strata, and consisting of at least sixty types of cells (Masland, 2012). It is a densely populated tissue, with a human retina containing roughly two hundred million cells. Figure 1.1 provides a schematic of the retina. The retina is composed of three primary layers; neurons within the retina interact in a broadly linear fashion. The photoreceptor layer lies at the back of the retina, and consists of two types of cells, rods and cones. Photoreceptors have a unique shape that consists of dozens of stacked membrane layers, each of which is embedded with any of several opsin proteins. Photoreceptors import a chromophore called 11-cis retinal from the retinal pigment epithelium (RPE), a pigmented, supportive tissue that provides the photoreceptor layer with nutrient support. When bound to opsins, 11-cis retinal can induce an electrical signal cascade in response to light. In the case of rod photoreceptors, 11-cis retinal is converted by light into all-trans retinal, enabling the

activated rhodopsin to drive hyperpolarisation of the cell – this hyperpolarisation causes an arrest of glutamate release at rod/bipolar synapses, which is transmitted as “vision” (Molday and Moritz, 2015). All-*trans* retinal is then recycled to 11-*cis* retinal through the RPE, a key enzyme of which is RPE65, notable for its dysfunction in Leber congenital amaurosis (LCA), discussed later. Rhodopsin, expressed in rod cells, is highly sensitive (reactive to a single photon) and responds to most visible wavelengths of light. Rods are active in low-light conditions; bright light results in a “bleaching” feedback mechanism that inhibits their signalling (Litman and Mitchell, 1996).

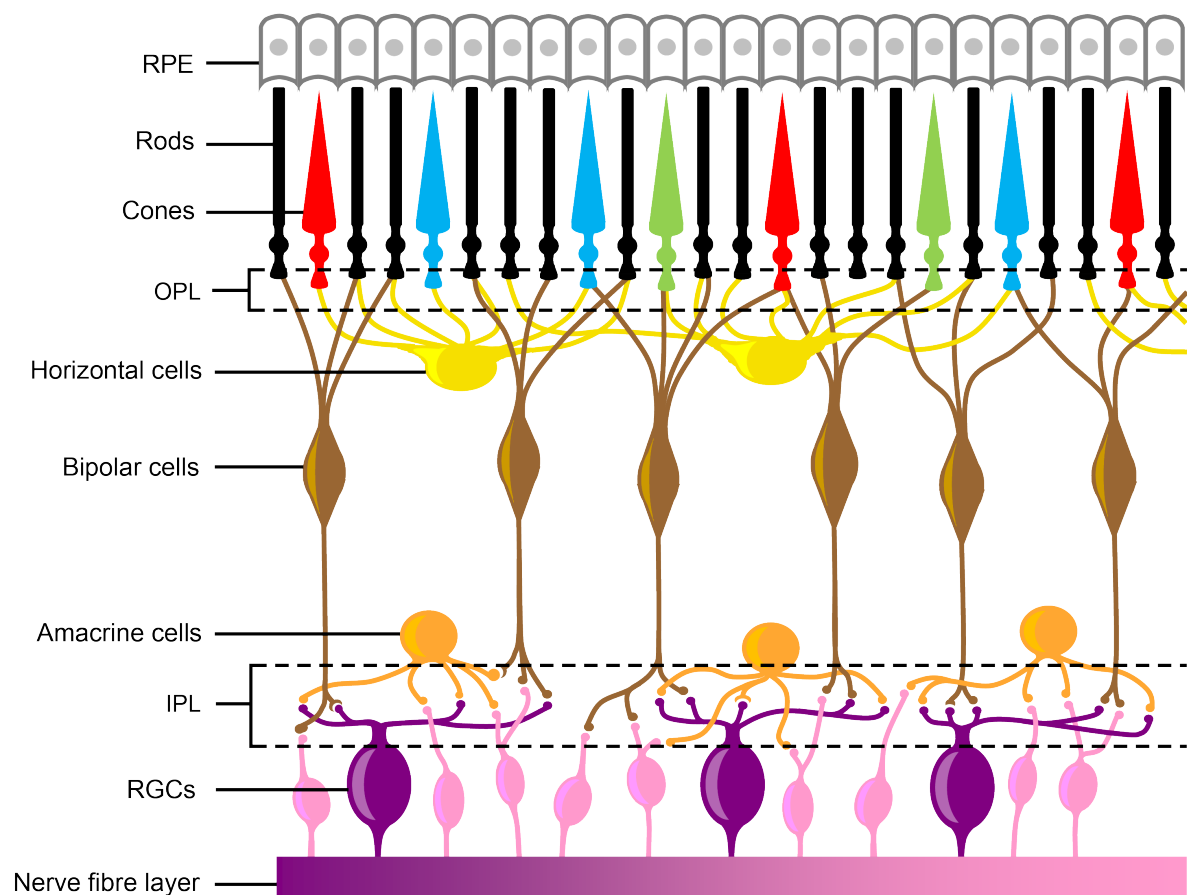


Figure 1.1: Schematic of the retina. Light enters the retina and is sensed by rod and cone photoreceptors located at the back of the eye. This sensation is propagated through connections made with bipolar cells in the outer plexiform layer (OPL). Bipolar cells synapse with ganglion cells in the inner plexiform layer (IPL), where the light signal is broken down into, among other things, edge and colour detection and direction-selective movement. Within this interface, inhibitory horizontal and amacrine cells modulate the signal based on inputs with alternate ON and OFF photoreceptors and bipolar cells. Ganglion cells coalesce into the optic nerve where information is transmitted to the visual cortex via the superior colliculus. RPE: Retinal pigment

epithelium. RGCs: Retinal ganglion cells. (Figure reproduced with permission from Daniel Maloney, Farrar laboratory, TCD)

Cones express cone opsins; these proteins are attuned to a specific band of wavelengths, and are less sensitive than rods. Mammals express two kinds of opsins that, broadly speaking, are sensitive to red and blue light, respectively. Primates express a third type of opsin that allows for discrimination between green and red. There are variations in rod density in different regions of the retina – a schematic of this is provided in Figure 1.2 (Østerberg, 1935). In humans, the greatest rod density is seen at approximately 20°, but rods are present throughout most of the retina in mammals. Cones, however, show spatial organisation that varies within mammals. Primates possess a central, cone-rich region called the macula (see Figure 1.2). The central pit of this region, the fovea, is populated almost exclusively by cones, and allows for highly accurate focusing on objects. The density of cones in the retina declines sharply with distance from the fovea. Many species (such as dogs, pigs, etc.) demonstrate “visual streaks”, relatively cone-rich regions located close to the centre of the retina. These streaks allow for enhanced diurnal vision, but to a lesser extent than primates (webvision.med.utah.edu).

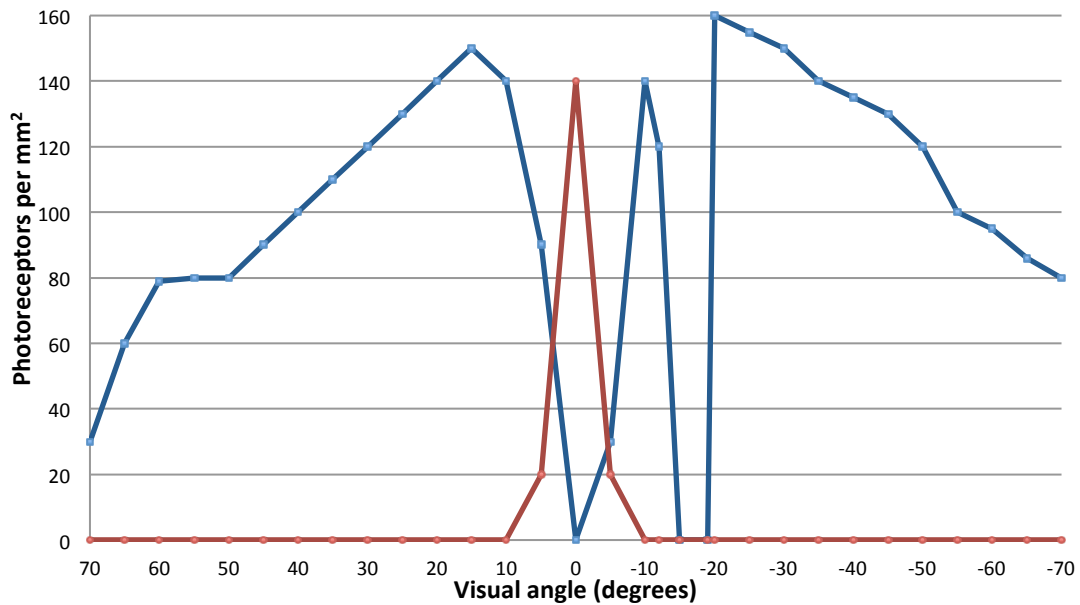


Figure 1.2: Density of rod (blue) and cone (red) photoreceptors across the retina. The highest rod density is seen at approximately 20° from fixation (fixation located at 0°). No rods or cones are found from -15° to -19°, owing to the optic nerve head (“blindspot”). Figure adapted from Østerberg (1935).

Photoreceptor dendrites extend into the outer plexiform layer (OPL), where they form synapses with bipolar interneurons and inhibitory horizontal cells. The light signal is split in the OPL into over a dozen parallel processes – photoreceptors will contact and be contacted by multiple bipolar cells, up to eighty in some rods (Euler and Masland, 2000). While in general cones are contacted by around a dozen bipolar cells (and vice-versa), in the fovea a single cone contacts a single bipolar cell (which in turn synapses with a single retinal ganglion cell; Kolb and Marshak, 2003). Importantly, the retina does not simply transmit pixels of light to the brain; rather, this light signal is decomposed in the retina into over a dozen parallel processes, from colour vision and edge detection to direction-selective motion (Wässle, 2004). The first stage of this decomposition takes place in the OPL. Photoreceptors continuously release glutamate when inactive; activation by light stems this release. Rods and cones can synapse onto two classes of bipolar cells, ON and

OFF. These are differentiated by their expression of glutamate receptors. ON cells express the “sign-inverting” mGluR6 receptor (they depolarise in response to a lack of glutamate from photoreceptors), while OFF cells express different kainite receptors, depolarising in the presence of glutamate (the negative signal; Pang et al., 2012). Horizontal cells act as inhibitors on rods and cones that serve to “average out” signal intensity on a given area (Masland, 2012). This allows for perception of objects of different brightness that doesn’t overexpose the retina, as well as heightening perception of edges.

Within the inner plexiform layer (IPL), bipolar cells synapse with retinal ganglion cells (RGCs) and amacrine cells. Here, a significant condensation of signal occurs; while a human retina contains approximately one hundred million photoreceptors, there are only one million RGCs. The IPL is divided into five distinct sublaminae (S1-S5), with synapses between OFF interactions and ON interactions being separated geographically (S1 and S2 host OFF synapses, S3-S5 ON). Amacrine cells in many ways act in a similar fashion to horizontal cells in the OPL, although some of the thirty or so classes of cells have more specialised functions. Direction selectivity of some RGCs is mediated by starburst amacrine cells (SACs) – cells with huge dendritic fields that synapse with bipolar cells across the field of the retina. SACs selectively inhibit RGCs based on activation of either ON or OFF bipolar cells that are geographically distal, effectively training those RGCs to respond only to light moving in a given direction (Zhou and Lee, 2008). One class of amacrine cells, AII cells, act as intermediaries between rod bipolar cells and the ganglion cell layer (GCL). Rod bipolar cells synapse with AII cells, which then activate local ON cone bipolar cells, thereby transmitting a signal (Demb and Singer, 2012; Marc et al., 2014). Indeed, despite the name, approximately 50% of the cells in the GCL are amacrine cells (Akopian et al., 2016; Jeon et al., 1998; webvision.med.utah.edu).

RGCs represent a diverse population of cells, each type of which samples individual aspects of vision, be it colour, edge detection, movement, and so on, receiving input from photoreceptors via bipolar and amacrine cells. Each of these role-defined cell types is thought to tile the retina – that is, each cell type has complete, uninterrupted coverage of the visual field. This allows the visual image to be decomposed into parallel processes, with at least 30 parallel outputs having been observed (Baden et al., 2016). RGCs integrate and relay the signal through to the brain's visual centres via the lateral geniculate nucleus and superior colliculus, where the image can be processed and formed. RGCs thus play a vital role in vision. However, the relatively small number of RGCs places a great deal of energetic strain on individual cells. This strain is compounded by the length of RGC axons (~55mm, vs. 10mm for photoreceptors), which requires significant energy expenditure in the form of axoplasmic transport of mitochondria and other cell elements (Carelli et al., 2009a). The combination of length and energy requirements is believed to cause RGCs to be vulnerable in a number of metabolic diseases (Baltan et al., 2010; Inman and Harun-Or-Rashid, 2017; Schmidt et al., 2008).

Disease	Cell Target	Treatment	Vector	Trial No.	Status
<i>Achromatopsia</i>	Cones	CNGB3	AAV	NCT03001310	Recruiting
				NCT03278873	Recruiting
		hCNGB3	AAV2-YF	NCT02599922	Recruiting
<i>AMD</i>	RPE, photoreceptors	sFLT01	AAV2	NCT01024998	Active
		sFlt-1	AAV	NCT01494805	Completed
		anti-VEGF fragment (RGX-314)	AAV8	NCT03066258	Recruiting
		CD59 (dry AMD)	AAV	NCT03144999	Active
		Endostatin, Angiostatin (Retinostat)	Lentivirus	NCT01301443 NCT01678872	Complete Recruiting
<i>Choroideremia</i>	Choroid, RPE	REP1	AAV2	NCT02077361	Complete
				NCT01461213	Complete
				NCT02553135	Active
				NCT02407678	Recruiting
				NCT02671539	Active
				NCT02341807	Active
<i>LCA</i>	RPE	hRPE65	AAV2	NCT00643747	Complete
				NCT00999609	Active
				NCT00481546	Active
				NCT00821340	Unknown
		hRPE65	AAV2/4	NCT01496040	Complete
		hRPE65v2	AAV2	NCT00516477	Active
<i>LHON</i>	RGCs	ND4 (GS010)	AAV2/2	NCT02946879	Recruiting
				NCT02781480	Recruiting
				NCT03293524	NYR
				NCT02652767	Active
		ND4v2	AAV2	NCT02652780	Active
				NCT03406104	Recruiting
<i>Retinoschisis</i>	Retina	RS1	AAV8	NCT02161380	Recruiting
				NCT03153293	Recruiting
		hRS1	AAV2-YF	NCT01267422	Complete
				NCT02317887	Recruiting
				NCT02416622	Recruiting

<i>Retinitis pigmentosa</i>	Photoreceptors	hMERTK	AAV2	NCT01482195	Recruiting
		PDE6B	AAV2/5	NCT03328130	Recruiting
		RLBP1 (CPK850)	AAV8	NCT03374657	NYR
	Inner retina (optogenetics)	ChrimsonR-tdTomato (GS030)	AAV2.7m8	NCT03326336	NYR
		ChR2 (RST-001)	AAV2	NCT02556736	Recruiting
<i>Stargardt disease</i>	RPE	ABCA4 (SAR422459)	Lentivirus	NCT01367444	Recruiting
				NCT01736592	Recruiting
<i>Usher syndrome 1B (RP)</i>	Photoreceptors	MYO7A	EIAV	NCT01505062	Recruiting
				NCT02065011	Recruiting
<i>XLRP</i>	Photoreceptors	RPGR	AAV	NCT03116113	Recruiting
			AAV	NCT03252847	Recruiting
			AAV2-YF	NCT03316560	Recruiting

Table 1.1: List of clinical trials being currently undertaken or completed using gene therapy to treat IRDs.

1.2.1 Inherited retinal and mitochondrial diseases

Evidence suggests that the retina is inherently vulnerable to a wide range of genetic diseases, the bulk of which arise from single base changes. At present over 260 genes have been implicated in causing retinal degeneration (Farrar et al., 2017). The vast majority of these genes, and indeed the majority of inherited retinal degenerations (IRDs), primarily affect photoreceptors, along with the RPE. As photoreceptors make up a significant fraction of retinal neurons (Masland, 2012), they have a unique, highly specialised shape and role, and the RPE is required for the cells' health, this does stand to reason.

One of the difficulties in dealing with IRD is that individual diseases may be the result of mutations in any one of dozens of genes. Retinitis pigmentosa (RP) represents the most common IRD, with roughly 1/3000 people affected worldwide, and can be caused by mutations in over sixty genes (Farrar et al., 2017). The onset of the disease is typically characterised by a loss of rods, leading to initial night-blindness, followed by a progressive loss of cones and as such, functional daytime vision (Campochiaro and Mir, 2017). The loss of cones, even with rod-specific RP mutations such rhodopsin and peripherin, is attributed to a number of factors, including increased oxidative stress (caused by a lack of oxygen uptake by rods) and starvation, caused by a loss of a rod-based support network (Bovolenta and Cisneros, 2009; Campochiaro and Mir, 2017). As a result of RP's genetic variability, autosomal dominant and recessive, as well as X-linked forms of the disease are observed, along with syndromic (and mitochondrial) versions of the disease such as Usher syndrome and NARP syndrome (Fahim et al., 1993; Farrar et al., 2013, 2014). Juvenile forms of IRDs affecting photoreceptors include Stargardt's disease and LCA; Stargardt's disease is primarily caused by mutations in the gene *ABCA4*, and has an incidence of around 1/10,000, while LCA, caused by mutations in at least half a dozen genes, has an incidence of roughly 1/50,000 (Allikmets, 2004). Of note, following

successfully conclusion of Phase III trials for RPE65-linked LCA, the AAV-based replacement drug Luxturna has been recently approved for clinical use in the US (Russell et al. 2017; sparktx.com). A summary list of ongoing and completed clinical trials in the retina is provided in Table 1.1.

While RGC-related IRDs are less frequent than those affecting photoreceptors, they represent an unmet clinical need, and as can be seen in Table 1.1, far fewer clinical trials are targeted at treating RGC-based disease. Of note, there are several diseases relating directly to mitochondria that cause disease in RGCs. These include single mutations in mitochondrial genes such as in Leber hereditary optic neuropathy (work on which involves much of this thesis), mutations in autosomal genes whose proteins are targeted to the mitochondria such as *OPA1* in dominant optic atrophy (DOA), and also as part of complex, multi-organ disorders such as Kearns-Sayre syndrome (KSS) or Leigh syndrome (Maresca et al., 2013). Mitochondrial disease, and in particular LHON, are expanded upon in the sections below. As a side note, glaucoma, while not primarily considered an IRD, is in effect caused by loss of ganglion cells through heightened pressure on the optic nerve. Further, increasing evidence suggests that oxidative stress caused by mitochondrial dysfunction may play a strong role in glaucoma-related RGC death (Almasieh et al., 2012; Risner et al., 2018; Williams et al., 2017). As the prevalence of glaucoma over the age of 40 is approximately 3.5% (Tham et al., 2014), it is worth considering whether prospective therapies targeting mitochondrial dysfunction in RGCs may be applicable to glaucoma and other such complex diseases.

1.2.2 Mitochondrial disease

Mitochondria are the prime source of energy generation in the cell. Excluded from the cytosol by a double membrane, mitochondria generate ATP by the creation of a proton gradient across the inner membrane of the organelle. This is accomplished by a complex suite of proteins termed the electron transport system (ETS). A diagram displaying the ETS is shown in Figure 1.3. Acetyl-CoA, the product of anaerobic glycolysis, is oxidised in the mitochondrial matrix as part of the tricarboxylic acid (TCA) cycle (partly mediated by Complex II), producing NADH. Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) transfer electrons to coenzyme-Q10 (CoQ) – the reduced CoQ transfers electrons through Complexes III and IV via cytochrome c – in the process of this reaction, protons are forced out of the matrix and into the intermembrane space. This potential difference is resolved by ATP synthase; ATP synthase acts as a “proton pump”, using the potential difference across the membrane to generate ATP from ADP by shuttling protons back into the matrix.

Mitochondria possess their own circular genome of 16.5kb (mtDNA), encoding 37 genes. These include 22 amino-acyl tRNAs, mitochondrial rRNAs, humanin, and genes for the 13 proteins highlighted in Figure 1.3 (revised Cambridge Reference Sequence, accession NC_012920). The genome encodes some of the ETS complex proteins, although the nuclear genome encodes the majority. Mutations in mitochondrial proteins can cause a wide range of multi-system disorders such as MELAS, MERRF, and NARP, as well as diseases that affect only a single organ such as Leber Hereditary Optic Neuropathy (LHON) (Farrar et al., 2013). mtDNA is more vulnerable to mutation than the nuclear genome, with mutation rates up to ten times higher (Ballard and Whitlock, 2004; Brown et al., 1979). This issue is compounded by the fact that mitochondria reproduce asexually,

and are inherited solely from the mother, which limits the extent to which deleterious mutations can be parsed out of a generation. mtDNA is subject to a certain degree of purifying selection in the course of female germ cell production (Stewart et al., 2008), however, the mechanisms by which this is achieved remain generally unknown.

This high rate of mutation helps explain why the vast majority of mitochondrial proteins are expressed in the nuclear genome, as Muller's Ratchet would indicate that asexually reproducing organisms eventually build up a highly deleterious mutational load. One way mitochondria minimise the impact of this damage is by having multiple genome copies; a single mitochondrion can have anywhere from one to ten mtDNA copies (Wiesner et al., 1992). Thus, mitochondrial mutations can be analysed in the context of genomic heteroplasmy when considering their severity. Indeed, varying levels of heteroplasmy between generations (either by chance, or possibly due to the aforementioned purifying selection) help to explain why, despite mitochondria being inherited uniparentally, many inherited mitochondrial diseases show incomplete penetrance (Tuppen et al., 2010). In particular, mitochondrial diseases disproportionately affect the eye. Approximately 50% of mitochondrial diseases have an ocular phenotype, either as a standalone disease (for example, LHON or dominant optic atrophy (DOA)). One explanation for this is that the energetic requirements of the eye are such that any loss of mitochondrial function may be sufficient to disrupt what is already a finely tuned balance (Carelli et al., 2009b).

1.2.3 Leber hereditary optic neuropathy

LHON is a mitochondrially-inherited disorder, caused by mutations in the genes encoding for Complex I proteins, NADH-ubiquinone oxidoreductase chain 1-6 (ND1-6). While in

theory, mutations in any of the mitochondrial Complex I subunits can cause LHON, three mutations – *MT-ND4* G11778A (OMIM 516003.0001), *MT-ND6* T14484C (OMIM 516006.0001), and *MT-ND1* G3460A (OMIM 516000.0001) – account for up to 95% of LHON cases (Mackey et al., 1996; Riordan-eva et al., 1995), with *ND4* alone accounting for approximately 70%. LHON is characterised by a bilateral loss of central vision, caused by death of RGCs and corresponding atrophy of the optic nerve. Vision loss typically occurs in the second or third decade of life; eyes can be affected simultaneously or with a lead-time of months or even years (Nikoskelainen et al., 1996). Following the acute phase of disease, patients are usually left with less than (20/400) vision. An uncommon feature of LHON is that fact that a spontaneous recovery of vision is observed in a minority of patients, in particular those with the *ND6* T14484C mutation (Kay et al., 2013; Mackey and Howell, 1992; McClelland et al., 2015; Pezzi et al., 1998; Stone et al., 1992). The reasons for this remain unclear, especially as the recovery rate is mutation-dependent; the *ND6* 14484 mutation has a recovery rate of 40-50%, while that of the *ND4* 11778 mutation is only around 4% (Man et al., 2003; McClelland et al., 2015). It may be that there is a significant lag between loss of vision (cell inactivation) and actual cell death by apoptosis.

LHON has an incidence of roughly 1/30,000. Notably, the disease shows a marked incomplete penetrance with a skewed sex ratio, with only 10% of women and 50% of men affected – this leads to 80% of those affected being male (Puomila et al., 2007). This occurs even in situations where the maternal mtDNA is homoplasmic for LHON mutations. Multiple reasons have been proposed to explain this discrepancy. The mitochondrial haplotype background of the patient appears to have some bearing on disease onset, with two sub-classes of haplogroup J (J1c and J2b) having a deleterious effect in 11778 and 14484 cases (Carelli et al., 2006). On the other hand, haplogroup H

appears to confer a degree of protection in 11778 cases (Hudson et al., 2007). Exposure to a number of chemicals known for their neurotoxicity, including alcohol and tobacco, may play a role in disease onset and severity, along with several drugs previously implicated in optic neuritis such as ethambutol and isoniazid (used for treating tuberculosis; Carelli et al., 2004; Sadun et al., 2003).

However, these examples do not explain the skewed sex ratio seen in LHON. In an attempt to understand the mechanisms behind this, multiple studies have been conducted involving the X-chromosome, but no definitive results have been found. An X-linked “two-loci” phenomenon has been hypothesised to explain the over-representation of males, but studies to look for this in a number of family pedigrees have failed (Hudson et al., 2005; Shankar et al., 2008). A potential weakness in this hypothesis is that it implies that in non-affected females, either 50% of cell death should still occur (due to random X-inactivation in cells), or a skewed, non-random X-inactivation would be seen. This has not been found to be the case (Hudson et al., 2007).

A study indicated that oestrogen may provide a causal link as to the lack of penetrance in females. Following from work establishing the presence of 17β -oestradiol (17β) receptors on mitochondria (Yang et al., 2004), Giordano et al., (2011) showed that, in cell lines and a cybrid model of LHON, 17β significantly increased the rate of mitogenesis. LHON cybrids created suffered from high levels of oxidative stress and energy deficits; 17β effectively reduced these, in part by the upregulation of superoxide dismutase 2 expression. Application of an inhibitor of the 17β receptor halted these effects. Building on this work, a later paper demonstrated a marked increase in mitogenesis in unaffected carriers of LHON versus affected and non-carrying cohorts (Giordano et al., 2014). Using mtDNA copy number as a marker, Giordano et

al. found that unaffected carriers had a far higher average copy number per cell over affected and control individuals. Affected LHON patients had on average double the mtDNA copy number compared to controls, while unaffected carriers had approximately triple (when tested in biopsied fibroblasts). Giordano et al. define a threshold of approximately 500 mtDNA copies/cell as a threshold that confers protection in LHON. Further, in one case a woman developed LHON only after onset of menopause. This research is backed up by another study in LHON patients and carriers, which demonstrated an inverse relationship between mtDNA copy number and LHON penetrance (Bianco et al., 2017). As before, particularly high levels of blood mtDNA copy number showed protection from disease symptoms. Together, these studies lend weight to the potential that oestrogen levels may be a prime determinant in LHON onset, and may inform on future therapeutic approaches. They also offer the possibility of LHON risk assessment before disease onset, using mtDNA copy number as an estimate.

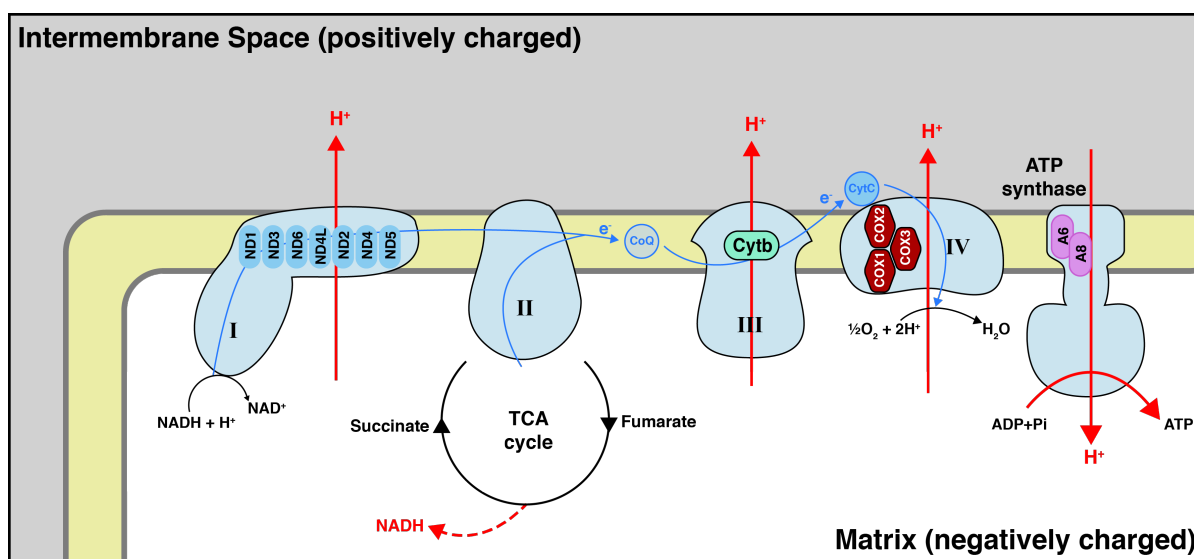


Figure 1.3: Diagram of the electron transport chain (ETS) of the mitochondria. Complexes I-IV are indicated by numerals. NADH (produced during the TCA cycle) is oxidised by Complex I to transfer electrons to Coenzyme Q10 (CoQ), in turn pumping protons (H^+) into the intermembrane space (IMS). CoQ, transfers electrons from Complex I and II through Complexes III and IV via Cytochrome C (CytC) in turn driving more protons into the IMS and reducing free oxygen to create water. ATP is generated by ATP synthase, using the proton gradient established by the complexes. Mitochondrial proteins highlighted: ND1-6, Cytochrome b (Cytb), Cytochrome oxidase subunit 1-3 (COX1-3), ATP synthase Fo subunits 6,8 (A6,A8).

1.3 Gene therapy in the retina

Gene therapy is defined as the delivery of some form of nucleic acid to treat a (usually genetic disease), either by addition of a functioning gene, silencing of an endogenous gene, or potentially modifying the host genome itself. The development of gene therapy is critical to the treatment of a range of inherited diseases with no known cure or treatment, or in the case of some conditions such as haemophilia, require repeated, expensive treatments (George et al., 2017). In the retina these diseases potentially number in the hundreds, and as discussed, have for the most part remained entirely refractory to conventional medicine (Farrar et al., 2017; Sahel et al., 2015). However, in recent years gene therapy has reached the point where research is actively being driven towards the clinic. In the retina, over three dozen viral gene therapies have been tested in clinical trials, with several having entered late-stage testing (clinicaltrials.gov). These therapies are summarised in Table 1.1 below, and as can be seen, are targeting a significant number of diseases. This may in part be due to the fact that, as most retinal cells are post-mitotic, targeting them with non-chromosomal DNA can offer viable long-term expression. Further, the retina is an accessible region to target and so allows for a higher degree of precision than systemic treatment.

The road to this point has been rocky at times. The first clinical trial for gene therapy was performed in 1980, to combat β -thalassaemia. However, soon after the trial was conducted, it emerged that the first gene transfer performed on humans had not actually been approved by the overseeing ethics committee (Wade, 1981). This was a setback to the field; it would be over ten years before another clinical trial was approved (Blaese et al., 1995; Sheridan, 2011). The first approved trial was for patients suffering from ADA-SCID, a deficiency in adenosine deaminase (ADA) that causes a near-complete lack of T and B-cell

functionality, and thus, a compromised immune system. The trial utilised a modified murine replicating leukaemia lentivirus (MoMuLV) called LASN in order to transduce a patient's cells with a functional copy of ADA (Blaese et al., 1995). The study was a modest success, with T-cell numbers in one patient remaining significantly elevated for the length of the study (although the patient continued to take recombinant ADA during this period).

A decade later, however, a number of other clinical trials went awry. In 1999, 18-year old Jesse Gelsinger died when an adenoviral therapy for ornithine transcarbamylase caused toxic shock and multiple organ failure (Raper et al., 2003). It later transpired that his liver function test results should have excluded him from the study at the outset. A year later, a trial for X-linked SCID appeared to show dramatic benefit when administered using a gammaretrovirus to bone marrow cells (Cavazzana-Calvo et al., 2000). The retrovirus, carrying a functional copy of interleukin-2, was applied *ex vivo* to the bone marrow of affected children. However, although the therapy showed impressive effect, of the 20 children treated, five went on to develop leukaemia, of which one eventually died.

Retroviruses replicate by inserting into the host cell's genome. In this case, the integration resulted in insertional oncogenesis, the activation of an oncogene near to the site of DNA insertion. At the time, animal studies had shown that this was an incredibly rare event, and existing data was not sufficient to indicate that up to a quarter of patients could suffer this outcome (Kohn et al., 2003). Although a significant proportion of patients (25%) experienced a significant side effect, the trial demonstrated that gene therapy could potentially provide significant beneficial effects. Indeed, as mentioned above, Strimvelis was approved for commercial authorisation in 2016. Strimvelis is an *ex vivo* gene therapy that treats ADA-SCID using a related gammaretrovirus as in previous studies. 75% of

those receiving the treatment no longer required treatment with ADA enzyme, and none of the 18 subjects who received the treatment have developed any malignancies in a 7-year follow-up (Aiuti et al., 2017). That said, the European Medicines Agency (EMA) has insisted that patients treated with Strimvelis are closely monitored to confirm the safety of the treatment.

Aspects of these studies led to tragic events, however after years of more in-depth research into viral vector safety and efficacy, gene therapy has undergone a renaissance. To date, nearly 2500 gene therapy clinical trials have been conducted (clinicaltrials.gov; abedia.com). Gene therapy offers the most promising approach to treating a variety of retinal degenerations. As mentioned, the retina itself is an excellent candidate for gene therapy – it is readily accessible, immune privileged via the inner blood retinal barrier (iBRB), and is composed primarily of non-dividing neurons. These factors combined enable the possibility of a far safer, more effective gene therapy (as discussed below).

1.4 Adeno-associated virus

Choice of vector is critical when considering a gene therapy. In recent years, gene therapies have increasingly used adeno-associated virus (AAV) as a delivery vector. AAV is a non-enveloped virus in the family *Parvoviridae*. It is replication incompetent – that is, in the absence of a helper virus (such as adenovirus), it lacks the capacity to replicate. AAV has never been shown to cause illness in humans, and approximately 60% of humans show pre-existing antibodies for the most common serotypes, in particular AAV2 (Calcedo and Wilson, 2013). The increasingly large array of available serotypes enables highly efficient targeting to a wide range of cells, including all retinal neurons.

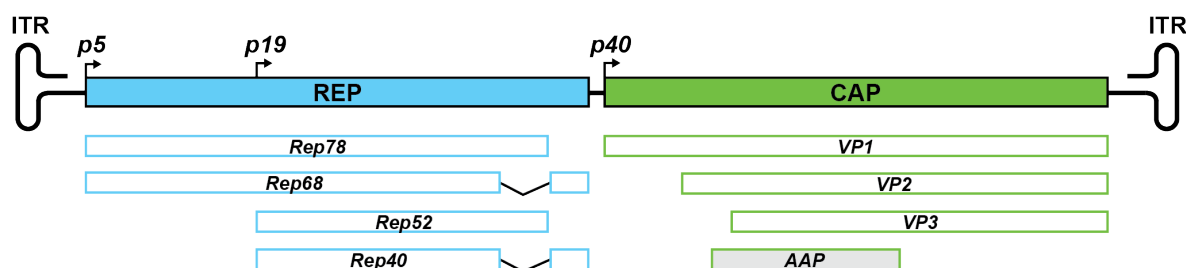


Figure 1.4: Schematic of wild-type AAV genome.

The genome of AAV is simple in structure, and is shown in Figure 1.4. The 4.7kb genome is flanked by inverted terminal repeat (ITR) regions, palindromic sequences that form hairpins structures and are necessary for AAV gene expression and genome replication (Bohenzky et al., 1988). Two gene sequences, REP and CAP (replication and capsid, respectively), are responsible for the production of the eight mRNAs that are required for AAV replication and capsid assembly (Gaj et al., 2015; Sonntag et al., 2010). The *Rep* genes are transcribed off two promoters, *p5* and *p19*, with optional intron splicing used to create different proteins. *Rep78* and *68* are responsible for replication of the virus' DNA, while *Rep52* and *40* are involved in virus packaging. The three capsid proteins *VP1*, *2*, and *3* are expressed from the *p40* promoter, with alternative splicing creating three different mRNAs. An AAV capsid is formed of 60 subunits in a 1:1:10 ratio of *VP1:VP2:VP3* (Gaj et al., 2015). Finally, a more recently discovered protein assembly activated protein (*AAP*) is expressed from a non-canonical translational start site (Sonntag et al., 2010). *AAP* promotes capsid assembly, and displays motifs that direct it to the nucleolus – however, it has also been shown that for some serotypes (4, 5 and 11), it is not required for capsid assembly (Earley et al., 2017). Engineered AAV vectors are produced by replacing the entire endogenous genome with the therapeutic construct of interest, retaining only the ITRs necessary for gene expression. In the standard triple transfection method of virus

production, this construct (as a plasmid) is co-transfected into a cell line, typically HEK293 cells with a second plasmid carrying the REP and CAP genes, and a third plasmid carrying helper genes from adenovirus that are required for viral replication (Ayuso et al., 2010; Chadderton et al., 2013; Qu et al., 2000). A specific variant of HEK293 cells, HEK293T, are most commonly used for AAV production. Two of the adenoviral helper genes required, E1a and E1b55k, are expressed by the 293T line, and so facilitate highly efficient AAV production (Wu et al., 2010). Using this method, very high titres, over 10^{13} vector genomes per millilitre (vg/ml), can be obtained. This is important as, in treating the retina, only a very limited volume can be administered as part of a therapy, owing to its size. As such, highly concentrated viral vectors enable the maximum dose delivery with the minimum applied volume. The human genome contains a region on Chromosome 19 that functions as an AAV integration site, titled AAVS1 (Deyle and Russell, 2009). This integration is *Rep* dependent, with only 0.1% integration efficiency even in wild-type AAV (McCarty et al., 2004). As such, it is not regarded as a significant concern for therapeutic AAV vectors lacking the *Rep* genes. A mouse orthologue of AAVS1 has also been identified, nearby the *Mbs85* gene whose promoter may drive expression of AAV genes once integrated (Dutheil et al., 2004).

There are a large number of AAV serotypes available for use in gene therapy; the choice of vector serotype is of paramount importance in designing a study. These serotypes have variously been isolated from humans and primates, and in recent years several have been generated through rational design and directed evolution. In terms of retinal gene therapy, there are several serotypes that have seen a large amount of use in both preclinical animal studies and human clinical trials. The majority of studies utilised the ITR sequences of AAV2, with the capsid genes of another serotype, and hence are often referred to as

AAV2/2, 2/5, and so forth. Extensive work has shown the efficacy in particular of AAV2, 5 and 8 in delivering a broad range of high-level transgene expression. AAV2/5 has been shown to effectively target photoreceptors in the RPE in, for example, mice (Palfi et al., 2012), in pigs (Puppo et al., 2013), in dogs (Beltran et al., 2017), in macaques (Le Guiner et al., 2014), and is currently in use for a human clinical treating RPE65-related Leber congenital amaurosis (LCA; clinicaltrials.gov; NCT02781480). AAV2/8 has shown similar success, and notably subretinal injection results in high level expression very quickly, within a week in one mouse model examined (Palfi et al., 2015). As with AAV2/5, 2/8 has been used extensively in a number of animal models, including mouse, pig and macaque (Burns et al., 2017; Manfredi et al., 2013; Mussolino et al., 2011; Vandenberghe et al., 2011), as well as being the serotype of choice for human clinical trials for RS1-linked retinoschisis and achromatopsia (clinicaltrials.gov; NCT02317887, NCT03001310).

Both of these serotypes efficiently transduce photoreceptors and the RPE when injected subretinally; however, their transduction efficiencies when injected intravitreally are quite low (Giove et al., 2010). The GCL and INL are protected by the inner limiting membrane (ILM) – a barrier composed of Müller glial cell feet that separates the retina from the vitreous (Woodard et al., 2016). AAV2/2 is a commonly used AAV serotype that can bypass the ILM, and has seen significant use in both pre-clinical and human clinical studies being used for both intravitreal and subretinal injection studies (Bennett et al., 2016; Chadderton et al., 2013; Koilkonda et al., 2010; Wan et al., 2016; Yin et al., 2011). AAV2/2 is discussed in greater detail in Section 2.1.1.

Increasingly, efforts have been made to increase the effectiveness of AAV vectors by modifying the capsids in a directed manner. One of the most successful approaches to this

has been the mutation of tyrosine residues on the capsid surfaces. It was initially shown in AAV2 that, although the virus is endocytosed efficiently, an enzyme called EGFR-PTK could phosphorylate the capsid's tyrosine residues (Zhong et al., 2007, 2008a). This phosphorylation causes ubiquitination of the virus, which then leads to proteasome-mediated degradation. The same group that performed these studies then demonstrated that, by introducing tyrosine to phenylalanine point mutations in the AAV capsid, viral gene expression was increased significantly (Zhong et al., 2008b). It was later shown that this heightened transduction efficiency is seen in the retina as well, and that Y-F mutations could enable AAV8 and AAV9 to transduce the GCL as well (Petr-Silva et al., 2009). The original 2007 Zhong paper highlighted and tested seven Y-F mutations on the AAV2 capsid. More recently, a group has tested the effect of using multiple mutations at once to drive transduction efficiency in the retina even higher. They showed that their multiple mutants (in particular the pentuple, sextuple and septuple examples) could transduce all cells types in the retina at once, even when injected subretinally (Petr-Silva et al., 2011). The triple mutant (Y444, 500, 730F) has proven to be highly effective in mouse, and has been successfully administered intravitreally in human clinical trials of an ND4-based therapy for LHON (Feuer et al., 2016).

Rational design has proven effective for increasing AAV's tropism and infectivity, both in the tyrosine mutant and others such as AAV8BP2, a virus with a modified stretch of amino acids that enables efficient bipolar cell targeting (Cronin et al., 2014). One group derived a pseudo-ancestral AAV capsid, termed Anc80L65, by reconstructing the evolutionary lineage of extant AAV serotypes (Zinn et al., 2015). The authors tested a number of derived capsids and found that Anc80L65 in particular demonstrated a highly efficient

transduction profile, targeting all major cell populations in the retina even from a subretinal injection.

Directed evolution can also be a powerful means of enhancing the transduction properties of an AAV vector. In order to overcome the intravitreal barrier to photoreceptor transduction, Dalkara et al. used repeated rounds of retinal transduction, sorting of photoreceptors, and error-prone PCR to generate a novel serotype. Beginning with capsids with random 7-mer insertions in the heparin binding domain, after four rounds of mutagenic selection they converged upon a single dominant mutant clone, which they termed 7m8 (Dalkara et al., 2013). The authors demonstrated that the 7m8 vector effectively transduces photoreceptors (along with all other retinal neurons), and at a rate four times higher than a comparable tyrosine mutant vector. Of note, however, a large number of viral particles (10^{11} vg) were injected per mouse retina. Commonly, intravitreal injections will use one or even two orders of magnitude less than this (Chadderton et al., 2013; Yu et al., 2012a). The ratio of photoreceptors to RGCs is approximately 100:1, but there is no way with this method of controlling where the virus goes and which cells are transduced, and hence there can be concerns that high viral doses can cause protein overexpression and cell death (Howard et al., 2008). Further, it has been noted that in non-human primates (NHPs), high doses of 7m8 are required to actually target photoreceptor cells to any significant degree (Ramachandran et al., 2016). That said, for low-expressing genes, or for constructs under the control of a cell-type specific promoter, 7m8 may be a means of targeting a range of cell types after intravitreal injection, including photoreceptors. Additionally, Ramachandran et al. did demonstrate excellent GCL expression with 7m8, even at reduced doses, in contrast to other studies with a wild-type 2/2 capsid.

One of the main drawbacks of AAV is its packaging capacity of 4.7kb. This is significantly smaller than other viruses used in gene therapy, such as lentivirus (8kb) and adenovirus (14-35kb; Ehrke-Schulz et al., 2016). There have been several efforts to produce AAV vectors with increased packaging capacity, but these appear to produce predominately incomplete genomes *in vivo* (Dong et al., 2010; Wang et al., 2012). An alternative approach has been the use of two vectors, each with half of the viral construct and either a splice donation or acceptance site. *In vivo*, ITRs readily concatemerise, and thus a full-length protein can be produced by the creation of an intron (Yan et al., 2000). While this leads to less efficient gene expression *in vivo* (compounded by the use of two vectors, halving the effective viral titre), expression of a therapeutic “large” gene can be sufficient to ameliorate a disease phenotype. This has been shown to have efficacy in the case of retinal degenerations, for example in mouse models of Stargardt disease and Usher syndrome type 1B (Trapani et al., 2014).

As a result of its non-pathogenicity, in the retina AAV elicits only a mild, humoral immune reaction – transient immunosuppression upon treatment with an AAV vector effectively neutralises this (Nathwani et al., 2014). Systemically, the pre-exposure to various AAV serotypes can pose an issue with respect to treating patients. AAV infection usually results in the generation of neutralising antibodies (Calcedo and Wilson, 2013). This can inhibit re-administration of therapy (in cases where a second dose is required) or even the initial application, if antibodies from a pre-existing infection exist. In the retina, these problems are less prominent, due to the immune privileged state of the eye. The eye is protected by the iBRB, as well as by a lack of lymph drainage channels in the subretinal space and vitreous cavity that would allow immune access (Streilein, 2003). Further, the aqueous

humour of the eye contains actively immunosuppressive proteins that ward off immune cell responses (Taylor et al., 1992; Taylor and Yee, 2003). As the volumes and viral titres injected into the eye are orders of magnitude lower than that required for systemic therapy, the immune response is correspondingly milder (Ertl and High, 2017). Subretinal injection in particular appears well tolerated based on the results observed in a number of human clinical trials (Bainbridge et al., 2015; Bennett et al., 2016; Russell et al., 2017), although it has been shown that very high doses of virus administered (10^{12} vg/eye) may evoke a potentially harmful immune response (Bainbridge et al., 2015).

In terms of repeat injection of AAV, it has been shown that treating the contralateral eye with an AAV vector following a previous administration is well-tolerated when injected subretinally (Bennett et al., 2016). However, despite the immunosuppressive nature of the aqueous humour, AAV injection into the vitreous humour elicits a stronger immune reaction (Willett and Bennett, 2013). A humoral immune response is generated with an initial intravitreal injection, and this response can significantly inhibit contralateral administration, potentially blocking transduction altogether, even in rodent models (Li et al., 2008). This offers a potential stumbling block for the development of therapies intended to treat both eyes of a patient. Indeed, clinical trials looking at intravitreal injection of AAV to combat LHON showed evidence of heightened immune responses in patients at doses required for therapeutic effect (Guy et al., 2017; Yang et al., 2016). In one of these studies, one patient was treated with the AAV therapy in their fellow eye, after significant benefit was observed in the first eye. However, no benefit was observed in the fellow eye and in fact visual acuity decreased following treatment after three months. A slight increase in neutralising antibodies was detected at three months, however this had

stabilised by six months. This transient increase may have inhibited the effect of the second injection.

While simultaneous treatment of both eyes may not elicit a heightened second response, common practice would dictate that only one eye is treated at a time, to guard against a bad reaction to the therapy. However, recently it has been shown that, even for a systemic therapy, lifelong AAV-mediated gene expression can be achieved by repeat administration using different capsid types in rodents (Bockor et al., 2017). While AAV immunity can prevent reapplication of therapy, this immunity tends to be highly serotype-specific, and so an identical gene construct in a different but applicable serotype may offer a solution. And, as discussed below, the increasingly large array of AAV serotypes available means that multiple capsids may be able to effectively target the same tissue.

1.5 Animal and Disease Models

In order to test potential gene therapies, it is critical that a relevant animal model is available. There are a number of selection criteria that determine how effective a model can be when developing a therapy for a specific disease. The initial requirement is, of course, being able to model the disease in the first place in an animal. As technology develops, it is becoming increasingly easier to generate transgenic animal models – in recent years, genome editing technology using CRISPR/Cas systems has emerged as a highly effective means of creating models for retinal degenerations (Arno et al., 2016; Wu et al., 2016). However, in practice, inducing the same mutation in animals that causes disease in humans does not always simulate the pathology well; it is sometimes seen that the mutation has a much milder, or on occasion quite different effect (Lin et al., 2012). In

these situations, analogous mutations causing similar disease to that in humans may be used, or indeed a chemically induced model – an example of this would be rotenone to induce Complex I deficiency to model diseases such as LHON (Chadderton et al., 2013; Zhang et al., 2002, 2006).

The weight of each selection criterion will vary based on the purpose of the model being used (for example, disease modelling versus biodistribution, requirements for anatomical similarity, if a model only exists in certain species, etc.). In general, ease of generation is a key requirement – quickly maturing species that frequently produce large litters are a valuable asset. In tandem with this, housing constraints must also be considered (in terms of practicality and cost). For example, many rodent facilities are specific pathogen free (SPF), and while zebrafish (*Danio rerio*) is a quickly reproducing species that is highly valuable for some retinal disease modelling, it requires a highly specific lab setup. The zebrafish eye, of course, also differs significantly from the mammalian retina. Ease of therapeutic administration is also a reasonable concern. For some studies, genetic relatedness to humans is of vital importance, particularly when determining the host's reaction to a therapy and the downstream effects that the therapy may cause.

The mouse has been the go-to model for retinal diseases for some time, owing to its rapid generation time and relative ease of maintenance. While a number of studies have used dog and pig (Beltran et al., 2015; Burns et al., 2017; Scott et al., 2014), and NHPs (primarily for pre-clinical safety testing and AAV serotype optimisation), rodents, and mice in particular, have seen the greatest use. As they are nocturnal mammals, they primarily rely on rods, and as such they have relatively few cones compared to diurnal mammals (web.medvision.utah.edu) – although, a transgenic, cone-only mouse model has been

developed in recent years (Samardzija et al., 2014). Their small size allows for easy handling with respect to histology. A notable downside is that their eyes are small, limiting the volumes of therapeutics administered and hence the behaviour of any viral vector injected will differ substantially from that performed on a human. In particular, intravitreal injection has been shown to be less efficient in NHPs when compared to mice (where it is possible to target the entire GCL, for example), which may influence therapies being tested *in vivo* (Koilkonda et al., 2014b; Ramachandran et al., 2016).

As rodent lifespans are short (<3yr), they make poor models for aging-related diseases such as Parkinson's disease (Beal, 2010). It has been suggested that the mouse does not model up to 40% of genes required for viability in humans (Liao and Zhang, 2008). For retinal gene therapy, canine models have been used for many years, in part because of the existence of a number of naturally occurring models for various forms of retinal degeneration. There are canine models for a number of diseases, including among others rhodopsin-linked retinitis pigmentosa (RP; Kijas et al., 2002), bestrophin-linked Best macular dystrophy (Guziewicz et al., 2007), and RPE65-linked LCA (Acland et al., 2001). Indeed, the LCA model was the first large animal rescue of retinal degeneration demonstrated, with profound physiological and behavioural recovery observed, maintained over several years (Acland et al., 2001, 2005). The facilities required for housing large mammals are complex and expensive, and dogs are no exception to this. Ethical concerns may also limit the experiments that an institution is granted permission to perform. That said, although canine models make up a minority of animal testing for IRDs, studies are still conducted, and even recently research groups have published results showing benefit in animal models for PDE6A and PDE6 β -related degenerations, as well as X-linked RP (Beltran et al., 2017; Burns et al., 2017; Pichard et al., 2016).

NHPs offer in many respects the closest retinal models to humans, possessing trichromatic vision, a macula, and significantly larger eyes than rodents. However, NHPs tend to be difficult to work with, as their long generation times and small litter sizes make doing studies on a large scale difficult or impossible. Associated costs are significantly greater with NHPs, and there is an increasing pressure to use them only where other species cannot serve as a good substitute. As such, the benefits of the mouse outweigh its drawbacks, and with proper experimental design should serve well. As an interesting side note, it has been suggested that the grey mouse lemur (*Microcebus murinus*), a protosimian roughly half the evolutionary distance between humans and mouse (~40-50mya) could serve as an effective primate mouse model (Ezran et al., 2017). They have a short generation time (gestation 2 months, maturity at 6-8 months, litter size 1-4), are small (30-50g) and are relatively easy to manage. Limited work has been done in relation to eye disease in the mouse lemur (Alleaume et al., 2017; Beltran et al., 2007). It is important to note also that the serotype of a given AAV vector may have a different tissue transduction profile from one species to the next, and transduction requirements for one species may not be mirrored in another. Therefore, to progress a therapy from preclinical evaluation to clinical studies, different serotypes of virus may be required, and in this regard NHPs may be essential.

Finally, a serious issue is the ethical considerations involved, as alluded to above.

Chimpanzees are the closest living relative to humans, and in terms of genetics, physiology and behaviour they mimic us most closely. However, for the purposes of animal experimentation, they are increasingly being phased out in favour of other NHPs, with the NIH in the US only responsible now for roughly 300 chimpanzees (sciencemag.org). This is in large part due to public campaigning on behalf of the animals, and the argument that

their level of intelligence should preclude them from experimentation. It is a complex argument, but one that has resulted in the vast majority of animal studies take place in small mammals such as mice and rats.

The prime directives instituted for animal welfare in scientific testing are referred to as the 3Rs – replacement, reduction and refinement (Prescott and Lidster, 2017). The purpose of these directives is to, where possible, reduce the numbers of animals being used (by replacing *in vivo* models with applicable *in vitro* ones, for example, and ensuring that the correct number of animals for an experiment is estimated beforehand), and maximise the use of those animals. A push for greater *in vitro* and even *in silico* models, where applicable, can allow for the same scientific rigour without the need for experimentation that can cause great distress to animals used. Where the use of animals is required – and it goes without saying that this need still exists – techniques performed on these animals should be refined to the greatest extent possible. This is important not only from the point of view of developing new technologies that minimise harm but also, critically, ensuring that researchers are sufficiently trained to carry out any work with animals without causing undue harm. The 3Rs are enshrined in both UK and EU research, and grants awarded and work carried out must adhere to these and other animal welfare guidelines set out by law.

Cell line models are highly useful for modelling genetic disease in several aspects. They are easily manipulated to induce a mutation (increasingly so with the prevalence of genome editing technology), can usually be grown to practically unlimited amounts and can be easily transfected or transduced with therapeutic gene vectors. A huge range of cell types are available, and by using induced pluripotent stem cell (iPSC) technology (Okita et al., 2007; Takahashi and Yamanaka, 2006), patient cells can be harvested readily and

transformed into the relevant cell type. Further, as the cells are incapable of suffering or “harm”, ethical constraints are lessened; indeed, to reduce harm, they can potentially be a superior alternative. However, the argument in favour of animal models is that a cell model is overly simplistic with respect to disease, and the complexities of modern therapies (including gene therapies). Cell models effectively act in a vacuum – the single line is divorced from the complex interplay of a living organism’s myriad types of cells.

However, with respect to retinal degenerations, recent research has produced retina-like structures (called retinal organoids) from human iPSCs. These “mini-retinas” grow in 3D cell culture, and have been shown to produce cell types morphologically and transcriptomically resembling many of the major cell populations of the retina, with a limited degree of intercellular connection occurring (Reichman et al., 2017; Sasai et al., 2012; Völkner et al., 2016; Wahlin et al., 2017). The organoids are small, with a circumference of only ~1mm, and take nearly a year to grow. Equally, while the cells resemble retinas in culture, there are distinct structural and transcriptional differences. However, they offer significant potential for their use in potentially modelling retinal degenerations without requiring an animal model. Indeed, one group has recently used them to show how a mutation in the gene CEP290 (implicated in Leber congenital amaurosis and Joubert syndrome) can cause dysfunctional cilia biogenesis in photoreceptors (Shimada et al., 2017). This research is still at an early stage, but shows great potential for reducing the need for animal models in retinal research in the future.

1.6 Summary

As the title suggests, there are a number of key objectives of this thesis that focus on the advancement of gene therapies for inherited retinal degenerations. Chapter 2 focuses on the mitochondrially inherited disease LHON. LHON is a disease caused by mutations in Complex I of the electron transport system, the means by which mitochondria produce the majority of ATP (and thus, energy) that the cell requires (Farrar et al., 2013). RGCs are the specific cell type affected in this disease, and to combat LHON the Farrar laboratory has developed a therapy that utilises a yeast protein called *Ndi1*. *Ndi1* is a single-unit Complex I substitute found in *S. cerevisiae* that effectively ameliorates the cell death caused by loss of Complex I function in a chemically induced mouse model of LHON (Chadderton et al., 2013). The goal of Chapter 2 was to test whether a codon-optimised version of *Ndi1*, *ophNdi1*, could achieve the same effect, and the extent to which it could do this even at a significantly reduced dose compared to the original *Ndi1* AAV2/2 vector.

Following from this, Chapter 3 discusses a strategy implemented to derive and test novel tissue-specific promoters for use in gene therapy and gene expression studies. Focusing on potential RGC promoters, Chapter 3 used data from microarrays studies performed on the retina (Kim et al., 2006), and a putative promoter *Nefh* was selected as a prime candidate. The expression level and tissue specificity of *Nefh* was tested in the context of an AAV2/2 vector driving EGFP expression, and was compared at each stage to a non-specific *CMV-EGFP* construct. Chapter 3 also includes an attempt to minimise the *Nefh* promoter, and the effect on expression and specificity in this instance (compared to the original promoter) was also examined.

Finally, in Chapter 4, the subject matter under study changes significantly. Recent studies have indicated that supposedly healthy cells demonstrate significant copy number variation (CNV) when analysed at the single cell level. While researchers have shown this occurs in neurons as well, no study has thus far demonstrated this in a pure, differentiated population of cells, nor has genomic integrity been assessed in the retina.

Using flow cytometry to sort single photoreceptor cells, in this chapter we have used ultra-low input whole genome sequencing (WGS) to interrogate the makeup of the genome of photoreceptors, and to determine whether megabase-scale CNVs exist in a large percentage of cells. An introduction to the background to this work can be found in Chapter 4 and was not discussed in this section.

Chapter 2: Effect of codon optimisation on a yeast gene *Ndi1* for the treatment of Leber hereditary optic neuropathy

2.1 Introduction

2.1.1 Therapeutic approaches for LHON

In the thirty years since the discovery of the genes implicated in LHON (Wallace et al., 1988), no therapy has been approved for use that effectively ameliorates disease symptoms. The current front-line treatment is a small molecule drug, idebenone (marketed as Raxone; Santhera Inc.). Idebenone is a synthetic analogue of Coenzyme Q10 – it retains the electron-carrying quinone group, but with a shorter, less lipophilic tail that ends in a hydroxyl residue (Giorgio et al., 2012). This affords the molecule a significantly higher solubility compared to Q10, and thus a greater capacity for supplementation (James et al., 2005). Reduced idebenone (freely reduced in the cytoplasm by NADH) directly passes electrons to Complex III, thereby bypassing Complex I and establishing a mechanism for improving symptoms in diseases such as LHON (Haefeli et al., 2011). Idebenone offers limited slowing of disease progression, having been tested in multiple trials and approved for EU markets (santhera.com). Evidence is conflicted as to whether significant improvement can be achieved with idebenone, however it does appear to at least slow the

decline of vision in patients (Klopstock et al., 2011, 2016; Lyseng-Williamson, 2016).

There are a number of clinical trials currently underway for ND4-linked LHON treatment (Guy et al., 2017; Yang et al., 2016).

LHON is characterised by a relatively rapid onset of disease; however, as this onset does not come until adulthood, it provides a large window for therapeutic intervention before cell loss has occurred. The incomplete penetrance of the disease does pose an issue with respect to this, as any prospective treatment should be limited only to those who will suffer the disease. However, the potential ability to detect innate protection against LHON as demonstrated by Giordano et al. (2014) could provide an effective screening process to determine which family members in an LHON pedigree would most benefit from intervention. This must also be weighed against the fact that, as was discussed above, LHON can manifest relatively late in life (particularly in post-menopausal women) and thus when and if to intervene must be considered carefully.

As LHON is caused by mutations in single genes, it is in theory amenable to gene therapy to correct the loss-of-function caused by Complex I malfunction. RGCs lie at the front of the retina, and as such they are in theory readily accessible by injection directly into the vitreous. AAV2 has been the standard vector for therapies involving RGCs – the serotype has been used in multiple therapies for a range of diseases, and has featured in clinical trials injected both subretinally and intravitreally (Bennett et al., 2016; Feuer et al., 2016; Russell et al., 2017; Wan et al., 2016). Data from humans suggest that the vector is well-tolerated in the eye from a safety perspective (Bennett et al., 2016; Russell et al., 2017). A number of other serotypes including AAV8 and AAVrh10 have been tested for use in intravitreal injection, with mixed results. In an initial study comparing these two serotypes

in mice, expression of both serotypes in the GCL was described as “patchy” (Giove et al., 2010). Both vectors are able to cross through the retina and transduce photoreceptors (Bush et al., 2016; Park et al., 2009), but this fact alone indicates that much of the virus bypasses RGCs altogether. AAV2 has a binding affinity for heparin sulphate proteoglycans (HSPGs) – ablation of HSPGs from cells causes AAV2 to lose binding affinity (Summerford and Samulski, 1998). RGCs are known to require HSPGs for axon guidance (Erskine and Herrera, 2007; Ogata-Iwao et al., 2011).

However, the ILM – the barrier composed of Müller glial cell feet that separates the retina from the vitreous – also carries HSPGs on its surface (Woodard et al., 2016). This may be noteworthy, as the ILM has been shown to prevent AAV access to the retina, in particular in larger mammals. In order to overcome this, studies have variously used ILM peeling, sub-ILM blebbing followed by injection into the bleb, altered capsids (discussed below), and vitrectomy (Boye et al., 2016a, 2016b; Costa et al., 2017; Kimura et al., 2016; Takahashi et al., 2017). All of these studies have shown success in increasing transduction levels of cells with AAV2 therapies. There are drawbacks to this method, however. All of these methods, although some more than others, risk greater damage to the retina of the animal (or indeed patient). In particular, ILM peeling requires surgery that is quite invasive relative to a single intravitreal injection. One of the reasons vitrectomy has been hypothesised to be effective in increasing transduction is that it may actually damage the ILM, causing pores to tear open (Costa et al., 2017). In the case of techniques such as sub-ILM blebbing, the bleb that results does not spread to the entire GCL, and so the area of treatment effect is limited as a result. An additional but not insignificant concern is the matter of complexity. Intravitreal injections are a commonly performed outpatient procedure for patients suffering from AMD (Garweg et al., 2018). It remains to be seen

whether introducing greater complications will make sufficient improvements to potential patient outcomes, and whether the increased risk is worth it.

As discussed in Chapter 1, there are a number of modified AAV serotypes that are capable of efficiently transducing RGCs, in particular the tyrosine mutant AAV2. However, of equal importance is the therapeutic itself – what is the vector carrying? Although LHON is an orphan disease, a number of groups, including our own, have made progress in recent years developing effective gene-based medicines for the disease. There are perhaps several reasons for this. The first is that LHON is a disease that is in a sense very amenable to treatment. It is mitochondrially inherited, and so it is easy to screen for potential patients. In addition, the delayed onset of disease symptoms enables the possibility of either prophylactic administration of a therapy, or close monitoring followed up with immediate treatment. A third reason is that, although LHON is solely an ocular disease, there are limited indications that for complex diseases such as Parkinson's disease and multiple sclerosis (MS), where the disease pathology may be driven at least in part by mitochondrial dysfunction (Bose and Beal, 2016; Nikić et al., 2011; Witte et al., 2014). As a result, the design of a therapy that improves mitochondrial efficiency and reduces oxidative stress may go some way towards also treating these serious and life-threatening diseases.

2.1.2 Disease models of LHON

As discussed previously, a relevant animal model is important for testing gene therapies. A number of different approaches have been taken to produce animal models for LHON. One method has been the transduction of mouse RGCs with mutant, human *ND4*. Endogenous *ND4* is expressed from the mitochondrial genome, and as such lacks a mitochondrial localisation signal (MLS). Further, two of its codons, 16 and 24, are TGA – TGA is a stop

codon in the nuclear genome, however it encodes tryptophan in the mitochondria (Yu et al., 2012b). As such, a wild-type *ND4* construct would not express as a result of an AAV transduction. Previously, an MLS from the ATPc gene had been fused with a mutant *ND4* (with amino acids modified for correct nuclear expression), and appeared to traffic well into mitochondria when administered intravitreally via AAV2/2 (Qi et al., 2007). This approach cause progressive RGC degeneration, along with optic nerve swelling and higher levels of reactive oxygen species (ROS). This model was updated by the use of a novel capsid – the VP2 capsid protein was modified with an MLS (along with EGFP), that the authors claimed directed the AAV capsids to localise within the mitochondria (Yu et al., 2012b). Indeed, the authors show punctuated, perinuclear EGFP expression, suggestive of AAV mitochondrial localisation, along with a degeneration model mimicking LHON. The same group later showed rescue by applying wild-type *ND4* in an AAV vector, these symptoms could be improved dramatically (Koilkonda et al., 2014a)

However, it is important to note that, although the paper demonstrates mitochondrial staining and EGFP tracking of the AAV capsids, it does not co-label the two. There is also a danger here in possibly generating a model that involves overexpression of a protein already expressed endogenously. Famously, a mouse model of amyotrophic lateral sclerosis (ALS) involves the overexpression of a misfolding mutant of SOD1 – however, it was later shown that overexpression of the wild-type protein can also induce the same phenotype (Graffino et al., 2013). While, in the initial study of *ND4* expression, the authors showed that wild-type protein had no effect on ocular phenotype, the later study did not appear to test this (Guy et al., 2009; Qi et al., 2007; Yu et al., 2012b). It is also interesting that a disease phenotype was observed in this study, given that endogenous *ND4* was still being expressed and that LHON is a disease believed to be caused by gene loss-of-

function. This must be considered in particular in view of the observation that wild-type ND4 administration appeared to ameliorate the disease in this model (Koilkonda et al., 2014a). Finally, the application of an AAV to elicit the disease is potentially risky, as there is potential for a second injection of AAV with the therapeutic resulting in a stronger immune response (Li et al., 2008), although such an approach may be better tolerated in rodents than in primates. As such, some of these results may need further clarification, and hence the use of this model to test therapies should be considered carefully.

The most direct route to produce a mouse model for LHON is to create a transgenic animal with the same (or similar) mutation that causes disease in humans. This approach does not always work for disease models, variously due to transcriptomic differences between mouse and human, subtle structural differences in the protein being mutated, and the length of time allotted for disease symptoms to manifest (mouse lifespan being maximally only three years). One group successfully produced a mutant model by inducing a mutation (P25L) in the mouse *ND6* gene (Lin et al., 2012). To create the model, mouse fibroblasts were randomly mutagenised, and cells that displayed respiratory defects were sequenced until one with a mutation in a mitochondrial Complex I gene was found. Mitochondria from these cells were then injected into enucleated fibroblasts and mice were screened and backcrossed until a homoplasmic line had been developed. According to the authors, the P25L mutation can be severe enough to cause Leigh syndrome when homoplasmic in humans. Leigh syndrome is a multisystem, fatal disease that can be caused by mutations in any of a number of genes, including in mtDNA. With the need to produce a phenotype rapidly, however, P25L produced noticeable results in mice. Affected mice show profound, LHON-like degeneration at 14 months, seemingly caused by massive ROS production, rather than a loss in ATP production. Electroretinogram (ERG) results showed significant

impairment of visual function compared to age-matched controls, as well as accumulation of abnormal mitochondria in cells and respiration defects. This model provides an interesting insight into the development of LHON at a molecular level, as well as providing a potentially valuable platform for testing therapies aimed at treating the disease. The long lead time in this model to disease onset provides a barrier from an ease-of-use point-of-view, although given that LHON does not develop in humans until the second or third decade of life, this offers a large window to test a prophylactic treatment. Of note, however, no research has since been published testing a gene therapy or otherwise using this rodent model.

A third approach to generating an LHON disease model is to chemically induce disease symptoms using an insult such as rotenone. Rotenone is an isoflavone compound widely used as an insecticide and pesticide. Rotenone acts by irreversibly inhibiting ubiquinone reduction undertaken by Complex I (which is involved in transferring electrons to ubiquinone from NADH), and as such, creates a phenotype that in many ways mirrors LHON (Zhang et al., 2002, 2006). Rotenone has the advantage of causing a rapid onset of disease, with high levels of optic nerve atrophy and RGC loss seen within weeks (Chadderton et al., 2013), and can be applied exclusively to the retina with an intravitreal injection. This allows for control of disease onset, and by modulating the concentration of rotenone injected, potentially the severity of disease as well. Rotenone has been used by our own group as a means of simulating LHON for testing gene therapies (Chadderton et al., 2013; Mansergh et al., 2014), as well as others (Park et al., 2007). It has also been used *in vitro* for screening the effectiveness of gene therapies and small molecule drugs on alleviating LHON symptoms, as well as *in vivo* in testing the effect of idebenone (Datta et al., 2016; Heitz et al., 2012; Daniel Maloney, Farrar lab - unpublished work).

Rotenone's prime disadvantage, however, is that it directly affects Complex I. As such, for therapies such as *ND4* (as mentioned above), the benefit observed may be extremely limited. Only rotenone insensitive therapies (those that do not directly ameliorate endogenous Complex I function) are suitable for use in this model. Secondly, data from our own group has demonstrated that the concentration of rotenone is important in controlling the level of damage to the GCL that is observed. Too low a dose can fail to produce a phenotype, while too much can almost obliterate the inner retina. At times there can also be some inconsistencies in the severity of disease phenotype induced when the same dose is applied from different preparations of drug (some evidence of this can be seen in the Results section of this chapter). Based on the above, it can be concluded that rotenone-induced Complex I deficiency is a highly useful model for LHON in specific contexts. It underlines an important fact – that even for a disease such as LHON whose heritability can be well determined, possessing multiple models of the disease is highly valuable. It also provides an extremely useful platform for testing a potential therapy in multiple models – this is highly valuable in ensuring that a therapy shows promise before progressing to clinical trials.

2.1.3 Specific therapies for LHON

Clinical trials have been conducted on LHON patients by three separate research groups to date (clinicaltrials.gov). All three of these use either the wild-type *ND4* gene, or a modified variant. Previous preclinical studies have demonstrated that human *ND4* administered to mice are protected from the deleterious effects of mutant *ND4* transduction. Several preclinical studies from one group have shown that, when a triple tyrosine mutant AAV2/2 vector with an MLS carrying wild-type *ND4* was intravitreally administered to mouse

retinas, it offered protection when mutant *ND4* was later applied in the same manner (Koilkonda et al., 2014a, 2014b; Yu et al., 2012a), as detailed previously. This application of the protective treatment before onset of disease is a frequently used approach for the testing of LHON therapeutics. The second injection, with the mutant *ND4*, occurred two days to one week after the first injection of the candidate therapy. Transduced retinas appeared significantly protected against mutant *ND4* insult, with mutant:wild-type transduction ratios of 1:4 and 4:1 both showing similar levels of protection. In one study, mutant *ND4* was applied to the retina (via electroporation) before the wild-type *ND4* was administered by AAV injection. The authors noted that symptoms appeared approximately one month post-electroporation, and as such, the therapeutic AAV vector was administered two weeks after the mutant gene (Cwerman-Thibault et al., 2015).

Pre-clinical safety studies for *ND4*-based therapies have also been performed in NHPs. Rhesus macaques injected with human *ND4* in a triple tyrosine mutant AAV vector were shown to have good safety profiles, with a rise in neutralising antibodies to AAV being the only significant safety concern shown (Koilkonda et al., 2014b). Of note, in a rat biodistribution study as part of this study, AAV particles were found in some rat lymph node samples, as well as in the optic nerves of macaques administered with high-dose AAV (2×10^{11} vg). Limited evidence of AAV transduction in NHPs was shown by immunohistochemistry (IHC) – EGFP-positive cells co-labelling with Brn3a were demonstrated in a single image, but the extent of transduction achieved was not alluded to (Koilkonda et al., 2014a).

Of the three groups conducting clinical trials for LHON, two have published results based on trial outcomes. One group (Gensight Biologics) has released preliminary data claiming significant improvement in a Phase I/II study, with sustained visual acuity increases seen

over a two-year period (trial ID NCT03293524). Of those groups who have published results, the first (based in the Huazhong University of Science and Technology; NCT01267422) have reported 9-month and 36-month follow-up data on patients injected with an AAV2/2 vector containing wild-type *ND4* (Wan et al., 2016; Yang et al., 2016). Patients received a relatively low dose as a safety precaution, with patients under 12 receiving 5×10^9 vg, and others 1×10^{10} vg. The study is notable, as the median age of patients at injection time was 17 years, with three of the nine patients being 10 or under. This notably reflects a particularly young onset of the disease (median 13 years of age) in this patient cohort. The visual acuity of six of the patients improved by 0.3 log MAR (median angle of refraction) or better at the 9 month point, with one patient reportedly able to read newspaper headlines. However, this had decreased to only four of the patients by 36 months. In the case of one of these patients, the uninjected eye improved dramatically, to a far greater extent than the treated eye (baseline acuity: treated 1.1 log MAR < untreated 1.0 log MAR; acuity at 36 months: treated 0.4 log MAR, untreated 0.1 log MAR). Spontaneous improvement in LHON has already been referred to above. In all cases, the retinal nerve fibre layer (RNFL) thickness was preserved in the treated eyes, while it continued to degenerate significantly in untreated eyes ($p < 0.05$). One patient showed a dramatic improvement at 9 months (increase of 0.9 log MAR) and at their request the second eye was injected. However, for reasons the authors did not investigate, the acuity of both eyes subsequently decreased, and they warned against injecting the contralateral eye for LHON until further studies were undertaken. This finding was in contrast to a previous study in Leber congenital amaurosis, in which an AAV gene therapy was administered subretinally (Bennett et al., 2016). In this instance, injection of the contralateral eye produced improvement, with no corresponding decrease in the first eye injected. However

in this regard intravitreal injection has been shown to elicit a greater immune response than subretinal injection, so this may at least in part explain these results (Li et al., 2008).

The second group of trials, carried out in the University of Miami (NCT02161380), was primarily done to test the safety of their LHON gene therapy; correspondingly, the dose administered was relatively low (low-dose: 5×10^9 vg; high dose: 2.46×10^{10} vg). This group, as with the previous, utilised a functioning *ND4* modified for nuclear expression, and with an MLS appended (Feuer et al., 2016; Guy et al., 2017) – the gene was administered using a triple tyrosine mutant AAV2/2 vector. Patients in this case were older (median age 30), and in many cases their baseline visual acuity was far worse, at only hand movement, finger counting or light perception levels. As such, acuity improvement tests were less well defined. That said, the authors reported a 12 month improvement over baseline over 0.24 log MAR; untreated eyes improved by 0.09 log MAR; this difference continued to increase to 0.96 between treated and untreated in one group (those with the disease for less than a year) and was found to be significant ($p = 0.001$). The drug appeared to be well tolerated, with two patients developing mild uveitis that resolved on its own. Conservation of RNLF thickness was maintained in the treated but not the untreated eye, as in the above study. Although early work, the suggestions of slight improvement, even in patients that had lost their sight several years before, is a positive indicator that supplementation of Complex I has significant potential as a strategy for LHON treatment.

2.1.4 Ndi1 as a treatment for LHON

The previous studies utilised *ND4* as their therapeutic gene, as *ND4* mutation is by far the most common form of LHON. However, a gene therapy that could treat all forms of LHON, including *ND4* mutations, would be highly valuable and could be relevant to other

Complex I deficiency disorders. *Ndi1* is a gene produced by a number of species of yeast that serves as a partial alternative to Complex I, transferring electrons to ubiquinone but without acting as a proton pump (Park et al., 2007), summarised in Figure 2.1. Importantly, unlike the 45 subunits of mammalian Complex I, *Ndi1* is a single protein that acts as a dimer (Iwata et al., 2012). *Ndi1* can be found in yeast species possessing a functional Complex I, as well as some such as *Saccharomyces cerevisiae* that do not. Of note *Ndi1* is expressed as a nuclear gene, and carries an endogenous MLS that effectively directs it to the mitochondria; this MLS also functions well in mammalian cells (Seo et al., 1998).

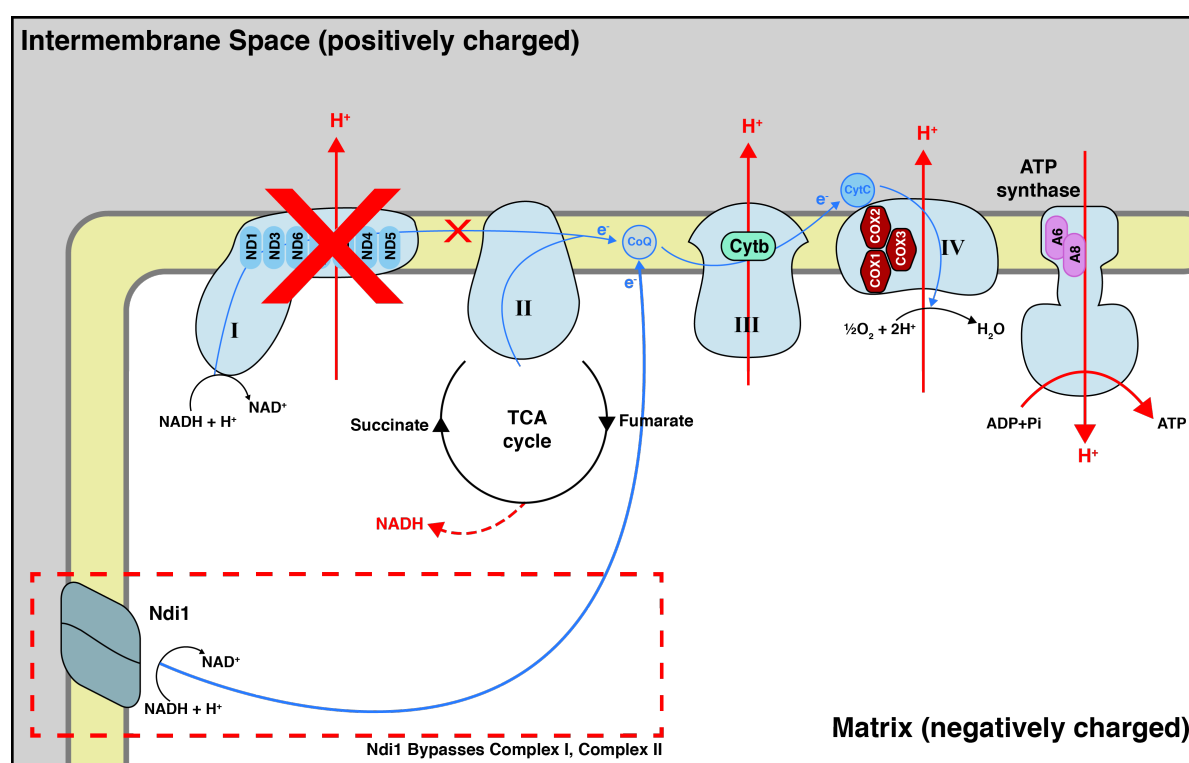


Figure 2.1: *Ndi1* function in mitochondria. In the absence of Complex I function (through mutation or inhibition), *Ndi1* has the capacity to directly transfer electrons to CoQ, enabling a reduction in reactive oxygen species and (although not acting a proton pump itself), enabling the pump action of Complexes III and IV.

Ndi1 was originally suggested as a treatment to overcome Complex I deficiency as early as 1998, where authors demonstrated that cells transfected with the gene could overcome Complex I inhibition but not inhibition of the other ETS complexes (Seo et al., 1998). It

was subsequently expressed *in vivo* in the rat brain, where it was shown to offer some protection against chemically induced neurodegeneration (Yagi et al., 2006). Functionally, *Ndi1* appears to alleviate the symptoms of Complex I inhibition or malfunction by relieving the pressure of oxidative stress. It has been shown *in vitro* that Complex I inhibition by rotenone causes cell death through the release of pro-apoptotic factors originating from mitochondria (Marella et al., 2007; Seo et al., 2006). This appears to be caused by an increase in ROS that drives pro-apoptotic kinase activity and cell death. The ability of *Ndi1* to direct electron transport to CoQ and thus Complexes III and IV in the absence of Complex I allows for restoration of energy production and continuing reduction of ROS. Indeed, *in vitro* at least, *Ndi1* has been shown to completely ameliorate the effects of Complex I inhibition (Marella et al., 2007). *Ndi1* later was shown to provide benefit in a rotenone-induced rat model of LHON (Marella et al., 2010). In this study, *Ndi1* was expressed using an AAV5 vector targeted to the superior colliculus, one of the regions of the brain where RGC axon terminals are found. Rotenone was administered using encapsulating microspheres, which slowly released the toxin within the superior colliculus over a period of several weeks. The group noted that the rats treated in such a way lost vision after two weeks, but RGC death was slower, in a manner that mirrored LHON disease progression. However, injections to the superior colliculus are not necessarily practical for use in humans. In contrast, intravitreal injection allows for more direct access to the RGC cell body, the major site of disease pathology in LHON and where the vast majority of gene and protein expression will take place.

Indeed, more recently a study from the Farrar research group demonstrated that *Ndi1* administered intravitreally using an AAV2/2 vector provides substantial protection against rotenone-induced insult in mice. When injected intravitreally, AAV-*Ndi1* showed

significant protection against rotenone damage as compared to a control, AAV-EGFP-injected eye (Chadderton et al., 2013). The numbers of RGCs in the GCL were 1.5-fold higher in AAV2-*Ndi1* treated eyes compared to controls, and the optic nerve was significantly thicker. Additionally, far less membrane debris was seen in the optic nerve in *Ndi1*-treated samples, as assessed by electron microscopy. Behaviourally, mouse functional vision was also improved in the treated eye, when testing the optokinetic response (OKR). OKR is a method for assessing visual acuity compatible with RGC disease models that uses a simulated rotating drum to analyse the limits at which a mouse can track changes in light (Prusky et al., 2004). *Ndi1*-treated eyes were found to be indistinguishable from wild-type eyes in OKR assays. This initial study demonstrated that, in principle, a trans-kingdom gene therapy (employing a yeast gene delivered via AAV) has the potential to effectively treat a disease such as LHON.

2.1.5 Optimisation of *Ndi1*

Ndi1 offers a potentially powerful therapy as a means of treating LHON, as well as potentially other diseases with mitochondrial involvement. However, where possible, optimisation of any gene therapy is a valuable and sometimes necessary approach. In particular, when dealing with virally delivered gene medicines, it is important to maximise the level of expression achieved using the minimum dose of viral vector. Preliminary work with *Ndi1* in our laboratory suggested that significant reductions in dose below that used in Chadderton et al. (2012) – approximately 3×10^9 vg – reduced therapeutic efficacy. Furthermore, while AAV has been shown to be well tolerated in the human eye, high doses of virus may potentially evoke a humoral and/or cellular immune response (Calcedo and Wilson, 2013; Li et al., 2008), and so minimising the effective viral dose in principle should maximise the therapeutic potential of the treatment. Codon optimisation offers a

potential means of increasing gene expression levels without modifying the protein expressed, by utilising the redundancy of the genetic code. While the majority amino acids are encoded by multiple codons, the balance of codon usage for a given amino acid varies from species to species (Gustafsson et al., 2004). As a simple example, although there are six codons for the amino acid leucine, in humans 41% of leucines are encoded by a CTG – in *S. cerevisiae*, that number is approximately 11% (genscript.com). There is a relationship between the frequency of a codon's use and the levels of corresponding tRNA produced (Rocha, 2004; Rudolph et al., 2016); as such, having a sub-optimal codon set could reasonably reduce that gene's expression. Codon optimisation has been employed in gene therapies delivered both systemically and to the retina (Fischer et al., 2017; Kosovac et al., 2010). While codon usage profiles between mouse and human are similar, there are vast differences between human and yeast. A key objective of the research undertaken by the TCD team has been to explore ways of optimising *NdiI* for future use in human gene therapies, with the resulting gene (*ophNdiI*) expressing at far higher levels in HEK293 cells than the original *NdiI* constructs. Some preliminary work on this was undertaken in HEK293 cells (Carrigan 2014, PhD thesis); this work demonstrated that, *in vitro*, *ophNdiI* expressed at a dramatically increased level compared to the original *NdiI*. Now, this work has been extended significantly to encompass *in vivo* evaluation in a disease model as part of the current study.

2.1.6 Summary and project objectives

The aim of this study was to assay *in vivo* the effectiveness of *ophNdiI* as compared to the original *NdiI* construct, the effectiveness of which has previously been characterised (Chadderton et al., 2013). *ophNdiI* has been shown to provide higher expression *in vitro* in HEK293 cells than the original *NdiI* (Carrigan 2014, PhD thesis). The aim of this chapter

was to assay whether *ophNdi1* would provide comparable increases in expression – and as such provide equivalent or superior protection – when administered *in vivo*. As in previous studies, the effectiveness of *ophNdi1* was assayed by incorporation into an AAV2/2 vector, expressed using a *CMV* promoter. This study was carried out by comparing *CMV-ophNdi1* to the original *CMV-Ndi1* vector at two reduced doses (6×10^8 and 1.2×10^9 vg/eye for *ophNdi1*, compared to 3×10^9 vg/eye for the original *Ndi1*), in a rotenone-induced mouse model of LHON. To test the protective effect of each vector, the optokinetic response (OKR) of treated mice was measured – this offered a means of assaying visual acuity and is valuable in situations where one eye is treated and the other left untreated. Combining this with direct measurements of RGC survival post-sacrifice, and RNA expression level *in vivo* for each vector, allows for a valid comparison between the effectiveness of expression and protection of *ophNdi1* and *Ndi1*, with *ophNdi1* administered at a lower dose.

2.2 Results

This study has been designed to examine the potential for improving upon candidate gene therapies – in this case *Ndi1* – by employing codon optimisation. Additionally, aspects of AAV production have been optimised by the incorporation of an enlarged backbone in the plasmid carrying the therapeutic transgene for AAV production, in order to maximise therapeutic AAV packaging. Human-optimised *Ndi1* (*ophNdi1*) engineered into an AAV vector has been evaluated and compared against the original *Ndi1* AAV2 vector construct (Chadderton et al., 2013) to measure the effect of optimisation on transgene expression, viral dosage and therapeutic efficacy. Having previously obtained beneficial effects using the original *Ndi1* design, a central question was whether *ophNdi1* might offer a superior therapy.

2.2.1 *ophNdi1* large backbone cloning

The optimised *Ndi1* gene, *ophNdi1*, was initially cloned into the larger plasmid backbone. This plasmid backbone (previously generated by Dr. Mary O'Reilly, Farrar laboratory, TCD) consisted of a pAAV plasmid enlarged in size using “stuffer” sequence from phage lambda. *ophNdi1* was excised from its original pAAV plasmid using restriction enzymes *MluI* and *XhoI*; the large backbone AAV plasmid was similarly cut with *SpeI* and *XbaI*; both constructs were then blunted using Klenow. Confirmation of the success of this initial digest can be seen in Figure 2.2. Both insert and vector were purified from an agarose gel and ligated together and colonies screened for candidate clones using standard methodologies (see Materials and Methods). Candidate clones were analysed and plasmids then sequenced to confirm correct insertion. A plasmid map depicting the construct generated can be seen in Figure W (see Appendix 4 for sequence). Subsequent to sequence

confirmation, a high-titre AAV2 preparation containing *CMV-ophNdi1* (AAV2-*CMV-ophNdi1*) was then produced (see Materials and Methods for details of AAV production methodologies employing triple transfection of plasmids in HEK293 cells). The cytomegalovirus (CMV) promoter was chosen as it is commonly used in preclinical and clinical studies targeting the retina using AAV. It is small (~500bp), which is highly useful for AAV vectors where size is a limitation, and it drives constitutive expression both *in vitro* and *in vivo*.

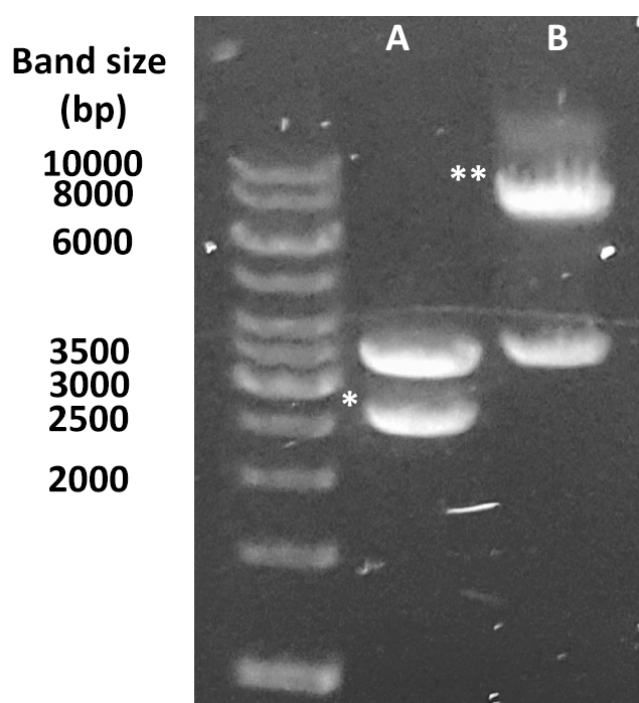


Figure 2.2: Restriction digest map of plasmid digest to clone *CMV-ophNdi1* (A) into the large backbone (B) for AAV preparation. * indicates the *CMV-ophNdi1* insert; ** indicates the large backbone vector.

2.2.2 *ophNdi1* mitochondrial localisation

Ndi1 is a protein that acts in the mitochondria, and as such mitochondrial localisation is critical to its function. To confirm the localisation of the Ndi1 protein expressed from *ophNdi1*, HEK293 cells were transfected with a plasmid expressing *CMV-ophNdi1*. It is notable that a slightly modified form of the *ophNdi1* plasmid with a c-terminal HA-tag was

used for this experiment as there was no available antibody for evaluation of *Ndi1* by immunocytochemistry (see Discussion of this chapter for more information). This HA-tagged *ophNdi1* was contained in a smaller, pAAV plasmid, rather than the enlarged backbone, for the purposes of easier transfection. HEK293 cells were co-transfected with a plasmid termed dsRed2 mito 7 (Addgene); the plasmid expresses a dsRed gene with an MLS appended, that has been shown to successfully locate to mitochondria within mammalian cells. Using an antibody to the HA-tag included in the construct, *ophNdi1* was shown to successfully colocalize with dsRed in cells transfected with both constructs (Figure 2.3). The punctuated pattern of expression within the cell is indicative of mitochondrial localisation (Badugu et al., 2008; Matthews et al., 2010). Figure 2.3c highlights a cluster of several cells with *ophNdi1* and dsRed colocalised to the mitochondria. Furthermore, the pattern of staining is distinct from EGFP lacking an MLS (Figure 2.4), where expression is diffuse throughout the cell. *ophNdi1* overlayed with an EGFP marker demonstrates a distinct difference in protein localisation throughout the cells. There is the possibility that the localisation pattern observed may be in part due to the HA tag itself, and not the MLS of *Ndi1*. A valuable control would antibody staining for an *ophNdi1* lacking the HA tag. Although this is not possible (owing to a lack of an effective *Ndi1* antibody), the use of another protein (such as dsRed mito) both with and without a HA tag would be useful in confirming the localisation.

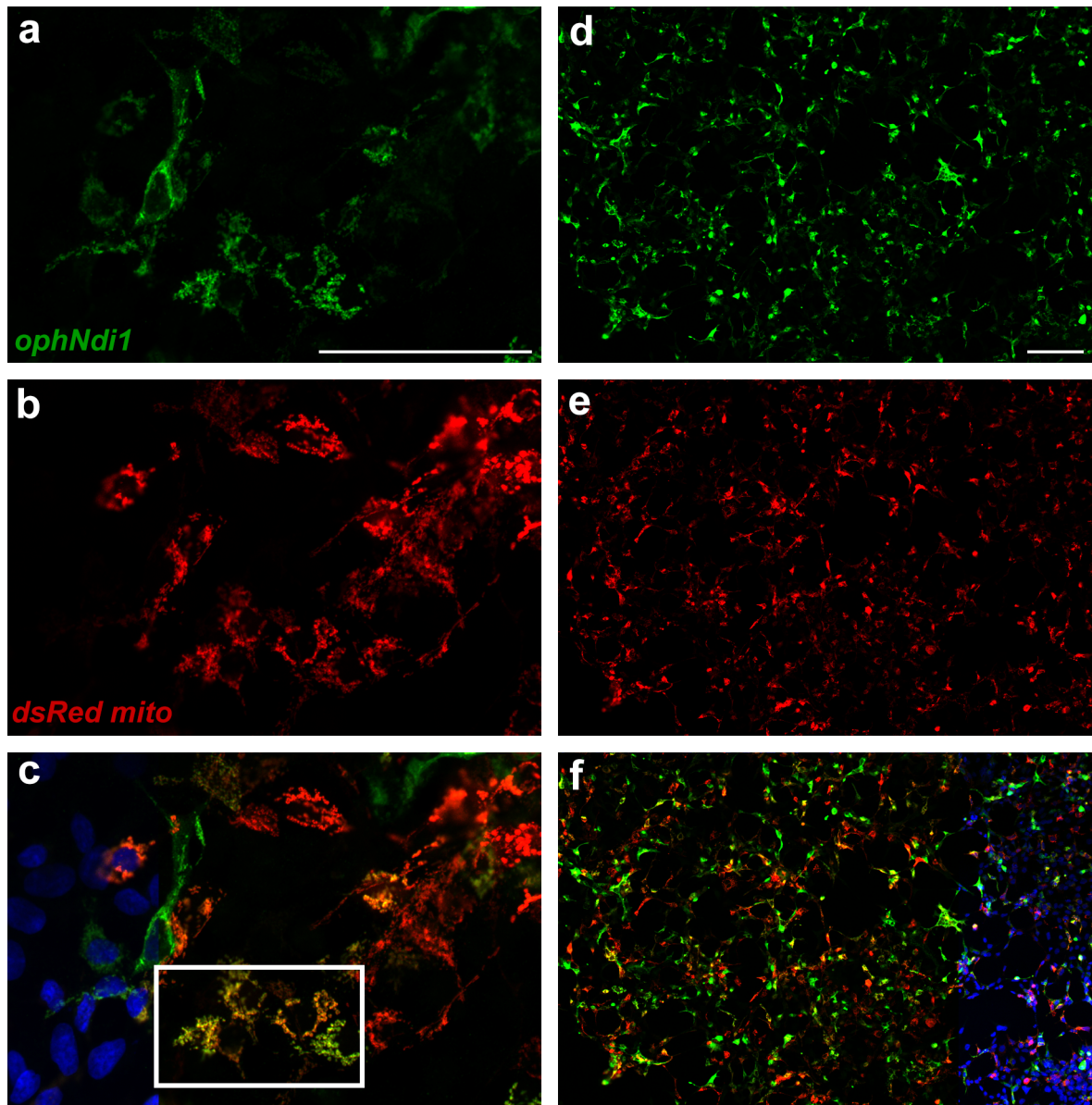


Figure 2.3: Immunocytochemistry analysis of HEK293 cells transfected with HA-tagged *ophNdi1* (green) and an MLS-conjugated dsRed marker (red). Two days post-transfection, cells were fixed and stained with an antibody targeting the HA tag of *ophNdi1*. DAPI was used as a nuclear counterstain (blue). Figure a-c show high magnification images focused on a representative cluster of cells (630x magnification), while d-f show a lower magnification giving a broader view of the staining pattern of the cells (100x). Cells transfected with both *ophNdi1* and dsRed appear yellow/orange in colour; the white box in c highlights a cluster of co-transfected cells. Scale bars: 30 μ m (a), 100 μ m (d).

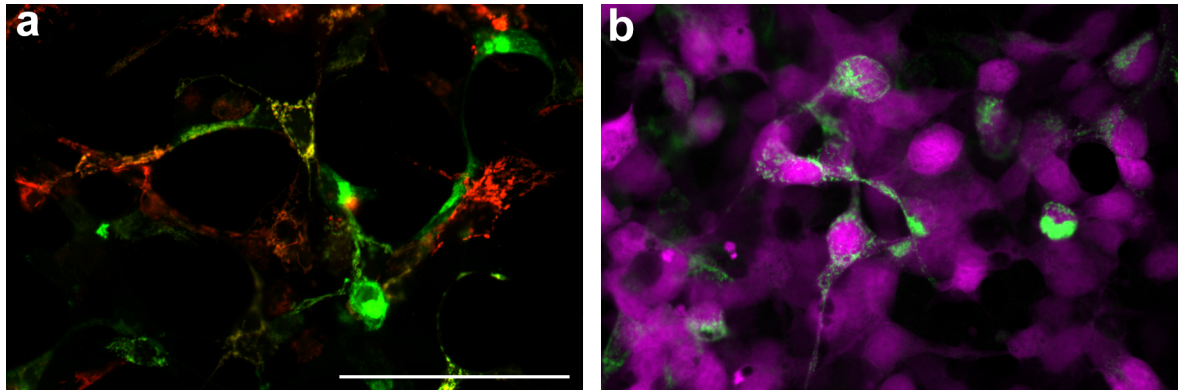


Figure 2.4: Further immunocytochemistry of *ophNdi1*-transfected cells. Figure a shows a representative high-magnification image of cells cotransfected with *ophNdi1* and MLS-conjugated dsRed. Figure b shows *ophNdi1* transfected with an EGFP marker lacking an MLS. In this image, EGFP has been coloured magenta, and HA-*ophNdi1* (originally stained with a red Cy3-conjugated antibody) has been coloured green. Scale bar: 30µm.

2.2.3 RNA expression

While aspects of the effectiveness of *ophNdi1* expression had previously been shown *in vitro* in HEK293 cells (Matthew Carrigan, PhD thesis, 2014), it was important to determine the extent to which codon optimisation might improve expression levels of the *Ndi1* gene *in vivo*. Adult wild-type 129 mice were injected intravitreally with either AAV-CMV-*Ndi1* (two separate AAV preparations were tested) or AAV-CMV-*ophNdi1* (each virus produced using the larger backbone), at the viral titres listed in Table 2.1. Three weeks post-injection, mice were sacrificed and RNA was extracted from the retinas (the n number of retinas used per vector are listed in Table 2.1). Transgene expression levels were measured using RT-qPCR (the primers used are listed in Materials and Methods). As seen in Table 2.1, *CMV-ophNdi1* expression levels far outstripped those of the original *Ndi1* vectors, despite a lower titre. The expression levels demonstrated by the original *Ndi1* vectors were at the edge of detection, and so could not be accurately quantified – however, notably there was a clear difference between the expression levels of the original *Ndi1* vectors and the codon optimised *ophNdi1* vector. Extreme variance in expression between *CMV-Ndi1* vectors, and indeed within each treatment group, was observed. This may be due to the low

number of RGCs found in the retina, as a proportion of total retinal cells; as a result, inconsistent levels of mRNA may have been extracted from each RGC population in question.

Viral Vector	Titre injected (vg)	n-number	Expression (copies/μl)	Standard deviation
<i>CMV-Ndi1</i> #1	4.5×10^9	3	297.10	442.18
<i>CMV-Ndi1</i> #2	4.5×10^9	4	43.30	48.83
<i>CMV-ophNdi1</i>	1.2×10^9	4	3813.75	2065.72
<i>Nefh_short-ophNdi1</i>	4.5×10^9	3	43.73	31.99

Table 2.1: List of viruses injected intravitreally for RNA expression analysis, and corresponding expression shown. All vectors utilised the AAV2 serotype. *Nefh_short-ophNdi1* was injected at the same time as the others listed as a comparison, but is relevant to Chapter 3 and is discussed there. n numbers given indicate number of retinas per group, each run in triplicate.

2.2.4 Preliminary rotenone batch test

Following from the data in Chadderton et al., (2013), the potential protective effect of a reduced *ophNdi1* vector dose against rotenone insult was assessed *in vivo* in wild-type 129 mice and compared to the benefit derived from the original *Ndi1* vector. Previous work with rotenone as an insult (a Complex I inhibitor) has shown occasional inconsistency in the effects obtained between batches of drug. In order to ensure the effectiveness of the batch to be used for these experiments, initially the effect of intravitreal rotenone injection in wild-type mouse eyes was assayed. Two weeks post-injection of 1.5nmol rotenone, injected and uninjected mice were sacrificed, and harvested eyes were cryosectioned as described in section 5.6. Sections were stained with NeuN to visualise RGCs in the retina (n=2 for uninjected eyes, n=6 for injected eyes). NeuN is a common neuronal cell body marker that, in the context of the retina, is relatively selective for RGCs (Buckingham et al., 2008; Templeton et al., 2009; Zhu et al., 2013). RGC counts were used to estimate

damage to the GCL; results are shown in Figure 2.5. Counts were taken from four sections spaced approximately 150µm apart from the central region of the retina (within 750µm either side of the optic nerve head). On average, rotenone-treated eyes showed a greater than two-fold decrease in the number of RGCs per millimetre (73 vs. 33; $p < 3.5 \times 10^{-7}$).

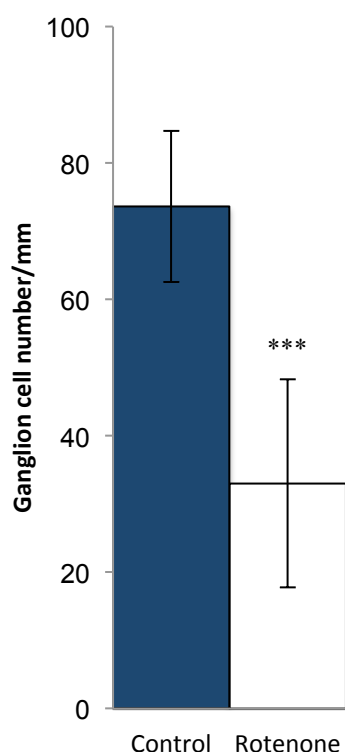


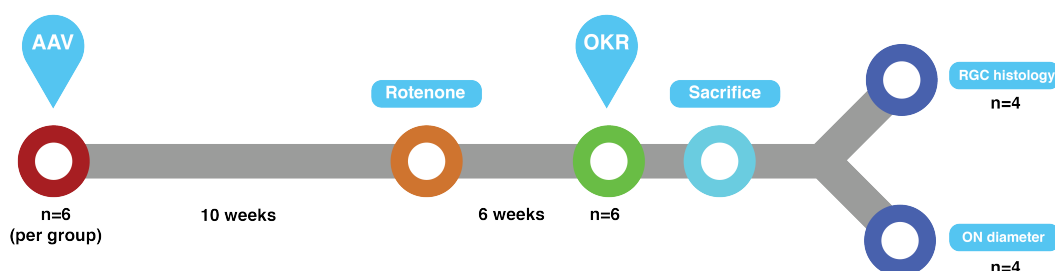
Figure 2.5: Bar chart representing mean ganglion cell counts per millimetre. Two weeks post-rotenone injection, retinas were cryosectioned and cell counts were performed in order to gauge effectiveness of rotenone as an insult. ***, $p < 10^{-7}$

2.2.5 Histological analysis

Having confirmed protein localisation of *ophNdi1* to the mitochondria (Figure 2.3), and that RNA expression levels were far higher than the original *Ndi1* viral vector (Table 2.1), the two *Ndi1*-based viruses were then compared for their ability to rescue the phenotype induced by rotenone insult in the mouse retina. Contralateral eyes of adult wild-type 129 mice were injected intravitreally with an AAV2/2 *Ndi1* construct or an AAV2/2 EGFP

control (The EGFP control vector was produced using the original pAAV backbone rather than the larger backbone). A summary timeline of experimental procedures is shown below in Figure 2.6.

(a) First experimental round



(b) Second experimental round

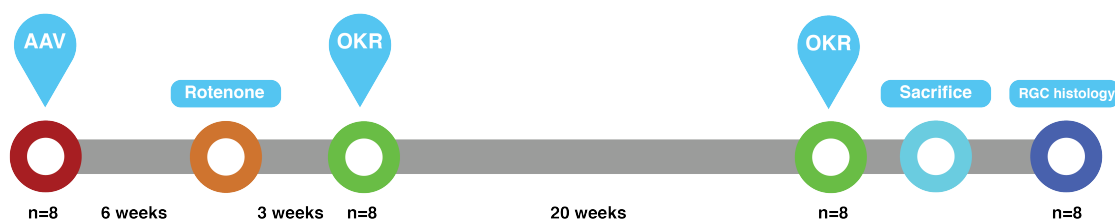


Figure 2.6: Experimental timeline of AAV and rotenone injections and analysis for the first (a) and second (b) rounds of assessment. (a) n-numbers refer to numbers of mice used per treatment group (original *Ndi1*, and two doses of *ophNdi1*, 6×10^8 and 1.2×10^9 vg/eye) with right eye treated and left eye serving as a paired, rotenone-only control. (b) n-numbers refer to number of mice used in sole treatment group, *ophNdi1* 1.2×10^9 vg/eye.

Two doses of the AAV-*ophNdi1* vector were used (6×10^8 and 1.2×10^9 vg/eye), amounting to one sixth and one third of the original AAV-*Ndi1* dose respectively (3.6×10^9 vg/eye). In *Ndi1/ophNdi1* treated eyes, AAV-EGFP was co-injected at a reduced dose (3×10^8 vg/eye) in order to visualise transduction of the retina (n=4 for each group). Ten weeks post-injection of AAV, 1.5nmol of rotenone was intravitreally injected into each eye. Six weeks post-rotenone treatment, the integrity of the retinas was examined. Histological analysis (Figure 2.7) showed that control eyes exhibited marked damage to the GCL as compared to eyes treated with any of the *Ndi1* constructs, which demonstrated greater levels of preservation of the retina. Lacking an effective *Ndi1* antibody, EGFP expression was used as a proxy for transduction; a representative example of this is given in Figure

2.7f. As previously, RGC counts were used to estimate the level damage to the GCL. Of note, all *NdiI* vectors, both the original and the optimised vectors, offered significant RGC protection compared to the controls. In particular, mouse eyes treated with either dose of the AAV2-*ophNdiI* vector provided benefit that exceeded that of the original AAV2-*NdiI* vector. *NdiI*-treated eyes showed a 1.4-fold increase in the number of RGCs compared to controls (*NdiI*: 68; rotenone only: 48, $p < 0.05$). The *ophNdiI*-treated samples showed a 1.8-1.9 fold increase in the number of cells, significantly greater than that observed in *NdiI*-treated eyes (1/6 *ophNdiI*: 93; 1/3 *ophNdiI*: 88 $p < 0.01$; t-tests were performed to generate p-value statistics, corrected for multiple testing using the Bonferroni method). Indeed, the RGC numbers observed in the *ophNdiI*-treated eyes showed no difference to that in wild-type, rotenone-free eyes. No significant difference was found in the number of RGCs between the two *ophNdiI* vector doses used in the study.

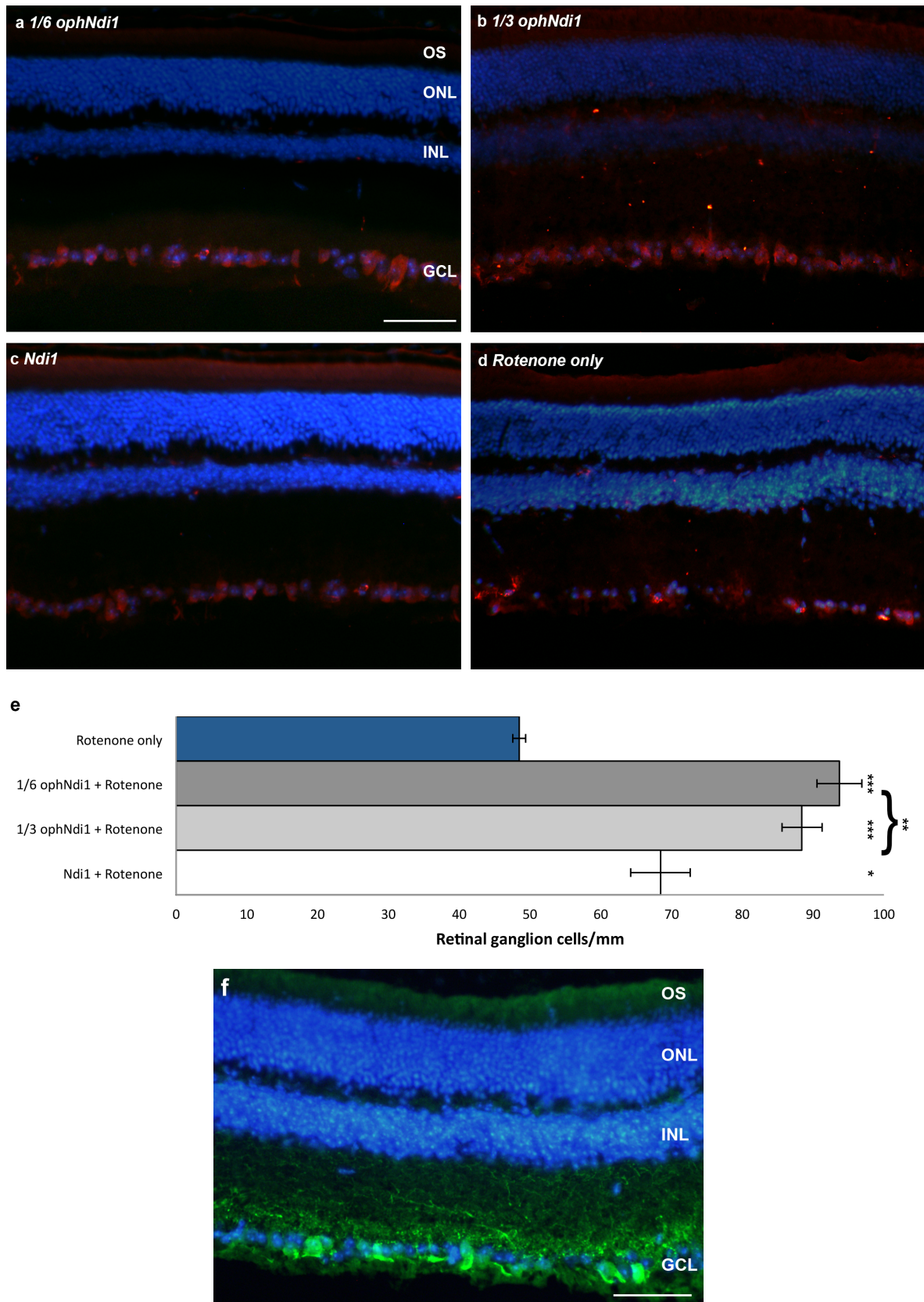


Figure 2.7: Histological analysis of RGC preservation. (a)-(d) Ganglion cell numbers per millimetre were counted on four sections roughly 150 μ m apart and averaged for each eye (each group n=4). All retinas were stained with rat α -NeuN to visualise ganglion cells; nuclei were counterstained with DAPI. Apparent green stain in (d) results from overexposure of DAPI filter. (e) Bar chart representing mean ganglion cell counts per millimetre (bars represent standard error). (f) Representative section of EGFP fluorescence (unstained)

with DAPI counterstain to confirm transduction. Eyes transduced with any of the Ndi1 retained significantly higher numbers of ganglion cells than rotenone only controls. Eyes from both ophNdi1 groups showed greater protection versus the original Ndi1. Abbreviations: OS, outer segment. ONL, outer nuclear layer. INL, inner nuclear layer, GCL, ganglion cell layer. Scale bar = 50 μ m. p-values: *, $p < 0.05$, **, $p < 0.01$. ***, $p < 0.005$

In order to assess the effect of AAV2-*ophNdi1* on the retina over a longer period of time, a group of mice injected with the higher dose *ophNdi1* vector were sacrificed five months post-rotenone injection (n=8 mice, right eyes treated and left eyes used as paired controls). Retinas were harvested and sectioned as before, and compared to the control contralateral eye. In this instance, Brn3a was used to stain RGCs on retinal sections for cell counting. Brn3a is a transcription factor that serves as a specific marker for RGCs in the retina (Schlamp et al., 2013). The neat, nuclear staining pattern observed allows for cleaner counts of RGCs and so was used in place of NeuN. As shown in Figure 2.8, treated eyes had a significantly higher number of cells preserved in the GCL as compared to control eyes (91.75 cells/mm vs. 67.33, $p < 0.05$). Notably, the control rotenone-only eyes had somewhat higher numbers of cells when compared to the previous set of experiments.

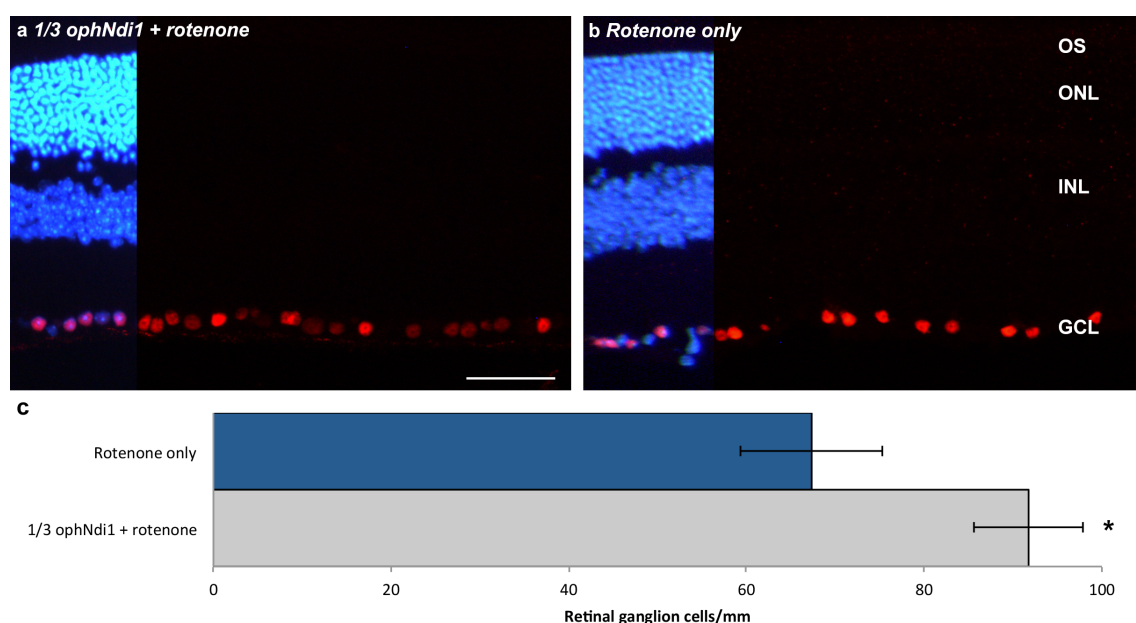


Figure 2.8: Histological analysis of RGC preservation using Brn3a as a marker. (a,b) Ganglion cell numbers per millimetre were counted on four sections roughly 150µm apart and averaged for each eye (each group n=8). Retinas were either transduced with *ophNdi1* and EGFP (a) or EGFP alone (b; EGFP not shown) – both groups were then treated intravitreally with rotenone. All retinas were stained with α-Brn3a in order to visualise RGCs for counting purposes. (c) Bar chart representing average number of RGCs counted per millimetre (bars represent standard error). Eyes transduced with *ophNdi1* (a) demonstrated significantly greater RGC preservation in the face of rotenone insult than those not (b). Abbreviations: OS, outer segment. ONL, outer nuclear layer. INL, inner nuclear layer, GCL, ganglion cell layer. Scale bar = 50µm. p-values: * < 0.05.

2.2.6 Optic Nerve analysis

The disease pathology observed in LHON patients involves both the GCL and also the optic nerve, a feature shared with the rotenone-induced mouse model. Ten weeks-post rotenone treatment, optic nerves from the same *Ndi1* treated and control mouse eyes discussed in Figure 2.7 were harvested and examined using light microscopy. 5mm of optic nerve proximal to the retina was sectioned, and four sections (approximately 0.4-1mm apart) were measured twice to gauge mean diameter.

Results from the optic nerve samples can be seen in Figure 2.9; animals treated with an *Ndi1* vector showed substantial reductions in optic nerve thinning relative to EGFP controls. Treatment with AAV2/2-*Ndi1* or AAV2/2-*ophNdi1* resulted in increased diameter of optic nerves 1.13-1.25-fold on average (p<0.05). The *ophNdi1* vector offered protection at least as effectively as the original *Ndi1* vector, even at the lower dose. No significant difference was found between the three treated groups (ANOVA). A slight trend indicating the higher dose *ophNdi1* offered greater protection was observed but could not be confirmed. The data shown here clearly demonstrates that the *ophNdi1* vector equalled or improved protection morphologically, of both RGCs and the optic nerve, compared to the original *Ndi1* vector at as low as one-sixth the viral load.

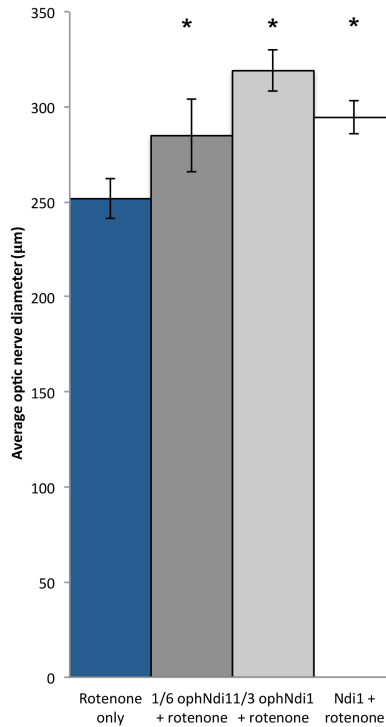


Figure 2.9: Optic nerve diameter following *Ndi1* treatment. Optic nerve measurements were made in four locations per retina and averaged (n=4 per group). Treated groups showed significantly higher protection of optic nerve diameter versus control. p-values: *, $p < 0.05$.

2.2.7 Optokinetic analysis

It is critical in developing a therapy that functional benefit is provided by the treatment; thus, wild-type adult mice treated with *Ndi1* and *ophNdi1* vectors were assayed for retention of spatial vision. The same vector doses were employed as per the prior experiments. Six weeks post-rotenone treatment, the OKR of mice was measured (n=6 for each group). The thresholds of spatial frequencies for both treated and untreated eyes were estimated using a stepwise approach; each animal was tested on four separate occasions to accurately gauge results. As shown in Figure 2.10, animals treated with *Ndi1* or *ophNdi1* vectors perceived significantly higher spatial frequencies than did those treated with rotenone alone (n=6 for each treatment group). Of the two AAV2/2 *ophNdi1*

groups, the higher dose group outperformed the original AAV2/2 *Ndi1* group in threshold limits (Figure 4(b); $p < 0.05$). Differences were 1.6-1.8-fold greater for treated eyes in the *ophNdi1* groups, and approximately 1.4-fold higher in the *Ndi1* group compared to control eyes (rotenone alone). Within each group, t-tests were performed on the treated eye versus untreated contralateral eyes. As the performance of the untreated eye varied between the treatment groups, the average differences between treated versus untreated eyes was also analysed (Figure 5(b)). Here again we see that there is a trend of increased protection in eyes treated with *ophNdi1* over the original *Ndi1* construct, although significance was only reached with the higher *ophNdi1* dose. The data further supports the heightened protective effect provided by *ophNdi1* over *Ndi1*.

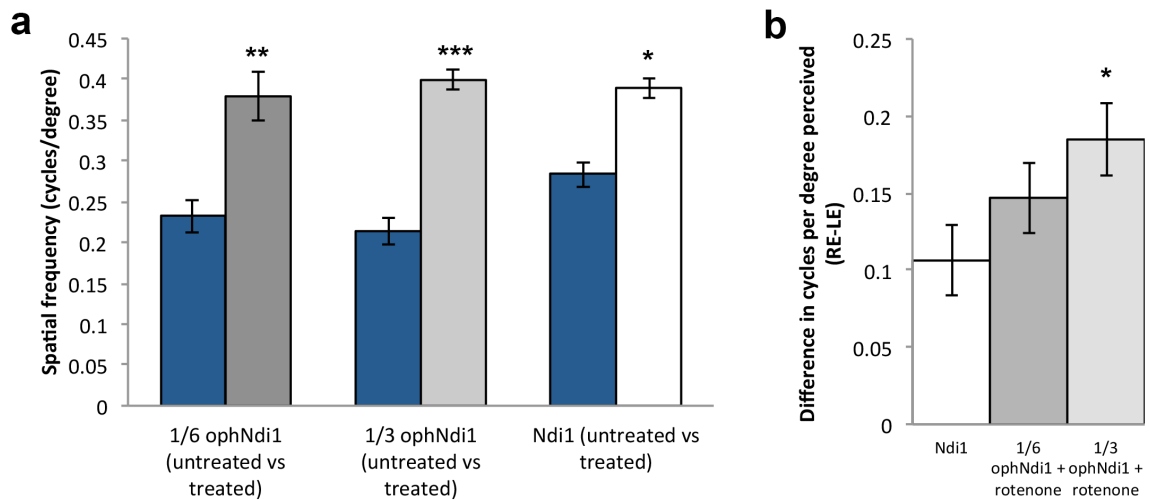


Figure 2.10: Optokinetic spatial frequency thresholds. (a) Treated eyes (grey, white) were compared to control (blue) eyes six weeks post-rotenone treatment (n=6 each group). OKR was measured in both treated and untreated eyes – left and right eyes in each case were considered pair-matched. (b) Difference in spatial frequency threshold observed for each treatment group. p-values: *, $p < 0.05$. **, $p < 0.005$. ***, $p < 0.001$.

While cell number and retinal integrity were preserved in the long-term subjects (Figure 2.8), it is imperative to ensure also that functional recovery does not diminish over time.

As such, mice treated with the higher dose *ophNdi1* vector were assessed three weeks

post-rotenone treatment, and again at five months (n=8). Mice tested three weeks post-rotenone were found to have a 1.7-fold greater threshold of perception in treated eyes versus non-treated eyes (Figure 2.11; $p < 10^{-7}$). When re-tested at five months post-injection, the untreated eyes had improved slightly, while the treated had declined – neither change was found to be statistically significant ($p > 0.10$). Of note, both treated and untreated eyes showed a slight but non-significant trend towards higher spatial frequency thresholds when compared to the initial experiments (Figure 2.11). This trend is in line with the higher numbers of preserved cells in the samples. However of particular note the benefit derived from treatment with AAV2/2 *ophNdi1* was maintained over the time thus far analysed (up to 5 months).

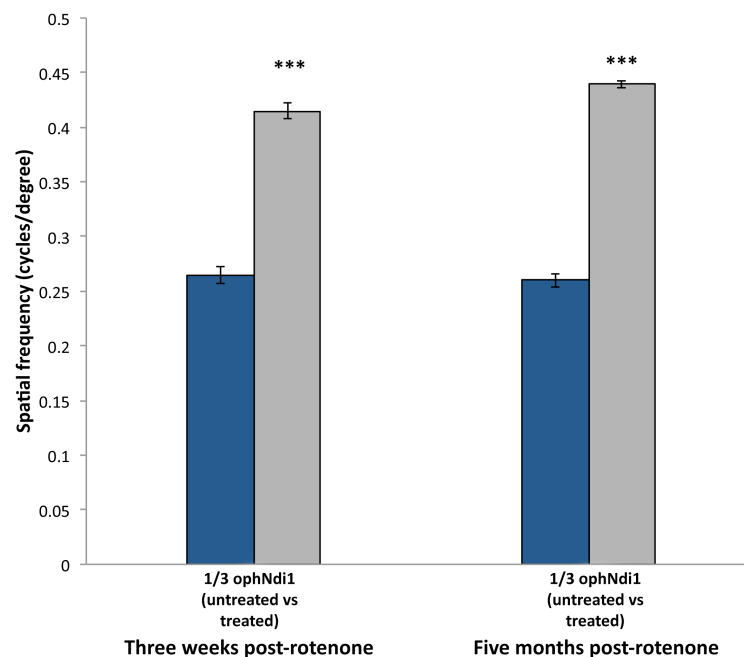


Figure 2.11: Optokinetic spatial frequency thresholds of mouse retinas tested at three week and five months-post rotenone insult. Retinas treated with 1.2×10^9 vg AAV-CMV-*ophNdi1* (light grey) were tested against untreated control eyes (blue). Three weeks post-rotenone, mice were acclimatised to OKR chamber and tested three times each (n=8). The same mice were tested again five months after rotenone was injected. Left and right eyes of each mouse were pair-matched for statistical analysis. *** $p < 10^{-7}$.

2.3 Discussion

Although many mitochondrial diseases can display an ocular phenotype, LHON is rare among retinal degenerations in that it is caused by single point mutations in mtDNA. Its uniparental mode of inheritance and delayed (although sudden) onset make it an attractive candidate for gene therapy. In recent years, several research groups have developed gene therapies for LHON, with results on human clinical trials already published, and recruitment for another trial already underway (Feuer et al., 2016; Yang et al., 2016; clinicaltrials.gov). These studies all utilise an AAV vector delivering *ND4*, the gene most commonly mutated in LHON patients. However, LHON is defined as an orphan disease, with ~150,000 sufferers worldwide. Given that the disease that can be caused by mutations in any of several different genes, a mutation-independent therapy that treats all forms of LHON may be preferable to the use of gene-specific replacement. Furthermore, while the approach evaluated in this chapter is directly relevant to LHON, a primary mitochondrial disorder caused by mutations in mitochondrially encoded Complex I subunits, this same approach may be relevant to a wide range of disorders where Complex I deficiency is one of the characteristic features of disease.

Ndi1 is a gene derived from *S. cerevisiae* that can act in principle as a single-protein replacement to Complex I, the respiratory complex that malfunctions in LHON. *Ndi1* has previously been shown to demonstrate effective neuroprotection in degenerative diseases such as multiple sclerosis, Parkinson's disease and LHON (Chadderton et al., 2013; Marella et al., 2009; Talla et al., 2013). It would, however, be of significant value to try to minimise the viral load required to provide a therapeutic dose through enhanced transgene expression, as is described in this chapter of this thesis. This would be optimal in terms of clinical management, vector production costs, and patient safety. Treatment regimes for

RGCs in diseases such as LHON will mostly likely involve an intravitreal route of administration. Prior studies suggest that, compared to subretinal delivery of AAV vector (typically used in targeting the photoreceptor layer and RPE), there can be an increased immune response when AAV is injected intravitreally (Li et al., 2008). As such, minimising the viral dose is particularly important for RGC gene therapies requiring intravitreal delivery such as *Ndi1*.

In the current study, we have compared an optimised version of *Ndi1*, *ophNdi1*, to the original construct when injected intravitreally in an AAV2/2 vector. HA-tagged *CMV-ophNdi1* successfully localises to the mitochondria; as demonstrated in Figure 2.3 *ophNdi1* shows very high levels of colocalisation with an MLS-conjugated dsRed protein. This colocalisation was shown to contrast significantly with expression of a marker such as EGFP lacking an MLS (Figure 2.4). Attempts were made by other members of the Farrar lab to use this HA-tagged construct to generate an AAV vector and examine expression and localisation of *CMV-ophNdi1 in vivo*. However, for reasons that remain unknown, anti-HA antibodies were unable to detect tagged *ophNdi1* protein *in vivo* via immunohistochemistry or western blot.

RNA analysis using samples from AAV2/2 treated mouse retinas demonstrated that the codon optimisation of *ophNdi1* resulted in a profound increase in expression compared to the non-optimised *Ndi1* gene (Table 2.1). The expression level provided by the *Ndi1* vector appeared quite low in this analysis – indeed, it was at the limit of detection. However, it is important to note that intravitreal administration of a viral vector such as AAV-*CMV-Ndi1* does not result in transduction of the vast majority of cells in the retina (namely, the photoreceptors). As such, it could be expected that the RNA expression

levels seen from a whole retinal RNA samples would be quite low. The RNA data support the view that *ophNdi1* vectors may provide more optimal expression in the context of mammalian cells.

Histological data suggest that the *ophNdi1* vector offers a significantly more effective therapeutic compared to the original *Ndi1*, with equivalent beneficial effects observed even when using only one sixth of the therapeutic dose. It was shown that AAV2/2-*ophNdi1* significantly protects against the damage caused by rotenone-induced Complex I deficiency at the viral doses used (Figure 2.7). Optic nerve thickness was also preserved in treated mouse retinas compared to untreated controls (Figure 2.9). Protection was maintained even as late as five months (Figure 2.8) when evaluated using the higher dose *ophNdi1*-treated retinas. This is perhaps not unexpected, as rotenone constitutes a single, punctuated insult to the retinal Complex I machinery. The biological half-life of rotenone has been reported as approximately 1-18 hours as reported by the US environmental protection agency (EPA; epa.gov). Long term expression studies of *ophNdi1* would be necessary to confirm that the viral vector can indeed provide protection over a longer period of time, as would be valuable in a gene therapy targeted towards LHON patients. Further, a limitation to the rotenone model not discussed is that it can be assumed that amacrine cells are at risk of damage and death due to rotenone exposure, potentially in a disproportionate manner to that observed in LHON. Although the extent of amacrine cell death was not examined in this thesis, as higher doses of rotenone can cause death of most of the retina (data not shown), amacrine cells located in the GCL, at least, should be endangered. Published research into LHON has not established whether amacrine cells die in the disease in humans, but as discussed previously, the high energy demands and long axons of RGCs are suspected to be a reason for specific malfunction in LHON (Carelli et

al., 2009b). As such, the role of amacrine cells and the risk posed to them must be considered.

Nevertheless, the data obtained at 5 months after a single vector administration is encouraging. Two staining methods were used for counting the numbers cells preserved when administered *CMV-Ndi1* or *CMV-ophNdi1* vectors in this study (NeuN and Brn3a; Figures 2.6, 2.7). While both staining methods produced similar counts, it is evident that Brn3a staining offers a cleaner means of counting cells – the Brn3a stain is highly punctuated relative to NeuN and offers a visibly clearer image in terms of gauging numbers of cells. Furthermore, there is limited evidence that NeuN may also stain some classes of amacrine cells found in the GCL (Schlamp et al., 2013). While these cells are geographically vulnerable to rotenone insult by intravitreal injection, for the purposes of an LHON disease model, a more specific RGC marker will provide more meaningful data in terms of delivering therapeutic protection to the target cell type. The protection offered by the AAV2/2 *ophNdi1* vector appears, at level of RGC preservation, to be indistinguishable from that of wild-type, untreated retinas (as can be seen in the rotenone study in Figure 2.5), highlighting the potential therapeutic value of the approach.

Behavioural tests in the form of OKR further demonstrated the effect of AAV2/2-*ophNdi1* at both reduced doses when compared to the dose of the original *Ndi1* vector used. Both *Ndi1* and *ophNdi1*-treated retinas showed a far higher threshold for visual tracking compared to untreated control eyes (rotenone alone), when tested using an OKR analysis chamber (Figure 2.10). A small but significant increase in benefit was shown in the high dose *ophNdi1* treatment group when compared to the original *Ndi1* group. In *ophNdi1*-treated mice tested at 5 months post-rotenone administration, this protection was seen to

be maintained over time (Figure 2.11). Previous studies have shown that OKR values in the region of 0.4 cyc/deg are to be expected for wild-type mice (Chadderton et al., 2013; Prusky et al., 2004). In the current study, retinas treated with either *Ndi1* or *ophNdi1* vectors retained levels of function when assayed using OKR that appeared equivalent to wild-type retinas. Overall, this data suggests that the *ophNdi1* vector performs highly effectively as a therapeutic against Complex I deficiency caused by rotenone administration, and that even with a relatively low titre administered (6×10^8 vg/eye), *ophNdi1* can equal the protective effects of the non-optimised *Ndi1* vector.

A limitation to the robustness of this work is the lack of dose matching between vectors in this study. The higher injection dose of *ophNdi1* used in this study and the dose of original *Ndi1* used represent the undiluted concentration of each vector prepared. It has previously been established by the Farrar group that lower doses of original *Ndi1* administered did not provide sufficient protection against rotenone insult (data not shown). As such, and to avoid the use of excessive animals in the experiment, the study was continued using the doses reported in this work. For greater rigour, it would be important to show the effect of each dose of *ophNdi1* compared to an equivalent dose of original *Ndi1*. Ideally this would be done beginning with *ophNdi1* administered at 3×10^9 vg, and working down. Further work being conducted by the Farrar group currently is accomplishing this.

Many inherited retinal degenerations, such as RP and Stargardt's disease, feature slow, progressive decline over a period of years. However, LHON patients typically experience no visual abnormalities in early life, before sudden and frequently bilateral onset of disease. Sudden and significant loss of vision is devastating for the patient; however, the

fact that decades can pass before symptoms disease manifest potentially offers a wide therapeutic window for intervention. One of the stumbling blocks to correct pre-symptomatic diagnosis and treatment of LHON is the incomplete penetrance the disease displays, especially with respect to females (where only ~10% of carriers develop symptoms). However, as mentioned before, recent work on mtDNA in LHON carriers, patients and unaffected controls does show a clear, significant trend in the relationship between mtDNA copy number and disease penetrance (Bianco et al., 2017; Giordano et al., 2014). This could potentially allow families with known maternal LHON mutations to undergo a risk assessment for the probability of disease onset. A sufficiently high probability of the disease manifesting may in the future offer the opportunity to treat patients prophylactically, before sight is lost.

There is currently no clinically approved means of estimating whether LHON symptoms will manifest in a carrier, or the severity of disease once onset has occurred. However, several studies have looked at possible biomarkers as a means of predicting whether a carrier will become a symptomatic patient. Neuron-specific enolase (NSE) levels, a marker of neuronal distress, have been shown to be raised in asymptomatic carriers of LHON mutations, for example in Yee et al. (2012). Average levels appeared to be approximately four times higher in unaffected males than in unaffected females. While the authors noted that NSE levels were far lower in actual LHON patients, they concluded that this could be due to the fact that RGCs had already been lost. It may be that in young carriers, NSE may offer a means of predicting disease onset, namely that higher NSE levels could indicate higher risk of disease. Indeed, NSE levels have also been suggested as a biomarker for diabetic retinopathy onset (Li et al., 2015). Phosphorylated neurofilament heavy chain (pNF-H) has also been suggested as a marker of degeneration

(Guy et al., 2008) – however, this marker seemed to coincide with visual loss (due to axon degeneration) and as such its use as a predictor may be limited. RNFL thickness as measured by optical coherence tomography (OCT) is a measure of LHON progression – initially, the RNFL thickens significantly, possibly before visual loss occurs, and then begins to thin over time as RGC axons die (Barboni et al., 2010). Barboni et al. suggest that this initial thickening may be a sign of impending visual loss, possibly offering up to a three-month window in which preventative treatment could be applied. As with NSE, it has been noted that RNFL thickness is substantially higher in LHON carriers (Yee et al., 2012). For a disease like LHON, intervening before the onset of RGC loss is ideal for preserving as much vision as possible. It is imperative that markers, including those discussed above, are developed in tandem with therapies for LHON such as *ophNdi1* to provide patients with the best outlook possible in treating their disease.

Summary and Future Plans

We have demonstrated that AAV2/2 *ophNdi1* offers a superior therapy to AAV2/2 *Ndi1* *in vivo* in a chemically induced mouse model, and moreover, that it is able to preserve RGCs and retinal function at or near wild-type levels when faced with a Complex I-directed insult (rotenone). There are several steps to take in bringing this research forward, some of which are discussed in later parts of this PhD thesis. While this study demonstrated excellent protection with the *ophNdi1* vector even at relatively low viral doses, it will be important to examine exactly how low a titre of vector can be administered in order to achieve the same level of protection as has been seen in this study. Furthermore, it would be valuable to test whether the expression vector could be optimised to a greater extent. This could be achieved through the use of more efficient capsids such as the tyrosine mutants (Section 2.1.1), or through the incorporation of

enhancers such as the β -globin intron, among others. Additional refinements such as these should aid in ensuring that maximum transgene expression levels can be achieved employing the minimum dose of viral vector.

While potent expression from viral vectors is usually desired, high-level expression of a transgene in cells other than the target cells of interest can at times be potentially hazardous. As such, using a construct that can restrict expression only to the target cell type (in this case RGCs) would be valuable. In this regard Chapter 3 of this thesis deals with the development of a new promoter, *Nefh*, that results in preferential RGC expression in the context of the retina (Hanlon et al., 2017). Combining this novel promoter with *ophNdi1*-based therapies developed as detailed in this chapter, and testing its efficacy *in vivo* (under the same conditions as detailed above) should be extremely useful in creating an optimised therapy. It should also be useful in exploring the value of the *Nefh* promoter for LHON gene therapies.

A notable absence from the results presented in this chapter is the lack of protein expression data. As *Ndi1* may be regarded as a relatively obscure gene, there is currently no commercially available antibody targeting the Ndi1 protein. Our laboratory has in the past generated with a service provider a custom anti-Ndi1 antibody that performed for western blotting *in vitro* but not for immunohistochemistry. We have generated an antibody can detect Ndi1 using western blotting from HEK293 cells transfected with *Ndi1* plasmid or AAV *Ndi1* vector. Recently, further optimisation of methodologies by members of our group Dr. Naomi Chadderton, Dr. Arpad Palfi, and Daniel Maloney has resulted in *in vivo* Ndi1 protein detection using protein from mouse retinas injected with AAV-*Ndi1* vectors. This method requires mitochondrial isolation and pooling of retinal

samples. *Ndi1* is a membrane-bound protein, which renders protein purification and antibody targeting more difficult, hence the need for mitochondrial isolation when the number of transduced cells is limited. We are currently in the process of testing a new antibody for immunohistochemistry that is being generated by a service provider, with some limited success thus far. It will be important, once a reliable antibody is to hand, to quantify the extent to which *ophNdi1* protein expression exceeds that of *Ndi1*. This should enable more precise estimates of the minimum therapeutic level of *ophNdi1* required, especially with respect to the inclusion of more efficient enhancers or capsids.

While the original goal of the *Ndi1* research project has been the exploration of novel candidate treatments for LHON, it is becoming increasingly apparent that mitochondrial dysfunction is a central factor in a range of neurodegenerative diseases, including Parkinson's disease, MS and many others (Bose and Beal, 2016; Nikić et al., 2011; Witte et al., 2014). Oxidative and respiratory assays carried out in our laboratory by Daniel Maloney have investigated the ability of *Ndi1* to ameliorate various effects and endophenotypes associated with mitochondrial dysfunction. *Ndi1*, and in particular *ophNdi1* appear to provide a support to mitochondria that enable them to weather a variety of insults included but not limited to Complex I deficiency associated with mitochondrial dysfunction, and thereby decrease oxidative stress (personal communication; results as yet unpublished). In principle, *ophNdi1* supplementation in complex diseases with mitochondrial involvement may provide a sufficient boost to the affected cells to prevent or lessen disease phenotypes. It remains to be seen what the extent of this effect will be and how it might be optimised in the future, however the potential therapeutic value of the approach supports further studies in a range of *in vitro* and *in vivo* disease models. The research described in the current chapter of this PhD

thesis is limited to work in HEK293 cells and in a rodent model of LHON. It is clear that an important future element to progressing such therapeutic strategies forward will involve exploration of the potential of these novel transkingdom gene therapies in larger animals to explore if such therapies may be well tolerated or otherwise in, for example, the non-human primates. However the work associated with this falls outside the remit of this PhD study.

In summary, we have codon-optimised *NdiI* to create *ophNdiI*, a therapeutic that provides substantial protection against rotenone-induced Complex I malfunction, a chemically induced model for LHON. AAV-*CMV-ophNdiI* equals or exceeds the protective effects of the original *NdiI* construct, even at highly reduced viral doses, and thus constitutes a significant advance of the state of the art.

Chapter 3: A novel retinal ganglion cell promoter designed for use in AAV vectors

3.1 Introduction

3.1.1 Gene therapy for RGCs

The growth of the field of gene therapy has brought with it a tremendous advancement in the sophistication of vectors and expression cassettes available for use. This rapid evolution is enabling the development of therapies that effectively target the retina, including RGCs. Increasingly, AAV vectors such as BP2, 7m8, tyrosine capsid mutants, and Anc80, among others are being produced, delivering excellent expression levels and expression throughout the retina (Cronin et al., 2014; Dalkara et al., 2013; Zinn et al., 2015). However, wide-ranging transgene expression can be a double-edged sword; in many situations, it would be advantageous to direct expression only to specific cell types, while possibly retaining the enhanced expression levels of these novel capsids. Proteins that are endogenously expressed only in certain cell types may cause unintended off-target effects if expressed elsewhere. Furthermore, although AAV is well tolerated in the human eye, restriction of transgene expression may reduce the chance of an immune reaction.

Several therapies that have been designed for retinal degenerations utilise cell-type specific promoters in their therapeutic approaches. These include rhodopsin (targeting rod

photoreceptors; Bennett et al., 1998; Millington-Ward et al., 2011; O'Reilly et al., 2007; Wert et al., 2013), rhodopsin kinase (rods and cones; Boye et al., 2010; Kay et al., 2013; Khani et al., 2007), *RPE65* (RPE; Bainbridge et al., 2008, 2015), *Hes1* (Muller cells; Ueno et al., 2017) and *RLBP1* (RPE and Muller cells; Choi et al., 2015). However, there is a lack of RGCs promoters that can suitably drive specific transgene expression when delivered via AAV vectors. A recently discovered 0.5kb promoter, *Mcp1*, drives expression in RGCs when those cells are subject to optic nerve crush – the gene from which the promoter was derived is involved in leukocyte recruitment in response to damage (Fujita et al., 2017). However, this may limit the use of *Mcp1* as a promoter in situations where physical damage does not occur, as in degenerative diseases such as LHON. As discussed in Chapter 2, several clinical trials have directed gene therapies towards RGCs (Feuer et al., 2016; Guy et al., 2017; Yang et al., 2016). These trials have all used constructs driven by ubiquitous promoters such as cytomegalovirus (*CMV*) or chicken- β -actin (*CBA*). Promoters such as these offer high levels of expression, and tend to be small in size (<1kb) – this makes them highly valuable when used in an AAV vector with an optimal packaging capacity of 4.7kb (Dong et al., 1996; Grieger and Samulski, 2005). However, these promoters drive gene expression well in a large number of cell types, and so off-target expression is common.

3.1.2 RGC-specific promoters

High-level RGC-specific expression in transgenic animal models can be achieved using the *Thy1* promoter (Feng et al., 2000; Saha et al., 2016). From the point of view of AAV vectors, however, the issue with *Thy1* is one of size. It has been shown that an enhancer element contained in the first intron of the *Thy1* promoter is necessary for both high level and RGC-specific gene expression (Aliç et al., 2016; Spanopoulou et al., 1991). However,

while the core promoter and enhancer elements are both small (~100-200bp each), approximately 6kb of spacing between the two elements is believed to be necessary for specific promoter function. This renders the *Thy1* promoter unsuitable for use in AAV vectors. A 0.48kb promoter derived from the human synapsin-1 gene (hSYN) can provide pan-neuronal expression in rodent and primate brains when utilised in adenoviral or AAV vectors (Diester et al., 2011; Kügler et al., 2003a, 2003b; Lopez et al., 2016). Recently, it was shown that in the rodent retina, intravitreal injection of a hSYN-driven AAV construct resulted in GCL expression (Gaub et al., 2014). However, in the context of the primate retina, hSYN-mediated expression only appears to significantly occur in damaged retinas or vitreolysed eyes (Yin et al., 2011). This may impact on the value of hSYN in human clinical therapies; nevertheless, its full therapeutic relevance remains to be established. Hence, the characterisation of a promoter that exhibits preferential RGC expression, appropriately sized for AAV, would be a valuable tool and a significant refinement for RGC gene therapies.

3.1.3 Summary and project objectives

Increasingly, RGC subtypes are being defined by differential gene expression, rather than morphological differences. Several groups have worked on establishing the transcriptional differences between different classes of RGCs using immunological, transcriptomic and transgenic methods (Rousso et al., 2016; Sanes and Masland, 2014; Sun et al., 2015; Sweeney et al., 2017). The human retina contains approximately 1.5 million RGCs, comprising ~1% of retinal neurons (Callaway, 2005). Knowledge of the different types of RGCs populating the GCL is still emerging; in 2012, it was reported that there were at least twenty classes of RGCs, and that number has risen to thirty (Baden et al., 2016; Masland, 2012).

The objective of this study was to identify new potential promoters that could provide highly preferential expression in RGCs, validating our method for isolating said promoters, and having done so to test those promoters for functional capacity. Transcriptomics offers a powerful means to analyse gene expression in different cell types. In order to identify potential RGC promoters, GCL-specific microarray expression data from post-mortem human retinas was used (Kim et al., 2006). Kim et al. isolated GCL populations consisting of 1,000 RGCs using laser-capture microdissection (LCM) and cell populations consisting of 1,000 cells from the remainder of the retina (termed outer retina, OR). The gene expression profiles of the two populations were compared. With this data, we assessed promoter conservation between mammalian species for genes that were highly expressed and highly enriched in RGCs, using data drawn from the UCSC database (Kent et al., 2002). Using as our basis the mouse alignment mm10, conservation of non-coding DNA sequence across species was used as an indicator of potential function. A number of highly conserved promoter upstream sequences were identified from genes shown to be both highly expressed and enriched in RGCs (Choudhury et al., 2016; Kim et al., 2006; Struebing et al., 2016).

In order to evaluate the lead candidate promoter, derived from sequence upstream of the *Nefh* gene, we aimed to compare expression specificity to a constitutive promoter *CMV*. Our approach was to compare the expression patterns of both promoters driving EGFP in an AAV2/2 vector. Owing to the large number of amacrine cells in the GCL, and the potential for off-target activity, expression of EGFP was assayed by colabelling with a number of markers for RGCs and amacrine cells. A final aim of this study was to test, using *Nefh*, whether isolating only the highly conserved regions of a promoter could

preserve expression level and specificity. This was tested, as before, in an AAV2/2 vector driving EGFP, and compared to the full-length *Nefh* promoter.

3.2 Results

3.2.1 Promoter characterisation pipeline

The objective of the current study was the characterisation and *in vivo* evaluation of an RGC promoter for future use in AAV-mediated gene therapies. A comparative evaluation of genes with highly enriched RGC expression was undertaken *in silico* and the lead candidate was investigated *in vivo* (Figure 3.1). Whilst gene expression profiles of RGCs are available, the promoters that drive this expression are ill defined. We chose several key criteria to identify candidate promoters using microarray data for RGCs (Choudhury et al., 2016; Kim et al., 2006; Struebing et al., 2016). Conservation data of regions upstream of the most enriched RGC candidate genes were obtained from the UCSC genome browser database (genome.ucsc.edu, mm10). In the study conservation of sequence across mammals (using the mouse genome as a base) was used as a proxy for presumed function *in vivo* to identify putative promoters. To ensure that any promoter chosen would be suitable for future use in AAV vectors, conservation analysis was limited to the immediate 2.5kb upstream sequence of genes. Based on the expression level of a gene in the GCL (EL_{GCL}) and the enrichment factor of that gene (EF), a gene score was generated to rank genes as candidates ($GS = EL_{GCL} \times EF$; Table 3.1; see Materials and Methods for details regarding the methodology employed). The basewise species conservation in the selected upstream sequences was plotted (conservation numbered between 0 and 1) and the five genes with the highest GS are presented (Figure 3.2). The list of species used in the analysis can be found in Appendix 1.

Rank	Gene name	EL _{GCL}	EL _{OR}	EF	GS
1	<i>NEFH</i>	21899.1	89.4	245	5.37x10 ⁶
2	<i>NEFM</i>	6984.1	31.7	220.6	1.54 x10 ⁶
3	<i>NEFL</i>	7841.1	50.5	155.3	1.22 x10 ⁶
4	<i>VSNL1</i>	4659.33	67.35	69.18	3.22 x10 ⁵
5	<i>SPARCL1</i>	5077	149.75	33.9	1.72 x10 ⁵
6	<i>SLC17A6</i>	1302.9	10.3	126.8	1.65 x10 ⁵
7	<i>TMSB10</i>	7124.3	324.6	21.9	1.56 x10 ⁵
8	<i>ANXA2</i>	2221.4	37.5	59.3	1.32 x10 ⁵
9	<i>STMN2</i>	4139.9	147.9	28	1.16 x10 ⁵
10	<i>PRPH1</i>	1238.5	18.4	67.5	8.36 x10 ⁴
11	<i>CRTAC1</i>	4478.6	347	12.9	5.78 x10 ⁴
12	<i>RBPMS</i>	832.5	12.6	66	5.49 x10 ⁴
13	<i>RAB13</i>	1802.7	59.7	30.2	5.44 x10 ⁴
14	<i>ATP1B1</i>	3803.3	299.2	12.7	4.83 x10 ⁴
15	<i>FABP3</i>	1054.6	24.9	42.4	4.47 x10 ⁴
	<i>BRN3A</i>	719.7	9.1	79.1	5.69 x 10 ⁴

Table 3.1: List of putative ganglion cell promoters. Human transcriptomic data of 1,000 cell populations from RGCs versus OR (Kim et al. 2006) was used to determine relative expression levels in the outer retina (EL_{OR}) and the GCL (EL_{GCL}). Enrichment factor (EF) for the GCL was calculated as $EF = EL_{GCL}/EL_{OR}$. A gene score (GS) was calculated as $GS = (EL_{GCL} \times EF)$ to provide an overall score. Genes are listed in order of GS. *BRN3A* expression is included at the bottom as a comparison.

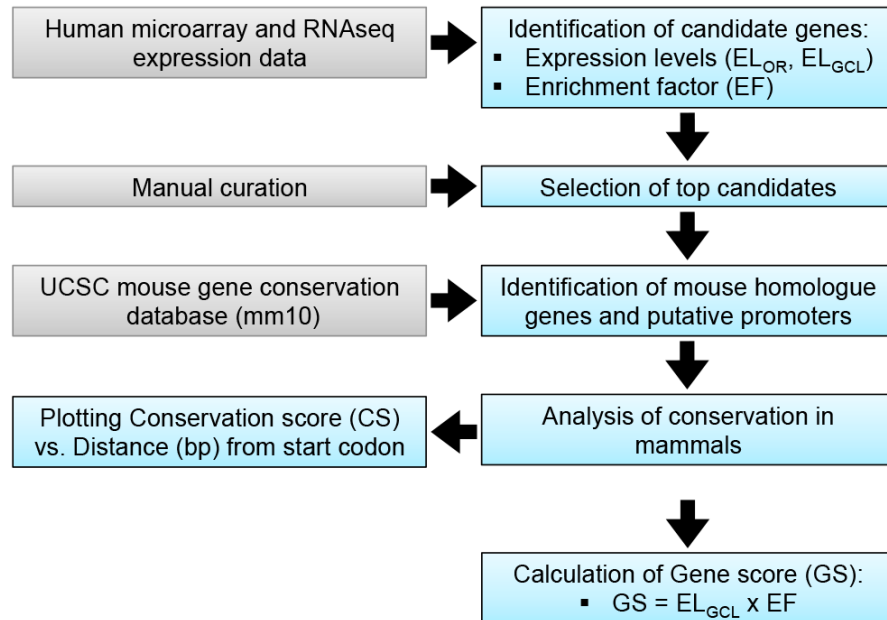
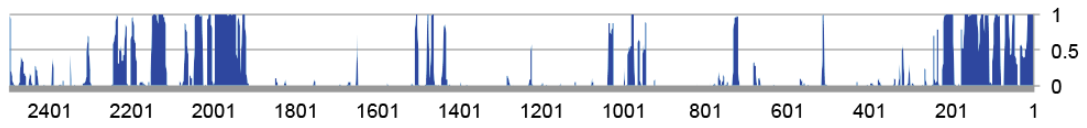


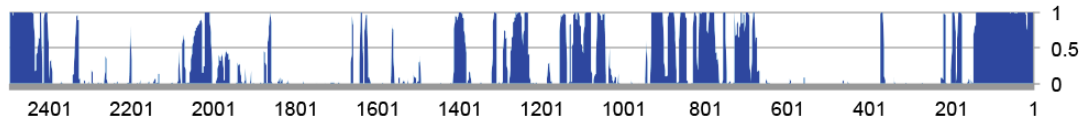
Figure 3.1: RGC promoter analysis. A: Putative promoter identification methodology. Transcriptomic data (Kim et al. 2006) was used to identify candidate genes, based on expression levels in the retina (EL_{OR}) and the GCL (EL_{GCL}). Enrichment factor (EF) for the GCL was calculated as $EF = EL_{GCL}/EL_{OR}$; top candidates were identified based on EF. A gene score (GS) was calculated as a means of discerning between candidates.

Following analysis, *Nefh* was deemed to be the most highly enriched gene in RGCs with an enrichment factor (EF) of 245-fold, as well as demonstrating an extremely high EL_{GCL} (21899.1; Table 3.1). Some of the mouse genes analysed showed greater average conservation across species in their 2.5kb upstream regions than *Nefh* (*Nefm* 0.289, *Stmn2* 0.292, *Crtac1* 0.349 vs. *Nefh* 0.185, where zero indicates a complete lack of conservation between mammals at that base and 1 complete conservation). However, due to their lower EF and EL_{GCL} scores, *Nefh* was deemed likely to drive higher levels of RGC-specific expression and hence to be a better candidate promoter for further analysis (GS: *Nefh* 5.37×10^6 vs. *Nefm* 1.54×10^6 , *Stmn2* 1.16×10^6 , *Crtac1* 5.78×10^4). *Tmsb10*, *Nefl*, and *Sparcl1* had lower scores than *Nefh* in all categories. *Brn3a*, a commonly used marker for RGCs (Kim et al., 2006; Nadal-Nicolás et al., 2014), was found to have an extremely high conservation within a 2.5kb upstream region, and a high EF (0.576, 79.1 respectively). However, its EL_{GCL} was found to be approximately 39 times lower than that of *Nefh* (719.7), and so was not included as a candidate promoter sequence for further work-up. The *hSYN* gene showed no significant GCL enrichment or expression in the Kim et al (2006) study.

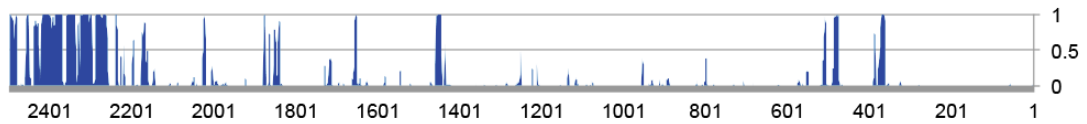
Nefh



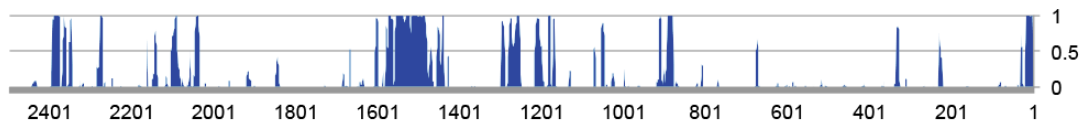
Nefm



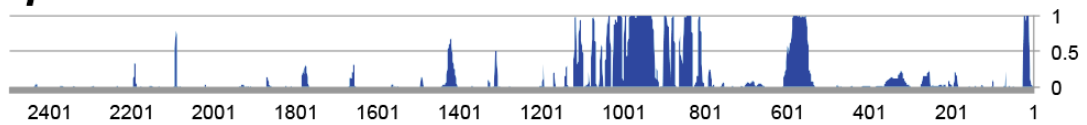
Nefl



Vsnl1



Sparcl1



Brn3a

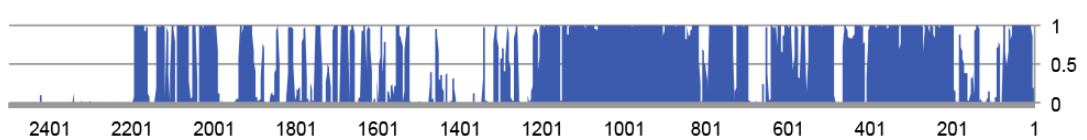


Figure 3.2: Analysis of 5' upstream sequence of candidate promoter sequences. Regions ~2.5kb upstream of the transcriptional start were analysed. The genes displayed represent the most highly expressed genes of the genes that are enriched >10-fold in RGCs. The y-axis represents conservation across mammals (CS), where 0 equals no significant conservation and 1 equals full conservation across mammalian species in the UCSC genome database. *Brn3a* is included as a control gene, with known RGC-specific expression.

3.2.2 *Nefh* promoter cloning

To explore the strength and specificity of the putative *Nefh* promoter, 2251bp of upstream sequence from the mouse homologue was cloned and used to drive expression of an *EGFP* reporter gene in an AAV2/2 vector (AAV-*Nefh*-EGFP) and transgene expression compared to that mediated by the *CMV* promoter (AAV-*CMV*-EGFP; Palfi et al., 2009, Chadderton

et al., 2012). Promoter sequence from the mouse *Nefh* gene was chosen for the study to ensure that function or non-function was not due to species incompatibility. The *CMV* promoter incorporated into AAV vectors has previously been shown to drive high levels of transgene expression in a wide variety of retinal cell types (Lebherz et al., 2008; Li et al., 2008; Mueller and Flotte, 2008), including RGCs (Chadderton et al., 2013; Tshilenge et al., 2016) and was used as a control vector for transgene expression. The *Nefh* promoter sequence was amplified from mouse genomic DNA using primers with *MluI* and *XbaI* sites incorporated, to allow for easy cloning into the same plasmid carrying *CMV*-EGFP plasmid, pAAV-MCS (digested with the same enzymes). In order to purify the amplified fragment, following PCR the reaction was run out on a gel and the 2.2kb fragment excised (Figure 3.3). This was necessary as the primers were not sufficiently specific, leading to the coamplification of a smaller, unrelated sequence. After ligation, confirmation of insert inclusion into the plasmid was carried out by restriction digest with *MluI* and *XbaI* (Figure 3.4). The plasmid was sequenced to confirm no mutations had been incorporated in the process of cloning. The *Nefh*-EGFP plasmid map is shown in Figure Wa. A virus was produced and titred as before (see Materials and Methods for titre details).

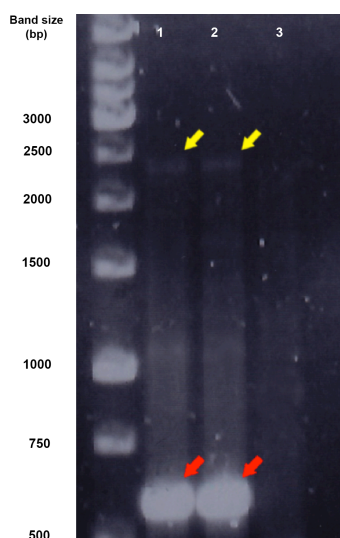


Figure 3.3: Agarose gel image of *Nefh* fragment amplified for PCR. Lanes 1 and 2 contain duplicate PCRs, while lane 3 is a DNA-negative control. The 2.2kb *Nefh* fragment is indicated by yellow arrows, while the non-specific fragment is shown with red arrows (approximately 600bp). Ladder band sizes are indicated on the left.

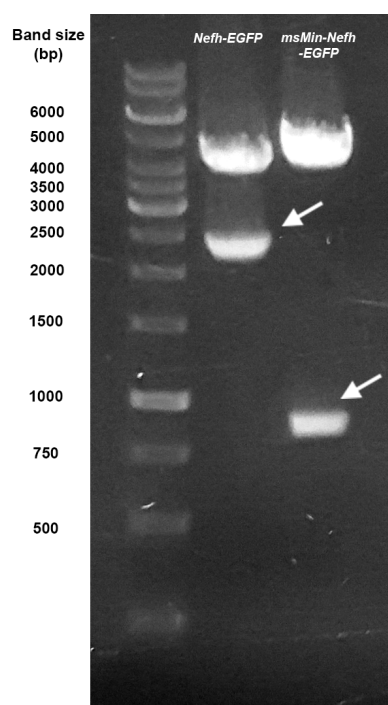


Figure 3.4: Agarose gel image of *Nefh*-EGFP and *msMin-Nefh*-EGFP plasmid restriction digests. Digestion of plasmids with *Xba*I and *Mlu*I confirms the presence of inserts, 2.2kb for *Nefh*, and 838bp for *msMin-Nefh* (indicated by white arrows). Ladder band sizes are indicated on the left.

3.2.3 *Nefh* promoter transduction profile

Adult wild-type 129 mice were injected with 3×10^9 viral genomes (vg)/eye of AAV-*CMV*-EGFP or AAV-*Nefh*-EGFP – fellow eyes were injected with either the *CMV* or the *Nefh* constructs (left eye, *CMV*, right eye, *Nefh*). Different groups of wild-type mice were injected either intravitreally or subretinally and EGFP expression evaluated (initial intravitreal vs. subretinal injection group – n=3 retinas per injection route for each of *CMV* and *Nefh* viral vectors (n=12 retinas total). Second expression study (Figures 3.7-3.10) n=6 retinas per viral vector injected, n=5 retinas per vector examined). The AAV2 serotype efficiently transduces RGCs; however, prior studies have shown that it will not transduce

photoreceptors when injected intravitreally. As such, it was necessary to confirm an absence of transgene expression in photoreceptors when *Nefh-EGFP* was administered subretinally, as AAV2 has the capacity to transduce these cells as well (Flannery et al., 1997; unpublished data). Figure 3.5 highlights examples of each type of intraocular injection with *CMV* and *Nefh* vectors. Of note in the retinal sections with intravitreally delivered vector (Figure 3.5a-d), expression of the *CMV*-driven EGFP construct extends into the INL and OPL of the retina, while the *Nefh*-driven EGFP is largely confined to the GCL.

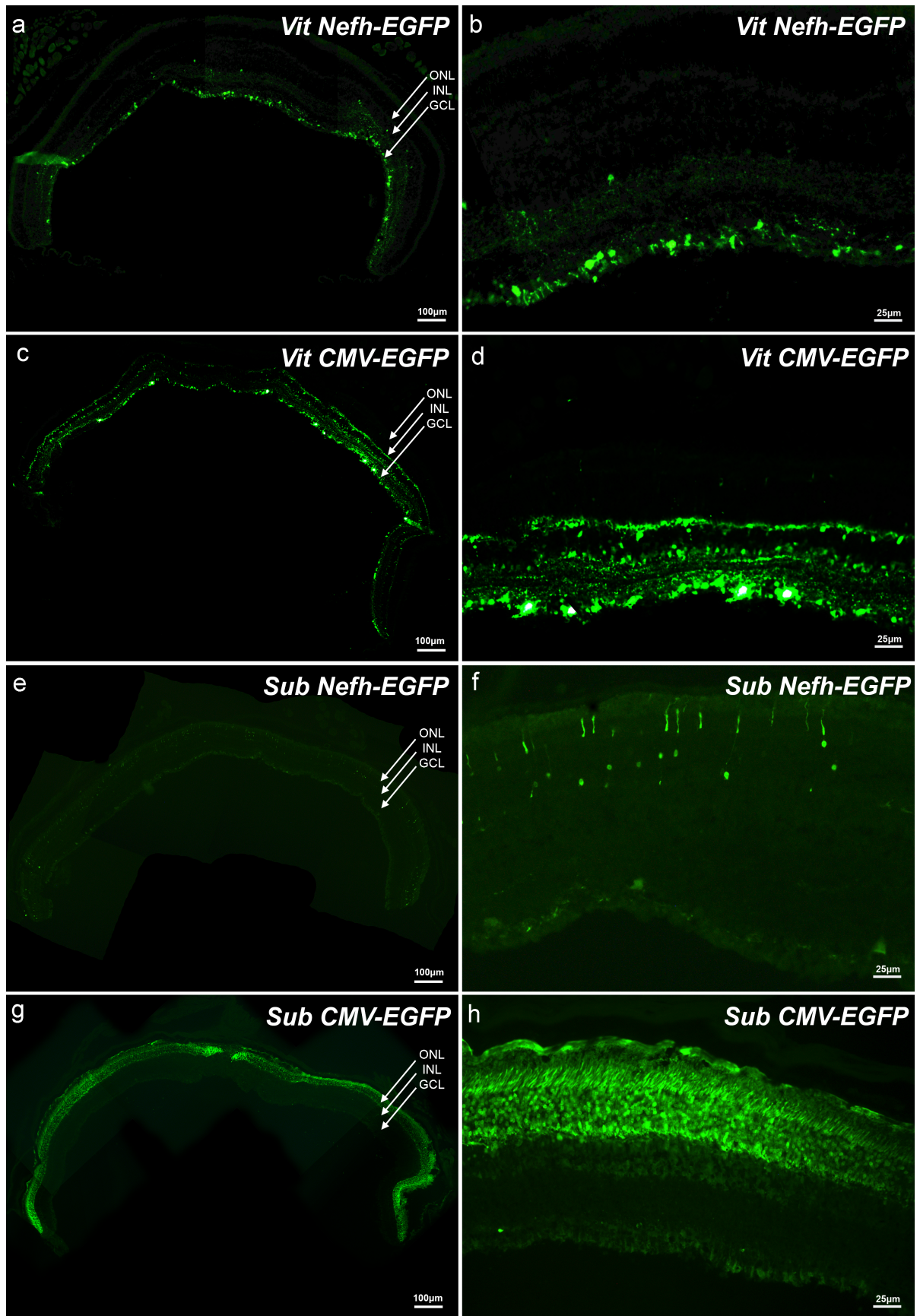


Figure 3.5: Analysis of *Nefh* specificity *in vivo*. Retinas injected either intravitreally (a-d) or subretinally (e-h) with AAV-EGFP vector were sectioned and imaged using a Zeiss Axioplan fluorescent microscope. The left column displays the whole retinal section, while the right shows higher magnification examples of those

sections. Scale bar 50µm. Abbreviations: ONL, outer nuclear layer. INL, inner nuclear layer. GCL, ganglion cell layer.

When a separate group of retinas were injected intravitreally, GFP expression levels within the GCL appear broadly similar; counts of transduced cells in the GCL show no significant difference between *CMV-EGFP* and *Nefh-EGFP* (*CMV*: 50.2% transduction, SD 14.1%, *Nefh*: 42.0% transduction, SD 11.2% – Figure 3.8). A highly significant difference was found between the numbers of transduced cells in the INL between the *CMV* and *Nefh*-driven constructs (*CMV*: 84.5% transduction, SD 34.2%, *Nefh*: 3.6% transduction, SD 2.9%, $p < 0.0001$ – Figure 3.8). Stray cells expressing *Nefh-EGFP* in the INL resemble displaced ganglion cells (Nadal-Nicolas et al., 2014). Subretinal *CMV-EGFP* shows wide expression, with near total transfection of the photoreceptor layer (Figure 3.5).

Comparatively, *Nefh-EGFP* shows almost no expression when administered subretinally, although sparse photoreceptor expression can be seen (counts not performed). This relative restriction of expression underpins the effectiveness of *Nefh* as a promoter that is highly preferential for RGCs.

Approximately fifty percent of cells in the GCL are RGCs with the other fifty percent being displaced amacrine cells (Akopian et al., 2016; Jeon et al., 1998; webvision.med.utah.edu). To delineate further the expression profile of the *Nefh* promoter, EGFP transgene expression was analysed in the GCL using antibodies targeting Brn3a, an RGC marker (Schlamp et al., 2013) and two amacrine cell markers, ChAT and GABA (Figure 3.6, 3.7) (Jeon et al., 1998; Wässle et al., 1987; webvision.med.utah.edu). Brn3a label was confined to the GCL in the retina and was used to explore the specificity of the putative *Nefh* promoter for RGCs (Figure 3.7A-E). In line with previously published data, 50% - 55% of all cells in the GCL were Brn3a positive (Figure 3.7 and Figure 3.8B;

Schlamp et al., 2013, Jeon et al., 1998). Figures 3.7 and 3.7 display representative images from eyes injected with the 3×10^9 vg/eye dose of *CMV-EGFP* and *Nefh-EGFP*, respectively. While *CMV-EGFP* and *Nefh-EGFP* expressed in comparable numbers of Brn3a-positive cells ($41.9 \pm 8.5\%$ *CMV-EGFP*, $39.5 \pm 12.7\%$ *Nefh-EGFP* and $33.10 \pm 11.2\%$ high dose *Nefh-EGFP*; Figure 3.6, 3.7 and Figure 3.8B), *Nefh* promoter-mediated EGFP expression in the GCL was observed in significantly fewer Brn3a-negative cells ($p < 0.001$, $12.1 \pm 3.3\%$ *CMV-EGFP*, $3.5 \pm 1.7\%$ *Nefh-EGFP* and $3.4 \pm 1.2\%$ high dose *Nefh-EGFP*; Figure 3.6, 3.7 and Figure 3.8B).

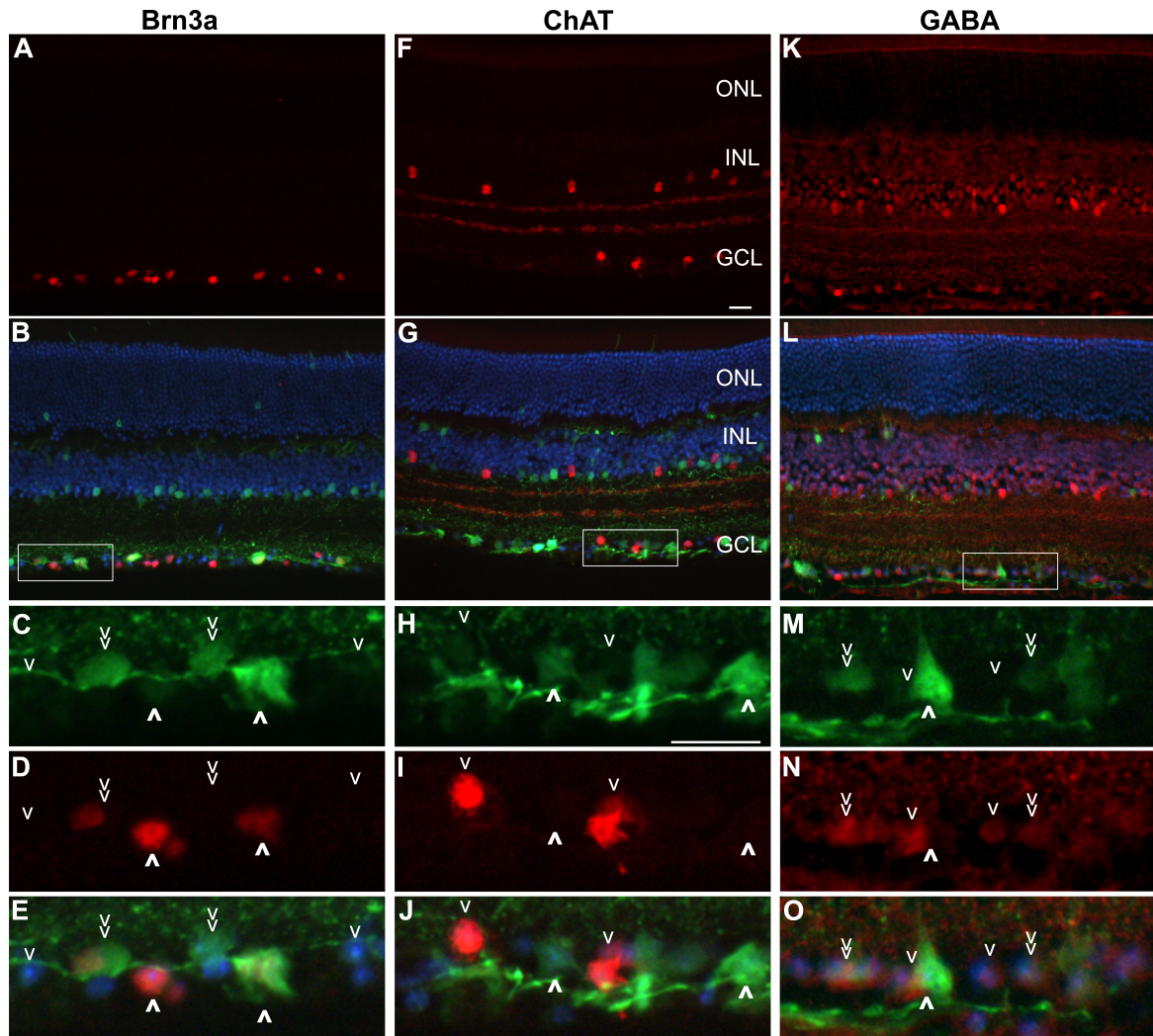


Figure 3.6: Immunohistochemistry analysis of AAV-*CMV-EGFP* transduced retinas. Eyes were injected intravitreally with AAV-*CMV-EGFP* (3×10^9 vg/eye). Transduced eyes ($n=5$) were fixed and cryosectioned 12 weeks post-delivery. Immunohistochemistry was performed for Brn3a (Cy3; A-E), ChAT (Cy3; F-J) and

GABA (Cy3; L-O) in combination with EGFP labeling (FITC). DAPI was used for nuclear counterstaining. Rectangles (in B, G and L) indicate positions of the enlarged areas. A, F and K: Cy3 label; B, G and L: Cy3, FITC and DAPI overlaid. C, H and M: FITC label; D, I and N: Cy3 label; E, J and O: Cy3, FITC and DAPI labels overlaid. C-E: Bold arrowheads: transduced Brn3a-positive cells. Regular arrowheads: un-transduced Brn3a-negative cells. Double arrowheads: transduced Brn3a-negative cells. H-J: Bold arrowheads: transduced ChAT-negative cells. Regular arrowheads: untransduced ChAT-positive cells. M-O: Bold arrowhead: a transduced GABA-negative cell. Regular arrowheads: un-transduced GABA-positive cells. Double arrowhead: transduced GABA-positive cells. ONL: outer nuclear layer; INL: inner nuclear layer, GCL: ganglion cell layer. Scale bars: 25 μ m (F and H). Data and figure generated in conjunction with Dr. Arpad Palfi, Farrar laboratory. ONL: Outer nuclear layer. INL: Inner nuclear layer. GCL: Ganglion cell layer.

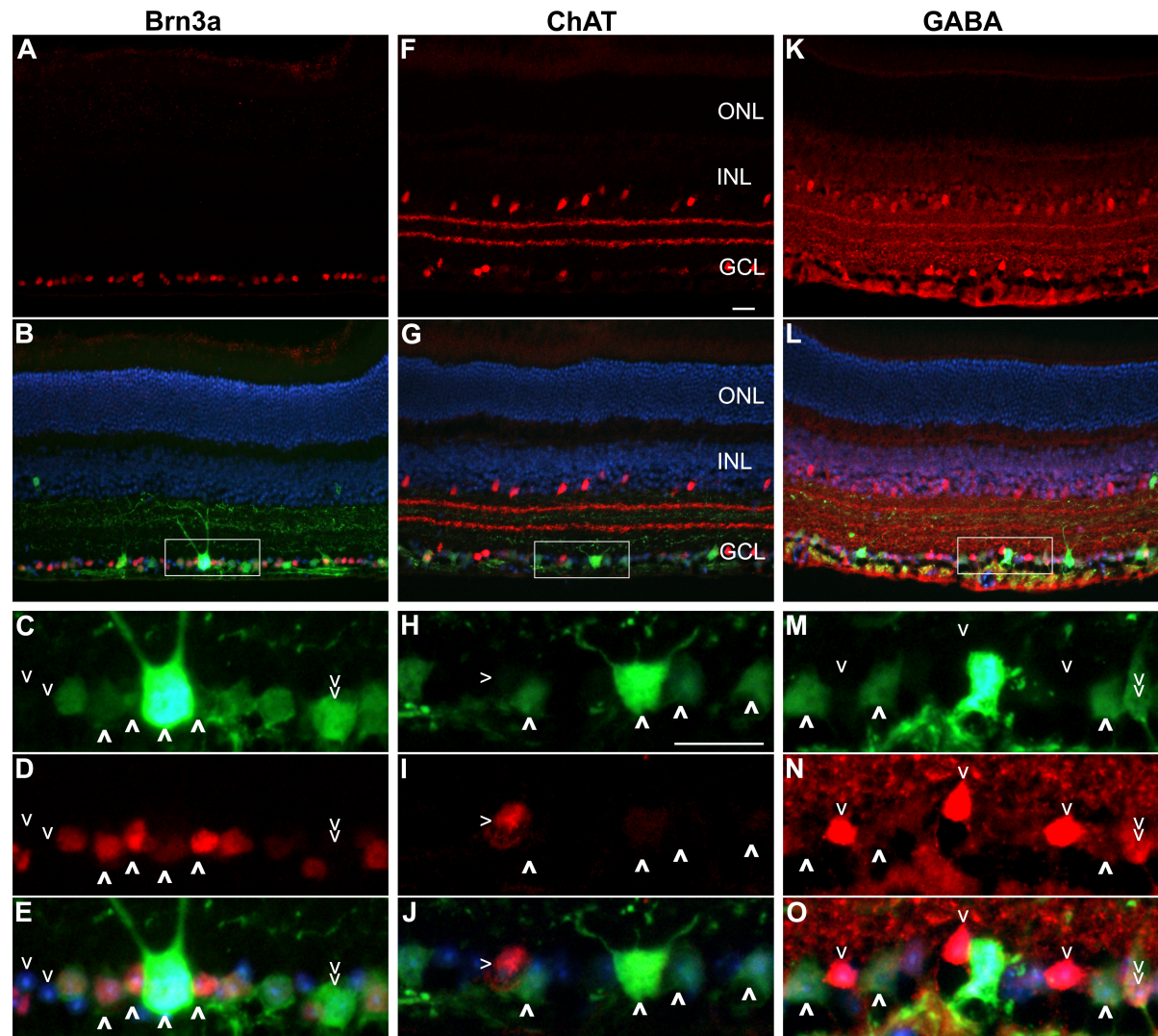


Figure 3.7: Immunohistochemistry analysis of AAV-*Nefh*-EGFP transduced retinas. Eyes were injected intravitreally with AAV-*Nefh*-EGFP (3×10^9 vg). Transduced eyes ($n=5$) were fixed and cryosectioned 12 weeks post-delivery. Immunohistochemistry was performed for Brn3a (Cy3; A-E), ChAT (Cy3; F-J) and GABA (Cy3; K-O) in combination with EGFP labeling (FITC). DAPI was used for nuclear counterstaining. Rectangles (in B, G and L) indicate positions of the enlarged areas. A, F and K: Cy3 label; B, G and L: Cy3, FITC and DAPI overlaid. C, H and M: FITC label; D, I and N: Cy3 label; E, J and O: Cy3, FITC and DAPI labels overlaid. C-E: Bold arrowheads: transduced Brn3a-positive cells. Regular arrowheads: un-transduced Brn3a-negative cells. Double arrowhead: a transduced Brn3a-negative cell. H-J: Bold arrowheads: transduced ChAT-negative cells. Regular arrowhead: a transduced ChAT-positive cell. M-O: Bold arrowheads: transduced GABA-negative cells. Regular arrowheads: un-transduced GABA-positive cells. Double arrowhead: transduced GABA-positive cells.

Double arrowhead: a transduced GABA-positive cell. ONL: outer nuclear layer; INL: inner nuclear layer, GCL: ganglion cell layer. Scale bars: 25µm (F and H). Data and figure generated in conjunction with Dr. Arpad Palfi, Farrar laboratory.

ChAT (Figure 3.6C,D, 3.7F-J) and GABA (Figure 3.6 E,F, 3.7K-O) markers were used to analyse subpopulations of amacrine cells. ChAT labelled cells in both the INL and GCL, resulting in prominent ‘double-layered’ staining within the IPL (Figure 3.7F-J); ChAT labelling identified approximately 17% cells in the mouse GCL (Figure 3.6, 3.7, Figure 3.8C), in line with previous data (Crooks and Kolb, 1992). GABA immunostaining resulted in widespread labelling in the mouse retina (Figure 3.7K-O); GABA labelling identified approximately of 15% cells in the GCL in our study (Figure 3.6, 3.7, Figure 3.8D). EGFP-expressing cells were significantly more likely to be ChAT-positive amacrine cells (Figure 3.7F-J) when EGFP expression was driven by the CMV promoter, compared to the *Nefh* promoter ($p < 0.05$, $7.5 \pm 4.6\%$ AAV-CMV-EGFP, $3.1 \pm 1.4\%$ *Nefh*-EGFP and $3.2 \pm 1.9\%$ high dose *Nefh*-EGFP; Figure 3.8C). Additionally a greater number of CMV-driven EGFP-positive cells were also co-labelled with GABA, however this represented a trend rather than reaching significance ($8.3 \pm 4.0\%$ CMV-EGFP, $5.8 \pm 2.4\%$ *Nefh*-EGFP and $4.0 \pm 3.7\%$ high dose *Nefh*-EGFP; Figure 3.8D).

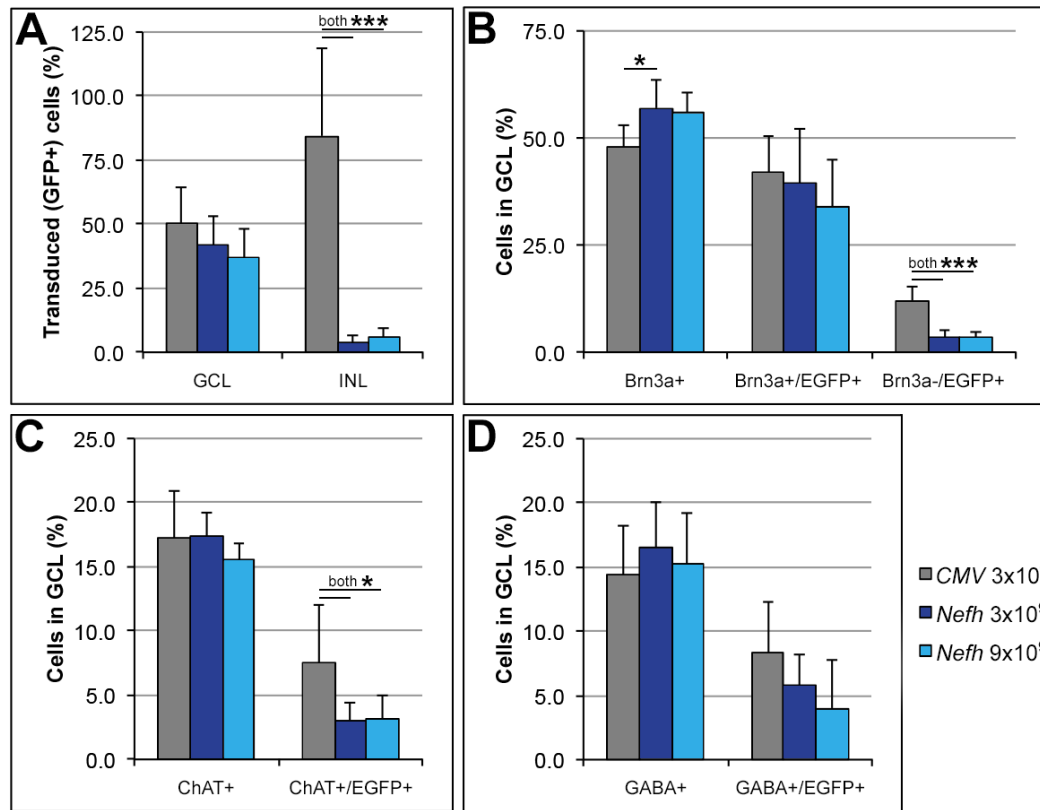


Figure 3.8: Quantification of *CMV* and *Nefh* mediated EGFP expression in vivo. Retinas were injected intravitreally with AAV.*CMV*-EGFP (3x10⁹ vg; A and D) or two different doses of AAV.*Nefh*-EGFP (3x10⁹ vg; B and E; and 9x10⁹ vg; C and F). Transduced eyes (n=4-5) were fixed and cryosectioned 12 weeks post-delivery. Immunohistochemistry was performed for Brn3a, ChAT, GABA and EGFP; DAPI was used for nuclear counterstaining. Manual quantification of labelled and co-labelled cells was performed on the immunolabelled retinal sections. A: Distribution of EGFP positive cells was determined in the ganglion cell (GCL) and the inner nuclear layers (INL). Additionally, co-localisation of EGFP with Brn3a (B), ChAT (C) and GABA (D) was determined in the GCL. ***: p<0.001; *p<0.05 (ANOVA). Data and figure generated in conjunction with Dr. Arpad Palfi, Farrar laboratory.

It was of interest to further characterise the expression profile of the *Nefh* promoter.

Melanopsin-positive retinal ganglion cells (mRGCs) are a specific class of RGCs found in the retina, distinguished by the fact that they express melanopsin, rendering them light-sensitive. They are known to have roles in non-visual light sensation, controlling the pupillary reflex, and circadian rhythm photoentrainment (Hannibal et al., 2017; Hannibal and Fahrenkrug, 2002; Hattar et al., 2002, 2006). To assess whether *Nefh* directed expression to mRGCs, sections of *Nefh*-EGFP-transduced retinas were colabelled with antibodies targeting GFP and melanopsin (Figure 3.9). Sections were taken at 150µm intervals, as above, and analysed using fluorescent microscopy (n=4). *Nefh*-EGFP was

found to express in melanopsin positive cells (Figure 3.9C-E, H-J); the size and shape of the cells varied considerably, however the cells were distinguished by their extremely wide dendrite arborisation. The number of melanopsin-positive cells varied considerably from section to section – mRGCs make up only 1% of RGCs, and so it is difficult to make accurate count estimates (Liao et al., 2016). The histology indicated that the vast majority of cells were transduced with EGFP and were melanopsin-negative, and a number of cells were melanopsin-positive but EGFP negative. This is in line with data displayed in Figure 3.8, which showed that approximately half of the GCL is transduced by AAV-*Nefh-EGFP*. The data conclusively demonstrates that *Nefh-EGFP* expression does effectively target mRGCs, a small but important subpopulation of RGCs.

As a second method of assessing preferential gene expression in RGCs from the *Nefh* promoter, a flow cytometry-based strategy was adopted. Adult wildtype 129 mice were intravitreally injected with 9×10^9 vg/eye AAV-*Nefh-EGFP* or 3×10^9 vg/eye AAV-*CMV-EGFP*. Three weeks post injection, retinas were taken, cells dissociated and analysed by cell sorting (Figure 3.10, Figure 3.11). Enrichment of RGCs was estimated by calculating the ratio of Thy1-positive:Thy1-negative cells in the EGFP-positive population (i.e., of all EGFP-positive cells, what proportion are Thy1-positive?). Interestingly, levels of Thy1 enrichment in these populations were significantly higher in *Nefh-EGFP* versus *CMV-EGFP* transduced retinal samples (Figure 3.11; 5.6-fold, n=12 versus 2.4-fold, n=9 respectively; $p < 0.001$). These data support the immunohistochemical observations above. To confirm that the Thy1 antibody did indeed enrich for Thy1-positive cells, Thy1 mRNA levels from sorted cells were assayed by RT-qPCR. Thy1-positive cells from n=12 retinas were sorted, along with non-antibody stained cells with similar scatter patterns from n=9 retinas. RNA from these retinas were pooled and assayed. Runs were standardised against

cell number rather than a housekeeping gene to reflect the possibility of differing expression levels between different cell types. Thy1 mRNA was found to be 3.23-fold higher in Thy1-positive cells than in non-antibody labelled retinal cells with a similar forward and sideways scatter (CT values of 32.618 and 33.477 respectively). Although numbers were not sufficient to perform statistical tests, this does indicate that the Thy1 antibody enriches for RGCs (Figure 3.10).

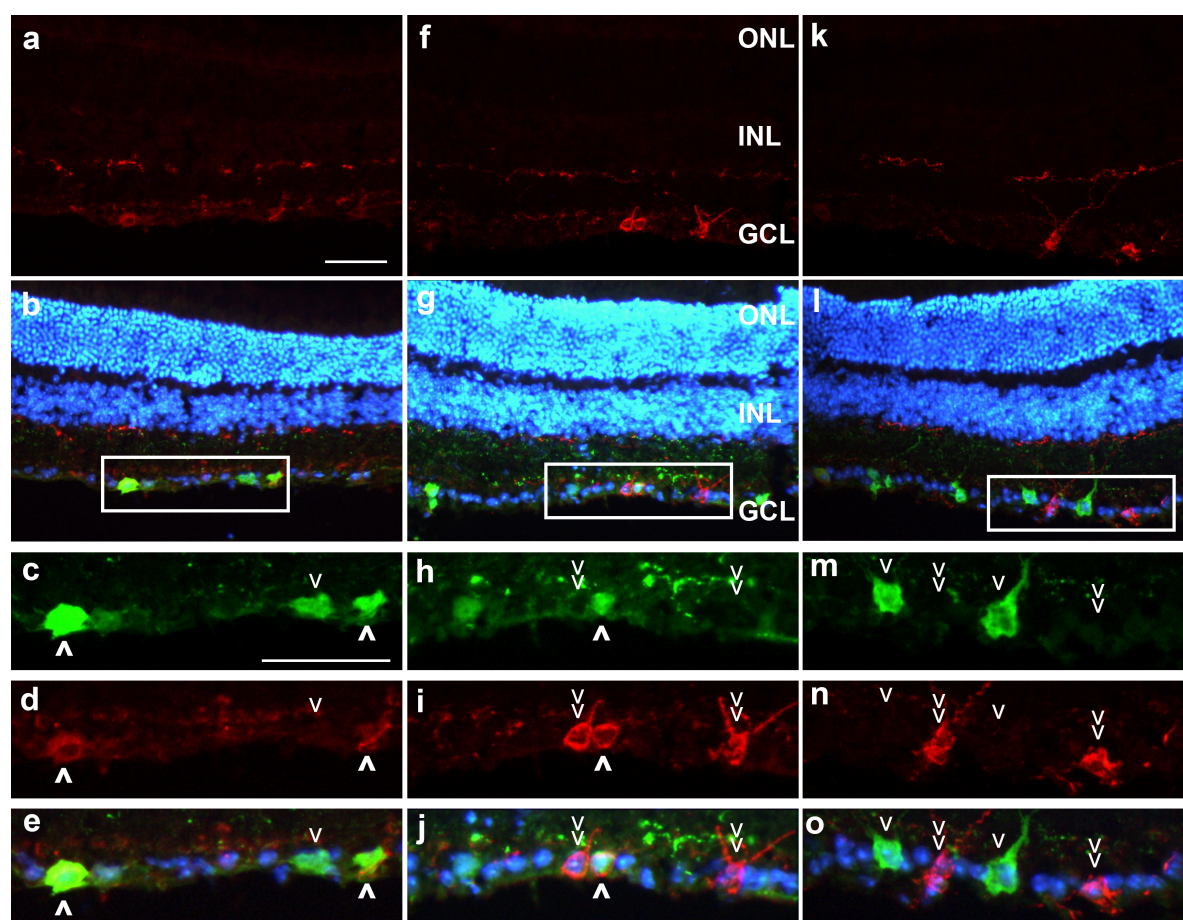


Figure 3.9: Immunohistochemistry analysis of melanopsin expression in AAV-*Nefh*-EGFP transduced retinas. Columns show examples of sections where coexpression of EGFP and melanopsin were observed (a-e, f-j) and where no coexpression was seen (k-o). Melanopsin was fluorescently labelled using Cy3, EGFP with FITC, and DAPI was used as a nuclear counterstain. Rectangles (in b, g and l) indicate positions of the enlarged areas. a, f and k: Cy3 label; b, g and l: Cy3, FITC and DAPI overlaid. c, h and m: FITC label; d, i and n: Cy3 label; e, j and o: Cy3, FITC and DAPI labels overlaid. (c-e, h-j, m-o) Bold arrows (^) indicate colabelled cells. Regular arrows (v) indicate transduced but melanopsin-negative cells. Double arrows indicate untransduced melanopsin-positive cells. ONL: outer nuclear layer. INL: inner nuclear layer. GCL: ganglion cell layer. Scale bars (a,c) 50µm.

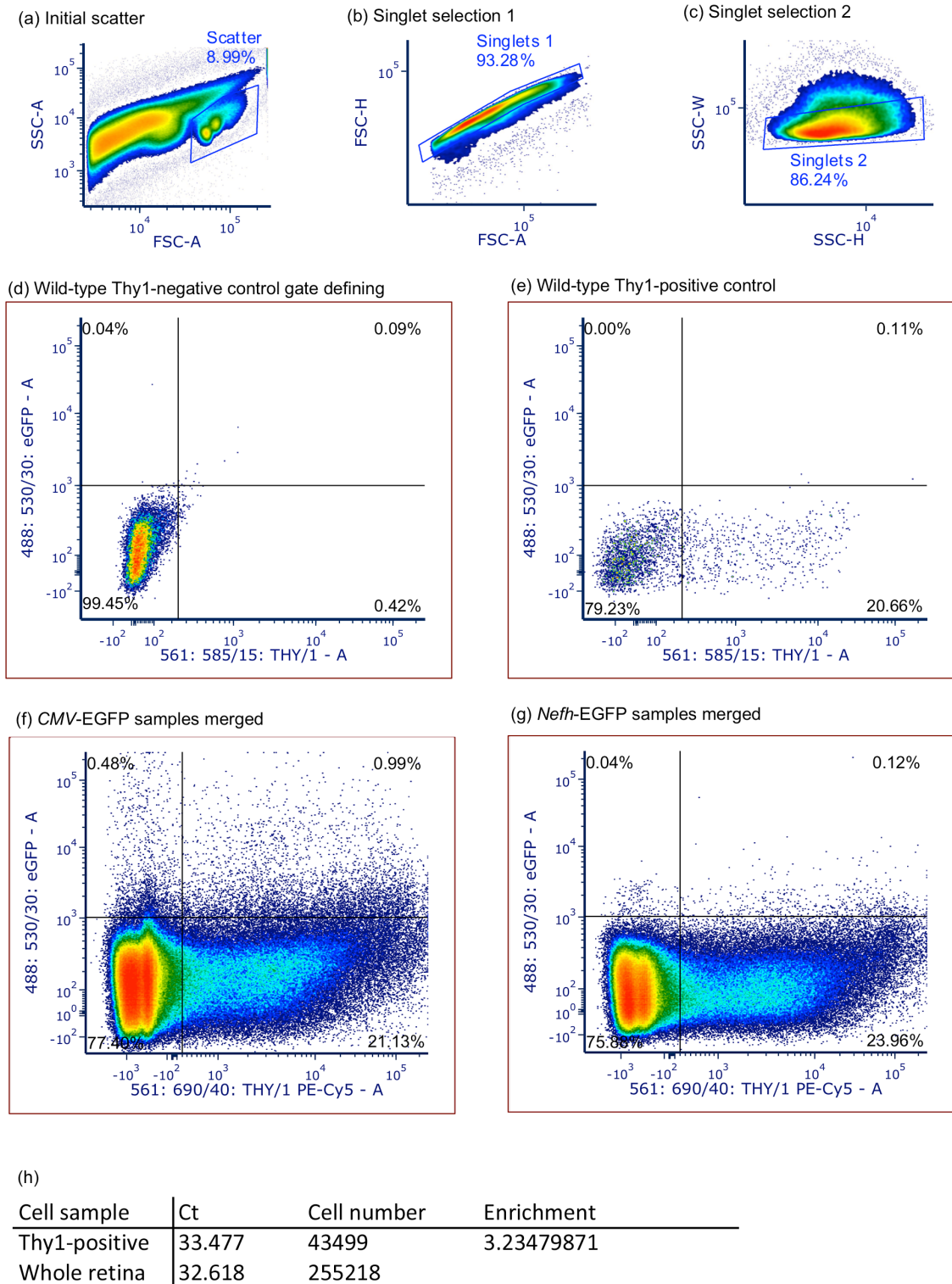


Figure 3.10: Flow cytometry analysis of *CMV* and *Nefh* promoter mediated EGFP expression. Eyes were injected intravitreally with either AAV-*CMV*-EGFP (3×10^9) or AAV-*Nefh*-EGFP (9×10^9 vg). Three weeks post-injection, retinas were dissociated and processed for flow cytometry analysis, using a Thy1 antibody conjugated to PE-Cy5. Nucleated cell populations were identified on the basis of DRAQ5 positive labelling (data not shown) and forward (FSC) and side (SSC) scatter (a), and singlets identified (b, c). Thy1 (x-axis) and EGFP (y-axis) gates were created based on wildtype retinas that had not been treated with Thy1 antibody

and wildtype retinas that had been treated with Thy1 antibody, representing Thy1-negative (d) and Thy1-positive (e) control samples. Using these pre-defined gates transduced retinal samples (n=6 per group) were sorted against EGFP and PE-Cy5 (Thy1; f, g). Percentage of cells in each quadrant are indicated. Enrichment values were generated by dividing the percentage of Thy1 and EGFP double positive cells by the percentage of EGFP-positive Thy1-negative cells. Thy1 positive cells (from n=12 retinal samples) and non-labeled singlets with a similar FFC/SSC profile (from n=9 retinal samples) were collected and pooled and *Thy1* mRNA levels were established by RT-QPCR (h). *Thy1* mRNA enrichment in Thy1 antibody positive cells was calculated from the ΔC_t value divided by the ratio of Thy1-positive cells to whole retinal cells.

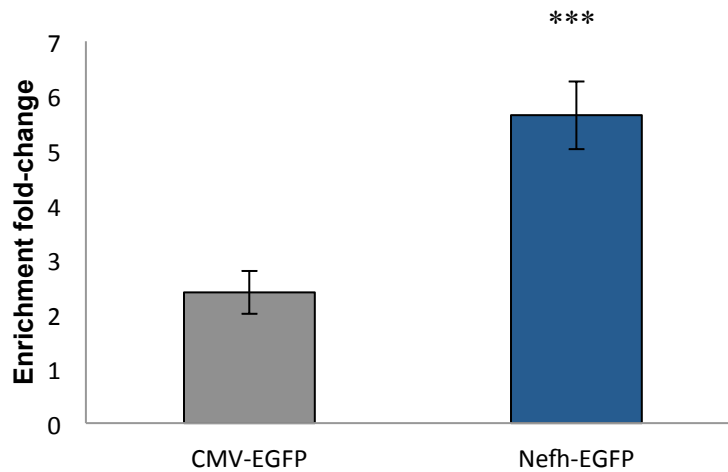


Figure 3.11: Estimation of Thy1-positive cell enrichment in flow cytometry experiments. Retinas transduced with either *CMV*-EGFP (n=9) or *Nefh*-EGFP (n=12) were dissociated and stained with a Thy1 antibody conjugated to PE-Cy5 as a marker for RGCs. Enrichment fold-change was calculated as the ratio of EGFP/Thy1 double-positive cells to EGFP-positive/Thy1-negative cells. The enrichment was calculated for each sample and then all samples were averaged. ***: $p < 0.001$.

3.2.4 Minimal *Nefh* promoter construction

The packaging capacity of AAV (4.7kb) is a barrier to the therapeutic delivery of genes in cases where either the gene itself or the expression cassette is too large. In order to optimise the *Nefh* promoter sequence for the potential expression of larger genes, regions of the promoter that showed the highest levels of conservation were isolated. Six regions (labelled A-F, Figure 3.12), 838bp in total, were synthesised as a potential hybrid *Nefh* promoter, titled *msMin-Nefh* (DNA synthesised by Integrated DNA Technologies). This was packaged with EGFP in an AAV2 vector (AAV2-*msMin-Nefh*-EGFP) and tested *in vivo* for expression efficacy and specificity. The putative promoter was synthesised with DNA restriction sites for *MluI* and *XbaI* enzymes, as with the PCR amplifying the original

Nefh promoter insert. The synthesised *msMin-Nefh* construct was digested with *MluI* and *XbaI*, as was pAAV.MCS *CMV-EGFP* (as with the original *Nefh* cloning methodology), and the two were ligated together. A digest with *MluI* and *XbaI* confirmed the presence of the insert (Figure 3.4) and the plasmid construct was sequenced to confirm perfect insertion.

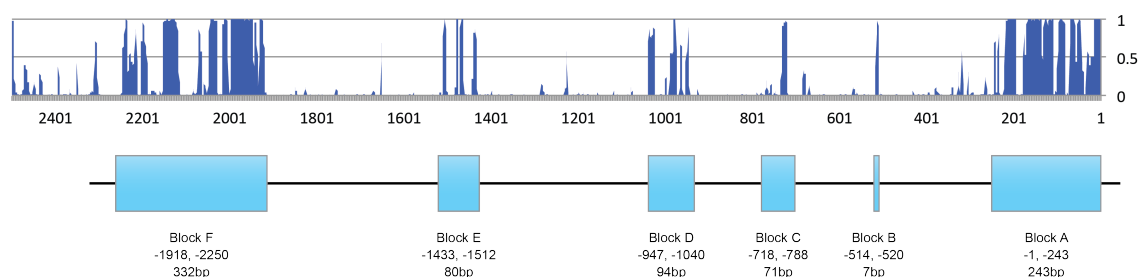


Figure 3.12: Schematic of *Nefh* conserved regions isolated for use in the *msMin-Nefh* promoter. Each region was chosen based on its conservation across placental mammals.

3.2.5 *msMin-Nefh* promoter transduction profile

AAV2-*msMin-Nefh-EGFP* was prepared as before (see Materials and Methods). A viral titre for this vector was obtained using the methods described in Chapter 5. Subsequent to the generation of this AAV vector, it was tested *in vivo* for the level and specificity of transgene expression compared to the 2.2kb *Nefh* promoter vector, as described below. Retinas were transduced by intravitreal injection of 3×10^8 vg of either *msMin-Nefh-EGFP* or *Nefh-EGFP* AAVs. In this case, a lower titre than previous experiments was used, as the AAV2-*msMin-Nefh-EGFP* titre obtained was a limiting factor (Table 5.2). It is notable that upon *in vivo* evaluation, the *msMin-Nefh-EGFP* vector showed a vastly different expression profile in mouse retina compared to the full length *Nefh* promoter construct. While the diluted *Nefh-EGFP* vector produced a similar transduction profile to that observed previously (Figure 3.14), *msMin-Nefh-EGFP* demonstrated very sparse, non-

specific EGFP expression in several different cell types across the retina (Figure 3.13).

EGFP-positive cells using the minimal promoter vector were found in both the GCL and INL.

Sections of transduced retinas were co-stained for EGFP and either Brn3a, ChAT or GABA as before. Retinas transduced with *msMin-Nefh-EGFP* showed as little as ten EGFP-positive cells in total. The full-length *Nefh-EGFP* vector, diluted to approximately one tenth for comparative purposes to ensure equal titres of both vectors were used, was found to express in fewer cells than the higher dose vector administrations, but still orders of magnitude higher than *msMin-Nefh*-driven EGFP expression (Figure 3.13, 3.14; n=4 retinas). Dilute AAV-*Nefh-EGFP* expressed in $15-25\% \pm 3-5\%$ of cells in the GCL (compared to approximately 50% using the higher dose, Figure 3.8), with expression in Brn3a-positive cells reduced as well ($14 \pm 5\%$, compared to 30-40% in higher dose injections). ChAT and GABA co-expression did not vary as substantially compared to the higher dose *Nefh-EGFP* administration (ChAT $3.3 \pm 1\%$, GABA $1.3 \pm 1.3\%$), although the low number of cells being transduced makes it difficult to interpret this. These numbers are illustrated in Figure 3.14.

msMin-Nefh appeared to direct expression in multiple cell types, however sparsely. In total, 2 *msMin-Nefh-EGFP* cells stained positive for Brn3a, 2 for ChAT, and none for GABA (n=4; Figure 3.13). The majority of EGFP-positive cells in *msMin-Nefh* retinas showed no co-labelling. The sheer lack of EGFP expression meant that counts for these samples could not be performed with any worth. Figure 3.13d shows an example of a cell found during analysis of transduced retinas. It appears to bear some similarity to bipolar cells as compared to that in the literature, based on morphology and positioning within the

middle of the INL (Euler et al., 2014). Overall, in this study the *msMin-Nefh* promoter sequence in its present form did not provide the robust and preferential RGC expression demonstrated by the full length *Nefh* promoter. While other forms of hybrid *Nefh* promoters were and continue to be considered (see Section 3.2.6 and Discussion), further research on this *msMin-Nefh* promoter construct was not pursued.

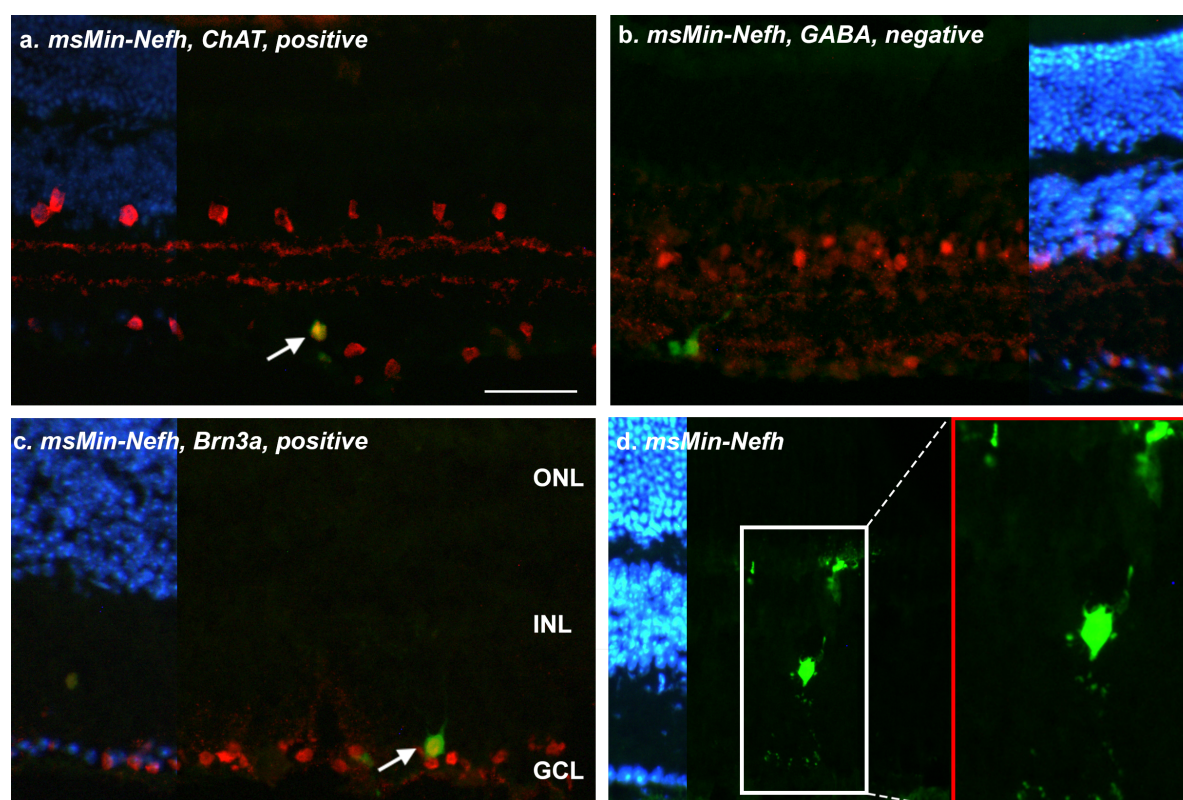


Figure 3.13: Immunohistochemistry analysis of AAV-*msMin-Nefh-EGFP* expression in transduced retinas. Eyes were injected intravitreally (3×10^8 vg). Transduced retinas (n=4) were fixed and cryosectioned 12 weeks post-delivery. Transduced retinal sections were colabelled with antibodies targeting GFP and either ChAT (a), GABA (b), or Brn3a (c). DAPI was used as a nuclear counterstain. White arrows (a, c) indicate co-expression of EGFP and either ChAT (a) or Brn3a (c). (d) Examination of a possible bipolar cell expressing EGFP, with a close-up highlighted (white box, red box). ONL: Outer nuclear layer. INL: Inner nuclear layer. GCL: ganglion cell layer. Scale bar: 50 μ m.

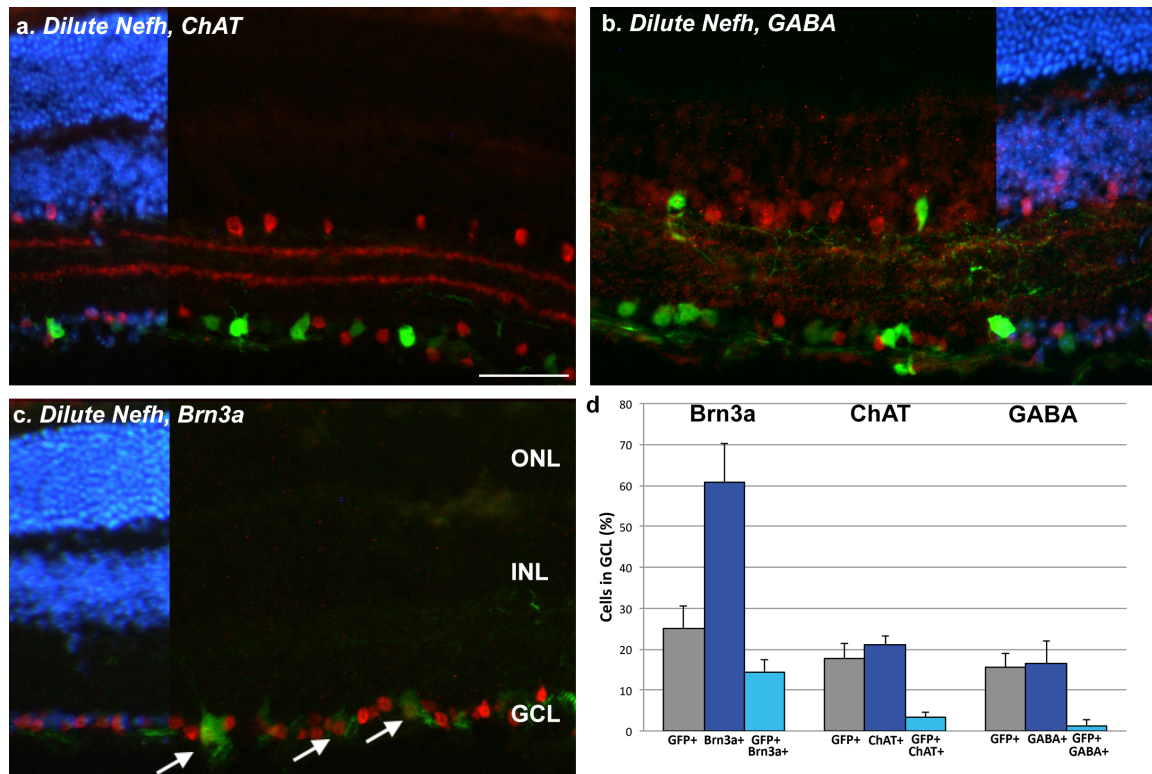


Figure 3.14: Immunohistochemistry analysis of dilute AAV-*Nefh-EGFP* expression in transduced retinas. Eyes were injected intravitreally (3×10^8 vg). Transduced retinas ($n=4$) were fixed and cryosectioned 12 weeks post-delivery. Transduced retinal sections were colabelled with antibodies targeting GFP and either ChAT (a), GABA (b), or Brn3a (c), as in Figure 3.7, 3.13. DAPI was used as a nuclear counterstain. (d) Quantification of dilute *Nefh*-driven expression in transduced mouse retinas. For each group (Brn3a, ChAT, GABA), manual quantification of labelled and colabelled cells was performed on the sections; two sections from each eye were taken for each stain ($n=4$, $n=8$ sections total, per stain). Percentages of cells positive for GFP were taken for each group, along with either Brn3a, ChAT or GABA. Co-localisation of these markers with GFP was also determined (indicated by white arrows). ONL: Outer nuclear layer. INL: Inner nuclear layer. GCL: ganglion cell layer. Scale bar: $50\mu\text{m}$.

3.2.6 AAV2-*Nefh-ophNdi1* constructs design and analysis

As discussed in the work regarding *ophNdi1* in Chapter 2, the combined use of *Nefh* with *ophNdi1* would be a valuable and important test for assessing the functionality of *Nefh* as a candidate promoter for RGC-targeted gene therapy. As such, two *Nefh* promoter sequences were cloned into the *ophNdi1* construct with an expanded backbone. However, previous cloning steps (undertaken in Chapter 2) resulted in a lack of available restriction digest sites for inserting *Nefh* into the large *ophNdi1* plasmid. In order to overcome this, a “patch” was designed and synthesised. This made use of two *BamHI* restriction sites in the *CMV*-

ophNdi1 plasmid, one found immediately after the ITR, and one within the *ophNdi1* CDS (Figure W). The patch added in several unique restriction enzyme sites (*PmeI*, *AflIII*, *MluI*) between *BamHI* and the beginning of the *CMV* sequence. The patch was successfully cloned into the *CMV-ophNdi1* construct. *Nefh* was then inserted by cutting both the promoter construct and the patched *CMV-ophNdi1* with *MluI* and *XbaI*, and ligating insert and vector together. Due to an error in production caused by a breakdown of the ultracentrifuge used to produce virus, generation of the full length AAV2-*Nefh-ophNdi1* virus was not completed in time for assays within this body of work. It is proposed that this vector will be use in future studies using rodent models such as that in Chapter 2 to assess its value as a means to direct gene therapies to RGCs.

In parallel with the *msMin-Nefh* study described above, a second minimal *Nefh* promoter was designed. It was of interest to explore whether a short *Nefh* promoter sequence (but longer than Region A of the *msMin-Nefh promoter*) extended 5' of the *Nefh* transcriptional start site would be of any functional use. Using a *BamHI* site found in the *Nefh* promoter sequence, a 480bp promoter region was cut out (using *MluI* as well; see Figure W), blunted using Klenow, and ligated into the patched *CMV-ophNdi1* plasmid. Subsequent to cloning, the 480bp *Nefh* viral vector construct was used to generate AAV (titled AAV2-*Nefh_short-ophNdi1*) using the preparation and purification methods referred to in the Materials and Methods. The vector was also titred as before, and injected *in vivo* and tested at the RNA level compared against AAV-*CMV-Ndi1* and AAV-*CMV-ophNdi1*. Given the low levels of expression achieved with the prior minimal promoter, it was decided initially to employ an RT-PCR based assay to provide insight into the relative expression achieved by the promoter (albeit not providing information on specificity). As discussed in Chapter 2, an antibody for *Ndi1* was not available, which eliminated the possibility of

immunohistochemistry. Whole retinal RNA was isolated and run in triplicate using RT-qPCR and analysed (n=3, 4). As can be seen in Table 2.1, the expression levels using *Nefh_short-ophNdi1* vector are far lower than that provided by *CMV-ophNdi1*, despite a five-fold higher titre. At the expression level demonstrated here, the true level cannot be accurately quantified. However, it is of note that the construct does appear to drive expression of the *ophNdi1* gene, and on an order of magnitude similar to the original *CMV-Ndi1*. This is compounded by the possibility that the *Nefh_short* promoter may provide a restricted expression profile relative to *CMV* and so a full understanding will require *in vivo* histological assays. Comparison to the full-length *Nefh-ophNdi1* would provide a more useful control, and as such this study is far from complete. While comparing expression levels of *Nefh_short-ophNdi1* to *CMV-ophNdi1* gives a general idea toward what degree *Nefh_short* functions, comparing to the full-length *Nefh-ophNdi1* vector will provide more insight.

3.3 Discussion

AAV has become one of the most commonly used vectors for gene therapy, with many clinical trials ongoing or completed and a number of gene therapies approved or seeking approval (clinicaltrials.gov). AAV is also the predominantly used vector in ocular gene therapies, with AAV2 currently the serotype of choice for RGC directed approaches (Bainbridge et al., 2015; Bennett et al., 2016; Feuer et al., 2016; Ghazi et al., 2016; Hauswirth et al., 2008; MacLaren et al., 2014; Yang et al., 2016; clinicaltrials.gov). Research in recent years has focused on improving the efficiency of AAV transduction and expression in the retina. The development of AAV vectors such as AAV7m8 and AAV8BP2 has improved levels of transduction in a wide variety of retinal cell types, and enabled consideration of intravitreal administration as a potential route of access to many retinal cells including photoreceptors (Cronin et al., 2014; Dalkara et al., 2013; Ramachandran et al., 2016). Various tyrosine capsid mutations in AAV have the potential to increase transgene expression levels by modulating capsid phosphorylation and ubiquitin proteasome-based degradation of viral particles during intracellular trafficking (Mao et al., 2016; Mowat et al., 2014; Petrs-Silva et al., 2009). Recent approaches to intravitreal delivery, including vitrectomy and sub-inner limiting membrane (sub-ILM) blebbing, have the potential to improve expression levels further (Boye et al., 2010; Tshilenge et al., 2016). However, a consequence of more efficient and broad transduction profiles may be greater potential for off-target effects. Confining expression of a gene therapy to only those cells affected by a disease represents a rational strategy; the potential reduction in immune responses may be an advantageous safety feature, as well as a means of aiding long-term expression.

In the current study, we have developed an approach to identify putative RGC promoters by analysing retinal transcriptomic data and referencing it against mammalian sequence conservation datasets to infer potential function. The expression levels of retinal genes were analysed, with high GCL enrichment and high absolute expression levels prioritised. Gene expression data in RGCs from the gene expression omnibus (GEO; ncbi.nlm.nih.gov/geo) was analysed in detail. Studies on expression from pre-natal or immature retina were omitted. In addition, samples where photoreceptor cell-specific gene expression was found to be high in RGCs were excluded as this indicated sample impurity. As such, the data from Kim et al. (2006) constituted the sole source of expression data used in this study, as to our knowledge no additional studies in the database suitably provided data on RGC gene expression enrichment in adult retina.

Conservation of the upstream sequence of these genes was evaluated in this context in order to establish lead candidate promoter sequences. Using this approach, we identified a number of potential promoters for use in RGCs. We proceeded to evaluate *in vivo* one of these promoter sequences, *Nefh*. *Nefh* showed significant conservation between species, high retina expression and RGC enrichment and that was of a suitable size for use in AAV-mediated gene delivery vectors where transgene capacity is limited. In this study, the *Nefh* upstream sequence efficiently drives transgene expression in RGCs following intravitreal injection of AAV-*Nefh*-EGFP into wild-type adult mice.

Following intravitreal delivery of either AAV-*Nefh*-EGFP or AAV-*CMV*-EGFP vectors, EGFP expression patterns were compared by histology. Serotype AAV2/2 was chosen for this study, given both its efficient transduction of mouse RGCs, as well as its well-established use and tolerance in the human eye, as has been observed in several clinical

trials (Bennett et al., 2016; Busskamp et al., 2010; Ghazi et al., 2016; Koilkonda et al., 2014a; MacLaren et al., 2014; Sengupta et al., 2016; Yang et al., 2016; Zhang et al., 2009). Both the *Nefh* and *CMV* promoters drove effective expression of EGFP in the GCL (Figure 3.5). Of note, the AAV-*CMV*-EGFP vector also resulted in expression in the INL, while AAV-*Nefh*-EGFP expression was predominantly confined to the GCL, with few EGFP positive cells observed in the INL (Figure 3.5, 3.7). Furthermore, when an increased dose of the AAV-*Nefh*-EGFP vector was administered, the levels of EGFP expression in the INL did not increase, highlighting the relative specificity of the *Nefh* promoter compared to *CMV*.

Fifty percent of the GCL is composed of amacrine cells (Akopian et al., 2016; Jeon et al., 1998; webvision.med.utah.edu). Analysis of EGFP expression in Brn3a-negative cells, as well as in GABA-positive or ChAT-positive amacrine cells, two major types of amacrine cells in the mouse GCL, demonstrated that AAV-*Nefh*-EGFP resulted in transgene expression in significantly fewer amacrine cells compared to the AAV-*CMV*-EGFP vector. While expression from the *Nefh* promoter was significantly restricted in ChAT-positive amacrine cells in the GCL compared to the *CMV* promoter, expression from both promoters was somewhat similar for GABA expressing amacrine cells in the GCL. This data supports the relative specificity of the *Nefh* promoter sequence in providing preferential gene expression in RGCs, and underlines its potential use for gene delivery to RGCs and its value for future gene therapies directed towards the retinal GCL. Of note, no significant difference was found between the numbers of transduced RGCs between the two doses of the AAV-*Nefh*-EGFP vector evaluated in mice. Previous studies have shown that only 40-60% of cells in the GCL are actually RGCs (Schlamp et al., 2013; Xiang et

al., 1996); it may be that saturation of RGC transduction is being reached even at the lower AAV-*Nefh*-EGFP dose assessed in this study.

RGCs represent a heterogeneous population thought to comprise in the region of 30 discrete types (Baden et al., 2016), which together represent just approximately 1% of cells in the retina. This has made isolation of pure populations of RGCs highly challenging within the field. Methods that have traditionally been used to enrich for RGCs, commonly using a Thy1 antibody, have included immunopanning (Barres et al., 1988), density gradient centrifugation (Kornguth et al., 1981), and magnetic cell separation (Shoge et al., 1999). More recently, flow cytometry-based methods with the Thy1.2 antibody have been used for RGC enrichment (Chintalapudi et al., 2016). These studies have highlighted that while the Thy1 antibody does indeed enrich for RGCs it does not exclusively label these cells, indicating that RGC-isolation methodologies still require further optimisation. In the current study we used Thy1.2-based flow cytometry to support the data obtained from immunohistochemistry. Similar to that observed in other studies, the antibody did not exclusively isolate RGCs, based on the percentage of Thy1-positive cells. However, in addition, there were indications at the RNA level that *Thy1* may be enriched in the flow-sorted population. Using flow cytometry we observed potential enrichment of Thy1-positive cells within EGFP-positive cell populations to be greater in AAV-*Nefh*-EGFP versus AAV-*CMV*-EGFP treated retinal cell samples. While the numbers of replicate experiments were not large enough to draw any firm conclusions from the data, flow cytometry analysis is in agreement with the histological data that indicates preferential gene expression in RGCs with the *Nefh* promoter.

mRGCs are one of the more unique subclasses of RGCs. Besides their expression of the photopigment melanopsin, they have extremely wide dendritic fields; although they represent about 1% of RGCs total, their arborisation is sufficient to effectively cover the entire visual field (Hannibal et al., 2017; Liao et al., 2016). There are five subtypes of mRGCs recognized (M1-M5), and these appear to be defined in part by their dendritic stratification within the IPL; like other RGCs (3.6, 3.7), they can be found in both the GCL and the INL (Liao et al., 2016; webvision.med.utah.edu). As mRGCs diverge significantly in many aspects from RGCs as a whole, it was of interest to evaluate whether the *Nefh* promoter effectively drove transgene expression in this cell type. As shown in Figure 3.9, AAV2-*Nefh*-EGFP does indeed transduce mRGCs as shown by costaining with antibodies targeting GFP and melanopsin itself. The small number of mRGCs in the retina makes it difficult to quantify actual numbers on a section-by-section basis, as most retinal sections have no mRGCs visible. The data generated so far in this study does show, however, that the *Nefh* promoter does drive gene expression in mRGCs, which are an important subclass of RGCs.

The final aspect of this study was to investigate the effects of minimising the *Nefh* promoter, by removing approximately 1.4kb of sequence, utilising only those regions that showed significant conservation of sequence across placental mammals (Figure 3.12). While AAV is a highly effective vector for gene therapy, the 4.7kb packaging constraint limits its versatility and the range of therapies that are compatible with it. By attempting to reduce the *Nefh* promoter size to under a kilobase, we sought to broaden its potential use as a promoter, particularly in AAV vectors, both in terms of enabling packaging with potentially larger genes and also allowing for the inclusion of enhancer elements that may increase expression levels if needed. However, the minimal promoter *msMin-Nefh*

demonstrated a significant departure in terms of both transgene expression level and specificity in the retina. The minimal *Nefh* promoter, when driving EGFP in an AAV2/2 vector, targeted very few cells in the inner retina (Figure 3.13). While there were too few cells transduced in total to give an effective statistical analysis, there seemed to be no clear preference for RGCs, or even the GCL. Finally, a second variant *Nefh* promoter, *Nefh_short*, was tested by *in vivo* RNA analysis. *Nefh_short* was compared to *CMV* in driving expression of *ophNdi1* (Chapter 2). The 480bp promoter did appear to drive expression of *ophNdi1*, but at a vastly reduced level compared to *CMV* (Table 2.1). Expression appeared broadly similar to that shown by *CMV-Ndi1*, although at a level where accurate quantitation does not hold. The absence of a full-length AAV-*Nefh-ophNdi1* vector makes judging the effectiveness of the promoter difficult. As mentioned in the previous section, evaluation of the expression level from this promoter is confounded somewhat by the fact that the RNA was isolated from whole retina. The studies to date are further inhibited by the absence of an *Ndi1* antibody, making evaluation of *Ndi1* expression via immunohistochemistry difficult. However, hypothetically if, unlike *msMin-Nefh*, *Nefh_short* drives more RGC-preferential expression, then the fact that fewer cells in total may be expressing *ophNdi1* would partly explain the reduced expression levels observed in the RNA analysis. Comparison of this promoter to the full length *Nefh*, along with histological studies as outlined in this chapter, will better enable us to examine the true expression pattern of the *Nefh_short* promoter construct.

There are several possibilities as to why the *msMin-Nefh* promoter underperformed compared to its full-length counterpart. The titre injected in this instance was far lower than that used in previous sets of experiments with the *Nefh* promoter. It is possible that with a higher AAV titre the expression profile of *msMin-Nefh* would become more

apparent. However, this was controlled for by the use of a diluted AAV-*Nefh*-EGFP vector to match the titre of the *msMin*-*Nefh* vector. It is notable that the diluted full-length promoter vector showed a level of expression lower than the full dose *Nefh* vector, but a highly similar pattern of preferential expression (Figure 3.14). What is apparent from this study is that something is lost in moving from the full-length *Nefh* to the minimal *msMin* variant. The mechanisms underlying this loss of promoter function may be structural (in terms of the three-dimensional structure of the 5' sequence), or may relate to the actual sequence and associated binding partners. In terms of structure, it has already been noted that the *Thy1* promoter used in transgenic lines for RGC-specific expression requires 6kb of spacing between the core promoter region and its RGC enhancer (Aliç et al., 2016; Spanopoulou et al., 1991). It may be that a similar situation has occurred in this instance with *Nefh*. The largest conserved regions of the *Nefh* promoter used in the minimal variant are at the beginning and the end of the promoter sequence; there may be a spatial requirement for full promoter function in this instance. However, additional experimentation would be required to address this. In terms of the sequence itself, it is possible that specific regions play a significant role in the specificity of the *Nefh* promoter, some of which may be species specific. The relationship between sequence conservation and promoter function, particularly with respect to tissue-specific genes, has been shown, particularly in the regions immediately upstream of the transcriptional start site (Farré et al., 2007; Suzuki et al., 2004). However, some studies have shown evidence of species-specific promoter divergence (Donaldson and Göttgens, 2006; Tirosh et al., 2008; Young et al., 2015). As such, it will be valuable to dissect the *Nefh* promoter region in order to discern what regions are required, and if issues such as spacing are critical in ensuring promoter function. While this is beyond the scope of this study, it will be beneficial in fully

understanding how *Nefh* functions, as well as fully evaluating the possibility of a functional but minimised promoter.

One method of determining how effective a promoter may be at delivering preferential expression is through the analysis of its sequence to search for transcription factor binding sites. A number of programs are available that can examine promoter sequence and search for putative binding sites. These have been based in recent on chromatin immunoprecipitation (ChIP) data and the ENCODE project as well as *in silico* approaches to predict binding sites, and include programs such as GTRD, PROMO and Factorbook (Messeguer et al., 2002; Wang et al., 2013; Yevshin et al., 2017). While an in-depth analysis of transcription factor binding sites was beyond the scope of this project, preliminary investigations conducted (with PROMO and GTRD) revealed a number of neuronal and general transcription activation sites. That said, some of these results were inconclusive, as the dissimilarity and uncertainty thresholds for calling putative binding sites appeared to differ between programs. As such, a comprehensive and sufficient analysis has not been done as of yet. However, in future, it is hoped that, were the pipeline established in this study to be reused, that analysis using one or more of these systems would be included (and ideally automated), as a further pre-test examination of whether a promoter sequence would be of worthwhile use.

The purpose of this study was two-fold. It involved the identification of candidate RGC promoters for potential use in AAV-mediated gene therapies, and moreover the validation of this methodology for the characterisation of putative promoter sequences. As sequencing costs continue to decrease, and techniques such as RNAseq become more widely adopted, access to transcriptomic datasets from a wide variety of cell types will

become more readily available. The availability of such large datasets will be a powerful resource, which, in a similar fashion to the present work, could be exploited to identify, characterise and validate new cell-type specific promoter sequences.

The current study used an AAV2/2 vector to facilitate the transduction of mouse RGCs. As discussed, however, it has been previously observed that while AAV2/2 is well tolerated in the human eye when administered subretinally, its transduction efficiency in primate RGCs is inferior to that of mice (Ivanova et al., 2010; Tshilenge et al., 2016; Yin et al., 2011).

The development of new capsid serotypes such as tyrosine mutants, AAV8BP2, and Anc80L65 (Petr-Silva et al., 2011; Ramachandran et al., 2016; Zinn et al., 2015), or new methods of administering AAV2/2 (as in the sub-ILM delivery of Boye et al. 2016) should aid in addressing this issue. It is important to note that the repertoire of AAV serotypes available for gene delivery is rapidly increasing. These improved viral capsids and delivery methods may increase the probability of detecting potential off-target transcriptional activity from tissue-specific promoters, including the *Nefh* promoter, beyond what was seen in the current study employing AAV2.

Intravitreal injection of AAV vectors enables efficient transduction of RGCs. RGCs are the primary target cell population for gene therapies for many disorders discussed in Chapter 2, including LHON, DOA and glaucoma, among others (Farrar et al., 2013). While intravitreal administration provides access to RGCs, it may more readily result in stimulating immune response(s) to vectors such as AAV compared to subretinal administration (Li et al., 2008). It would therefore be valuable to minimise the therapeutic vector dose used, and to confine transgene expression to the target cells of interest, thereby potentially limiting undesired side effects.

Observations regarding patterns of cellular loss in end stage photoreceptor degenerations have highlighted the retention of certain retinal layers. While the photoreceptor layer frequently degenerates, many other retinal cells remain relatively intact, including bipolar, amacrine, horizontal and RGCs. These observations have been elegantly juxtaposed with the identification of light sensitive molecules from organisms such as algae and archaeobacteria. Optogenetics is the expression of these molecules, provided as a gene therapy or protein, in non-light sensitive neurons thereby introducing a capacity for light detection. RGCs represent one key target cell population for optogenetics (Farrar et al., 2014; Gaub et al., 2014). Hence, the *Nefh* promoter may also be of value in the design of future optogenetic-based gene therapies for IRDs.

Conclusions and future work

The above discussion highlights the potential utility of the *Nefh* promoter sequence identified in the current study, in providing preferential RGC transgene expression and in the design of future gene therapies for disorders involving RGCs. The next logical step, as discussed in the previous chapter, would be to test the efficacy of full-length *Nefh* in driving a therapeutic gene for an RGC disease model. A construct combining *Nefh* and *ophNdi1* would provide a good evaluation of this novel promoter's function – whether the level of transgene expression provided is sufficient to protect RGCs against damage in the LHON model used in Chapter 2. Indeed, this study would follow a similar format to that of Chapter 2. Testing *Nefh* in the setting of other disease models and therapeutic strategies – such as an optogenetic intervention for photoreceptor disease – would also be worth pursuing in the future.

While the *Nefh* promoter functions well in directing highly preferential gene expression to RGCs in the mouse retina, the use of a mouse promoter for a gene therapy may be at times suboptimal when considering clinical application (although promoters such as chicken- β -actin have been used in human clinical trials, as discussed previously). As such, testing of the human *NEFH* promoter in a similar vein as detailed in this chapter would be highly valuable for the further characterisation of this novel promoter, but falls outside the work of this PhD thesis. The human *NEFH* promoter region follows a similar layout to its mouse equivalent, and can be seen in Appendix 5. Of note three of the conserved regions from the mouse promoter (A, D and F) align significantly to the human promoter. There are also regions of conservation within human *NEFH* that do not map to mouse. An interesting difference in the human promoter is that the region immediately 5' of the transcriptional start site is extremely GC-rich (up to 80%). While this has been shown to be an indicator for transcription factor binding and promoter activity (Khuu et al., 2007), it can at times make handling the DNA difficult, as it is refractory to both PCR and synthesis methods of amplification. However, despite this potential difficulty, it is important to test this promoter region, to assay its effect on gene expression, and also to examine the regions of the promoter as has been attempted with *msMin-Nefh*.

Additional work remains with regard to the characterisation of more minimal *Nefh* promoter sequences that can provide preferential gene expression in RGCs. One aspect of *Nefh* promoter function that would be of interest to determine is whether the spacing of promoter elements is a key element in determining expression level and specificity, as has been found to be the case for the *Thy1* promoter. One simple approach to attempt to address this would be the production of a hybrid *Nefh* promoter sequence that includes the most proximal and distal conserved regions, with “junk” DNA in the middle. The necessity

of the smaller conserved regions, and indeed the non-conserved regions, could then be assessed based on the results of such a study. It may be that many regions within the *Nefh* promoter are required for tissue-specific function, and that further minimisation is not possible in a significant way, however it would be worthwhile testing to establish if this is the case. A smaller promoter with similar efficacy to the full length *Nefh* characterised in this study would be a valuable addition to the gene therapy toolbox, and it is a possibility that is worth pursuing.

Chapter 4: Megabase-scale copy number variation in rod photoreceptors

4.1 Introduction

4.1.1 Integrity of the genome

One of the hallmarks of tumours, especially in late stage cancer, is that they frequently show high levels of copy number variation (CNV; Lucito et al., 2003). The assumption that the healthy nuclear genome remains intact was soon overthrown as well, with microarray experiments showing significant differences in copy number across the genome between individuals (Sebat et al., 2004). This has also been shown in neurons (Rehen et al., 2005). The impact of this genomic instability remains to be fully elucidated, although in cancerous tissue it may offer a selective advantage in cases where oncogenes and tumour suppressor genes can be duplicated and deleted, respectively. In the context of neuronal genomes, which are not actively dividing, it may be that the loss of some regions of DNA may have a less severe impact on cell function. Understanding what, if any, DNA is lost in neurons may provide fundamental insights into the biology of neurons, including retinal neurons.

One field in which this may be highly relevant is that of induced pluripotent stem cells (iPSCs). iPSCs were developed first in 2006 by exposing mouse fibroblasts to a range of pro-stem cell factors, which “forced” differentiated cells back into a nascent, stem cell-like state, that could then be maintained (Takahashi and Yamanaka, 2006). Work had been done on reprogramming adult cells back toward stem cell fates, but had previously required cells’ nuclei to be replaced by nuclei from stem cells (Wilmut et al., 1997). However, iPSC technology enabled for the first time stem cells of an organism (and thus, in theory, cells of any kind) to be derived solely from adult host cells. This has enabled for the generation of cell and animal models of a number of diseases with greater ease than before, and raises the possibility (as discussed below) of creating cells for transplantation that do not suffer from the prospect of host rejection (Yamanaka, 2012).

Cellular transplantation has been researched in the retina, with some successes, for many years. Much of this work has taken place with photoreceptors, using both embryonic stem cell-derived approaches, and more recently using iPSCs (M’Barek et al., 2017; Mandai et al., 2017; Pearson et al., 2016; Schraermeyer et al., 2001; Welby et al., 2017). The extent to which these photoreceptors or proto-photoreceptors integrate has been questioned in recent years, however the work still represents an exciting opportunity for treatment. This work is at a much earlier stage for RGCs, due in part to added complexity arising from the number of RGC classes that exist, the complexity of wiring in the inner retina and the length of RGC axons. However, work on this has developed in recent years, including methods such as using scaffolds to enable the growth of longer axons *in vitro* in preparation for potential transplantation (Yang et al., 2017). This work is now extending towards transplantation, with some studies achieving significant increases in visual ability

after optic nerve crush/RGC depletion, and others not (Becker et al., 2016; Cen et al., 2018; Divya et al., 2017). While gene therapy has made great strides in recent years, and the number of trials delivering functional vision recovery has grown, the technology cannot recapitulate post-mitotic cells that have already died. The ability to generate neurons through iPSCs may thus offer another avenue toward restoration of vision in patients with IRDs, apart from or, in the case of generating iPSCs from the patients themselves, alongside a corrective gene therapy.

A concern that arose alongside the development of iPSCs was that only a very small fraction of cells – approximately 1% – would develop stem cell characteristics. Further, many iPSC populations generated are found to have genomic rearrangements, including aneuploidy and CNVs (Liang and Zhang, 2013; Martins-Taylor and Xu, 2012). It would be valuable for the purposes of studying iPSCs for patients suffering from IRDs to understand the extent to which CNVs and genome rearrangements naturally occur in cells. That is to say, is this genomic instability somehow a side-effect of the iPSC creation process, or is the minute assessment of genomic integrity of iPSCs simply revealing the baseline variation already inherent in “healthy” cell populations? Understanding whether this occurs in the retina may help to answer questions such as these. Furthermore, recent data suggesting that the genome may vary significantly between neurons (discussed in detail below) is of fundamental biological interest. At minimum, this requires further validation in a range of different neuronal types to confirm that this is indeed the case.

4.1.2 NGS and single-cell sequencing

A significant technical limitation of high throughput genetic technologies is that microarrays and some sequencing technologies require large amounts of input DNA. While

these studies have shown systemic mutations, they lack the resolution to see differences in DNA sequence on a local scale, between individual cells. However, the advent of single-cell whole-genome sequencing (SCS) has enabled the genome to be examined at unprecedented levels, even on low-output sequencers such as the Illumina Miseq, among others. At a single-cell resolution, sequencing coverage is too low to gain a full understanding of the genome. However, for applications such as read-counting and CNV analysis, single cell DNA sequencing can perform well. For example, it has been used to probe minute differences in tumour population copy number (Navin et al., 2011).

In 2013, McConnell et al. used SCS to demonstrate mosaic CNV in human brain neurons. The group isolated neurons from recent (<24hrs) human post-mortem brain tissue, and showed up to 40% of cells had deletions or duplications of megabase-scale regions of the genomes of these neurons (120 neurons were analysed in this study). They found the CNVs to be random in nature, with no single region overrepresented in their findings (McConnell et al., 2013). However, the authors failed to distinguish between different types of neurons in their study. Taking a section of tissue and isolating cells based on whether they expressed the neuronal marker NeuN, they were unable to distinguish between the myriad different types of neurons that are present in the human frontal cortex. Thus, it could not be conclusively proven from this study that the CNV pattern found in neurons is random. Besides the above research, other recently published studies follow the pattern of taking regions of brain tissue for single cell analysis, but sorting only for neurons, despite the acknowledgement in the literature that the lineage and transcriptomic nature of associated types of neurons can be vastly different (Cai et al., 2014; Harbom et al., 2016; Upton et al., 2015). Analysis of a single type of cell could shed light on the arrangement of the genome in neurons.

4.1.3 Approaches to dealing with low-plexity data

One of the primary challenges in SCS is that the plexity of the data being generated from individual cells is very low. Navin et al. (2011) reported an average coverage of roughly 0.06x. This is compounded by the very real threat of severe amplification bias, as a usable amount of material is generated from a single cell's nuclear DNA. In recent years, more sophisticated sequencing chemistry has significantly increased the number of reads achievable from a desktop sequencer, and new machines offer greater depth as well. This is still limited by the amplification step, however. The low read depth means that accurate coverage of regions of any length is next to impossible. Indeed, the noise inherent in the sequence data is such that steps must be taken in order to produce a signal. Repetitive regions are a significant confounding factor with respect to this.

A number of methods for amplifying DNA from single cells have been developed in recent years – three of these have become the methods that a majority of kits available on the market make use of. One of the most standard ways, often simply called whole genome amplification (WGA) or name for the associated brand, consists of three steps – fragmentation, annealing, and PCR. In kits such as the Sigma Genomeplex (WGA4) or GE Genomiphi, the DNA is enzymatically fragmented initially, followed by a one step polymerase reaction to anneal compatible primers to the end of the fragments (which exists in a range of roughly 200bp-2kb; Sigma). These primers are then used to amplify the genome. A second method uses multiple displacement amplification (MDA). MDA utilises random hexamer primers which anneal across the genome, and the high fidelity ϕ 29 polymerase to amplify from those hexamers. The general idea is that this allows for greater evenness across the genome, rather than an enzymatic fragmentation reaction which may

require certain sequence combinations not found, for example, in repetitive regions of DNA. The third major method uses multiple annealing and looping-based amplification cycles (MALBAC). MALBAC involves amplifying DNA in such a way that the resultant amplicons have compatible ends that form looped structures. This prevents amplicons being used for further amplification. While this means that the reaction takes place on a linear rather than exponential timescale, it significantly reduces the possibility of amplification bias, as errors included in amplicons cannot be subsequently propagated. A study was recently conducted comparing these methods of amplification, and their suitability for single cell CNV analysis (Ning et al., 2015). The research group found that MALBAC had a slight bias towards high GC content, although the authors note that that may be a symptom of the other methods faring more poorly at high GC amplification. Both WGA and MALBAC showed high reproducibility in terms of the spacing of reads mapping to the genome, following sequencing of amplified DNA (r value 0.88-0.92). MDA, however, fared quite poorly, with the cell-to-cell correlation value ($r=0.02$) indicating that the proportions of regions of DNA being amplified vary widely between samples. This is likely due in part to the random nature of the hexamer primer annealing, which would involve less systemic bias in the system but far higher variability. As such, the suitability of MDA for single-cell NGS is debatable.

Several different approaches have been taken in order to handle data for detecting CNVs and somatic aberrations. As can be seen below, many of these have been designed for use with single nucleotide polymorphism (SNP) microarrays. However, at a basic level, low-coverage sequence data mirrors SNP arrays. Methods for modelling CNVs essentially come down to counting the density of reads in a given area, compared to regions either side of that area – in theory this can be as easily done with WGS sequence reads as it can

with SNP array density. A key conceptual difference between a microarray and a low-coverage sequencing reaction is that a SNP array consists of single-base information designed to be evenly dispersed throughout the genome, while low-input sequencing achieves a random snapshot of regions that would be expected to be relatively even across the genome by chance. Hence, for dealing with SCS data, a greater focus must be placed on reducing bias (caused by such factors as repetitive regions and the inherent mappability of different genomic regions).

4.1.4 Methods for detecting CNVs

There are many methods used for assessing CNVs as seen in the literature and in the collection of available programs used to perform the analysis. The majority of these programs can be adapted for use with SCS data. One method used by proprietary software produced by Illumina (BeadStudio – microarray data only), as well as open source alternatives such Control-FREEC (Boeva et al., 2012; Winchester et al., 2009) is B allele frequency (BAF). BAF involves the generation of genome-wide expected values, based on normalised outputs using the data being analysed. The BAF itself is then calculated as the difference between that expected value in a given region and the observed SNP density in that region. The BAF ratio can be described as the proportion of total allele signal for a given region explained by one of those two alleles. Calculating those ratios (between 0 or 1) for the two alleles allows the interpretation of copy number between 0 and 4 (where 0 is indicated by a loss of clustering in that region). Copy numbers beyond this are not suitable for BAF analysis, and for noisy data BAF is unsuitable, as the parameters for delineating CNV changepoints are insufficiently strict (Alkan et al., 2011; Boeva et al., 2012).

Hidden Markov Models (HMMs) are used by a number of programs, including CANOES, EXCAVATOR2 and ADTex (Zare et al., 2017), and represent one of the major classes of algorithms for detecting CNVs. They are particularly prominent in whole exome sequencing (WES) data analysis. The basis of HMMs is to attempt to stochastically model the “hidden” state of the system based on observed data using probability distributions (Seiser and Innocenti, 2014). Based on the read count density across the genome, a transition matrix is generated (effectively a table that determines the probability of a copy number shift) with the underlying assumption that each given region being analysed is dependent on adjacent regions, that is, copy numbers of adjacent regions are assumed to be the same. Different programs take different approaches to upstream preprocessing, based on the noise of the data. HMMs are particularly seen to apply to WES as the target capture nature of WES allows for regions to be examined in isolation as a means of reducing noise. One program of note that takes a unique approach to HMM-based CNV analysis is CoNIFER (Krumm et al., 2012). CoNIFER uses singular value decomposition (SVD) to eliminate noise. It takes the assumption that, as the copy number of a given region will take an integer value (i.e., a whole number), this key parameter actually accounts for a relatively small amount of variation. As such, it decomposes the principal components of the data into three data matrices, from which the strongest degrees of signals are tagged as noise and removed. It then rebuilds the matrices and uses HMM to detect CNVs. This is a highly efficient means of reducing noise, however CoNIFER is specifically designed for captured regions as in WES and so is unsuitable for SCS analysis.

The other algorithm employed, like HMM, in a large number of programs is circular binary segmentation. Like CoNIFER, CBS takes the assumption that a given single cell will have

an integer number of copies of any given segment of DNA, and this can be used to smooth the genome to better define copy number of a region. This is the same method used by McConnell et al. (2013). CBS uses an underlying model of discrete changes in copy number mapped against a series of permutation distributions, with significant deviations from these distributions used to define change-points in copy number (Olshen et al., 2004). These change points can then be followed in either direction to define copy number in a region, until a second change-point is encountered. This brackets this region as having a deviated copy number from the norm, and is repeated across each chromosome in such a way that multiple CNVs may be defined. The noise inherent in the data can then be used to define error, beyond which a CNV is called real or not. This analysis is recursive, repeatedly breaking the regions of a chromosome up into segments until every element of the chromosome has a copy number that either matches adjacent ones or has a defined, significant deviation. The “circular” aspect that is a departure from binary segmentation (Sen and Srivastava, 1975), allows for greater resolution in detecting a single large breakpoint, by joining segment ends to perform a second test of likelihood that the copy number differs (Olshen et al., 2004; Winchester et al., 2009). Navin et al. (2011) and McConnell et al. (2013) created windows (termed “bins”) of variable size throughout the genome, with a defined length of uniquely mappable sequence per bin (50kb-500kb). Reads in a given region were then mapped to these bins. McConnell et al., using this method, defined a significant CNV by an arbitrary number of bins with the same defined copy number. This method is computationally more intensive than other means, and can result in non-integer copy numbers, but with appropriate normalisation and interpretation allows for a simple and accessible approach to SCS data analysis.

4.1.5 Summary and Project Objectives

The aim of this study was to examine the genomic integrity of retinal neurons at an individual level. It must be noted that this research diverges significantly from work in previous chapters, with a focus on AAV and gene therapies for IRDs. However, understanding the biology of retinal neurons at an elemental level is of fundamental interest. Understanding the extent to which the retinal genome can vary without harm may aid in future technologies for treating retinal disease. As such, the possibility that this was occurring was worth investigating.

To date, studies attempting to address such issues have not used a single, purified cell population. Rod photoreceptors represent the vast majority of cells in the retina (approximately 70% of cells). A transgenic mouse model exists that expresses EGFP exclusively in photoreceptors (under control of the rhodopsin promoter; Chan et al., 2004). The presence of EGFP in a single cell type enabled the isolation through flow cytometry of a pure population of single cells for SCS analysis. Following DNA amplification and sequencing on an Illumina MiSeq, sequence files were analysed using a pipeline that involved alignment, mappability mapping, and read counting, followed by implementing CBS to detect CNVs in single-cell samples. Using this approach we have demonstrated megabase-scale CNVs in mouse photoreceptors. We have shown that, in our initial sample, 40% of cells sequenced had significant CNVs. Finally, although there appears to be no fixed pattern, two cells were found from one mouse with identical duplications, possibly suggesting that CNVs may develop before the post-mitotic stage of photoreceptor life cycle.

4.2 Results

4.2.1 Photoreceptor isolation and amplification

Rho-EGFP^{+/-} mice on a combined BALB/c and 129 background were used for the purpose of isolating photoreceptors. Adult mice (mean age 6 months) were sacrificed, and their retinas were dissociated and sorted using flow cytometry as described (Materials and Methods), with single, live, EGFP-positive cells sorted into different tubes. DNA was initially amplified using a Genomeplex WGA4 kit, followed by sequencing preparation using an Illumina Nextera kit. As the input DNA quantity was so low (and the possibility of sorting error resulting in no cell present), following amplification with WGA4, samples were run on an agarose gel stained with SYBR-gold to confirm the presence of DNA. An example of this gel analysis can be seen in Figure 4.1. Following Illumina adapter ligation with Nextera, samples were sequenced on a MiSeq (150-read single end) and analysed.

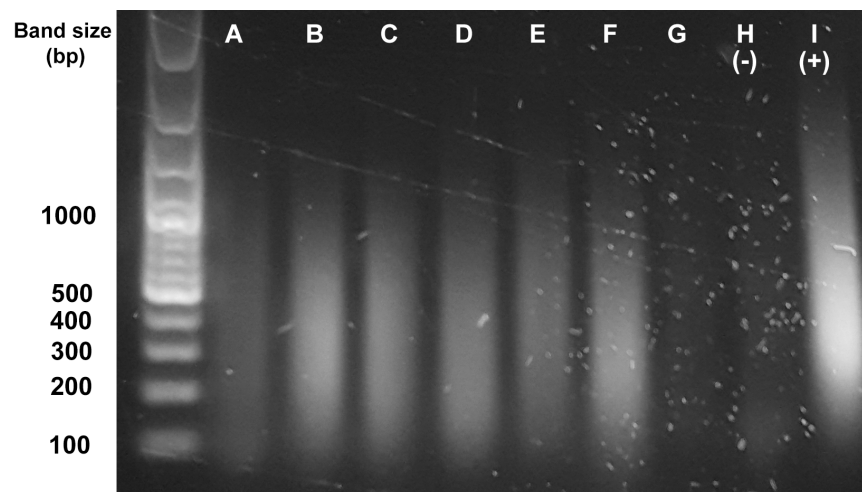


Figure 4.1: Agarose gel showing samples post-WGA4 amplification but before Illumina adapter ligation. A-G are single-cell samples, while H is a negative control and I a positive control. As can be seen from this gel image, no DNA was detected in G.

4.2.2 Analysis strategy

In order to study the integrity of DNA copy number at a cellular level, SCS analysis is required. In this study, DNA amplified from individual mouse photoreceptors was analysed for CNVs using a CBS algorithm. Individual rod cells were isolated by flow cytometry using EGFP as a marker, providing a pure, fully differentiated single neuronal population for sequence analysis. Following flow sorting, cells' genomes were amplified and sequenced on an Illumina MiSeq next generation sequencer. Individual cells do not provide sufficient material to sequence every base of a genome. However, by read mapping to a reference genome – in this case mm10, the most recent assembly – it is possible to examine genome sequence for duplications and deletions at a megabase scale. A flow diagram of the multiple steps used to undertake single cell whole genome sequence analysis process path from retinal tissue to single cell in sequence analysis is displayed in Figure 4.2.

Initially, sequence reads were aligned against an index reference genome (GRCm38/mm10) using Burrows-Wheeler Alignment (Li and Durbin, 2009) and duplicates were removed using the Picard Tools suite. The numbers of reads per region were then counted using a program written in Python (SeqSeg; Appendix 3) and used to estimate copy number of a given chromosomal region. The genome was divided into 500kb windows into which reads were binned; a consecutive CNV of six windows or more was required to be counted as a real event.

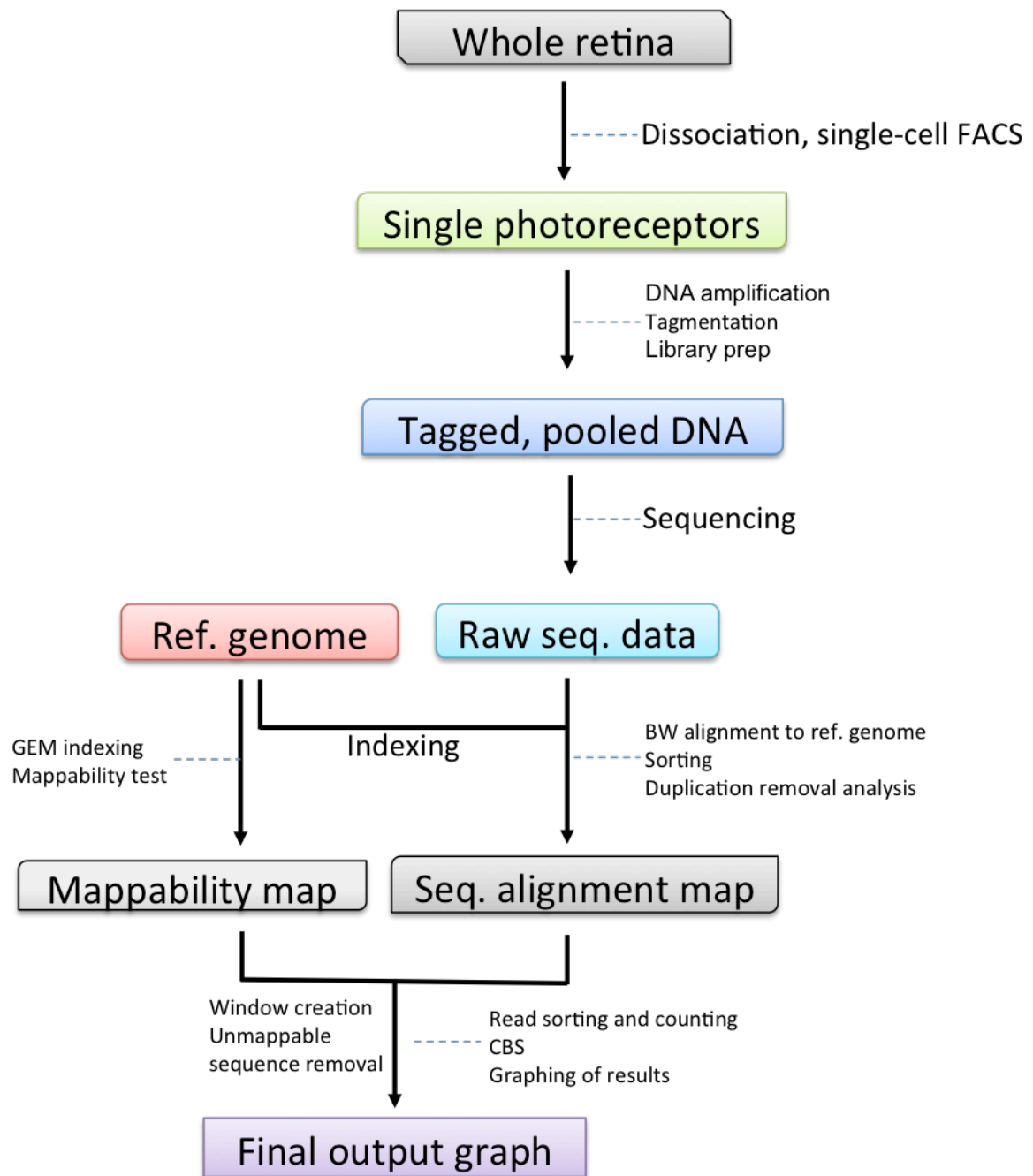


Figure 4.2: Flow diagram of single-cell whole genome sequencing process and data analysis.

The read coverage for each sample was very low, and varied considerably from sample to sample. Given the potential difficulties and costs associated with the approach, in these preliminary studies 20 individual flow cytometry-purified retinal cells were sequenced. Compounded with the issue of non-uniform genome mapping, the sequence coverage was too low to reliably find deletions manually. In order to address the issue of noise within the sequence data, as well as meaningfully define changepoints indicating CNVs, an approach was taken as highlighted in Figure 4.2. The Genome Multitool (GEM) mappability algorithm (Derrien et al., 2012) was used to define uniquely mappable sequence, that is, sequence that maps to a single locus of the genome. SeqSeg was modified to call from another program written in-house (WindowMaker) that defined windows based on a region covering 500kb of uniquely mappable sequence, utilising the output from GEM. From this, SeqSeg sorted the reads from sequence files into these bins. In order to curtail noise from the low coverage and to find cohesively deleted/duplicated regions, a segmentation algorithm called Circular Binary Segmentation (CBS) as discussed above was employed (algorithm was employed using DNACopy software in R; Olshen et al., 2004). As gains and losses for a given region are discrete, the CBS algorithm uses the read data provided to split a given chromosome into regions of equal copy number in order to account for the noise that results from random low-plexity read sampling. This allows for the construction of a map of each chromosome with estimated copy numbers of different regions displayed. CBS was originally designed to receive input from microarray data; the output from SeqSeg was modified slightly to mimic a format suitable for analysis, as detailed in the DNACopy reference manual.

4.2.3 Sequencing results

Of the twenty individual cells sequenced and analysed, eight samples showed CNVs across at least six windows of 500kb of uniquely mappable sequence. The full set of sequence plots can be found in Appendix 2. Half of those cells exhibiting CNVs showed evidence of only one; the other were found to have as many as five (Figure 4.6a). The distribution of CNVs found across mouse chromosomes is summarized in Figure 4.6b. Figures 4.3, 4.4 and 4.5 show examples of chromosome CNV maps, with alternating chromosomes highlighted in green and blue. Figure 4.3 and shows maps of whole genome plots. Figure 4.3a shows a genome with no detectable CNVs, while Figure 4.3b shows several deletions and duplications, none of which match the minimum requirement of significance to be counted as a potentially ‘real’ event. Figure 4.4 shows a sample with a single CNV branch point; the dotted lines indicate one and two median absolute deviations (MADs), respectively. Figure 4.4b shows the chromosome from Figure 4.4a containing the observed CNV. All cases where CNVs of 3mb or greater were found were duplications rather than deletions. In several cases there were deletions seen, however in almost all cases the copy number observed was less than the 2 MAD threshold set, and where the CNV exceeded that threshold, it was not sufficiently long to be considered as real. MAD was chosen as a measure to define real events as it provides a robust, sample-specific measure of variability, while effectively controlling for outliers that may have escaped earlier quality control measures.

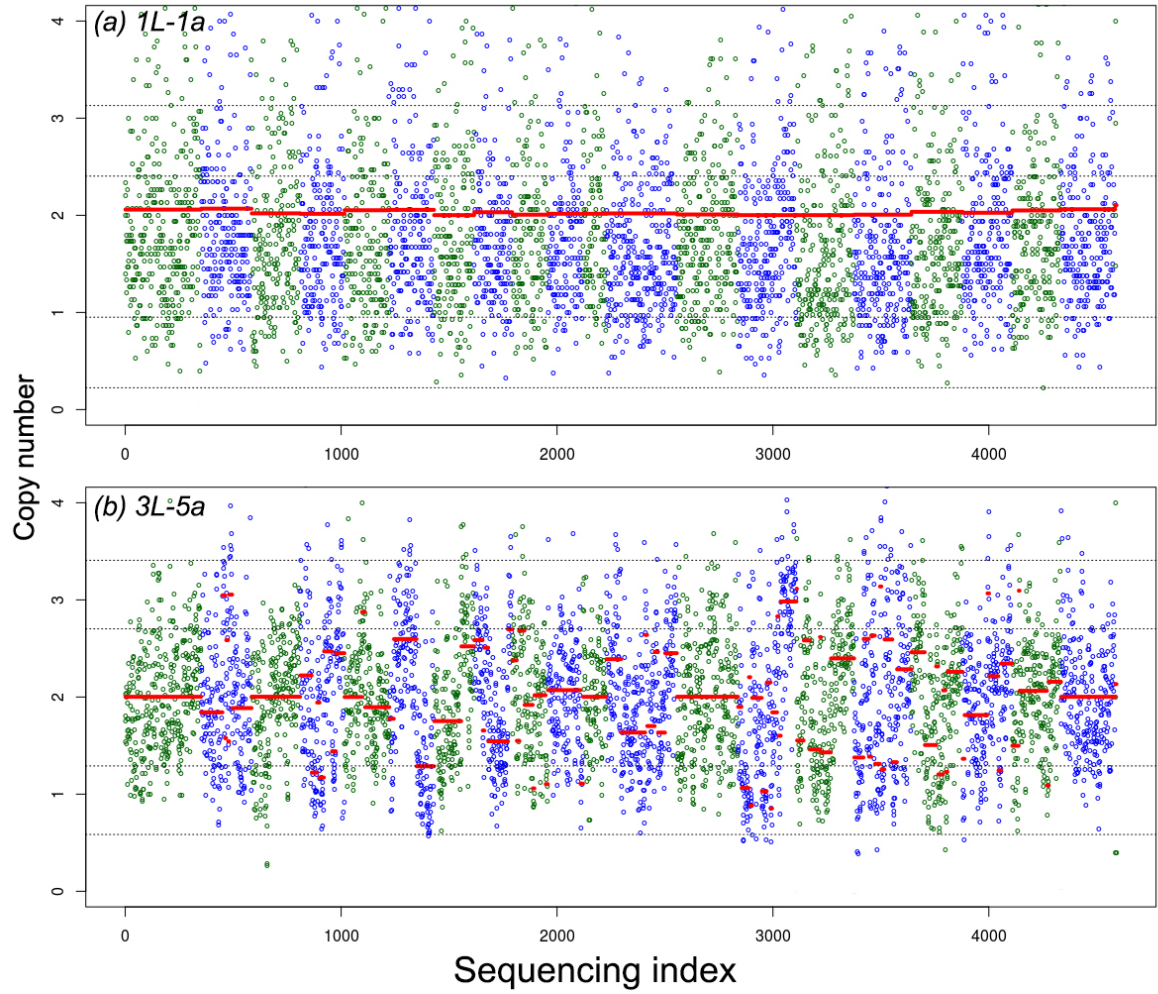


Figure 4.3: Plot of copy number across the genome for two samples. Alternating chromosomes are highlighted in blue and green, with the red line indicating consensus copy number in that section of the genome (divided into 500kb windows of uniquely mappable sequence). The sequencing index represents the list of windows. Dotted lines indicate one and two MADs from the consensus copy number. CNVs are defined as copy number breakpoints (indicated by breakages in the red line) of at least six 500kb windows in a row. Only those CNVs greater than two MADs from the consensus copy number are counted as real.

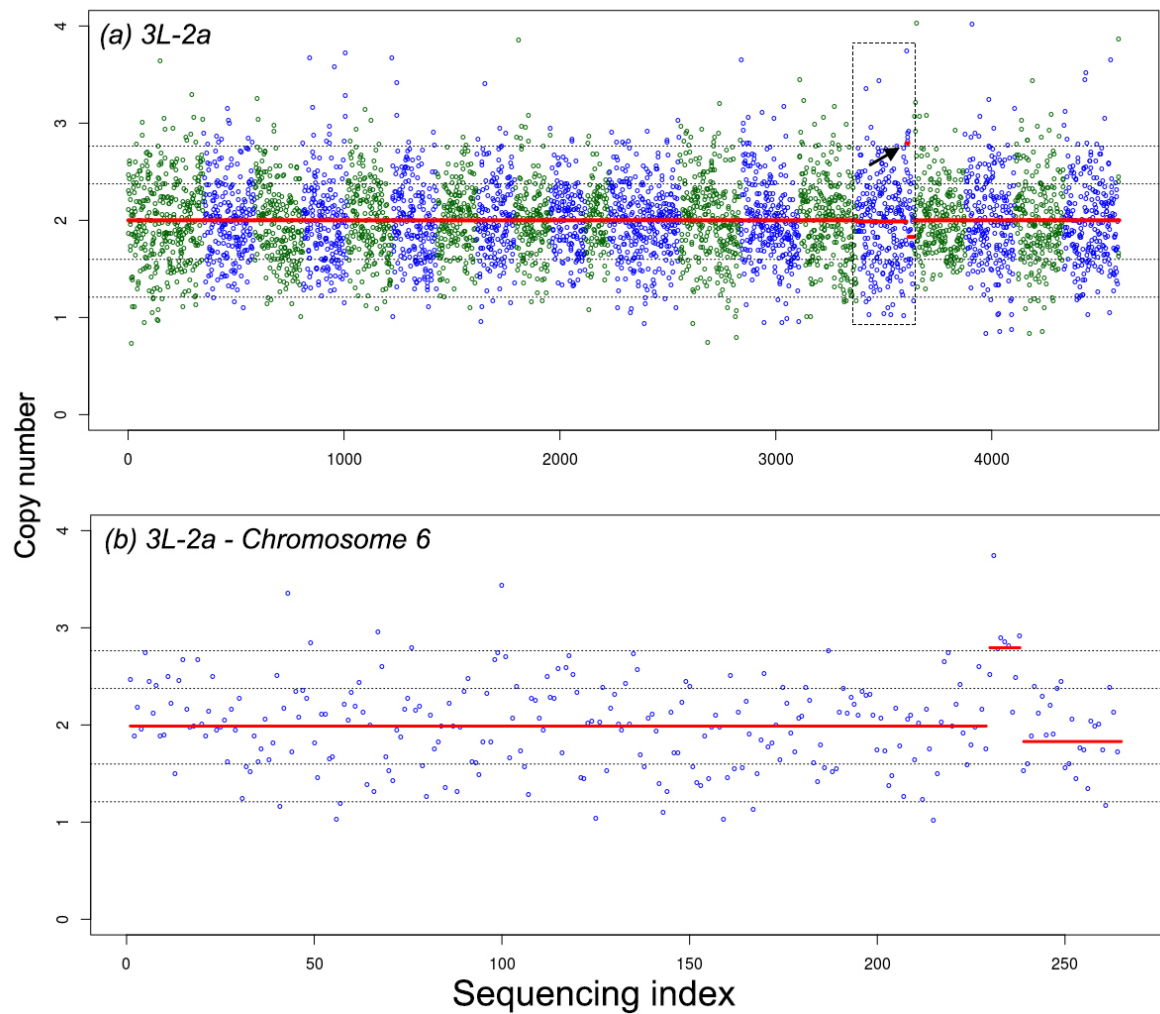


Figure 4.4: Plot of copy number for one sample, with significant CNV highlighted (b). (a) The entire genome copy number plot is displayed. The dotted black rectangle indicates the area highlighted in (b). The black arrow indicates a significant CNV greater than six windows, and greater than 2 MADs from the median.

Of those cells with CNVs, two samples showed extremely similar patterns of CNVs; the plots of those samples are shown in Figure 4.5. Three duplications achieving significance were shared in identical genomic regions (Figure 4.5, black arrows). Further, one duplication too short to be considered real was also shared (purple arrows). Of note, both samples shared a large, ~10mb heterozygous deletion in the same region of chromosome 12, that, owing to the noisiness of the data, could not be considered real. A third photoreceptor sequenced from the same mouse (fellow eye) displayed evidence of five duplications (Appendix 2h), however none of these matched the set of CNVs seen in 3R-1a

and 3R-2a. One pair of samples (1R-1a, 3L-1a; Appendix 2n,o) showed erratic patterns of average copy number in each bin; these samples had the lowest numbers of high quality reads mapped (0.0002-0.0004x coverage), which may have contributed to the incomplete mapping – these samples and associated data were not considered further due to low number of high quality reads.

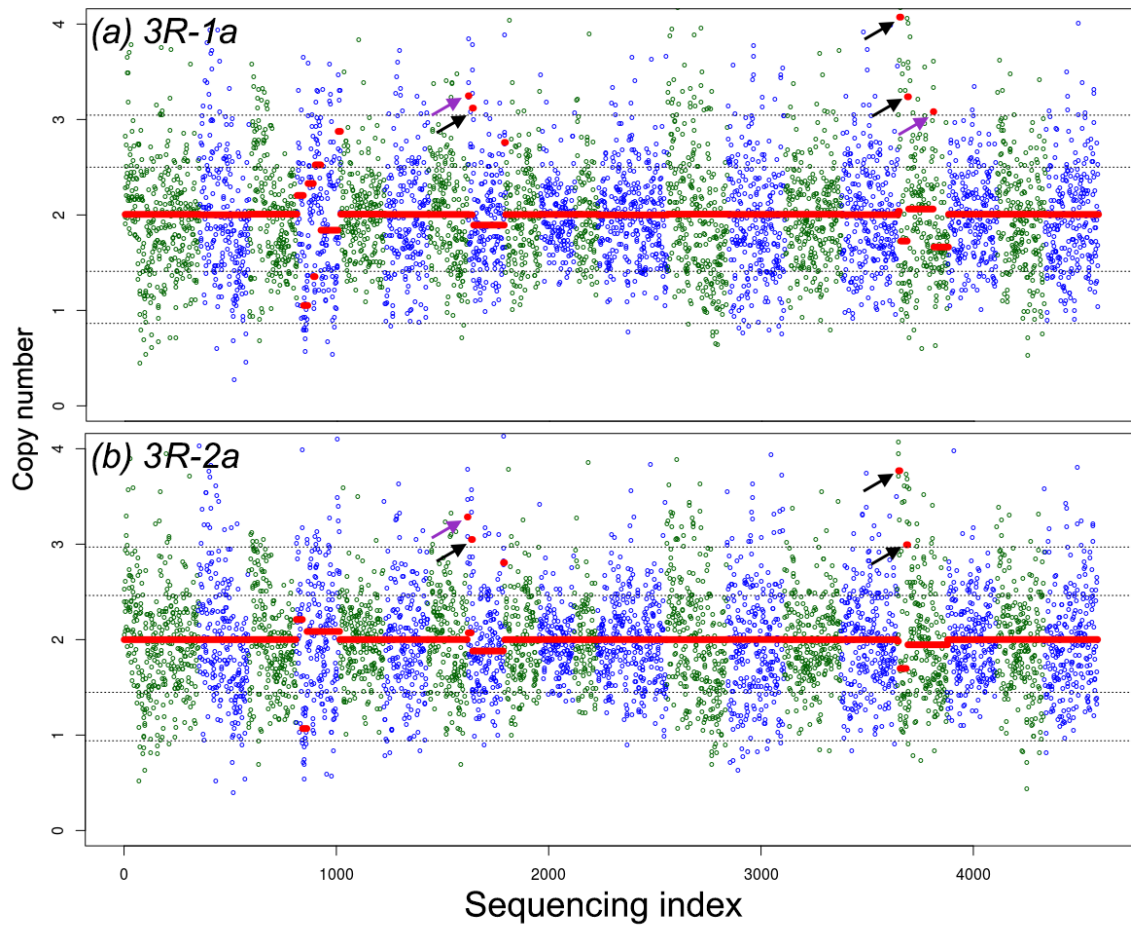


Figure 4.5: Plot of copy number across the genome for two cell samples (1a and 2a) sequenced from the same eye (designated 3R). Black arrows indicate CNVs of six or more windows with a copy number of at least two MADs from the median. Purple arrows indicate CNVs of less than six windows, too short to be considered.

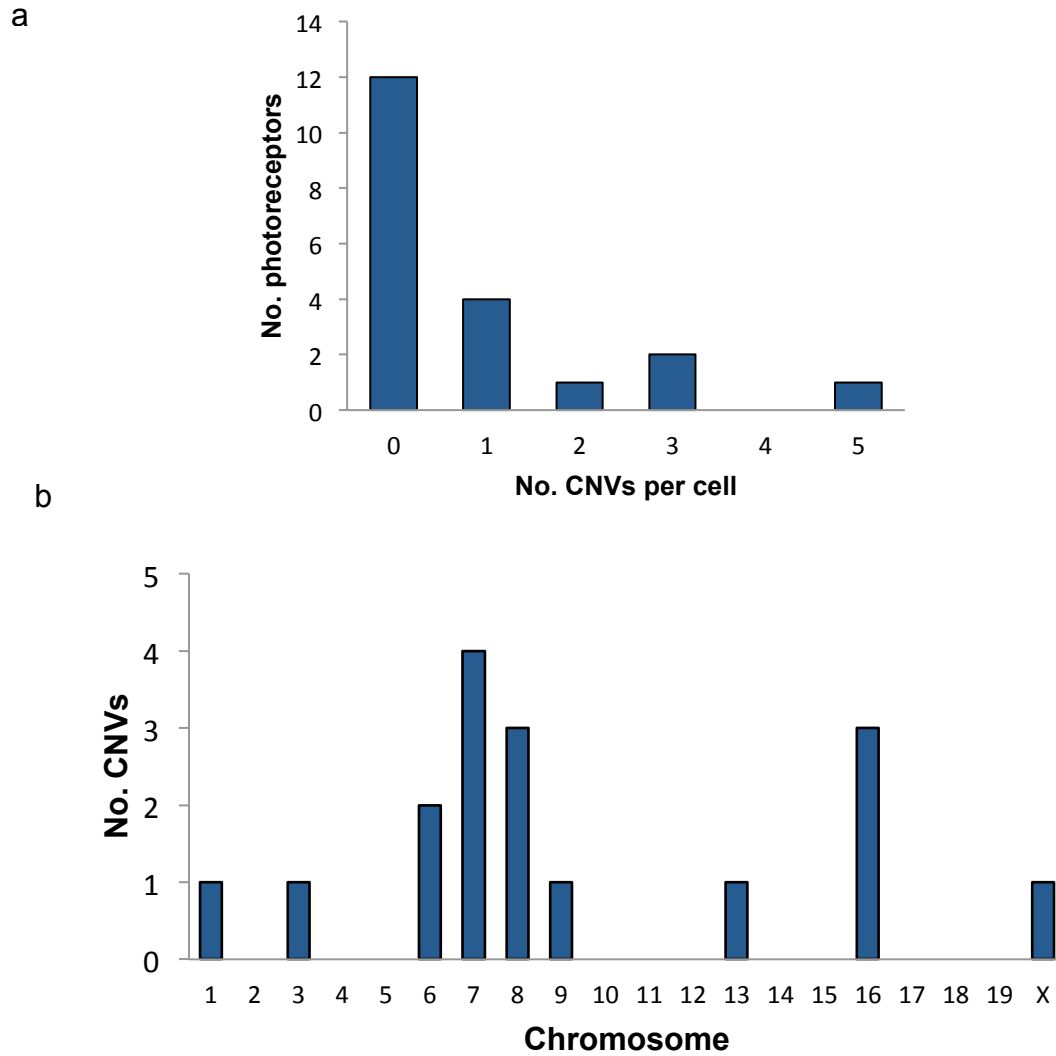


Figure 4.6: (a) The number of significant CNVs found in the corresponding number of sequenced photoreceptors. Significant CNVs were defined as six or more consecutive windows of 500kb uniquely mappable sequence. The copy number displayed by the putative CNV was also required to be at least 2 median absolute deviations (MADs) from the median copy number (normalised to 2; magnitude of MAD was defined by the variance in a given sample). (b) The number of CNVs found per chromosome across the 20 cells sequenced.

4.3 Discussion

The increasing sophistication of WGS and the growth of SCS have enabled interrogation of the genome at a higher resolution than ever before. A key use of this technique in recent years has been to examine aneuploidy and chromosomal instability in tumour samples (Navin, 2015). However, these analyses are predicated on the idea that the genome of every healthy cell is identical. Indeed, the field of gene therapy relies on the idea that a known mutation – and that mutation alone – is causative in disease. The idea, then, that there may be inconsistencies in genomic integrity, even in post-mitotic cells such as neurons, is worth investigating given its relevance to so many fundamental biological concepts. It is important to address the fact that neurons are not unique in featuring somatic megabase-scale CNVs. It has been demonstrated in multiple studies that actively dividing cells such as fibroblasts show evidence of CNVs (Knouse et al., 2016). However, buildup of mutations is considered a normal aspect of somatic cells as division takes place – for non-dividing cells such as neurons to have deletions and duplications at the scale of whole chromosomal regions is of considerable interest. The studies into the existence of mosaic CNVs in neurons by, for example McConnell et al., (2013) and Cai et al., (2014), together with the availability of a mouse line expressing a reporter gene in specific retinal cells, prompted the investigation of genome instability in such retinal neurons.

In this study, we have isolated single rod photoreceptor cells from transgenic mice expressing EGFP under the control of a rhodopsin promoter (*Rho-EGFP^{+/-}*). Using single-cell whole genome amplification, we sequenced the amplified genomes and probed for the presence of CNVs. Sequencing data was aligned to a reference genome and putative CNV signal was isolated using a CBS algorithm. Of the twenty single-cell samples sequenced thus far, eight have demonstrated the presence of CNVs sufficiently long in size to be

considered as potentially real. All of the significant CNVs found in the eight samples were duplications. Of those seven, two demonstrated highly similar patterns of duplication, as well as similar but non-significant patterns of deletion.

The results from this study parallel data seen in other work such in terms of the proportion of cells displaying CNVs. 40% of cells show evidence of at least one CNV, which is similar to the proportions observed in other studies in the literature. However, in contrast to previous work, all of the CNVs found in this study were duplications, with no deletions passing the minimum significance threshold seen. In the McConnell et al., (2013) paper, deletions were observed approximately twice as frequently as duplications. There are several possible reasons for this to have occurred. The tolerance threshold for deletions in rod photoreceptors may be different compared to cortical neurons. Indeed, it may be that the transcriptional equilibrium of mouse cells in general may be more easily able to deal with increased copy number. Equally, the sequencing depth may not have been sufficient to faithfully produce signal from the available material; higher coverage sequencing may offer sufficient data to resolve deletions. That said, the read coverage (approximately 0.06x) match quite closely with previous work seen in papers such as McConnell et al. (2013) and Navin et al. (2011).

This study used flow cytometry to isolate single photoreceptors, made possible by using a Rho-EGFP mouse line. A significant proportion of single-cell studies conducted in recent years have used flow cytometry for cell isolation, as it affords potentially more precise sorting of single cells (Gross et al., 2015). There is the possible risk of capturing two cells instead of one in each sort – however, correct parameters for isolating only singlets reduces this risk substantially, and affords greater precision than other methods such as serial

dilution (Hu et al., 2016). The outer segments of photoreceptors can snap off when handled indelicately (Parinot et al., 2014), however, flow cytometry has long been validated as a method for photoreceptor isolation, and the Farrar group has extensive experience with the technique (Akimoto et al., 2006; Parapuram et al., 2010). Upon sorting, cells were immediately frozen in dry ice and stored at -80°C, and the lysis step of DNA amplification was performed before cells were allowed to warm again. Negative and positive controls were used during amplification, to ensure that nuclear DNA was amplified.

The sheer paucity of starting material in SCS renders the technique vulnerable to PCR amplification and bias. While steps were taken to minimise this (including exclusion of regions known to contain repetitive DNA or sequence that cannot be accurately mapped at low coverage), it may not be possible to eliminate these biases entirely. There are a number of reasons that we may cautiously interpret the data as likely robust and not, for example, as artefacts of uneven amplification. Previous studies have demonstrated the reliability of SCS when sequencing known clonally related cells such as tumour samples or eight-cell embryos (Navin et al., 2011; Vanneste et al., 2009). Additionally, the CNVs detected are an order of magnitude larger than the PCR amplification products used at any step of this study. Some of the major studies done in previous years do also address this, as the issue of CNVs appearing as the result of artefacts of sequencing such small quantities of DNA cannot be understated.

As coverage within a given sequencing instance is not even, resequencing of the amplified DNA may help in confirming the findings. While it is possible that amplification bias is a factor in the results seen, resequencing would be valuable in order to confirm that the results are not due in part to sequencing artefacts.

A finding from this study that is unique among previously published work is that two of the cells isolated from one retina (3R-1 and 3R-2) displayed near-identical patterns of CNVs, both in significant duplications and in deletions approaching the threshold of significance (Figure 4.5). A third sample from the same animal, but the fellow eye, failed to show a similar CNV pattern. Previous studies have taken populations of neurons that are only distinguished from a non-neuronal population; it is possible that this similarity stems from using a pure, monotypical cell type. Repeated sequencing of the “sister” neurons will be required to confirm the deletion pattern, alongside sequencing of other photoreceptors isolated from the same retina and from the same mouse. If it can be conclusively shown that at times different neurons display identical patterns of CNVs, it may shed light on at what point during development this genome instability can occur. The existence of even a handful of cells with similar deletion and duplication patterns would suggest that the CNVs form before the post-mitotic stage of rod photoreceptor development. A larger study using multiple pure cell types isolated from the retina should in principle allow for a more precise timing of such events. Again, caution must be used in interpreting these preliminary results, as until samples are resequenced, possibly at higher sequencing depth, it is still possible that some of the CNVs observed may be a function of error relating to the sequencing process. Moreover, it is somewhat counter-intuitive to suggest that such large changes to genomic integrity would be well tolerated, although this may be more the case in the context of non-dividing cells such as neurons.

The high amplification requirement in SCS adds a second potential problem for accurate, high-resolution sequencing – over amplification. As amplification proceeds, the primer concentration relative to the number of DNA strands in the reaction decreases

exponentially. This eventually causes the effective PCR efficiency to decline, with the majority of DNA strands separating during denaturation without reannealing. As these single strands share a small set of common adapters used in the sequencing process, imperfect annealing of non-complementary strands occurs. The “daisy chains” of heteroduplex DNA that form are difficult to quantify with intercalating fluorescent probes (as used in the Tapestation 2200 and Qubit), as their incorrect sizing leads to inaccurate quantitation. Adding too high a concentration of primers can be equally unwise, however, as they are difficult to remove from the sample before sequencing and can interfere with the reaction. qPCR methods allow for more accurate quantification as they begin with a denaturation step, but accurate fragment sizing is still needed to correctly calculate concentration. NGS relies on precise quantities of DNA loaded onto flow cells for the sequencing reaction.

Future Work

The work detailed in this chapter of this thesis represents only an initial study on the nature of CNVs in retinal neurons. The initial plan in designing this study was to sequence about one hundred cells, more than are presented here. Unfortunately, cost becomes a factor with that degree of scale. Using the Miseq, sequencing costs per cell were roughly €400 – however, more than one run was disrupted by inaccurate estimation of DNA concentration, as discussed. Additionally, resequencing of samples (in particular those samples with similar CNV patterns) would be valuable to confirm the fidelity of CBS in analysing low-coverage sequencing data. Sequencing samples in greater depth, such as those with CNVs approaching the threshold of confidence, may lead to greater insight on the true state of their genomic makeup. However, the time and cost required for such additional studies resulted in it being beyond the scope of this project. The cost of sequencing could be

reduced substantially by switching to a HiSeq X or Novaseq – although it would not be possible to run the samples ourselves, it could enable the parallel sequencing of approximately 100 samples at once, reducing the sequencing cost per cell. It would be of particular interest to perform an in-depth study using dozens of cells from single retinas, and looking for cells that display identical copy number patterns as seen in Figure 4.5.

A primary requirement to demonstrate that the single-cell sequencing and analysis techniques used are accurate is the development of suitable controls. While these methods have been validated in a number of previous works, nevertheless it is essential that, as part of this work, the same be done. There are two key methods of controlling these experiments that would help to determine whether the results generated by these techniques could be trusted. The first would be the sequencing at single-cell level of cells with known CNVs, to see a) if the CNVs could be reliably detected and b) whether other, non-existent CNVs appear as artefacts. However, this study (and, more so, previous work) indicates that even in cells with supposedly known genomic makeups, CNVs and differences can be found. As such, a second, highly useful control would be the sequencing at a single-cell and multi-cell level of a clonally amplified population. Ideally, a cell line with known or suspected CNVs would be amplified from a single cell – from this amplified population, a number of individual cells would be sequenced, as well as the bulk population, to see whether the pattern of CNVs (if any) matched. Together, these two controls will be required to confirm that the preliminary results described in this work are more than simple artefacts of error.

New methods of single-cell DNA preparation are constantly being developed, both by companies such as Illumina, who release more advanced sequencing chemistry on a

regular basis, and by researchers themselves. One group recently tested an entirely new method of DNA sequence amplification, linear amplification via transposon insertion (LIANTI; Chen et al., 2017). LIANTI uses a roundabout way of amplifying DNA that relies on transposon insertion. Tn5 transposons modified with T7 promoters are inserted randomly throughout the genome; an RNA polymerase is then introduced which generates pseudo-mRNA from these promoters, which is reverse-transcribed into DNA. The logic behind the approach is to reduce errors by removing the aspects of *in vitro* DNA replication that cause errors. As with MALBAC, LIANTI uses a non-exponential approach to amplification, as only the original DNA copy of sequence can be used to create RNA. This means errors in replication cannot propagate. Secondly, as this method is primer free, amplification errors from non-specific binding are not a factor. Despite the seeming complexity of the method, the authors demonstrate that, compared to other methods such as MDA, MALBAC and degenerate oligonucleotide primer PCR (DOP-PCR), LIANTI has an order of magnitude less variation in the read outputs for single-cell sequence analysis. As a result of this, Chen et al. were able to examine CNVs on the order of 50kb in size. Incorporating new methods such as these into this body of work may enable a far more in-depth study, not only for the more accurate detection of CNVs but also allowing for a larger study to be done. As the amplification method results in less noise per sample, it is possible that lower input quantities into a sequencing reaction may still yield usable data at the CNV window size we are examining.

Concluding remarks

IRDs are the most common form of sight loss among people of working age in the world, affecting approximately one in 3000 people worldwide (Sahel et al., 2015). For the vast majority of cases, there is no cure for these disorders, and treatment, if available at all, is usually in the form of steps taken to slow disease progression. However, in recent years the field of retinal gene therapy has made significant advances, and increasingly therapies are being tested in clinical trials with some successful outcomes (Bennett et al., 2016; Feuer et al., 2016; Guy et al., 2017; Russell et al., 2017; Yang et al., 2016). AAV in particular has seen much use for delivery of genes to the retina, due to its lack of pathogenicity and the wide array of serotypes available for targeting specific types of cells.

As the field continues to mature, it is imperative that the therapies that are developed are optimised to the greatest degree possible. There are many aspects involved in this, from transgene design and choice of viral serotype, to refinement of manufacturing processes, and many others. The refinement of gene delivery technologies so that the most efficient and relevant therapeutic expression can be achieved is a key part of optimisation. This can be accomplished through, variously, the development of highly efficient AAV capsid serotypes, optimisation (including codon optimisation) of constructs and vectors so that maximum expression yield is obtained for the minimum viral load, and whole-construct enhancements such as the use of scAAV. A second important optimisation involves directing AAV vectors to transduce cells and express transgenes in as narrow and specific a target cell range as is required. While AAV is significantly less immunogenic than many other viruses used for gene therapy, the possibility of an immune reaction can never be underestimated, and so it is important that any putative therapy is as targeted towards the cell type of interest as possible. As before, design of new capsids is important in this

regard, as the AAV capsid is responsible for tissue tropism of the prospective therapeutic. Many novel capsids (such as tyrosine mutant AAV, AAV 7m8 and Anc80L65) are typified by their expanded expression profile, rather than being directed at specific cell types (Dalkara et al., 2013; Zhong et al., 2008a; Zinn et al., 2015). As such, the development of novel, tissue-specific promoters is an important enhancement for the advancement of man AAV gene therapies. Several tissue-specific promoters have already been developed in recent years. Restricting transgene expression to specific cell types using these promoters may reduce potential off-target effects associated with the AAV-delivered therapeutics.

An additional progression in AAV-based gene medicines is the development of therapies with as broad a therapeutic spectrum as possible. One issue with the rate of gene therapy advancement is that the cost to bring a novel therapy from pre-clinical models to human trial is high, with years of research and animal testing frequently required, without guarantee of success. Thus, the development of therapies that can treat multiple forms of a disease, or indeed multiple diseases, would be highly valuable. For example, rhodopsin-linked RP alone can be caused by at least 200 mutations in the rhodopsin gene. To circumvent this mutational heterogeneity, a mutation-independent treatment strategy has been proposed; research performed by our own research group has shown that this is a highly viable approach in mouse models of the disease (Millington-Ward et al., 2011). As another example, LHON can be caused by mutations in any of a number of mitochondrial Complex I genes. Hence, the development of a therapy that can provide benefit independent of the dysfunctional gene would be valuable, as distinct from current clinical trials which seek to treat only the most common *ND4* mutant variant (Guy et al., 2017; Yang et al., 2016).

A number of goals were achieved in the course of this PhD research. The overarching aim of this thesis was the optimisation and advancement of AAV-mediated therapies for inherited retinal disease. The focus of the work described in Chapter 2 was to develop and assess a codon-optimised version of the yeast gene *Ndi1*, and test whether *ophNdi1* could function as well as the original *Ndi1* in an *in vivo* setting. The results from Chapter 2 consistently demonstrate that AAV-*ophNdi1* effectively protects the GCL against rotenone-mediated Complex I dysfunction. Both morphological and behavioural assays showed the significant benefit *ophNdi1* offers, and that even at a reduced viral dose, the *ophNdi1* vector equals or exceeds the benefit shown by AAV-*Ndi1*. *ophNdi1* has the potential to provide real treatment to the degeneration of RGCs caused by LHON. More work is required to fully establish the therapeutic potential of *ophNdi1*, as well as its relevance in LHON as distinct from animal models. As a first step, testing AAV-*ophNdi1* in a setting such as the genetic LHON mouse model as developed by Lin et al., (2012) would be valuable in demonstrating the vector's effectiveness. Notably, the phenotype in this model is quite slow and hence, while a valuable resource, the future generation of additional genetic models for LHON would also be useful. One of the key advantages AAV-*ophNdi1* has over therapies currently undergoing clinical trial for treating LHON is that, as *Ndi1* acts independently of endogenous Complex I, it has the potential to treat the disease regardless of the causative gene. Indeed, some forms of Leigh syndrome are caused by more severe mutations in the mitochondrial *ND6* gene (also implicated in LHON), and so *ophNdi1* may be able to ameliorate the symptoms of that disease. It may be that *ophNdi1* provides a more general benefit to cells subject to damage, offering potential disease targets for using this approach by augmenting oxidative phosphorylation and achieving a corresponding reduction in stress. As such, there may be potential to use

ophNdi1 in more general conditions that involve mitochondrial dysfunction, such as Parkinson's disease or multiple sclerosis.

In Chapter 3, it was established that the *Nefh* promoter can successfully drive preferential transgene expression in RGCs when delivered intravitreally via AAV2 using EGFP as a reporter gene. Histological assays evaluating the range of expression within the retina and coexpression with markers of RGCs and amacrine cells (Brn3a, ChAT, GABA) were conclusive in showing that *Nefh* offers a significant advantage over the *CMV* promoter in terms of preferentially targeting RGCs. Secondly, these results validated the pipeline approach taken in this study. Expression data combined with conservation of sequence were used to identify potential promoters for use in AAV gene therapy. The continuing advancement of NGS, along with the development of tools to analyse genome and transcriptome data has resulted in vast quantities of data being available for use. Central repositories such as GEO have made access to this data easier than ever before. In terms of this project, this access allows for relatively easy examination of transcriptomic profiles across a range of cells types, including many distinct cell types in the retina (ncbi.nlm.nih.gov/geo). The pipeline described in Chapter 3 could be used to exploit this data to develop novel promoter for other cells types than discussed here, and so has a broad appeal.

The next logical step of the research mentioned so far is the combination of the *Nefh* promoter with *ophNdi1*, with a goal of creating an effective treatment for LHON that provides preferential expression of the gene therapy in RGCs. It is unfortunate that, owing to some initial technical errors and time constraints, the full-length AAV-*Nefh-ophNdi1* vector was not tested to any significant degree. However, extensive testing of AAV-*Nefh*-

ophNdi1 would most likely involve a combination of the experimental approaches taken in Chapter 2 and 3. Histological analysis for both RGC survival, and to confirm the expression pattern of AAV-*Nefh-ophNdi1* as compared to AAV-*CMV-ophNdi1* would enable assessment of the function of the promoter for driving expression in RGCs and thereby preventing damage in a rotenone-induced model of LHON. Once the antibody for *Ndi1* that is currently being developed is fully characterised (as referred to in the Discussion of Chapter 2), analysis can be done to confirm preferential expression of the *Nefh-ophNdi1* vector. The extent to which AAV-*Nefh-ophNdi1* provides protection relative to the *CMV* vector, as well as how low the viral dose can be while providing that protection, will provide a valuable test of the functionality of the *Nefh* promoter to drive expression of a gene therapy in RGCs.

It is currently proposed that development of the minimal *Nefh* promoter constructs discussed in Chapter 3 will be continued in TCD. Multiple constructs, examining some or all of the conserved regions of the promoter, may provide insight into the minimal requirements for high-level, preferential RGC expression. The *Nefh_short* promoter is an additional step towards that, and though full evaluation of this promoter was beyond the scope of this project, it will form a key aspect of the work going forward. The gene expression profile provided by the *msMin-Nefh* promoter leaves much to be desired. However, further exploration of the different regions of the *Nefh* promoter may shed light on why the level of function of *msMin-Nefh* seemed to be so reduced. In terms of optimising therapies for use in AAV, achieving the minimal size of the *Nefh* promoter while retaining functional and preferential expression will be key in its optimisation, particularly given the 4.7kb transgene capacity of AAV.

The final portion of this PhD thesis questioned the nature of the genomes of retinal neurons, and to what extent those genomes may vary from cell to cell. While the research in this chapter is very divergent from AAV-mediated gene therapies for retinal disease, developing a greater understanding of the biology of retinal neurons is of significant fundamental interest. Moreover, knowledge regarding which regions of the genome may be modified (subject to CNVs) while allowing photoreceptor survival may help us to identify potential “safe harbours” for genome editing of photoreceptors. Genome editing as a technology will undoubtedly be adopted in future generations of gene therapies for IRDs. As discussed, transplantation of retinal neurons may in the future be used as a treatment for patients that have already suffered terminal cell loss. It is not out of the realms of possibility to suggest that this may be used in parallel with gene therapy to deliver more comprehensive means of treating IRDs. The development of iPSCs for creating autologous stem cells usable in a treatment such as this only raises that likelihood. However, the inconsistency of the genomic integrity of iPSC lines developed raises questions as to their reliability and safety for future use on patients. Understanding the extent to which CNVs may develop in neuronal cells – and ideally, when – may help to shed light on what might be the cause of this, and indeed if there is a safe threshold for genomic rearrangement. This research may be later extended to RGCs in the future but it was focused initially on rod photoreceptors due to the homogeneity of the cell type and the availability of the *Rho-EGFP* mouse for convenient isolation.

Megabase-scale CNVs have been reported both in healthy, actively dividing cells such as fibroblasts and non-dividing mature brain neurons (Cai et al., 2014; Knouse et al., 2016; McConnell et al., 2013). Although only a preliminary exploration of twenty cells, the results indicate that rod photoreceptors do contain CNVs and at similar rates to that

reported in the literature. In particular, while the pattern of CNVs in the cells analysed appeared generally random, two cells sorted from the same retina showed nearly identical patterns of CNV events, including those that approached but did not meet the threshold set for significance. This initial study brings up a number of questions to be addressed – are these CNVs random, is there a unified cause for them or a number of different possibilities, have they potential to be deleterious? To attempt to answer these questions, a far larger study must be undertaken, with sufficient cells sequenced that a bigger picture of the pattern of CNV occurrence in post-mitotic photoreceptors may emerge. Sequencing multiple cells from the same sorted retinas will be important in determining whether coincidence of CNVs is rare, and may possibly inform on how early in the life cycle of photoreceptors these mutations occur. A significant possibility in terms of whether severely deleterious CNVs can occur is that they can and do, but those cells in which a deleterious event has occurred do not survive to adulthood and so are not found in screens such as this one. This work has the potential to significantly add to our understanding of what makes up neurons at a basic level. This may even lead to new insights into how mutations of all kinds develop in the retina, and what steps occur at the molecular level to mitigate these mistakes.

Final words

Several gene therapies have now been approved for commercial market, including one for retinal disease – in effect, gene therapy has crossed the line from possibility to reality. In the last decade, hundreds of pre-clinical studies have been published demonstrating AAV-based gene therapies for retinal disease, with dozens of clinical trials completed or underway. There are still problems to be solved, in optimising viral vectors and therapeutic

genes, along with non-technical hurdles such as determining the best windows for intervention, and acquiring the funding and ethical approval for studies involving what can often be very rare diseases. Indeed, as discussed throughout Chapter 4, fundamental knowledge of the nature of the retinal genome is far from complete. More research must be conducted at every level, from basic to clinic, in order to ensure that any therapies that will be produced in the future will be as effective *and* as safe as possible.

That said, technological advances, even over the course of this PhD research, have been significant. The growing sophistication of NGS technology means that diagnosing patients, for example, is cheaper and easier than ever. It has also allowed the interrogation of genomes down to the single cell level with ever-increasing clarity. An expanding array of prospective treatments, along with new and augmented viral serotypes continually being developed, the field of gene therapy is taking considerable steps forward. Moving from the bench to the clinic is difficult, more so with novel technologies, but if recent strings of successes in clinical trials and with the approval of therapies for market are any indication, the future looks bright.

Chapter 5: Materials and Methods

5.1 Cloning of plasmids

2251bp of mouse *Nefh* upstream sequence were amplified from genomic DNA with *MluI* and *XbaI* sites attached (a list of primers can be found in Table 5.1). *Nefh* was cloned into a pre-existing pAAV.MCS *CMV-EGFP* plasmid by digesting with *MluI* and *XbaI* restriction enzymes (New England Biolabs) and ligating fragments together using T4 DNA ligase (Roche). The *msMin-Nefh* promoter was synthesised with *MluI* and *XbaI* sites matching those of *Nefh*, and was ligated in accordingly (Integrated DNA technologies; Figure W).

A large backbone using “stuffer sequence” had previously been prepared in the lab group (Dr. Mary O’Reilly), using sequence from phage lambda. To incorporate *CMV-ophNdiI* into the large backbone, the backbone plasmid was cut with *XbaI* and *SpeI* restriction sites. The *ophNdiI*-containing plasmid was cut with *MluI* and *XhoI*, and both insert and vector digest overhangs were blunted (DNA Polymerase I, Large (Klenow) Fragment; New England Biolabs).

CMV-NdiI was excised from its carrier plasmid with *MluI* and *NotI* restriction enzymes (New England Biolabs) – identical sites were used in the large backbone and the two were ligated as before. *ophNdiI* was then inserted into the large backbone by removing *NdiI* with *XbaI* and *NotI*.

In order to incorporate the *Nefh* promoter sequence into the expanded backbone plasmid containing *ophNdiI*, several extra restriction enzyme sites were incorporated into the

CMV-ophNdi1 plasmid. A patch was designed and synthesised that consisted of the entirety of sequence that existed between two *BamHI* sites found in *CMV-ophNdi1* (Figure W), but with additional restriction enzymes sites (seen in Figure Wf). The patched *CMV-ophNdi1* sequence was then digested with *MluI* and *XbaI* (as was the *Nefh-EGFP* plasmid) and *Nefh* was ligated in in place of *CMV*.

Primers	Purpose	Sequence
<i>Nefh</i>	PCR amplification	F: 5' AGATCATACGCGTGCCACACCTTTACCTTCAC 3' R: 5' AGATCATTCTAGAATGGCCGGAGCAGGTGCG 3'
<i>EGFP</i>	qPCR	F: 5' TTCAAGGAGGACGGCAACATCC 3' R: 5' CACCTTGATGCCGTTCTTCTGC 3'
<i>Ndi1</i>	qPCR	F: 5' CACCAGTTGGGACAGTAGAC 3' R: 5' CCTCATAGAAGGTAACGTTC 3'
<i>Thy1</i>	qPCR	F: 5' TGAACCAAAACCTTCGCCTG 3' R: 5' AGCTCACAAAAGTAGTCGCC 3'

Table 5.1: List of primers used in the PhD thesis. All primers used were synthesised by Sigma-Aldrich.

5.2 Cell line maintenance

HEK293 cells were cultured in 150mm circular dishes in DMEM+ containing 10% foetal calf serum, 1% 2mM sodium pyruvate and 1% 2mM L-Glutamine (Thermo Fisher Scientific). Cells were incubated at 37°C, at a CO₂ concentration of 5%. Passage of cells was performed by media aspiration and gentle washing with PBS, followed by addition of 2ml trypsin to detach cells. 10ml of DMEM (as prepared above) was added and cells were split into new plates at appropriation dilutions.

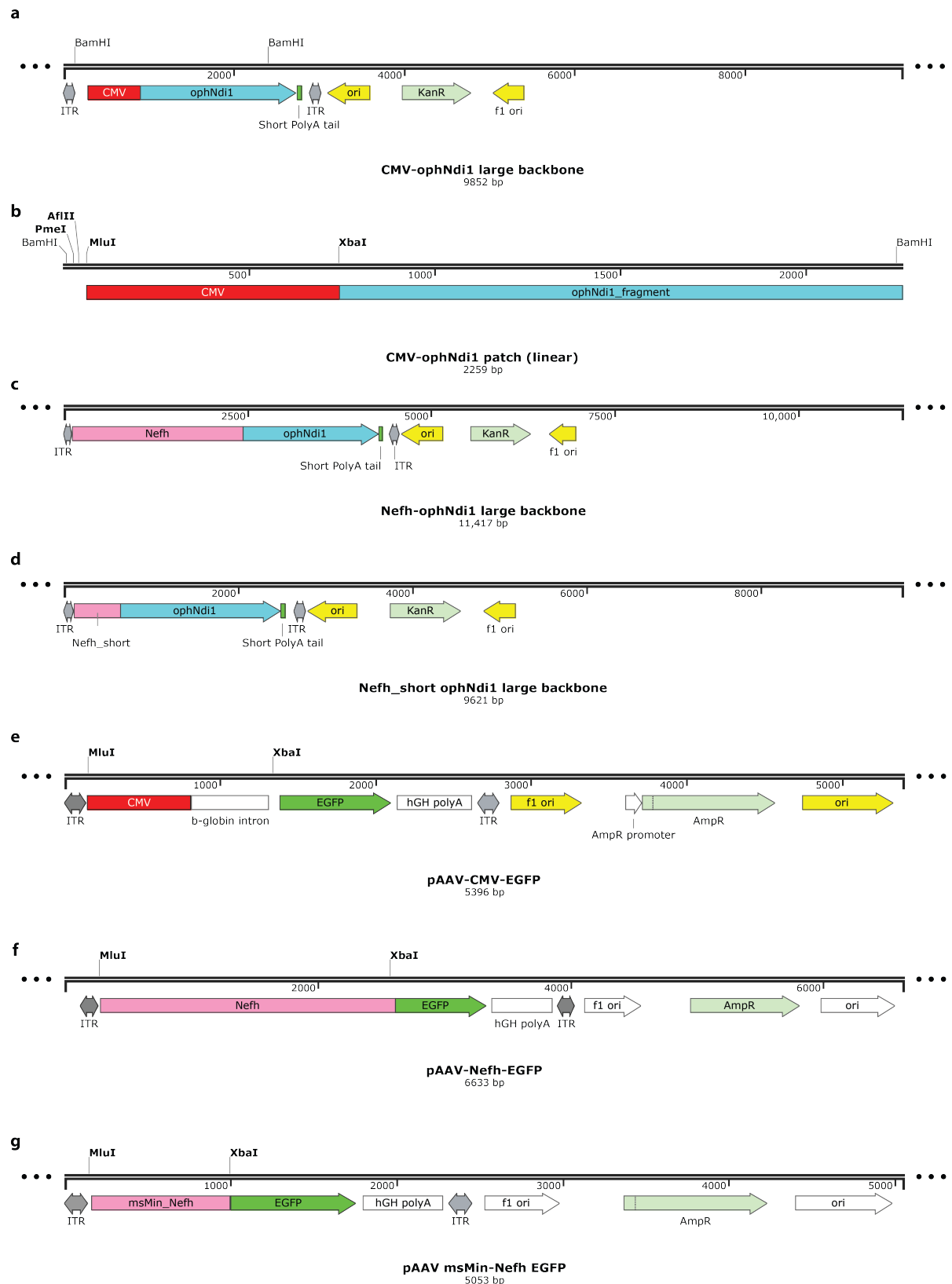


Figure W: Linear maps of plasmids used throughout this thesis.

5.3 Plasmid transfection

For transfections other than viral preparations, cells were transfected using Lipofectamine 2000 (Invitrogen) and OptiMEM (Thermo Fisher Scientific), as per manufacturer's instructions. For viral preparations, transfection reagents were homemade as follows: 150mM NaCl (pH 5.5, adjusted with HCl – transfection buffer) and 150mM NaCl (pH 5.5, adjusted with HCl) containing 0.01g PEI per 100ml.

5.4 AAV production

Recombinant AAV2/2 viral vectors *CMV-EGFP*, *CMV-Ndi1*, *CMV-ophNdi1*, *Nefh-EGFP*, *msMin-Nefh-EGFP*, and *Nefh500-ophNdi1* were generated as previously described (Chadderton et al. 2013). 100 150mm plates containing HEK293 cells (grown in DMEM containing foetal calf serum, sodium pyruvate and L-Glutamine as in section 5.2) were transfected with three plasmids – the vector construct of interest, a plasmid expressing AAV2/2 viral capsid proteins, and a helper plasmid to enable virus production. 1.25mg of viral capsid, 2.5mg of helper plasmid, and 1.25mg of vector construct (2.05mg for the large backbone constructs) were used in the transfection. 72 hours post-transfection, cells were harvested and lysed by three freeze thaws (freeze at -80°C, thawed at 37°C). The cell pellet was combined with the retained media and incubated in a solution containing PEG8000 and sodium chloride overnight. The virus-containing solution was then centrifuged, and the pellet resuspended in PBS (0.005% sodium deoxycholate; Thermo Fisher Scientific). A caesium chloride (CsCl) gradient was prepared – 27.415g of CsCl was added to 50ml water (CsCl 1.4), and 40.825g added to another 50ml of water (CsCl 1.6). CsCl 1.6 was layered slowly upon CsCl 1.4 in a polyallomer tube. 16.344g of CsCl was added to the viral solution, and this was layered slowly upon the CsCl to complete the

gradient. Tubes containing virus were spun at 28,000rpm overnight (Beckman Coulter Optima L-100 XP ultracentrifuge – SW28 bucket rotor).

Following this, the gradient solution was drained into a series of 1.5ml tubes – samples that read between 3.368 and 3.373 were taken, pooled, and spun again overnight at 59,000rpm (70.1 Ti rotor). This process was repeated once. Samples were then dialysed against PBS over approximately 12 hours in a slide-a-lyzer (Thermo Scientific) – they were then processed for titring.

5.5 Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

In all instances a general protocol for RT-qPCR was followed; exceptions and additional steps are listed below. A list of primers used can be found in Table 5.1. Samples were analysed on a StepOnePlus (Applied BioSystems) using either a Kapa SYBR FASTQ qPCR kit (Kapa; SCS samples) or a QuantiTect SYBR green reverse transcription qPCR kit (Qiagen; all other runs). qPCRs were run twice, each time in triplicate, to ensure consistent results; SCS qPCR standards were run in duplicate. For qPCR experiments beginning with RNA, a reverse transcription step was included; qPCRs run to calculate viral titres and single-cell sequencing sample concentrations were run without this step.

5.5.1 Viral titring

Viruses were titred as above, with some pre-processing. Before being run, samples were treated with DNase I (Sigma-Aldrich), which was then inactivated, and proteinase K (to lyse viral capsids). A standard curve was generated using plasmid used to construct the

virus, and served as a copy number known control. A list of titres for viruses used in this PhD thesis can be found below in Table 5.2.

Viral Vector (AAV2/2)	Titre (vg)
<i>CMV-NdiI #1</i>	1.5×10^{12}
<i>CMV-NdiI #2</i>	1.5×10^{12}
<i>CMV-ophNdiI</i>	3.48×10^{11}
<i>CMV-EGFP (Ch. 2)</i>	6×10^{11}
<i>CMV-EGFP (Ch. 3)</i>	1×10^{12}
<i>Nefh_short-ophNdiI</i>	2×10^{12}
<i>Nefh-EGFP</i>	3×10^{12}
<i>msMin-Nefh-EGFP</i>	1.07×10^{11}

Table 5.2: List of titres calculated for AAV vectors generated in the course of this thesis.

5.5.2 Single-cell Sequencing

Samples were quantified using qPCR. The protocol was carried out according to manufacturer's instructions; standards and primers used were those included in the kit (proprietary sequence).

5.5.3 Flow cytometry qPCR

For qPCR performed on samples isolated from flow cytometry, extracted RNA was pooled from n=12 or n=9 retinal samples, and samples were then run in triplicate. CT values were standardised to known cell number (gathered from flow cytometry) rather than standard housekeeping genes. As the cell populations being compared were different, there was a possibility that standard housekeeping genes may be expressed at different levels in these populations, thus making a standardisation unreliable.

5.6 Animals, injections, histology

Wild-type 129 S2/SvHsd mice, bred in-house, were maintained under specific pathogen-free conditions. *Rho*-EGFP mice were generously donated to the lab by Prof. JH Wilson. Intravitreal injections were carried out by Prof. Paul F. Kenna. Mice were anaesthetised using ketamine and medetomidine (750µg, 10µg per 10g body weight) injected intraperitoneally, and pupils were dilated using 1% cyclopentolate and 2.5% phenylephrine. A puncture was made in the sclera under local analgesia (amethocaine), and rotenone/AAV was delivered using a 34-gauge microneedle. 0.6µl of 2.5mM rotenone in dimethyl sulfoxide (DMSO) or 3µl AAV2/2 at various doses (described below) was administered to the vitreous over 120 seconds. Following the procedure an anaesthetic reversal agent (atipamezole hydrochloride, 100µg per 10g body weight) was administered intraperitoneally. Animals were killed by CO₂ asphyxiation; death was confirmed by cervical dislocation. Eyes and optic nerves were enucleated from mice. Eyes were fixed in 4% PFA for two hours at room temperature or overnight at 4°C. Following this, the eye was dissected along the limbus and the lens removed. Eyes were cryoprotected in a sucrose gradient and frozen in OCT. To freeze, eye cups in moulds filled with OCT were placed in an isopropanol bath; this bath was placed in a dish containing liquid nitrogen, until each

was fully frozen. Following this, eyes were sectioned through at 12-14µm through the sagittal/transverse planes (orientation in these planes was not noted).

All sections were stained in a similar manner. Slides were blocked in 5% donkey serum/0.3% Triton X-100 (Sigma Aldrich) for one hour and stained with primary antibody overnight at 4°C or room temperature for two hours. Following this, slides were washed in PBS and stained with secondary antibody for two hours at room temperature. Slides were washed again, stained with 1µg/ml 4',6-diamidino-2-phenylindole (DAPI) to visualise nuclei, washed again, and coverslips and mounting medium applied. A full list of antibodies used can be found below in Table 5.3.

For IHC cell number counts, the Photoshop count tool was used. For the counts performed in Chapter 2, four sections from each retina approximately 150µm apart were analysed for NeuN or Brn3a-stained numbers (n=4-8 retinas per group). For the counts performed in Chapter 3, two transduced sections approximately 300µm apart were analysed for each marker (n=4-5 retinas).

Antibody name	Company, order code	Use	Stains	Dilution used
<i>Brn3a</i>	SCBT; sc-31984	<i>IHC</i>	RGCs	<i>1/200</i>
<i>EGFP</i>	Abcam; ab13970	<i>IHC, flow cytometry</i>	EGFP	<i>1/1000</i>

<i>NeuN</i>	Abcam; ab104224	<i>IHC</i>	Neurons (RGCs)	1/100
<i>ChAT</i>	Millipore; AB144P	<i>IHC</i>	Amacrine cells	1/500
<i>GABA</i>	Sigma; A2052	<i>IHC</i>	Amacrine cells	1/2000
<i>Melanopsin</i>	ATS; UF006	<i>IHC</i>	ipRGCs	1/5000
<i>Thy1</i>	eBioscience; 53-2.1	<i>Flow cytometry</i>	RGCs	1/100
<i>HA</i>	Abcam; ab9134	<i>ICC</i>	HA tag	1/200

Table 5.3: List of antibodies used in the course of this research.

5.7 Optic nerve sectioning and measurement

Following enucleation, approximately 5mm of optic nerves proximal to the retina was removed and fixed in 4% PFA for two hours at room temperature. As the technique for successfully preparing optic nerves for sectioning differs from that of retinas, a schematic of sample preparation for sectioning is outlined in Figure 5.2 below. Using a forceps, nerves were straightened, and placed into wells of a 96-well plate filled with OCT. These were snap frozen as above. Following freezing, a bubble of air develops at the bottom of the well; using a scissors, the air-filled region of the well was removed. A plastic blunt instrument was used to gently push the OCT block out through the top of the well. This allowed for easy and consistent mounting and sectioning of the optic nerves. Sections were stained with DAPI for easier visualisation. Optic nerve diameters were measured using the Ruler tool in Photoshop, blind. The short side (minor axis) was determined by eye and measured in each instance. Each measurement was taken twice on separate occasions, and averaged – four section from each optic nerve

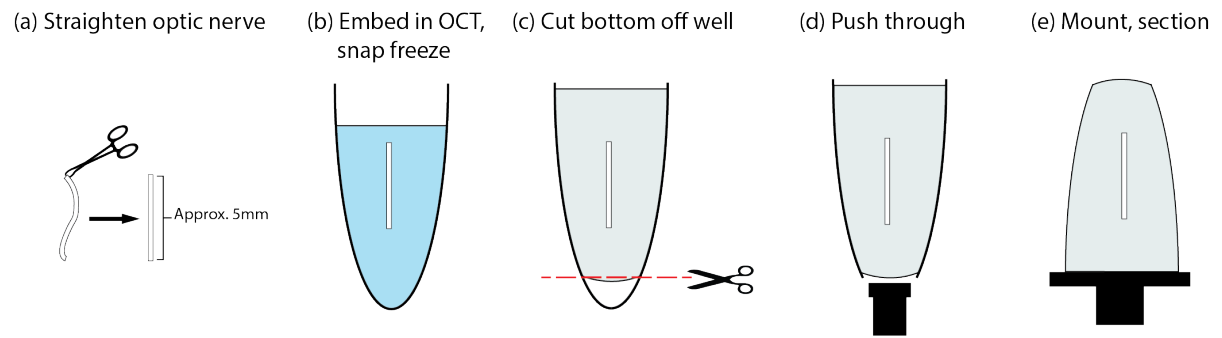


Figure 5.2: Schematic of optic nerve sample preparation for sectioning.

5.8 *In silico* RGC promoter analysis

Human genes whose relative expression was enriched in the RGC layer by over 10-fold compared to relative expression in OR were selected (Kim et al., 2006). Genes were assessed based on GCL expression level (EL_{GCL}) compared to the OR expression (EL_{OR}), termed the enrichment factor ($EF = EL_{GCL} / EL_{OR}$; Kim et al., 2006) and the 15 genes with the highest ELs were selected for further investigation. A gene score ($GS = EL_{GCL} \times EF$) was used to rank genes for suitability as potential promoters. Further analysis was performed on mouse genomic data, as a mouse promoter was the desired output. Data from the UCSC genome browser (mm10 mouse mammalian conservation track; UCSC; Kent et al., 2002) were used to establish conservation upstream of the transcriptional start site of candidate genes; results from analysis of 2.5kb upstream of the start site are presented (Figure 3.2). An *in silico* pipeline (Python) was developed to isolate basewise conservation data from UCSC (conservation data ranged from 0 to 1 for a given base, where 0 represents no significant conservation between mammals and 1 indicates complete conservation). The forty mammalian species and their sequence assembly names that make up this conservation data are listed in Appendix 1. This was plotted in a graph in order to

visualise conserved regions. *NEFH* was chosen as having the highest GS of the genes analysed. Using the parameters defined above, the mouse *Nefh* upstream region was selected for evaluation *in vivo*, given the expression profile of the gene and conservation of its 5' upstream sequence.

5.9 Retinal dissociation for flow cytometry

In preparation for cell sorting, retinas were excised from mice sacrificed as above. Retinas were dissociated in a solution of trypsin (Sigma-Aldrich; 10mg/ml PBS) with DNase (Sigma-Aldrich; 10mg/ml PBS) and washed with PBS. A trypsin inhibitor was added after ten minutes of dissociation to prevent over-digestion (Roche; final concentration 20mg/ml). For the case of EGFP-positive photoreceptors, cells were sorted based on EGFP fluorescence with non-expressing controls used to gate. Where staining was required for cell sorting (in the case of RGC sorts), dissociated cells were blocked with donkey serum and stained with primary antibodies. Cells were washed with PBS and stained with secondary in the dark. Both staining steps took place at room temperature for 45 minutes. Washing and centrifugation were performed between steps, with due care taken to preserve cell integrity as much as possible. Where DRAQ7 was used as a cell death marker, substance was added immediately prior to cell sorting.

5.10 Flow cytometry cell sorting

To isolate RGCs, cells were labeled with anti-Thy1-PE-Cy5, (CD90.2, Rat Thy1.2, 53-2.1 1:100; eBioscience Inc., San Diego, CA). DRAQ5TM (BioStatus, Leicestershire, UK) was used to sort nucleus-positive cells after which cell populations were sorted on the basis of

forward and sideways scatter, and subsequently two stages of singlet selection. From these, retinal cells expressing both EGFP and Thy1 were identified (BD FACSAria IIIu high speed cell sorter, BD Bioscience, San Jose, CA). EGFP was excited by a 488nm laser and the emission was collected using a 530/30 band pass filter. Thy-1 PEcy5 had been measured exciting the probe with a 561nm laser and collecting the signal with a 690/40 nm band pass. QC of the cell sorter had been done with BD CS&T beads and the drop delay had been adjusted using the BD Accudrop beads (RUO), following manufacture specifications. EGFP-positive cells expressing Thy1 were represented as a percentage of the total EGFP positive cells. Data has been reanalyzed with the FCSExpress 6 Flow software (DeNovo Software). To isolate EGFP-positive photoreceptors, cells were again sorted by forward and sideways scatter, along with singlet selection. EGFP-positive cells were sorted as above. Propidium iodide was used to exclude non-living cells. Statistical analysis (Student's *t*-test) was performed using Microsoft Excel and $p < 0.05$ was considered statistically significant.

5.11 Single-cell sequencing preparation

In order to isolate single cells, transgenic *Rho-EGFP* mice (expressing EGFP only in rod photoreceptors) were used. *Rho-EGFP*^{+/-} mice on a 129 S2/SvHsd background were sacrificed, and their retinas enucleated as before. Cells were sorted by flow cytometry; in this case, however, single cells were sorted into individual tubes. Single cell DNA was amplified using a Whole Genome Amplification kit (WGA4; Sigma-Aldrich). In order to prepare samples for sequencing, a Nextera sample prep kit was used according to manufacturer's instructions (Illumina). Briefly, DNA was "tagmented" (broken into 100-1000bp fragments and simultaneously ligated with adaptors). To those adaptors, 7nt

barcodes unique to each sample were attached by PCR. From this, all DNA was denatured and sequenced on an Illumina Miseq.

5.12 Optokinetic response (OKR)

The OptoMotry system (VOS, OptoMotry, Cerebral Mechanics, Alberta) was used to measure the OKR spatial frequency thresholds for treated and untreated eyes. Briefly, OptoMotry is designed to measure the mouse tracking response to black/white gratings. The mouse is positioned on a platform inside a chamber of four 17-inch screens, simulating a spinning drum. A camera placed above the mouse allows monitoring by the researcher of the mouse's eye and head movements. The drum is focused on the mouse's head and yes/no indicators are used to judge whether a tracking response is seen. A staircase system is used to gradually increase the grating frequency until the spatial frequency threshold, the point at which the mouse no longer tracks, is reached. Each reversal (a 'no' indication) halves the step size of the frequency. The test terminates when the step size differences reduce to below the hardware's capacity (0.003 cycles per degree (c/d)). Clockwise and anticlockwise drum movements test the mouse's right and left eyes, respectively, with the direction changes during the test randomised throughout.

5.13 Whole genome sequence analysis

Initially, sequence reads were aligned against an index reference genome (GRCm38/mm10) using Burrows-Wheeler Alignment (BWA; Li & Durbin 2009), and duplicates were removed using the Picard Tools suite. Genomic DNA is not uniformly mappable, resulting in mismatches and areas of overly high read mapping. The Genome

Multitool (GEM) mappability algorithm (Derrien et al., 2012) was used to create read windows containing equal quantities of uniquely mappable sequence, that is, sequence that maps to a single locus of the genome. These windows were set at 500kb. Per McConnell et al., a consecutive CNV of six windows or more was required to be counted as real. Following this, the numbers of reads per region were counted using a custom Python program called SeqSeg (see Appendix 3). SeqSeg calls another custom program WindowMaker, which parses the mappability output of GEM. WindowMaker defines windows of 500kb of unique sequence based on GEM's basewise classification of mappability. SeqSeg takes these assigned windows, into which non-duplicate sequence reads (mapped with BWA and Picard Tools as above) are binned. These bins are sorted in order by chromosome, and the read count for each bin is then output into a format readable by the R program DNACopy (see below).

In order to curtail noise from the low coverage and to find cohesively deleted/duplicated regions, a segmentation algorithm called Circular Binary Segmentation (CBS) (Olshen et al., 2004) was employed (algorithm employed using DNACopy software in R). As gains and losses for a given region are discrete, the CBS algorithm uses the read data provided to split a given chromosome into regions of equal copy number in order to account for the noise that results from random low-plexity read sampling. This allowed for the construction of a map of each chromosome with estimated copy numbers of different regions displayed. The program output into the CBS algorithm was modified to mimic a format suitable for analysis.

5.14 Statistical Analysis

Where not otherwise noted, all statistical analysis was performed using the R programming language and software environment, as well as the RStudio package. Statistical tests used were two-way t-tests or, where stated, one-way ANOVA. Multiple comparisons used t-tests were corrected with the Bonferroni method.

Chapter 6: Appendix

Appendix 1: Table of species used in promoter analysis

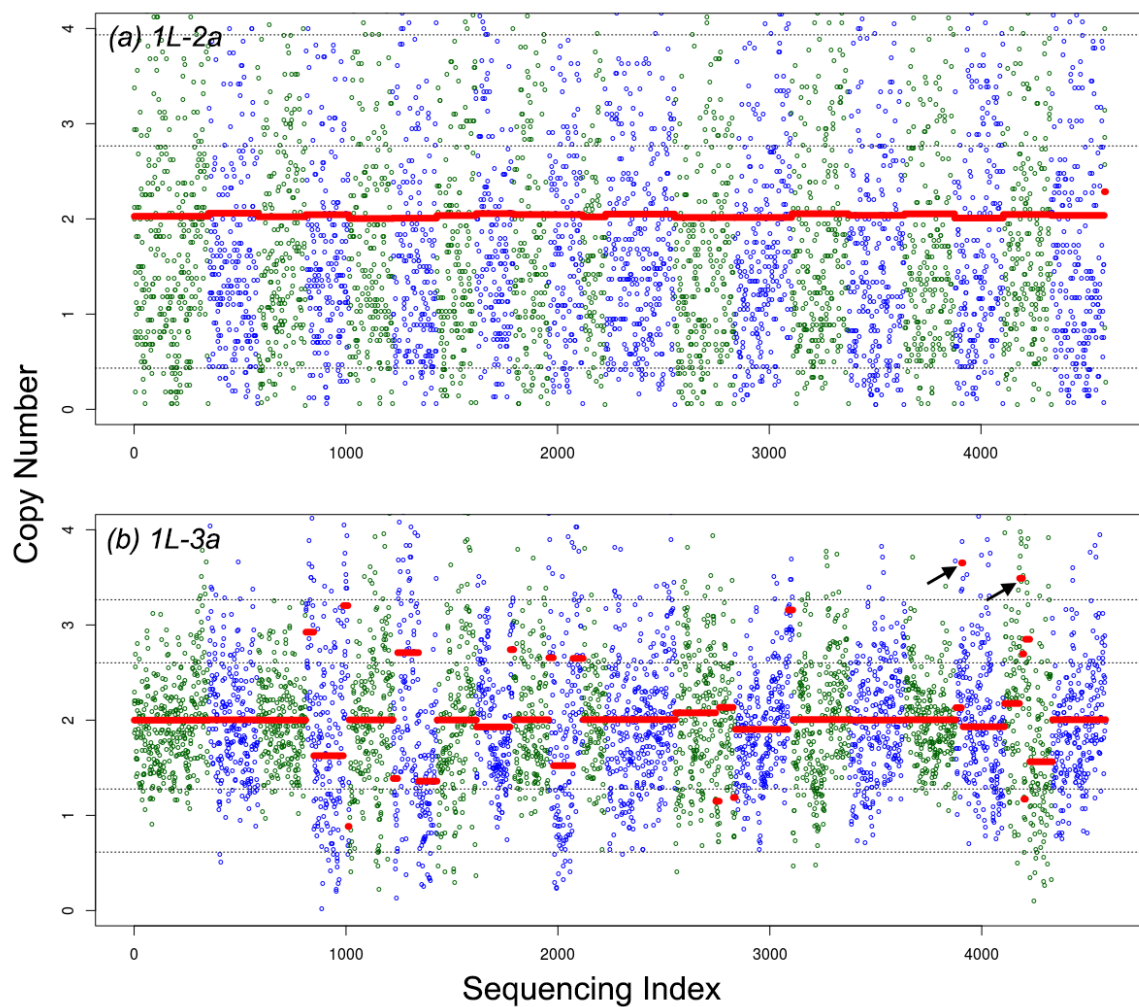
Animal	Species	Assembly Date	Assembly Name/details
Mouse	<i>Mus musculus</i>	Dec. 2011	GRCm38/mm10 reference
Guinea pig	<i>Cavia porcellus</i>	Feb. 2008	Broad/cavPor3 Syntenic net
Kangaroo rat	<i>Dipodomys ordii</i>	Jul. 2008	Broad/dipOrd1 Reciprocal best
Naked mole-rat	<i>Heterocephalus glaber</i>	Jan. 2012	Broad HetGla_female_1.0/het Gla2 Syntenic net
Pika	<i>Ochotona princeps</i>	Jul. 2008	Broad/ochPri2 Reciprocal best
Rabbit	<i>Oryctolagus cuniculus</i>	Apr. 2009	Broad/oryCun2 Syntenic net
Rat	<i>Rattus norvegicus</i>	Mar. 2012	RGSC 5.0/rn5 Syntenic net
Squirrel	<i>Spermophilus tridecemlineatus</i>	Nov. 2011	Broad/speTri2 Syntenic net
Tree shrew	<i>Tupaia belangeri</i>	Dec. 2006	Broad/tupBel1 Reciprocal best
Marmoset	<i>Callithrix jacchus</i>	Mar. 2009	WUGSC 3.2/calJac3 Syntenic net
Gorilla	<i>Gorilla gorilla</i>	May. 2011	gorGor3 Syntenic net
Human	<i>Homo sapiens</i>	Feb. 2009	GRCh37/hg19 Syntenic net
Mouse lemur	<i>Microcebus murinus</i>	Jun. 2003	Broad/micMur1 Reciprocal best
Gibbon	<i>Nomascus leucogenys</i>	Jun. 2011	GGSC Nleu1.1/nomLeu2 Syntenic net
Bushbaby	<i>Otolemur garnettii</i>	Mar. 2011	Broad/otoGar3 Syntenic net
Chimp	<i>Pan troglodytes</i>	Feb. 2011	Pan_troglodytes-2.1.4/panTro4 Syntenic net
Baboon	<i>Papio hamadryas</i>	Nov. 2008	Baylor 1.0/papHam1 Reciprocal best
Orangutan	<i>Pongo pygmaeus abelii</i>	Jul. 2007	WUGSC 2.0.2/ponAbe2 Syntenic net
Chinese rhesus	<i>Macaca mulatta</i>	Oct. 2010	BGI CR_1.0/rheMac3 Syntenic net
Squirrel monkey	<i>Saimiri boliviensis</i>	Oct. 2011	saiBol1 Syntenic net

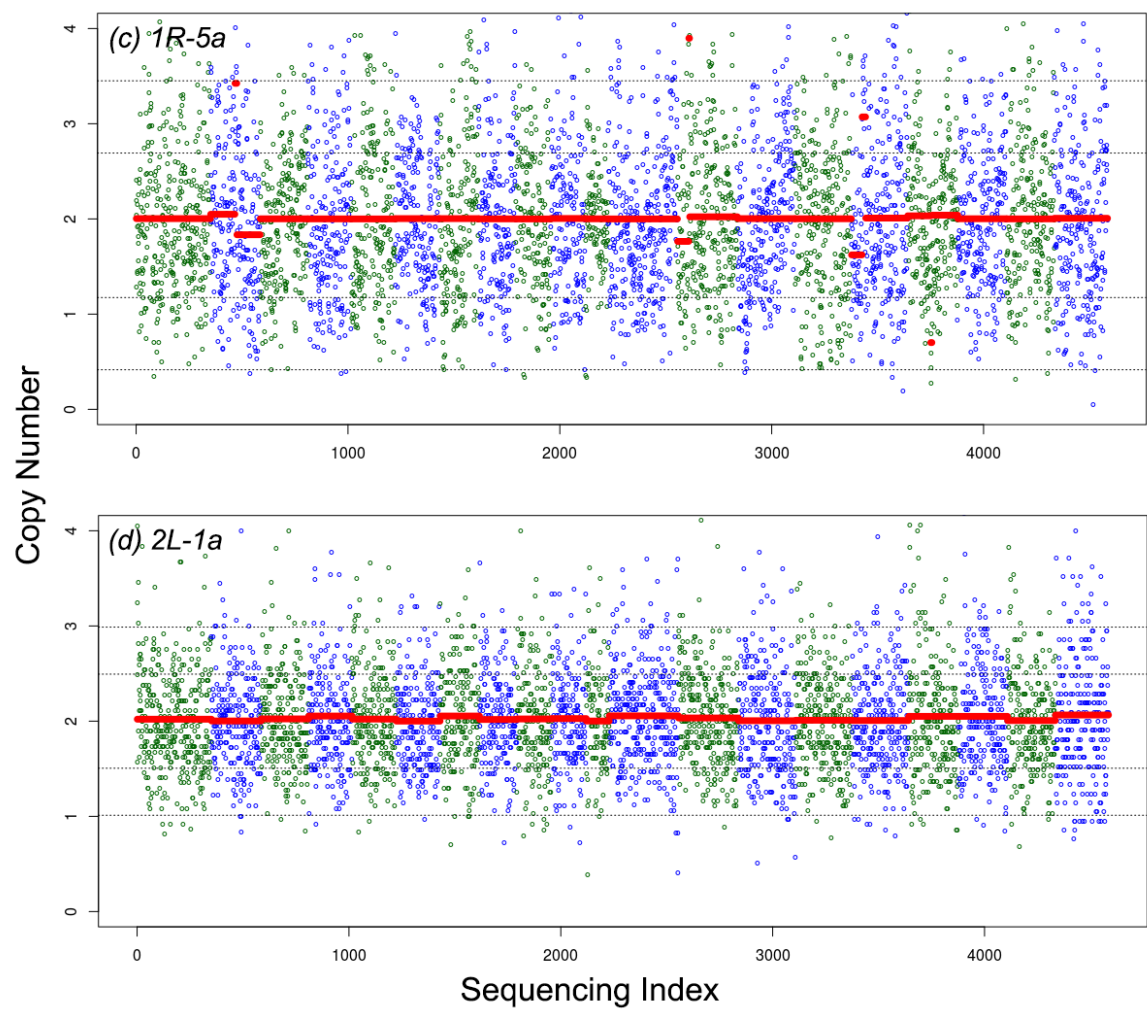
Tarsier	<i>Tarsius syrichta</i>	Aug. 2008	Broad/tarSyr1 Reciprocal best
Panda	<i>Ailuropoda melanoleuca</i>	Dec. 2009	BGI-Shenzhen 1.0/ailMel1 Syntenic Net
Cow	<i>Bos taurus</i>	Oct. 2011	Baylor Btau_4.6.1/bosTau7 Syntenic Net
Dog	<i>Canis lupus familiaris</i>	Sep. 2011	Broad/canFam3 Syntenic net
Sloth	<i>Choloepus hoffmanni</i>	Jul. 2008	Broad//choHof1 Reciprocal best
Armadillo	<i>Dasypus novemcinctus</i>	Dec. 2011	Armadillo/dasNov3 Reciprocal best
Tenrec	<i>Echinops telfairi</i>	Jul. 2005	Broad/echTel1 Reciprocal best
Horse	<i>Equus caballus</i>	Sep. 2007	Broad/equCab2 Syntenic net
Hedgehog	<i>Erinaceus europaeus</i>	Jun. 2006	Broad/eriEur1 Reciprocal best
Cat	<i>Felis catus</i>	Sep. 2011	ISGSC Felis_catus 6.2/felCat5 Reciprocal best
Elephant	<i>Loxodonta africana</i>	Jul. 2009	Broad/loxAfr3 Syntenic net
Microbat	<i>Myotis lucifugus</i>	Jul. 2010	Broad/myoLuc2 Reciprocal best
Sheep	<i>Ovis aries</i>	Feb. 2010	ISGC/oviAri1 Reciprocal best
Rock hyrax	<i>Procavia capensis</i>	Jul. 2008	Broad/proCap1 Reciprocal best
Megabat	<i>Pteropus vampyrus</i>	Jul. 2008	Broad/pteVam1 Reciprocal best
Shrew	<i>Sorex araneus</i>	Jun. 2006	Broad/sorAra1 Reciprocal best
Pig	<i>Sus scrofa</i>	Aug. 2011	SGSC Sscrofa10.2/susScr3 Syntenic net
Manatee	<i>Trichechus manatus latirostris</i>	Oct. 2011	Broad v1.0/triMan1 Syntenic net
Dolphin	<i>Tursiops truncatus</i>	Oct. 2011	Baylor Ttru_1.4/turTru2 Reciprocal best
Alpaca	<i>Vicugna pacos</i>	Jul. 2008	Broad/vicPac1 Reciprocal best

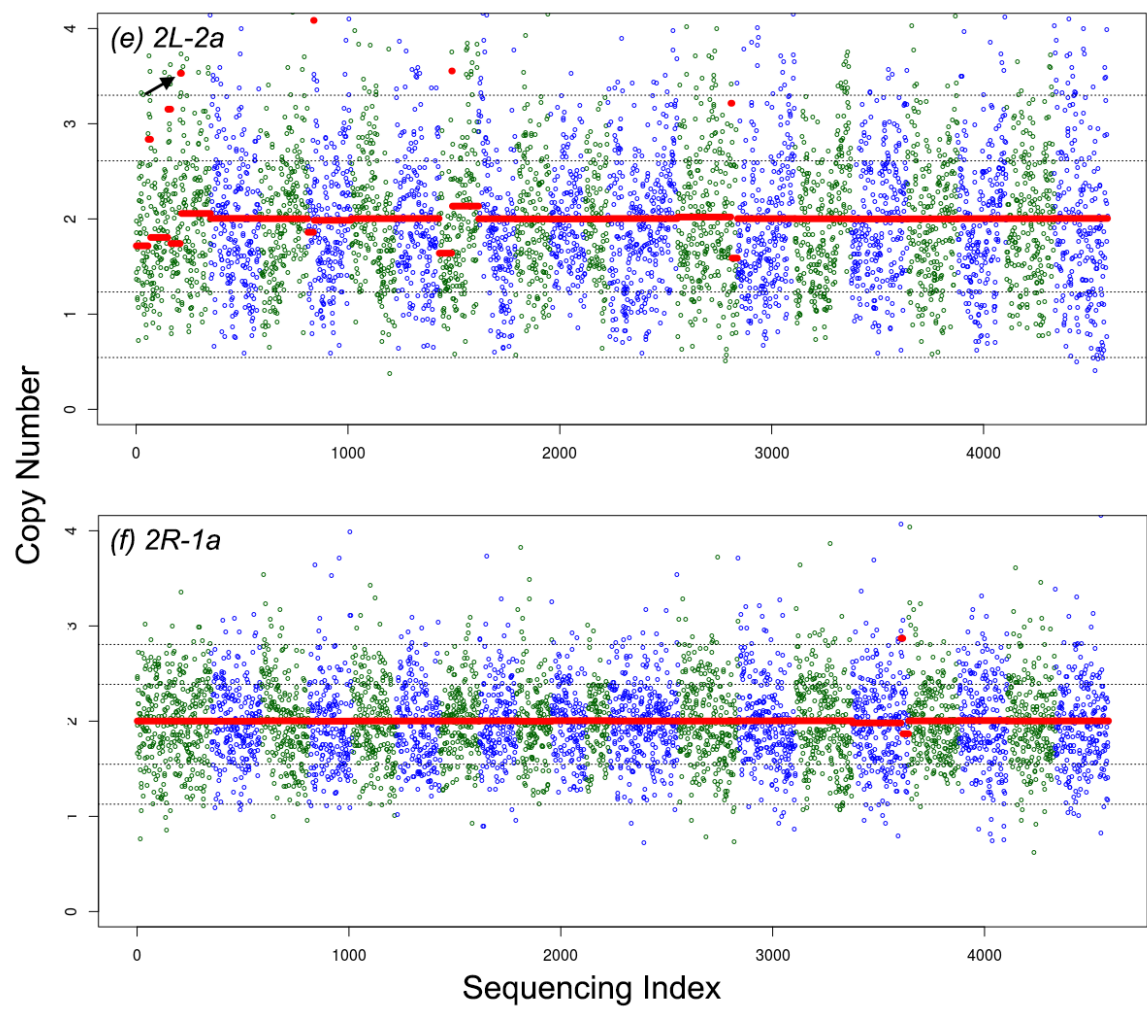
List of animal sequences used for conservation alignment. A placental mammal species alignment using the UCSC genome database (genome.ucsc.edu; phastConsElements60wayEuarchontoGlires) was used for the conservation alignment seen in Figure 2. Species are grouped as Glires, Primates, and other placental mammals, with species names, then most-recent sequence assembly dates, and assembly details listed.

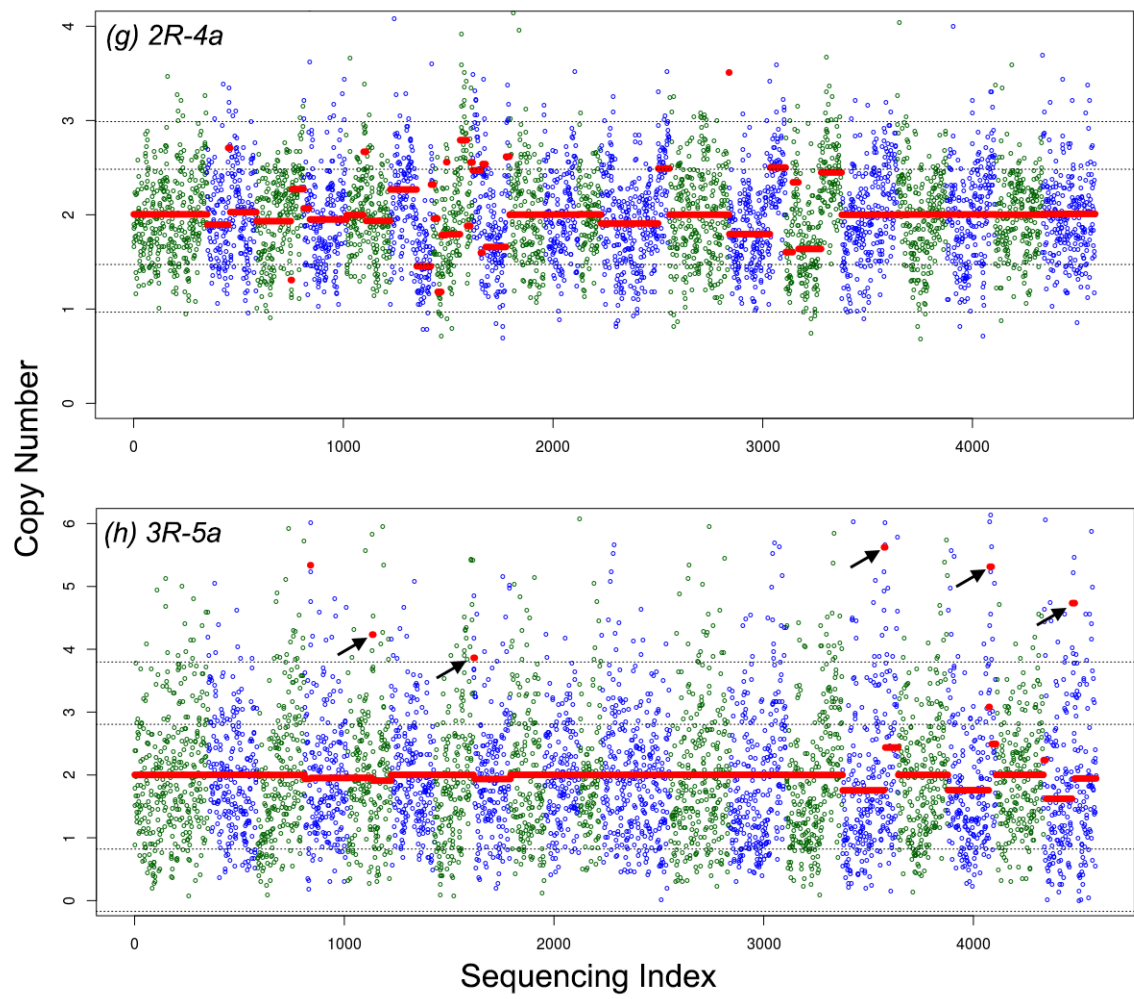
Appendix 2: Single Cell Sequencing Plots

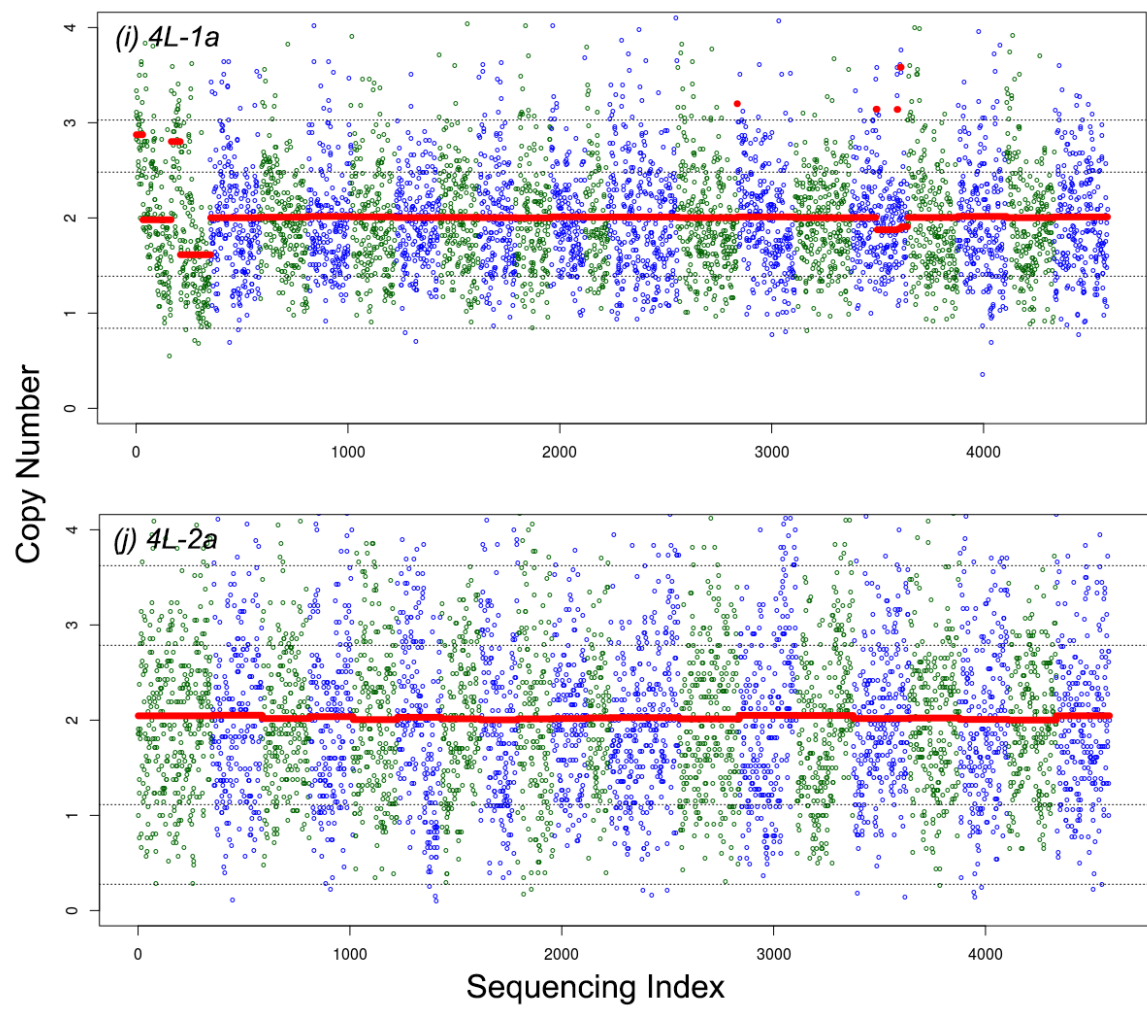
Plot of copy number across the genome for each sample sequenced. Alternating chromosomes are highlighted in blue and green, with the red line indicating consensus copy number in that section of the genome (divided into 500kb windows of uniquely mappable sequence). The sequencing index represents the list of windows. Dotted lines indicate one and two MADs from the consensus copy number. CNVs are defined as copy number breakpoints (indicated by breakages in the red line) of at least six 500kb windows in a row. Only those CNVs greater than two MADs from the consensus copy number are counted as real, and are indicated with black arrows. Chromosomes are ordered 1, 10-19, 2, X – Y chromosomes were excluded from analysis.

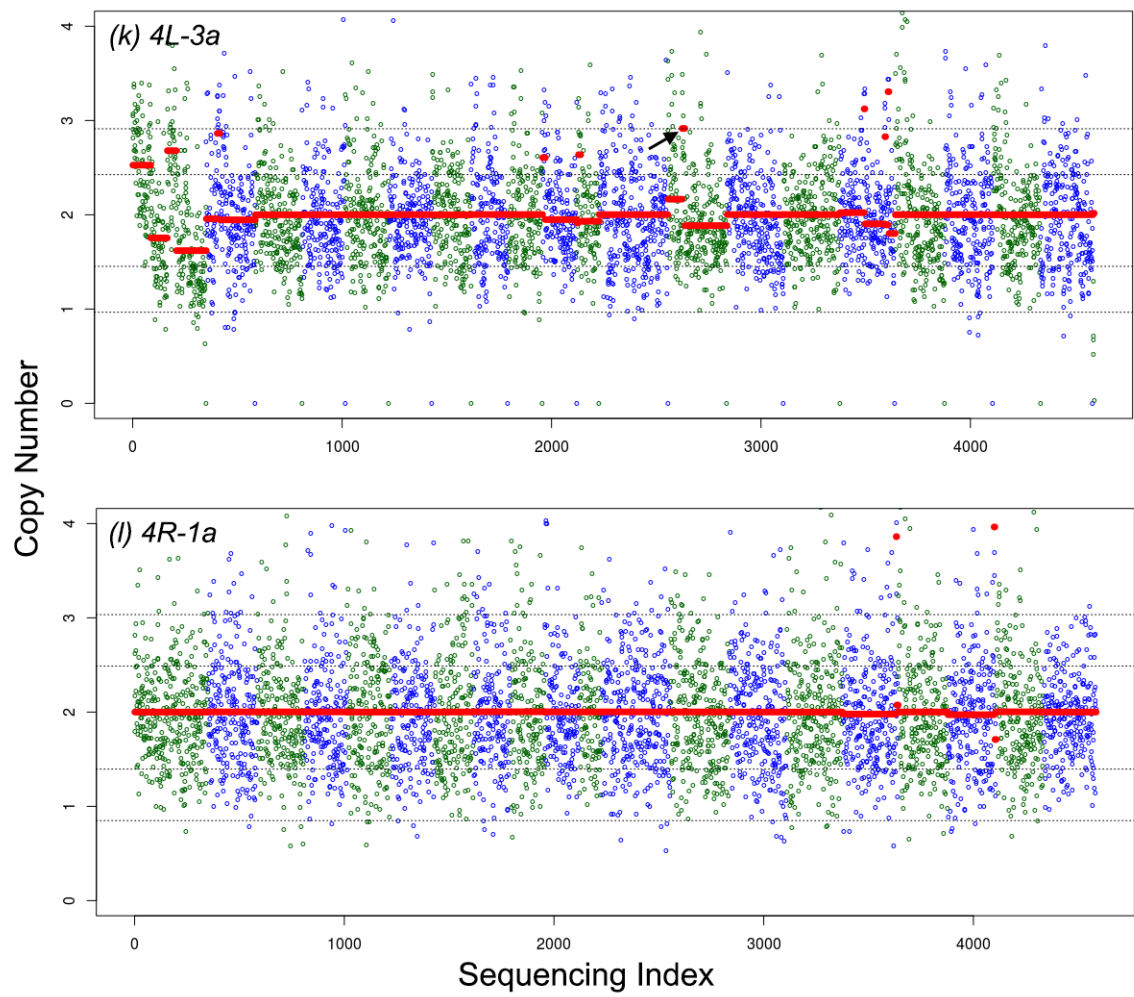


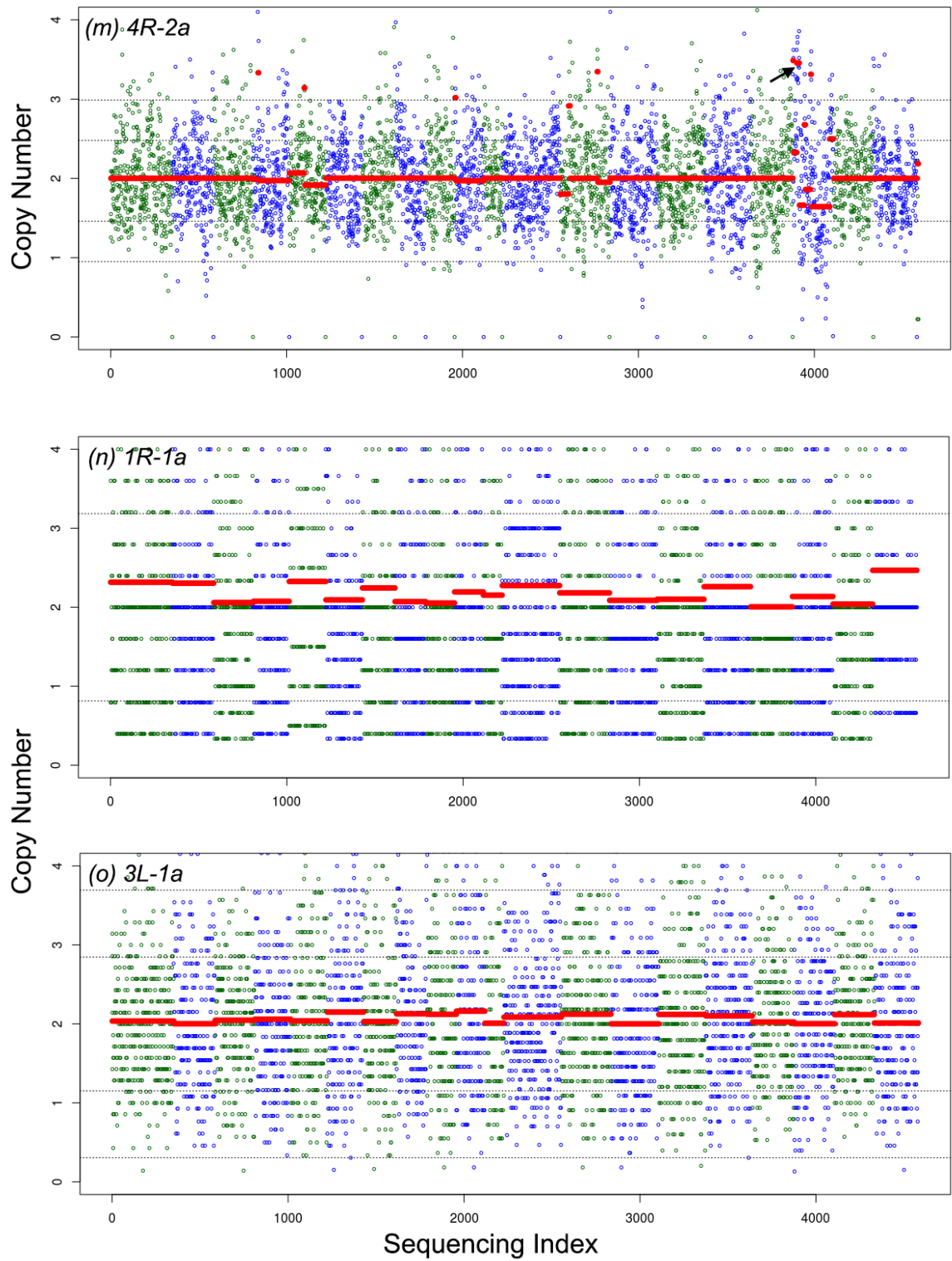












Appendix 3: Source code for single-cell sequence analysis

The final step before CBS is applied to single-cell data is to sort the sequence reads into appropriate bins. This was done using the programs shown below. The main program, SeqSeq.py, calls WindowMaker.py to import the list of windows of uniquely mappable sequence that serve as bins. The SAM file to be analysed is called from the command line when SeqSeq.py is run.

SeqSeq.py

```
1. #!/usr/bin/python
2.
3. #Opens the file, feeds in all the chromosome names.
4. #Takes input from K2_theWindowMaker for 500kb windows.
5. #Counts the number of reads in a given window, and assigns that to a dictionary.
6.
7. #Gets the average window value.
8. #Gets the copy number values in microarray format.
9. import pdb
10. import sys
11. import math
12. import cPickle as pickle
13. f = open(sys.argv[1], 'r') #open file to be read
14. ChromDic = {} #ChromDic keys will be chromosomes, values will be dictionaries of
    read lists per chromosome
15. for line in f: #pulls out chromosome names from fastq file based on the line
    headers
16.     if line[0:3] == "@SQ":
17.         if not 'chrM' in line: #Don't bother with mDNA
18.             chrcolumns = line.split("\t")
19.             ChromDic[chrcolumns[1].rstrip("SN:")] = {}
20.         if not line[0] == "@": #Speeds things up by a few seconds
21.             break
22. from K2_theWindowMaker import WindowList #imports file containing the windows
    to be used for read counting in each chromosome
23. CurrentChrom = ""
24. f.close() #closes the file so it can be opened again.
25. f = open(sys.argv[1], 'r') #open again
26. for line in f:
27.     if not line[0] == "@": #if the line is one of the pertinent ones, split it into
    columns
28.         columns = line.split("\t")
29.         if columns[2] == '*': #Skips a line if chromosome can't be assigned, or if
    it's the mitochondrion/y chromosome, or if the mapping quality is less than 10.
30.             continue
31.         if columns[2] == 'chrM':
32.             continue
33.         if columns[2] == 'chrY':
34.             continue
35.         if int(columns[4]) < 10:
```

```

35.         continue
36.         if not CurrentChrom == columns[2]:#Resets the current chromosome if the c
hromosome changes, and resets the WinLimit counter.
37.             WinLimit = 0
38.             CurrentChrom = columns[2]
39.             ChromLoc = int(columns[3]) #Position in chromosome is columns[3]
40.             if not WinLimit in ChromDic[CurrentChrom]: #If it doesn't exist, make it
exist
41.                 ChromDic[CurrentChrom][WinLimit] = 0
42.             try:
43.                 if ChromLoc <= WindowList[CurrentChrom][WinLimit]: #If the position i
n chromosome is within the window bracket, add one to the read count
44.                     ChromDic[CurrentChrom][WinLimit] += 1
45.                 else: #If not, move on to the next window and start again
46.                     WinLimit += 1
47.             except: #A Python telomere -
included to stop the code running off the edge of the chromosome and breaking
48.                 WinLimit -= 1
49.
50. ChromAvg = {} #This block of code calculates the chromosomal average, to exclude
outliers that have escaped the duplicate removal phase from PicardTools.
51. ChromCount = 0
52. for chromosome in ChromDic:
53.     ChromAvg[chromosome] = 0
54. for chromosome in ChromDic:
55.     for item in ChromDic[chromosome]:
56.         ChromCount += ChromDic[chromosome][item]
57.     try:
58.         ChromAvg[chromosome] = ChromCount/len(ChromDic[chromosome])
59.     except:
60.         pdb.set_trace()
61.     ChromCount = 0
62.
63.
64.
65. for chromosome in ChromDic: #Excludes outliers that warp the mean chromosome copy
number. Without this, single outliers that escaped duplicate removal
66.     for item in ChromDic[chromosome]: #can force the whole chromosomal copy numbe
r down by one or more when the chromosome is normalised.
67.         if ChromDic[chromosome][item] > 5*ChromAvg[chromosome]:
68.             ChromDic[chromosome][item] = ChromAvg[chromosome]
69.         elif ChromDic[chromosome][item] == 0:
70.             ChromDic[chromosome][item] = ChromAvg[chromosome]
71.
72.
73.
74. ChromReads = {}
75. for chromosome in ChromDic:
76.     logcounter = 0 #This bit is to turn the read-out into a microarray-
like data set so DNACopy can use the data.
77.     SumOfBins = 0 # It gets the (read count divided by the average chromosome-
wide read count)
78.     for interval in ChromDic[chromosome]:
79.         logcounter += 1 #counts the number of windows, basically
80.         SumOfBins += int(ChromDic[chromosome][interval]) #sums the value of each
window
81.     ChromReads[chromosome]= SumOfBins / logcounter #Gets the average window v
alue, corrected for the fact that there are two copies going into each amplificat
ion reaction.
82.
83.
84.
85. for chromosome in ChromDic: #Print out everything.
86.     for interval in ChromDic[chromosome]:
87.         if ChromDic[chromosome][interval] == 0:
88.             ChromDic[chromosome][interval] += 1

```

```

89.         if not chromosome == 'chrY':
90.             try:
91.                 print chromosome + "," + str(interval)+","+str(2*(float((ChromDic
[chromosome][interval])/ChromReads[chromosome]))) #Prints out in microarray forma
t
92.             except:
93.                 pdb.set_trace()

```

WindowMaker.py

```
1. #Used Genome Multitool (GEM) to get mappability score across the whole genome (ie
   , relative uniqueness of reads, bases, etc.)
2. #Excluded all bases that didn't have a mappability score of 1 (score of 1 denoted
   by "!"), and counted those that did.
3. #Once 500,000 "!"s are hit, shuttle off that bracket as a 500kb window and move o
   n to the next.
4. #Files are awkward, as a 0 is denoted by a blank space. So there's a lot of them
   at the start and end of a file.
5.
6. g = open('/home/killian/Scripts/mm10_gem.mappability.mappability') #Opens mappab
   ility file. There was only going to be one so the file location is hard-
   coded in.
7. UniqueCounter = 0
8. ChromCounter = 0
9. WindowList = {} #Sets up chromosome dictionary
10.
11.
12. for line in g:
13.     if line[0:4] == '~chr': #If line is starting a new chromosome: reset everythi
        ng, start the count again.
14.         CurrentChrom = line.lstrip('~').rstrip()
15.         WindowList[CurrentChrom] = []
16.         ChromCounter = 0 #
17.         UniqueCounter = 0
18.         continue
19.
20.     for character in line.rstrip('\n'): #Strip tabs away
21.         ChromCounter += 1 #True base number calculator
22.         if character == "!":
23.             UniqueCounter += 1 #Unique base number calculator
24.         if UniqueCounter == 500000:
25.
26.             WindowList[CurrentChrom].append(ChromCounter)
27.             UniqueCounter = 0
```

Appendix 4: DNA sequences

DNA sequences for *ophNdi1*, the *Nefh* promoter, *msMin-Nefh*, and *Nefh_short* are inserted below. In the case of promoters, the final base (3') of the sequence corresponds to -1 of the transcriptional start site of the *Nefh* gene.

>*ophNdi1* (1839bp)

```
GGTGTCAGTTTCATCACATCATCGAATTACACGTTTACCCAAGAGAAGAACTAAAAACCA
CTATGCTGAGCAAGAACCTGTACAGCAACAAGCGGCTGCTGACCAGCACCAACACCCTCGT
GCGCTTCGCCAGCACCAGATCCACCGGCGTCGAAAACAGCGGGGCTGGCCCCACCAGCTTC
AAGACCATGAAGGTCATCGACCCCCAGCACAGCGACAAGCCCAACGTGCTGATCCTGGGCT
CTGGCTGGGGCGCCATCAGCTTTCTGAAGCACATCGACACCAAGAAGTACAACGTCAGCAT
CGTGAGCCCCAGAAGCTACTTCCTGTTCACCCCCTGCTGCCCAGCGCCCCTGTGGGAACC
GTGGACGAGAAGTCCATCATCGAGCCCATCGTGAAC TTCGCCCTGAAGAAAAAGGGCAACG
TCACCTACTACGAGGCCGAGGCCACCAGCATCAACCCCGACCGGAACACCGTGACCATCAA
GAGCCTGAGCGCCGTGTCCAGCTGTACCAGCCCGAGAACCACCTGGGCCTGCACCAGGCC
GAGCCCGCCGAGATTAAGTACGACTACCTGATCAGCGCCGTGGGCGCCGAGCCCAACACCT
TTGGAATCCCCGGCGTGACCGACTACGGCCACTTCCTGAAAGAGATCCCCAACAGCCTGGA
AATCCGGCGGACCTTCGCCGCCAACCTGGAAAAGGCCAACCTGCTGCCTAAGGGCGACCCC
GAGCGGAGAAGGCTGCTGAGCATCGTGGTCTGTTGGGCGGAGGCCCCACAGGCGTGGAAGCTG
CTGGCGAGCTGCAGGACTACGTGCACCAGGACCTGCGGAAGTTCCTGCCCGCCCTGGCCGA
GGAAGTGCAGATTACCTGGTGAAGCCCTGCCCATCGTGCTGAACATGTTTCGAGAAGAAG
CTGAGCAGCTACGCCAGAGCCACCTGGAAAACACCTCCATCAAGGTGCACCTGAGAACCG
CCGTGGCCAAGGTGGAAGAGAAGCAGCTGCTGGCCAAGACCAAGCACGAGGACGGCAAGAT
TACCGAGGAAACCATCCCCTACGGCACCTGATTTGGGCCACCGGCAACAAGGCCAGACCC
GTGATCACCGACCTGTTCAAGAAGATCCCCGAGCAGAACAGCAGCAAGCGGGGCCTGGCCG
TGAACGACTTCCTGCAGGTCAAAGGCAGCAACAACATCTTCGCCATCGGCGACAACGCCTT
TGCCGGCCTGCCTCCTACAGCCCAGGTGGCCCACCAGGAAGCCGAGTACCTGGCCAAGAAC
TTCGACAAGATGGCCCAGATCCCCAATTTCCAGAAGAACCTGTCCTCCCGGAAGGATAAGA
TCGACCTGCTGTTTCGAGGAAAACAACCTTCAAGCCCTTCAAGTACAACGACCTGGGCGCCCT
GGCCTACCTGGGCAGCGAAAGAGCCATTGCCACCATCAGAAGCGGCAAGCGGACCTTCTAC
ACCGGCGGAGGCCTGATGACCTTCTACCTGTGGCGGATCCTGTACCTGTGTACCTGAGCAT
```

GATCCTGAGCGCCCGCAGCAGACTGAAGGTGTTCTTCGACTGGATCAAGCTGGCCTTCTTC
AAGAGGGACTTCTTCAAGGGCCTGTGAATGAAATTAACATGCCCTTTTCTGGAAAAAGGAA
AAAAGGTGGTAGGCACCGTTTTTTTCTGAGTTTGCATCCTTTTTTTTCTAAAACCCTCTAA
ACAAAACCTAACACACACACACACGACACAAAAAATGCACATGATGTTTTATTATTTATAT
ATTCCCCTTTTTTTCGAAATGATGCTTGACTAATGCACAAGCAAAAACCCCGACAAGATT
GGTTGATCA

>*Nefh* full length (2295bp)

GCCACACCTTTTACCTTCACACACACCCCACTCCTATCATTCATGTGTGCTGTCAACTGCT
TGTCAGACTTCTCACCCCCAAGAAGGGCATGTGCATTCTGCAGACAACTGAAGAGACTCGA
AGGAACAAGAATCTAATAACAAAAATCCAAGCAGTATGGGAGATAAATGGGGAAGCCATGT
GGGCGTAAGGGGGTAGAGGTCTGCATCCCAGTCCCCCTCCCCATGGCATCTGCAGTGCCTCC
CAGCCTTTCTGACCCCTGCAAAGAGCAGCATGACTGGACCTTTAAATTGGGAAAATGCTTC
ATCATGTTCTGCTCCATCATGAAAACTAGAGTCTCCTCCCCCTCCTCCCTAGTGCCTCT
CCTGGCCTGCAGCCAGGGGCTGGGAATGAGACACAGGACAGGAAAGGGATCTCTTTTAGGG
AATCTATCAGTTCTCCTCCTAGGGATCCCTCCAAAAGAGAAAACCACAGCAAACTGGGGTG
CAGTGAGGCTTGAGGTAAGTGCCTGGGAGAAGTTCTGATCTGAAGAAGTCTATACTGGTTT
CCAGAGCTTGTCAGTGGGCATTGGAGTGGGGCTCTCTCTGCTCCGGGAAGAGGTTTGCAGG
GAGAAAGAACTTCACAGAGAGCCAGGCACTGGACAGGACATGCAGGGGTGGGTCACTTACA
TACAACCGTAGGTGTTTTGAGCCCGTCATATGACTCATCCAATCCTCCCCTGTACCGCAC
AGAGGGACTGCTTGGAAGCTATGGAACCTCCCTACTCCGTTAGGCATAGATTTAACCCCT
TCCCATCCGAGGAGCGGCTGCTGTCCGTGGTGCTGAAGCGATAGCGGCACGGGCGGCTCCG
TCCACTAACACCGCTTTTGACCGGAAAACCAAACCAAGAACGAGCCGTATAATAAGCAAG
AGTCCAAGTCTAAGCCCCTCCGCCGTCCCCGCCCTTTTACCTGAAGCCTCAGTAGGGCTC
ATGATGGAGGTCGGTGGACTTTGGTACTGAAAAACCACTCCACCACTTCCTCGGAGCATGA
AAGGGGATGCTTACGGCAGTACTGGTTCATCTATTCTGGAAAAGGAATGAGATGCCAAGAT
AAAGCAGAAAAATCGGGCAAGGAAGGGAGAAAGACAAAGTTCTCAGGTGAGAGGAACTGGT
TACTATTCCGACTGGCAATATGTGGGTTCTCCTCCCCAAAATCAGCCAGACATTTCCCAAG
TTCGAACCTCCTAGGGGCACATGGGAGCTTGAGCTGCATCTTGTCTCTTGCACACAAGGG
AAAACCAAACATAGGAGAACAATTTGTACAAGGTCATTCAGCTAGCGAAGCACAGAAGC
TAACCCACCCCTGTGGCAGAACTTGGCTTCGGTGTGAGGCTCTTGCTGCCTACTGAGGGA
CCCCCTGTTCTTCGTAGGCAGTTTTCTTTCCGGGCAAGAGGAGACTCCACTTTCCAGTCG
TGGCCACTGGAATTTTTAGAGAGCACACGTTCTCTCACCCAGCGCTCCCTTTCTCCGTC
TGCAGTGTCTCCTTCTCAGGGTAGCTTTGCGGTCTTTCAAACCTCCACGCCCCACCCAAC

CCCAACCCCGAAGCCAGCTGTACAGTTCCTTAAGCCCCTTTGGGTGGCCAGGGCCGCTGT
AGTATCTGGGGAACACTGCACCGCCAGCTAGAAGGTCCCCATTTATCATCAGTAGCATCCA
TCATGCAACCCCATACAGAATCCCTTCGTGGGTGACTGCAGTCTGCACTCCTCATCTCAAG
GTCCTCTCTAACTATCAGGGAACCAACCCTGTGCTGCTTCTCAAGTGGGGGTGTCCTCTCA
TAGTAATCACTGCAGTCTCCCACTGCTTCAACCCGAAGGCGCCCTGACCCATCAGTTCTGC
AATCCTCTCCCTATTTCCAGTGCCCTCTCTTATTCTGAGGGTCTTATTCTGACTAATAGGG
TCTTCCGACATGCACCTGGAGGTCTGCACTTGTCCGCTCCGGAAGTCCTTTACTCCTTGGT
CTGACCTCGGGAGGCTCTACTGACGATGCGTCGATTCCCCTTCACTCCTGGGTGCTCCCC
CCAGCCCCGCCCCTCTCACTGCGGAGAAGCCGGTCGGCCCGGGGCCGCGGGGGAGGAGGTG
GAGAGGGTGGGGCCCTCCTCCCCAGCCCCCACTGCCGAGGGGCCGGACCGGGCCACCGCG
GATATAAAAGAGCCGGAGTCCCAGAGCTGCCGCAGTGCTGCCTGCCCCGTCCCAGCCCCGC
ACTCCCGCTCCGCTGGCGGCCGCACCTGCTCCGGCCAT

>*msMin-Nefh* (828bp)

TGTGCTGTCAACTGCTTGTGAGACTTCTCACCCCCAAGAAGGGCATGTGCATTCTGCAGAC
AACTGAAGAGACTCGAAGGAACAAGAATCTAATAACAAAAATCCAAGCAGTATGGGAGATA
AATGGGGAAGCCATGTGGGCGTAAGGGGGTAGAGGTCTGCATCCCAGTCCCCTCCCCATGG
CATCTGCAGTGCCTCCCAGCCTTTCTGACCCCTGCAAAGAGCAGCATGACTGGACCTTTAA
ATTGGGAAAATGCTTCATCATGTTCTGCTCCATCATGAAAACTAGAGTCTCCTCCCCCTC
CTCCCTAGTGCCTCTCCTGGCCTGCAGATTTAACCCTTCCCATCCGAGGAGCGGCTGCTG
TCCGTGGTGCTGAAGCGATAGCGGCACGGGCGGCTCCGTCCACTAACTGCATCTTGTCTCT
TGCACACAAGGGAAAACCAACATAGGAGAACACAATTTGTACAAGGTCATTCAGCTAGCG
AAGCACAGAAGCTAACCCCGCGCTCCCTTTCTCCGTCTGCAGTGTTCTCCTTCTCAGGGTA
GCTTTGCGGTCTTTTCAAACCTCCACGCCCTCTAACTCTTCACTCCTGGGTGCTCCCCCCCCA
GCCCCGCCCCCTCTCACTGCGGAGAAGCCGGTCGGCCCGGGGCCGCGGGGGAGGAGGTGGAG
AGGGTGGGGCCCTCCTCCCCAGCCCCCACTGCCGAGGGGCCGGACCGGGCCACCGCGGAT
ATAAAAGAGCCGGAGTCCCAGAGCTGCCGCAGTGCTGCCTGCCCCGTCCCAGCCCCGCACT
CCCGCTCCGCTGGCGGCCGCACCTGCTCCGGCCAT

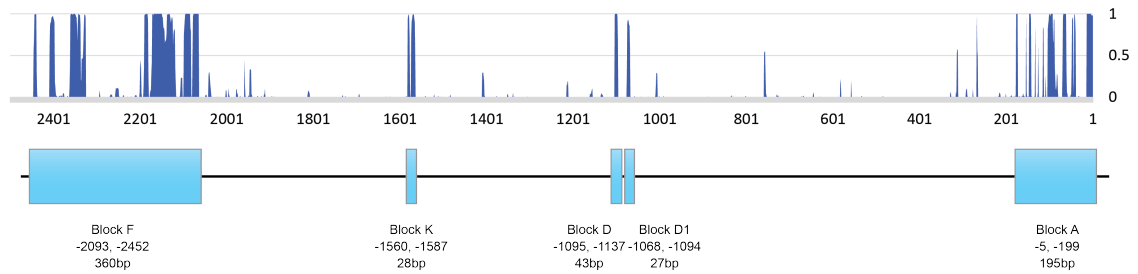
>*Nefh_short* (450bp)

GCCACACCTTTTACCTTCACACACACCCCACTCCTATCATTCATGTGTGCTGTCAACTGCT
TGTGAGACTTCTCACCCCCAAGAAGGGCATGTGCATTCTGCAGACAACTGAAGAGACTCGA
AGGAACAAGAATCTAATAACAAAAATCCAAGCAGTATGGGAGATAAATGGGGAAGCCATGT
GGGCGTAAGGGGGTAGAGGTCTGCATCCCAGTCCCCTCCCCATGGCATCTGCAGTGCCTCC

CAGCCTTTCTGACCCCTGCAAAGAGCAGCATGACTGGACCTTTAAATTGGGAAAATGCTTC
ATCATGTTCTGCTCCATCATGAAAACTAGAGTCTCCTCCCCCTCCTCCCTAGTGCACTCT
CCTGGCCTGCAGCCAGGGGCTGGGAATGAGACACAGGACAGGAAAGGGATCTCTTTTAGGG
AATCTATCAGTTCTCCTCCTAGG

Appendix 5: Human *Nefh* conservation diagram

Schematic of human *Nefh* promoter conserved regions. Blocks A, D and F correspond to equivalent regions in the mouse *Nefh* promoter. Blocks D1 and K show significant conservation (20 bases or more of conservation >0.8) with some mammals but not with mouse.



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