

Comparing optogenetic approaches to visual restoration in a model of retinal degeneration

Michael James Gilhooley

Merton College

Submitted for the degree of Doctor of Philosophy, Trinity term 2019

Supervisors:

Professor Mark Hankins

Professor Susan Downes

“Comparing optogenetic approaches to visual restoration in a model of retinal degeneration”

Michael James Gilhooley

Merton College

Submitted for the degree of Doctor of Philosophy, Trinity term 2019

Abstract

The inherited retinal degenerations (IRDs) are the most common cause of irreversible visual loss in the young - but therapeutic options have traditionally been limited. While genetically heterogeneous, the IRDs are linked by a final common pathway of visual loss secondary to photoreceptor death, with cells of the neural retina surviving relatively intact. The technique of optogenetics (inducing light sensitivity by exogenous expression of light sensitive proteins within cells) is developing as a therapeutic method to stimulate these surviving cells: returning light signals to the degenerate retina and ultimately restoring lost vision.

The aim of this thesis was to compare different optogenetic tools and cellular targets for optogenetic visual restoration in a model of inherited retinal degeneration in order to inform future translational work.

Three novel mouse models of retinal degeneration, additionally devoid of native melanopsin and incorporating cell specific *Cre* recombinase expression were devolved and validated to allow cell population specific targeting of genetic constructs both delivered transgenically and by using intravitreal injections of adenoassociated viral vectors (AAVs).

These models were used to isolate ON-bipolar cells from dissociated degenerate retina in the first comparison of gene expression profiles in such cells to their wildtype counterparts. This demonstrated a lack of changes likely to preclude these attractive cellular targets for therapeutic optogenetics.

Cell targeted delivery of a candidate optogenetic tool (melanopsin) using these models was compared to conventional (non-specific) AAV delivery. This showed restoration of retinal electrophysiological light responses on *ex vivo* multiple electrode array recordings with kinetics differing between delivery approaches. Targeted delivery of three candidate tools (melanopsin, rhodopsin and ReaChR-channelrhodopsin) were similarly compared with markedly differing response characteristics demonstrated between tools

Finally, translatable, compressed ON-bipolar cell specific promoters were used to produce a potentially translatable cell specific AAV delivery system. This was demonstrated to successfully deliver functional optogenetic tools to a degenerate retina *in vivo*.

Together, these data indicate that the described mouse lines form a useful model system with which to compare optogenetic vision restoration approaches. In addition, while no one optogenetic tool or target was demonstrated to be overall superior to another, the diversity of responses seen could in themselves represent a great opportunity to improve the quality of responses restored by this technique as it moves towards clinical translation for the treatment of inherited retinal degenerations.

Declaration

I declare that this thesis is my own original work, other than where indicated in the text, and it has not been submitted toward a research degree at this university on any other.

Of note, as indicated in Chapter 4, the dissociation of retinae, FACS sorting and validation of these cells was carried out by Dr. D. Hickey.

The Gene array experimental work was carried out by Cambridge Genomic service (as indicated in the text).

Michael James Gilhooley
6th August, 2019

Amended with requested minor corrections,
24th November, 2019

Dedication

For A.V.L.D.

With Love

Table of Contents

Chapter 1 – General introduction	5
Introduction.....	5
The Retina.....	5
Gross Anatomy	5
The neural retina	7
Intrinsically photosensitive ganglion cells.....	16
The range of retinal sensitivity	19
The Retina in disease	19
The inherited retinal degenerations	19
Survival Cell Stimulation.....	20
Stages of retinal degeneration	21
Morphology and Synaptic Remodelling	22
Remodelling of neurotransmission.....	24
Implications of neural plasticity in degeneration	25
Treatment strategies for Inherited retinal degenerations	26
Retinal Prostheses	26
Stem cells	27
Gene replacement therapy	29
Adenoassociated virus	30
Optogenetics	32
Questions.....	34
Thesis Aims	35
Chapter summary	36
Chapter 2 – Material and Methods	37
Animals.....	37
Mice.....	37
Tissue Preparation for genotyping.....	39
Polymerase chain reaction for genotyping.....	39
Gene array	42
Systematic literature review and prioritisation of candidate genes	42
Adeno-associated Virus Vector Production.....	43
Cloning	43
Plasmid Preparation.....	44
Cell culture	44
AAV production	44
Co-transfection.....	45
Cell lysis.....	47
Virus Purification	47
Adeno-associated virus validation	48
Titre.....	48
Purity.....	49
<i>In vitro</i> transduction ability	52
Intravitreal Injections	53

Immunohistochemistry.....	54
Cryosections	54
Retinal flat mounts	55
Validation of antibodies using transfected HEK293T cells	56
Immunocytochemistry.....	59
Fluorescence light microscopy	59
Confocal Microscopy.....	60
Behavioural Assays	62
Pupillometry Assay: the post illumination pupil response	63
Optokinetic response assay.....	67
Novel object, visual context recognition assay.....	69
Behavioural Battery Protocol	71
Running Wheel Activity Monitoring.....	73
Multiple Electrode Arrays	73
Data Analysis	76
Statistical analysis.....	78
<i>Chapter 3 – Results: development and validation of mouse lines</i>	<i>79</i>
Introduction.....	79
Choice of Species: practicality	79
Models of retinal degeneration: a phenotype analogous to the human disease.....	80
A model without native melanopsin: a retina with no native light responses	83
Expressing proteins of interest in required cell populations	84
Transgenic indicators of Cre expression.....	89
Experimental design and results	90
Description of breeding approach	90
Validation of the transgenic mouse lines	97
Discussion	109
Limitations of the lines produced	109
Conclusion	113
<i>Chapter 4 Results: Bipolar cell gene changes in degenerate retinae.....</i>	<i>114</i>
Introduction,.....	114
Experimental design and results	116
Summary of experimental approach	116
FACS isolation, validation and RNA extraction	116
Comparison of gene expression	120
Photoreceptor “carry over”	120
Identifying candidate genes for further characterisation: database approaches.....	126
Identifying candidate genes for further characterisation: literature review	130
Further characterising candidate genes: Immunohistochemistry	130
Discussion	141
Gene expression in context	141
Commonalities in differentially expressed genes: metabolic stress	143
Identifying Candidate Genes	144
Further characterisation of selected candidate genes.....	145
Conclusion	148
<i>Chapter 5 – Results: Comparing non-specific and targeted delivery of melanopsin in the degenerate retina</i>	<i>150</i>

Introduction.....	150
Note on nomenclature.....	152
Experimental design and results	153
Production and <i>in vitro</i> validation of hOPN4 viral vectors	153
<i>In vivo</i> validation of hOPN4 viral vectors	157
Retinal coverage	161
Retinal Electrophysiology.....	176
Behavioural Responses	194
Circadian entrainment – a pilot experiment	204
Discussion	208
AAV delivery	208
Electrophysiology: Kinetics.....	210
Electrophysiology: sensitivity and amplitude of responses.....	213
Behavioural Responses	215
Implications for further research and translation.....	219
Conclusion	219
<i>Chapter 6 – Results: Comparing specific delivery of optogenetic tools in the degenerate retina.....</i>	221
Introduction.....	221
Vertebrate Opsins.....	221
Alternative tools	223
Microbial Opsins.....	223
Channelrhodopsin variants	226
Comparing optogenetic tools	230
Note on nomenclature.....	232
Experimental design and results	233
Production and <i>in vitro</i> validation of viral vectors.....	233
<i>In vivo</i> validation of viral vectors	238
Retinal Coverage.....	251
Retinal Electrophysiology.....	251
Irradiance response curves: dynamic range.....	283
Behavioural assays.....	284
Discussion	287
An idealised tool	287
Comparison of tools.....	289
Forms of response and kinetics.....	289
Sensitivity & dynamic range	289
Magnitude – firing rates.....	291
Strengths and weaknesses of each tool	292
Conclusion: an idealised tool	293
<i>Chapter 7 – Delivery of Melanopsin to the degenerate retina using compressed L7 promoters.....</i>	295
Introduction.....	295
AAV delivery & bipolar cell specific promoters: challenges	295
The L7-6 compressed promoter	296
L7 mini-promoters	299
Experimental design & Results	301

Preparation of plasmids	301
Preparation of viral vectors	304
<i>In vivo</i> validation of compressed L7 promoters in degenerate retina	305
Functional validation of compressed promoter targeted Melanopsin	313
Discussion	317
Differences in restored hOPN4 responses depending of method of delivery	317
Ple155 mini-promotor in the degenerate retina	318
Conclusion	319
<i>Chapter 8 – General Conclusion.....</i>	321
Overview	321
Implications, future directions and suggestions for further work	324
Melanopsin coupling.....	325
ReaChR responses.....	325
Rhodopsin responses.....	326
Retinal processing.....	326
Viral delivery.....	327
Models without melanopsin.....	327
Conclusion	329
<i>Chapter 9 – Appendices</i>	330
Appendix 1 - Sequencing primers	331
Appendix 2 - Commercial laboratory cloning reports (Genescript Ltd)	332
Appendix 3 - Bipolar Cell Gene array report	333
Appendix 4 - Surgical note proforma	334
Appendix 5 – Two-way ANOVA and post hoc test result tables for MEA responses	335
<i>References.....</i>	336

Chapter 1 – General introduction

Introduction

The inherited retinal degenerations (IRDs) are an important group of diseases linked by a final common pathway of vision loss due to photoreceptor death. An emerging approach to their treatment involves the stimulation of surviving retinal neurons, with the aim of returning light sensitive signals to the brain. One method of such stimulation is by the introduction and expression photosensitive genes to these cells: “optogenetics.”

While not directly involved in the disease process, changes are likely to occur in surviving retinal cells during IRDs, as they lose their light signal input from photoreceptors (deafferentation). It remains unclear however, if changes may limit the functional utility of the optogenetic approach. In addition, the optimum choice of optogenetic “tool” and cellular target for the restoration of functional vision has not been systematically determined. In this Chapter the anatomy of the retina and clinical problem will be introduced; potential treatment strategies will be discussed and important unanswered questions articulated to guide the investigation described in this thesis.

The Retina

Gross Anatomy

The eye is a highly-specialised organ: embryologically part of the central nervous system, it can be thought of as a modified extension of the brain (Figure 1-1). Its primary function is to both transduce light signals from the environment and initiate the process of extracting useful information to pass to retinorecipient visual areas.

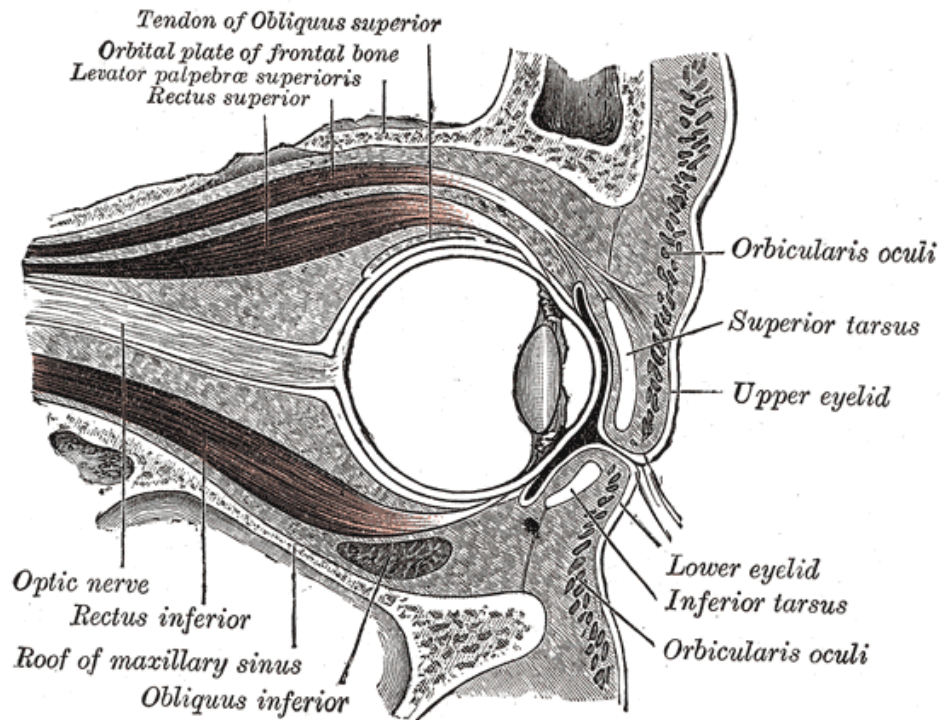


Figure 1-1 The context of the globe within the adnexa. Modified from(1)(Fig. 888)

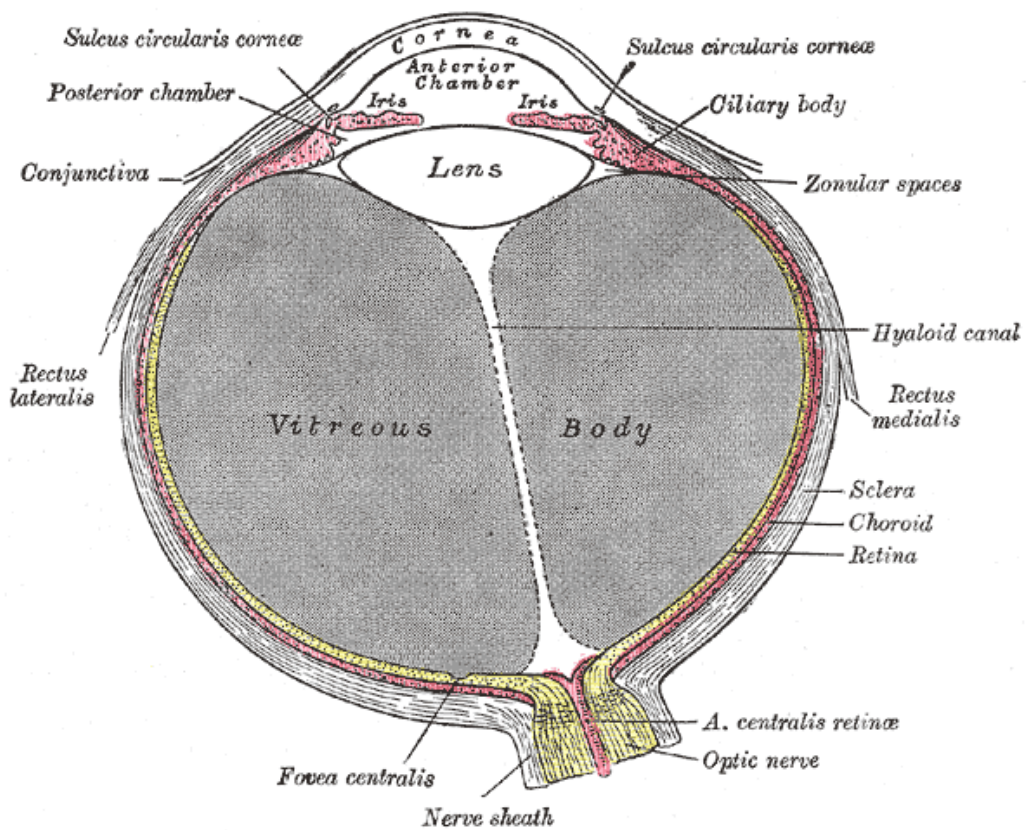


Figure 1-2 The gross anatomy of the globe Modified from(1) (Fig. 869)

This process begins with the optical preparation of light inputs by the anterior segment of the eye, focusing it onto the retina(2)(Figure 1-2). Incident light passes through the retina and is channelled to its base where the photoreceptor cells (rods and cones) make use of chromophore-opsin photopigment complexes to transduce light signals: photon absorption isomerises the chromophore (11-cis retinal) to its all-trans form leading to a conformational change in the opsin partner, initiating second messenger signalling. This amplifies the light signal, ultimately resulting in hyperpolarisation of the photoreceptor's membrane potential (Figure 1-3)(3, 4). This suppresses the dark-release of glutamate and so begins onward neural transmission. Chromophore (all-trans retinal) is regenerated in the retinal pigment epithelium and Müller cells(5)(Figure 1-4).

The neural retina

Retinal neural processing is effected by a complex interaction between multiple cell types which fall into five main classes: photoreceptors, bipolar cells, ganglion cells; horizontal cells and amacrine cells, supported by Müller cells and astrocyte glia(6). The neural cells are arranged into a hierarchy with both a 'vertical' flow (from photoreceptor, via bipolar cell to retinal ganglion cell and optic nerve) as well as a 'horizontal' flow of information (Figure 1-5) with information moving laterally aided by horizontal and amacrine cells. This lateral flow helps to enhance the prominence of important visual features (for example with lateral inhibition & centre-surround responses) and so aid in extracting useful information from images(6) even before they reach the retinorecipient brain.

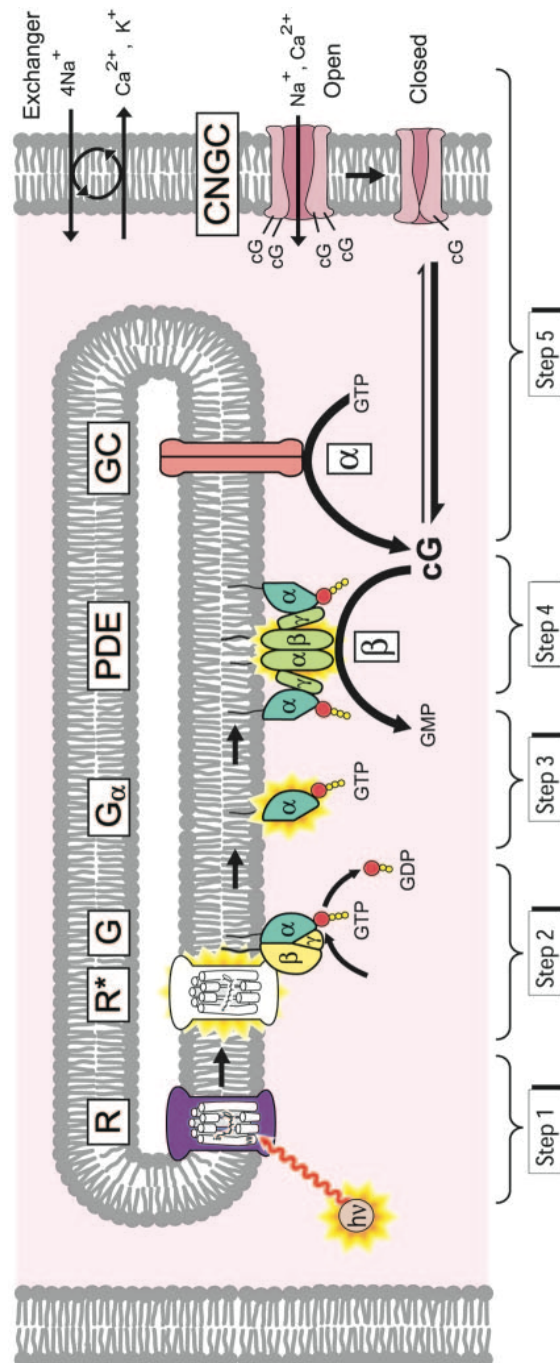


Figure 1-3 The rod phototransduction cascade:

After Rhodopsin (R) absorbs a photon of light (h), the activated rhodopsin (R^*) repeatedly contacts molecules of the heterotrimeric G protein (G), which catalyses the conversion of GDP (Guanosine diphosphate) to Guanosine triphosphate GTP, producing the active form $G\alpha$ -GTP (G^*). Two G 's bind to the inhibitory γ subunits of the phosphodiesterase (PDE), thereby activating one or both of the corresponding α and β catalytic subunits, which then catalyze the hydrolysis of cyclic GMP (cGMP). The consequent reduction in cytoplasmic concentration of cGMP leads to closure of the cyclic nucleotide gated channels and blockage of the inward flux of Na^+ and Ca^{2+} , thereby reducing the circulating electrical current. The Na^+/Ca^{2+} , K^+ exchanger is not directly involved in activation; however, during the light response, it continues to pump Ca^{2+} out, so that the cytoplasmic Ca^{2+} concentration declines, aiding in recovery of the response by stimulating guanylyl cyclase (GC) activity.

Figure and modified legend from (4)

Deactivation of each of the elements leads to quenching of the rhodopsin response: R^* is phosphorylated by rhodopsin kinase and bound by arrestin to deactivate, the spent all-trans retinal entering the visual cycle for regeneration. Transducin deactivates as GTP hydrolyses to GDP, an inhibitor of the PDE(7).

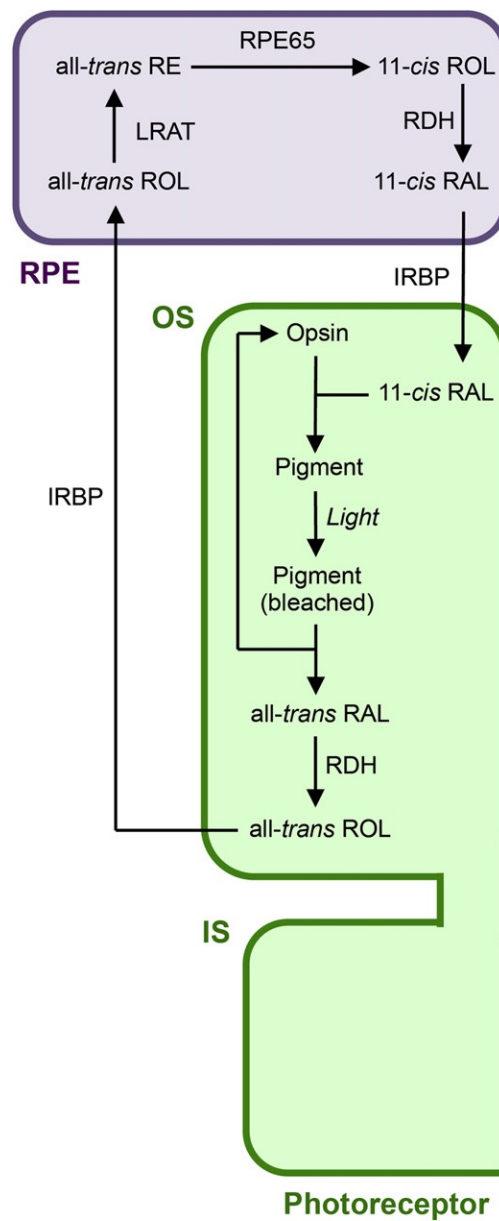


Figure 1-4 The classical visual cycle sees rhodopsin regenerated by transport to the retinal pigment epithelium (RPE) from photoreceptors. An accessory pathway exists in Müller cells and is particularly associated with pigment regeneration in cones(5).

RAL - retinal, ROL - retinol, RDH - retinol dehydrogenase, LRAT - lecithin:retinol acyl- transferase, RE - retinyl-ester

OS – outer segment, RPE65 - retinal pigment epithelium specific protein, 65kD (or Retinoid Isomerohydrolase – RPE65).
Figure modified from (5).

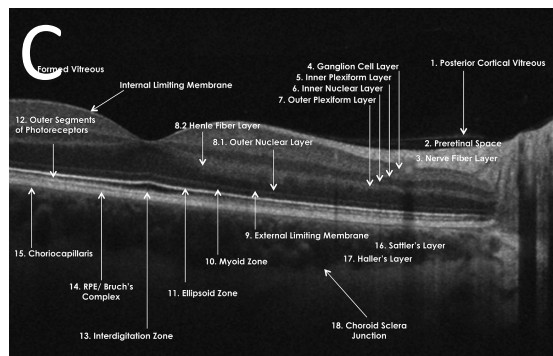
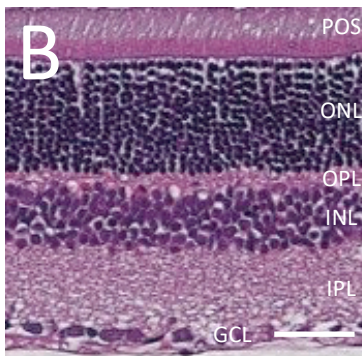
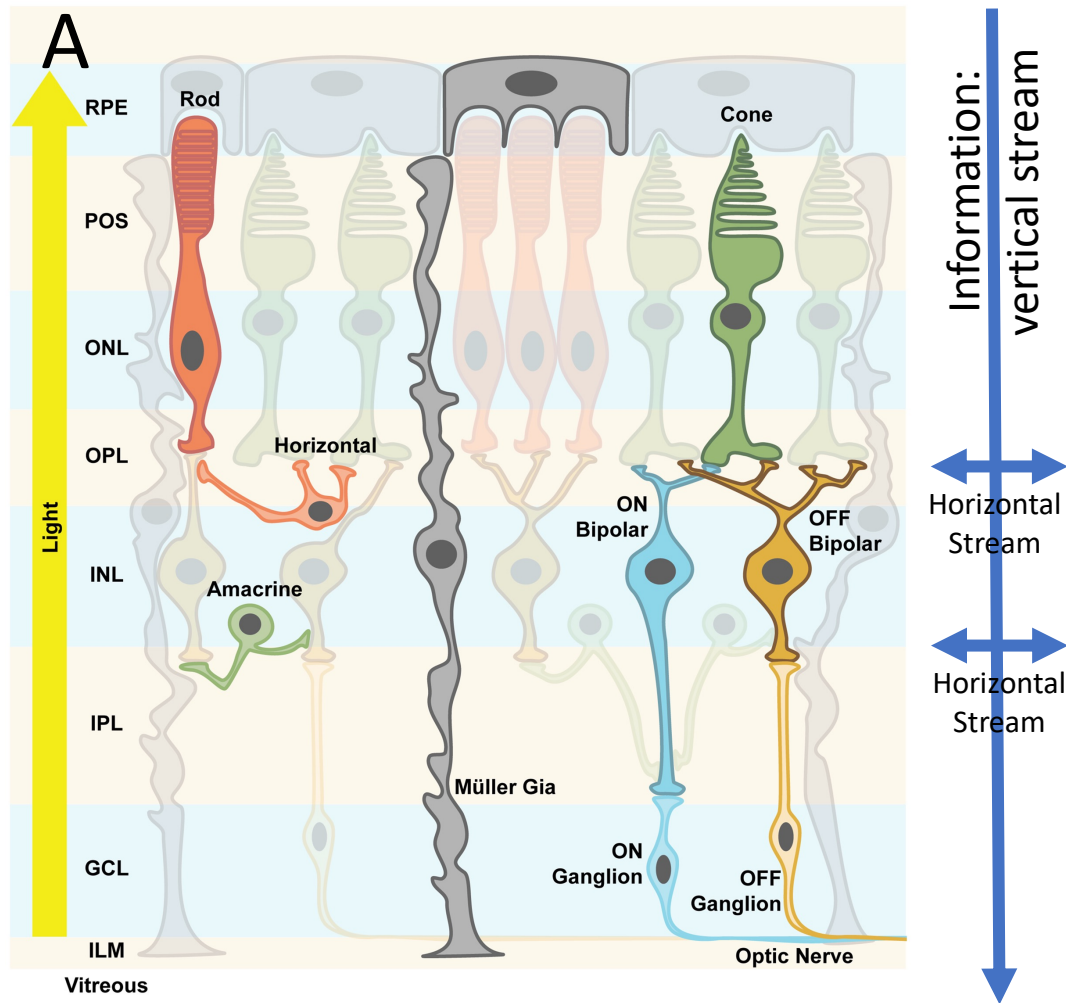


Figure 1-5 Representations of the cellular architecture of the healthy retina. A. A cartoon representation of the relations and interactions of the major cell classes in the healthy retina. Information flows both vertically from photoreceptor towards the optic nerve, but is also modified by a “horizontal” exchange of as transduced light signals begin to be neutrally processed. B. A section of wildtype mouse retina stained post mortem with Haematoxylin and Eosin representing the same view as A. C. In vivo optical coherence tomography of a human eye annotated with consensus names for anatomical landmarks(8). NB. As per clinical convention the orientation of this image is inverted ie. The ILM is superior and the RPE inferior in the image.

ILM - inner limiting membrane, GCL – Ganglion Cell Layer, IPL – Inner Plexiform Layer, INL – Inner Nuclear Layer, OPL - Outer Plexiform Layer, ONL – Outer Nuclear Layer, POS – Photoreceptor outer segments, RPE – retinal pigment epithelium. Scale bar 20µm. A. Figure modified from (9); B. modified from (10), C. Figure modified from (8).

Retinal neurons: bipolar cells

Three classes of neuron (bipolar, horizontal and amacrine cells) process retinal input received by the photoreceptors, in preparation for transfer to higher centres by ganglion cells. The presence of a second order sensory neuron (the bipolar cell) is unique to the visual system(7) (other sensory systems having direct second order projections) and is highly adapted to the unique complexity of visual processing. Bipolar cells lie in the centre of the retina (Figure 1-5), interact (as a class) with all other retinal neurons – (Figure 1-7) and have relatively short axonal lengths. These properties allow these cells to quickly and effectively encode and transmit information preferentially using graded membrane potentials(11) (while still retaining the ability to firing action potentials(12)).

Within this class, specialisation has developed to further meet the demands of processing visual information within the retina. Up to thirteen bipolar cell subtypes have been described in the mouse: one type preferentially communicating signals of rod origin and up to twelve of cone origin(11, 13, 14)(Figure 1-8). The rod bipolar cells are of the “ON” type: they respond to a light induced fall in photoreceptor glutamate release by depolarising their membrane potential (by way of the metabotropic mGluR6 receptor coupled to an inhibitory $G_{i/o}$ G-protein second messenger system, in turn acting on TRPM1 channels (Figure 1-6)(15-19). There is more diversity in the cone bipolar cells with types 1,2,3& 4 using excitatory ionotropic glutamate receptors (AMPA and Kainate in varying proportions(20)) to hyperpolarise in response to light(11, 21, 22) and 5,X,6,7,8 & 9 displaying ON responses(11).

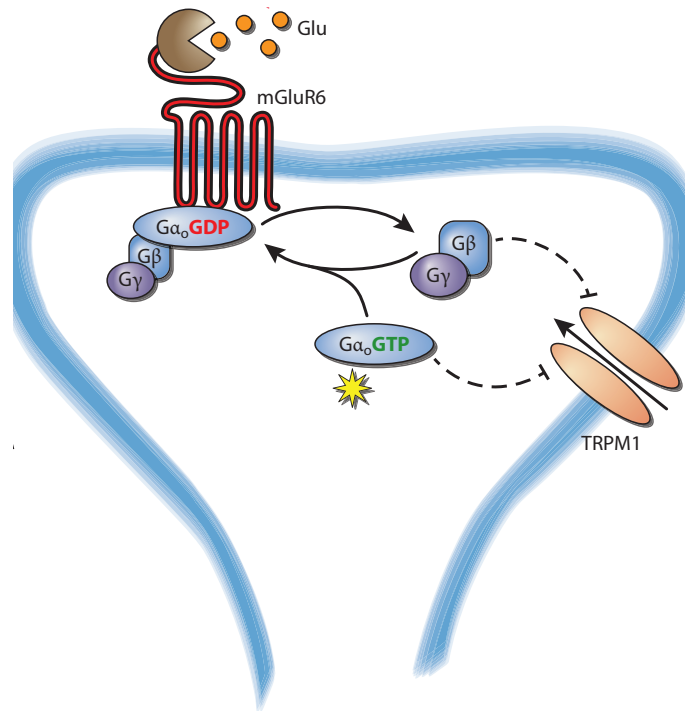


Figure 1-6 – ON-bipolar cells (such as rod ON-bipolar cells) respond to glutamate released from photoreceptors in the dark by hyperpolarising their membrane potential. Higher glutamate concentrations (glu) lead to activation of the metabotropic mGluR6 receptor which is coupled to an inhibitory $G_{i/o}$ G-protein second messenger system, this in turn acts on TRPM1 channels to reduce channel opening. The opposite occurs when light induces a fall in photoreceptor glutamate release (15-19). Figure modified from(23).

This diversity in responses is reflected in diversity of morphology and lamina of projection in the inner plexiform layer(24) (Figure 1-8) - there is also a variety of different response kinetics and field sizes – contributing to parallel processing in the cone pathway(11). Divergent processing in the cone pathway – cones synapse with multiple diffuse bipolar cells within their reach – is the mainstay of parallel processing within the cone system, allowing enhancement of different visual features(11) – for example by lateral inhibition. Rod bipolar cells however receive input from many rods – allowing for a higher signal to noise ratio due to convergent processing – adapted to their role in vision at low light intensities(25).

Intra-retinal neurons: Amacrine cells

Bipolar cells interact with another retinal neuron class in the inner plexiform layer: the amacrine cell and with around thirty subtypes thought to exist(26), there is again huge diversity in function of these cells which are generally inhibitory (GABA or glycinergic (27)). Their common function lies in the modulation of signals passing from bipolar cells to ganglion cells – enhancing centre surround responses for example (6, 28). However, two amacrine cell types are particularly important to the rod bipolar cell: the A11 and A17 types. Rod bipolar cells do not directly synapse with retinal ganglion cells, but rather synapse with A11 amacrine cells, which, in turn, relay signals via gap junctions to ON-cone bipolar cells and via sign inverting glycinergic synapses to OFF-cone-bipolar cells. The A17 amacrine cell is intimate in the regulation of this process (Figure 1-9)(11).

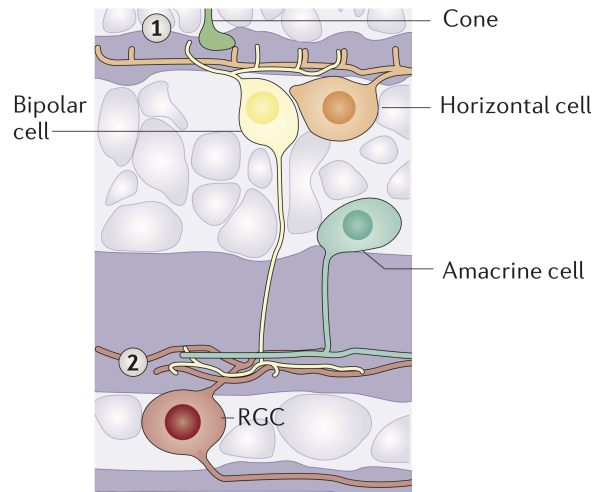


Figure 1-7 Retinal bipolar cells are regulated by both horizontal and amacrine cells. Figure modified from (11)

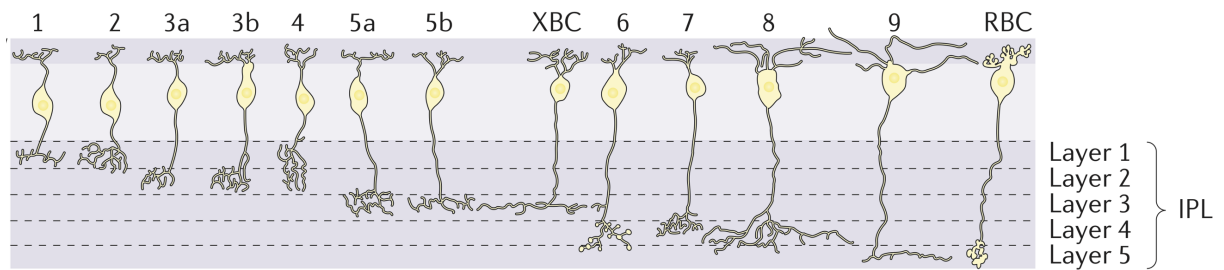


Figure 1-8 The ten main classes of bipolar cells in the mouse and their projections in the inner plexiform layer. Cone types 1 to 4 project to OFF sublaminae while cone types 5 to 9 project to ON sublaminae - as does the sole Rod ON-bipolar cell (RBC). IPL – inner plexiform layer. Figure modified from (11).

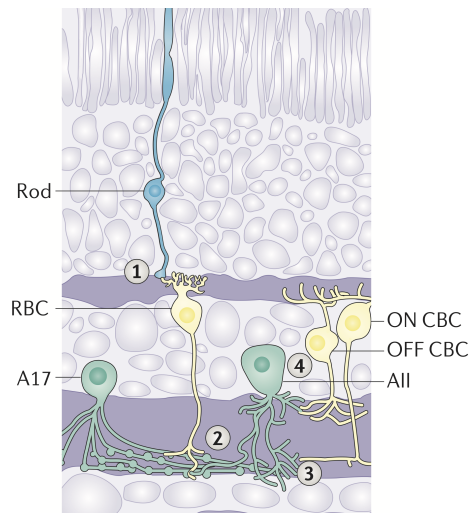


Figure 1-9 The Rod pathway. Rods converge their outputs onto rod bipolar cells (1). This cell however, does not directly synapse with retinal ganglion cells, but rather with A11 amacrine cells (2) which relay signals via gap junctions to ON-cone bipolar cells and via sign inverting glycinergic synapses to OFF-cone-bipolars(4); the A17 amacrine cell is intimate in the regulation of this process (3). Figure and legend modified from (11).

Intra-retinal neurons: horizontal cells

Horizontal cells, resident in the inner nuclear layer and extending their dendrites out into the outer nuclear layer are also heavily involved in the lateral inhibitory regulation of bipolar cell signalling(6). With GABAergic inhibitory transmission prominent, three classes of horizontal cells are found in humans, and one in the mouse retina(29) which characteristically slowly hyperpolarise in response to light signals.

Gap junctions between horizontal cells are common: increasing the size of their receptive fields; as too are synaptic connections between these cells, OFF bipolar cells and cones. A further important interaction between horizontal cells and the “vertical” flow of information in the retina occurs at the photoreceptor ribbon synapse(30). Here, ON-bipolar cell endings along with those of horizontal cells are surrounded by the photoreceptor spherical; the ‘ribbon’ - within the photoreceptor terminal - is a row of electron dense primed glutaminergic neurotransmitter vesicles(31, 32). This close cellular apposition and ‘pre-priming’ are evolved to allow particularly quick vertical photoreceptor to bipolar cell transmission, modulated by the horizontal cell to aid centre surround responses and so contrast sensitivity(33).

Retinal ganglion cells

The retinal ganglion cells (RGCs) are the output cells of the retina – they project to multiple centres in the brain with their axons forming the optic nerve where they encode information as trains of action potential firing(34). Over 30 subtypes of ganglion cell have been described(34), based not only on the nature of their firing in response to light, but also by morphology and genetics. All RGCs receive processed input of photoreceptor origin, via the retinal neurons described above at synapses in various layers of the inner plexiform layer (see Figure 1-5)

Differing inputs to and properties of individual RGC subtypes allow a diversity of outputs – with RGC responses to specific visual features. Examples include motion in a particular direction or centre-surround opponency, where activation by light of ON-bipolar cells inputting to the centre of an RGC's receptive field gives a maximum RGC response only when accentuated by darkness activating a concentric ring OFF-bipolar cells (or vice-versa).

The neural retina - hierarchy

This anatomic (and conceptual) hierarchy is reflected at the physiological level – for example, as discussed above, “ON” bipolar cells are equipped with inhibitory (sign inverting) metabotropic glutamate receptors (mGluR6) causing depolarisation(6, 17, 19, 35-37). Conversely, “OFF” bipolar cells express sign preserving, ionotropic (AMPA, kainate) excitatory receptors allowing hyperpolarisation in response to the reduced glutamate caused by increasing light levels(11). Integration of such “vertical” opponent signals is modulated by the lateral contributions from horizontal and amacrine cells leading to the centre-surround receptive fields found in retinal ganglion cells(7, 38, 39). Direction selectivity and colour opponency(40) can be similarly achieved drawing from signals originating from different cell populations. Together this can allow useful basic information to be efficiently highlighted in the visual scene, *before the signal has reached the brain*(38-40). Indeed, while much is still unknown, compared to other neural and sensory systems, this process is relatively well understood at the anatomical, histological and physiological levels and therefore can provide an ideal model in which to examine processes to manipulate neural systems.

Intrinsically photosensitive ganglion cells

In addition to this classical neurosensory system – originating from rod and cone based phototransduction – a third photoreceptor system has been described in the vertebrate

retina: that of the melanopsin expressing, intrinsically photosensitive ganglion cells (ipRGCs)(41, 42). This system was first proposed with the finding that *Pde6b*^{rd1/rd1} mice (lacking functional rods and cones) maintained 'non-image forming' responses to light – circadian entrainment and pupillary responses – not present following optic nerve transection(43).

While there is emerging evidence of a wider role for ipRGC signalling in image forming(44-48), its most characteristic function would appear to be in the circadian and pupillary system where its integration and coding of general irradiance levels of a much longer time course than the rod & cone systems is useful(46, 49). As may be expected, given the apparent differing function of the system, the development and signal transduction cascade appears quite distinct from that of the classical photoreceptors(50, 51) (Figure 1-10).

Melanopsin is a seven transmembrane G-protein coupled opsin that binds cis-retinal, coded for by the *Opn4* gene(41). Increasing evidence suggests that the most important signalling cascade in the native environment of the ipRGCs is via G_{naq/11} pathways leading to membrane depolarisation via TRPC ion channel opening(51-53) (50, 54, 55)(Figure 1-10). However, recently, coupling in other rarer (non "M1") ipRGC subtypes has been described as using alternative (cyclic nucleotide mediated) pathways (56, 57) – suggesting, even in its native environment, melanopsin coupling is promiscuous.

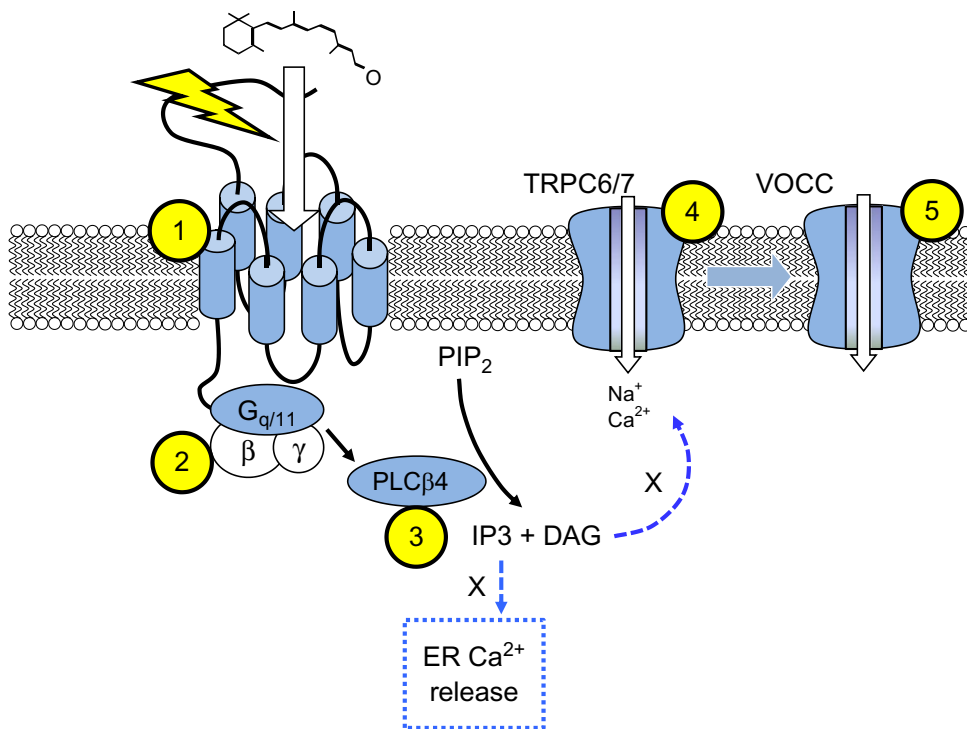


Figure 1-10 Proposed melanopsin signalling in the (M1 type(56, 57)) ipRGC .

A photon is absorbed by the chromophore, leading to a conformation change in melanopsin (1). This activates the a multimeric G protein ($G_{q/11}$) (2) and in turn PLC (3); a drop in PIP_2 concentrations lead to opening of TRPC channels (4) (this process is likely independent of both endoplasmic reticulum Ca^{2+} release and DAG concentration(50)). The ensuing depolarising of the membrane activates voltage gated calcium channels and an action potential is fired (5).

PLC - Phospholipase C, PIP_2 - Phosphatidylinositol biphosphate, IP_3 - Inositol triphosphate, DAG – Diacylglycerol; ER – Endoplasmic reticulum, TRPC – canonical transient receptor potential, VOCC – voltage operated calcium channel. Figure modified from (50).

Melanopsin has also been demonstrated to be able to couple to generic second messenger systems when expressed outside its native environment in various cell types: for example in vitro when expressed in neuro2a cultured cells it can cause a calcium dependant depolarization in response to light(58, 59) (see Chapters 3 and 5). In vivo, expression of melanopsin in drosophila rhabdomeres can induce responses with very fast kinetics , presumably by coupling to fly $G_{q/11}$ pathways withing these cells and illustrating the importance of host cell environment and coupling, rather than the opsin's intrinsic properties, in determining the speed of its response(60).

Like other mammalian opsins, melanopsin binds 11-cis retinal (which isomerises with photon capture to begin the phototransduction cascade(61)). However, it additionally possess intrinsic photoisomerase activity(54, 62) and so does not rely on the visual cycle for pigment regeneration (perhaps a function of its distance from the RPE when resident in the RGC layer).

The range of retinal sensitivity

The sophistication of these neuro-retinal systems leads to an impressive functional range: the human retina is able to perceive light intensities over a range of 10 orders of magnitude from circa 10^{-6}cdm^{-2} (including single photon responses)(63) and a spectrum from around 400 to 700nm(64). Indeed, this represents a challenging feat to retain or restore in a state of disease.

The Retina in disease

The inherited retinal degenerations

In the developed world, the inherited retinal degenerations (IRDs) represent the most common single cause of vision loss in the working age population: accounting for 20.2% of Certificates of Visual Impairment (CVI) issued to adults under the age of 65 in the United

Kingdom. This comes with enormous economic, social and personal cost(65-69). There is phenotypic heterogeneity from patient to patient with markedly different age and speed of onset, eventual severity of vision loss and association with other clinical features (such as in Usher's, Refsum's or Bardet-Beidel syndromes). This is reflected in an equally large underlying genetic heterogeneity with mutations in nearly three hundred genes implicated in IRDs (68, 70) – making efforts to develop treatments for each of these individual genetic errors difficult.

However, vision is generally lost in these diseases through a final common pathway at the cellular level, with a mutation leading to loss of photoreceptor function. In addition, the accumulation of toxic by-product within photoreceptors as a result of the mutation can also result in progressively deteriorating cell function over time towards its eventual death. Where death of rod photoreceptors is prominent, cone survival can be affected by falls in levels of rod derived cone viability factor leading to secondary cone death(71, 72): hence the clinical syndrome of Rod-Cone dystrophy. Conversely, where the mutation leads to cone loss predominantly, the clinical picture can be of a Cone-Rod dystrophy(73).

Survival Cell Stimulation

Given this common aetiology, based on loss of rod and cone photoreceptors, but without a *direct* insult in the neural retina, it has been hypothesised that stimulation of these surviving retinal neurons could return perception of light: that as vision is lost with the death of photoreceptors, all that need be done is to replace the light dependant signal that they provide. Indeed, this concept has been demonstrated clinically with the use of retinal prostheses to electrically stimulate surviving retina in IRD patients which can return a level of visual perception to allow independent navigation(74). This impressive success however has often been communicated to give the impression that the neural retina is *unaffected* by

the loss of its photoreceptor input cells and would remain completely functional if only the light sensitive input was returned.

Given the plasticity seen in other areas of nervous system following deafferentation, this would seem unlikely, indeed, there is growing evidence that the neural retina markedly remodels following the loss of the photoreceptors(75). As technologies to stimulate survivor cells mature (with the eventual aim of increasing resolution of restored images) consideration of retinal remodelling will become much more important: does the neural retina truly survive in a form that will allow the restoration of naturalistic visual perception?

Stages of retinal degeneration

Retinal remodelling during the inherited retinal degenerations is an area of active research, with multiple changes described both in humans(76) and animals. The *Pde6b^{rd1}* mouse(77, 78) (see Chapter 3, a mouse with retinal degeneration) is perhaps the best studied model and so will form the basis for much of the following discussion. Neuro-retinal changes in retinal degeneration have been reported not only at the morphological & synaptic connection level (79-82) but also in electrophysiological responses (82-86), receptor(87-90) and gene expression(91). Consensus has settled around dividing the process of remodelling into four distinct phases (85)(Table 1-1) with an initial pre-disease state (phase 0), followed by a latent period of photoreceptor stress with changes in biochemistry and early anatomical changes (phase 1), a second period characterised by large photoreceptor death, marked changes in cell morphology, intraretinal connections as well as physiological and receptor expression changes (phase 2), with the transition to the final stage (phase 3) characterised by the loss of ordered retinal layer and the formation of a “seal” of glial cells to maintain the blood retinal barrier.

Morphology and Synaptic Remodelling

Morphological and synaptic changes are present even early in degeneration with photoreceptor outer segments becoming truncated with dendrites beginning to retract from bipolar and horizontal cells with concurrent neurite extension(81-83). As degeneration progresses, gross morphological changes become apparent as normal lamination is lost with photoreceptor death and migration of neural cells within the retina(85); dendrite retraction continues with many being lost altogether(79).

Neurite sprouting also continues with deafferented cells making abortive attempts to regain input: for example rod bipolar cells can be seen forming synapses at the cone pedicle after death of their associated rods(92-94) while dying rods can be seen to send neurites out throughout the retina(85). By late (phase 3) degeneration, most photoreceptors have been lost and native dendrites of retinal neurites have retracted. Glial and vascular activity is prominent as hypertrophic Müller cells form a seal between the RPE and remnant retina: the glial seal(80). While there is some evidence that returning afferent light signalling to the retina, may be able to reverse some of this synaptic and morphological remodelling at an early stage of degeneration(95) – it may be more difficult to do so once such advanced glial changes have occurred.

Phase 1	Phase 2	Phase 3
Photoreceptor stress • Truncation of rod outer segments • Truncation of cone outer segments • Photoreceptor cell death begins • Rod neurite extension • Dendrite retraction Transmission alternation • Amino acid neurotransmitter changes • Reduction and transposition of mGluRs on ON-bipolar cells • Aberrant expression of functional iGluRs on ON-bipolars Glial cell activation begins • Conservative reactive gliosis	Photoreceptors • Large scale rod death • Cone death increases Transmission • iGluR aberrant receptor expression downregulated after most of cones are lost • Downregulation in amacrine cell NMDA activation characteristics Changes in neural retina • Retraction and loss of dendrites and neurite extension of bipolar and horizontal cells • Cell kinesin • Inner retina cell death begins Glial Activation • Collapse of photoreceptor layer leads to glial seal formation • Conservative reactive gliosis in outer retina subsides; but continues in the inner retina	Photoreceptors • Rod bipolar cells make peripheral contacts with cone pedicles if they survive. Transmission • Further loss of remaining glutamate receptor function Changes in neural retina • Sprouting and formation of new synaptic sites • Cell kinesin disrupts orderly retinal layers in the inner plexiform layer; pigmented epithelial cells invade the inner retina • Progressive retinal cell death Glial Activation • Vessel invasion • Glial seal completed • Patchy loss of Müller cell glutamine synthetase immunoreactivity, expression of potassium channels and other evidence of proliferative gliosis

Table 1-1 Retinal changes evident in the *Pde6b^{rd/rd}* retina (a murine model of retinal degeneration). Modified from (85).

Remodelling of neurotransmission

Remodelling in the degenerating retina, would appear not to be limited to morphology of cells, but to their internal workings – particularly their release and responses to neurotransmitters. As the dendrites of horizontal and amacrine cells retract during degeneration, levels of their inhibitory neurotransmitters (GABA and glycine) are seen to fall across the retina (96). This could be particularly concerning for the health of surviving retinal cells as such a reduction in inhibitory signals, without a similar drop in excitatory transmission, can lead in other neural systems to excitotoxicity(97) and ultimately cell death.

Indeed, marked changes in glutamatergic transmission have been observed in the degenerating retina. With the loss of photoreceptors down regulation and redistribution of the metabotropic glutamate receptors (mGluR6, Figure 1-6) away from the synaptic cleft of ON-bipolar cells is seen. Concurrently (depolarising) ionotropic glutamate channels(90, 98) are upregulated in the same cells, to the degree that these cells functionally reverse their “class” ie. switch from “ON” to “OFF” type responses(87, 89). These ionotropic receptors themselves are in turn then lost, but not until late in the process of degeneration(85).

Possible down regulation of mGlu6 in ON-bipolar cells is significant beyond electrophysiological class switching: mGlu6 expression is localised to ON-bipolar cells, therefore its promoter (*Grm6*) has been widely used as tool to achieve bipolar cell specific expression of genes (such as optogenetic tools) delivered by viral vector(99-106). This may contribute to difficulties in achieving useful levels of expression of optogenetic tools in the ON-bipolar cells of degenerate retinas(105) as part of an experimental approach to treating inherited retinal degenerations in animal models.

However, little is known regarding changes in gene expression in this cell type during degeneration – are levels of *Grm6* (the gene coding for the mGluR6 receptor) expression actually reduced (with resulting implications for the use of the *Grm6* promoter to deliver genes to ON-bipolar cells) or are the functional changes seen(98) due rather to redistribution of receptors away from the synaptic cleft?

Implications of neural plasticity in degeneration

While indeed much is still unknown regarding changes to neural cells of the retina during degeneration – especially at the genetic level of individual cells – it can be appreciated that where extensive study has been made (for example in terms of anatomy) marked and wide-ranging changes are apparent. Whether or not these are detrimental to clinical survivor cell stimulation approaches remains uncertain: therefore, further investigation of individual cell types (such as ON-bipolar cells) at the level of gene expression are important to ensure that they retain expression profiles likely to be conducive to survivor cell stimulation. Reassuringly, in animal models, various objective and subjective quantifiers of visual function including electrophysiology, pupil and other behavioural paradigms appear to return with survivor cell stimulation(74, 107).

Interestingly in clinical trials, human retinal survivor cell stimulation is being reported to achieve good cortical fMRI responses in visual tasks both after gene replacement therapy(108) and visual prosthesis implantation(109). Together with improvements in functional visual paradigms in previously ‘no perception of light’ (NPL) patients(110), this suggests that the balance of plasticity is at least favourable enough to give a crude level of vision some years after the initial loss of light sensitive inputs.

Treatment strategies for Inherited retinal degenerations

Indeed, these findings do confirm that stimulation of the surviving retina in the IRDs may provide an option to return light perception to those blind from IRDs. Several approaches have been used to tackle this problem with varying outcomes, advantages and disadvantages. In the following section, we will further explore the most promising of these: retinal prostheses, stem cell approaches, gene replacement therapy and optogenetics.

Retinal Prostheses

The approach with the longest history is that of direct electrical stimulation of the retina, with reports as early as the 1950s of attempts to induce the perception of light (termed phosphines)(74). Implantable electrode arrays are now commercially available(74, 111, 112) which stimulate a small area of the diseased retina in a retinotopic fashion introducing a degree of spatial fidelity to percepts achieved by patients.

Stimulation in implants under clinical evaluation is achieved by two main routes: subretinally, such as in the IMI (113, 114) and epiretinally such as in the Argus II® system(74, 111) (Figure 1-11). The latter uses a spectacle mounted camera to transduce visual scenes, which are then processed to extract and enhance relevant details (such as edges) and remove information that cannot be communicated by the array (such as colour)(111) (Figure 1-12). The signal is then communicated along a surgically implanted cable to an array on the surface of the retina delivered via a scleral tunnel after vitrectomy thus allowing stimulation of surviving retinal ganglion cells.

Conceptually, the ON-bipolar cells are the natural target for survivor stimulation: they are the highest order surviving retinal neuron after photoreceptor loss and so the epiretinal (and so RGC dominant) stimulation of this implant could be seen as a disadvantage compared to a subretinal stimulator(74). However, the less difficult surgical approach and low energies required to stimulate the cells when the array is apposed directly to the retinal nerve fibre layer (RNFL) is advantageous for survival of the electrodes, with persevering safety at five years following implantation in a phase I trial(110, 111, 115, 116). Practically, In the size of the array itself will limit the degree of visual field that can be restored, but in addition, the spacing and size of electrodes on such implants are large in comparison to either bipolar or RGC receptive fields, and therefore unlikely to lead to single cell stimulation regardless of where the implant is placed.

These metrics are limited by the issue of current spread and the harshness of the operating environment for electronic equipment(74). Therefore, while retina prostheses have proven the principle of survivor cell stimulation in the degenerate retina, an active search continues for methods that can move towards single cell stimulation in such a way as to preserve as much intrinsic retinal processing as possible.

Stem cells

An alternative approach to stimulating surviving cells in the degenerate retina is provided by stem cell transplantation. Stem cells are capable of indefinite self-renewal and differentiation into a variety of specialised daughter cells types (117).

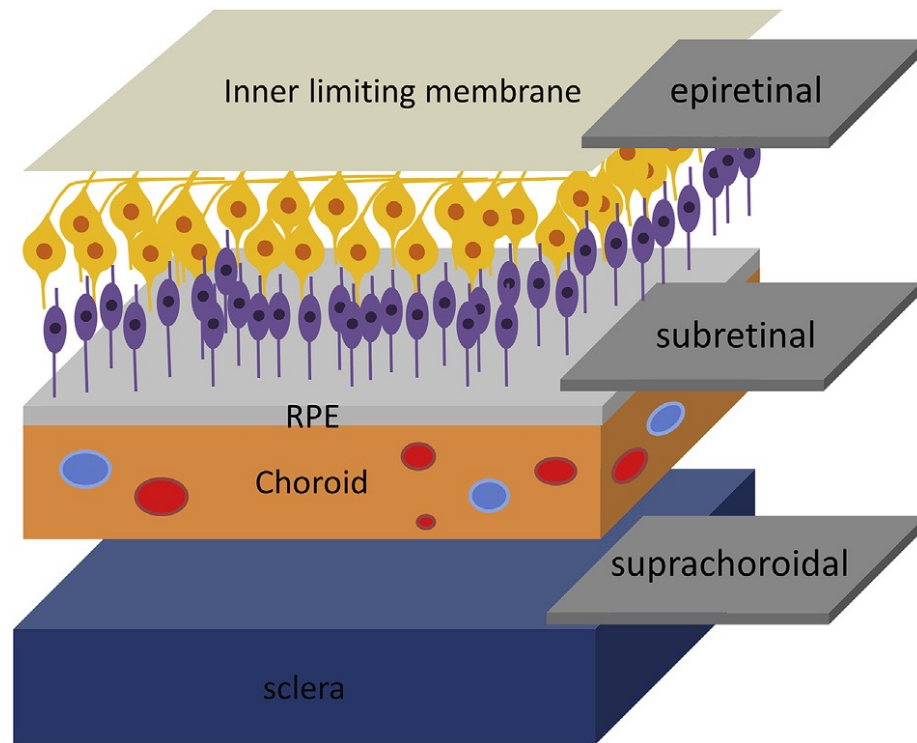


Figure 1-11 The Epi- and subretinal planes in relation to the neural retina (adapted from (74))

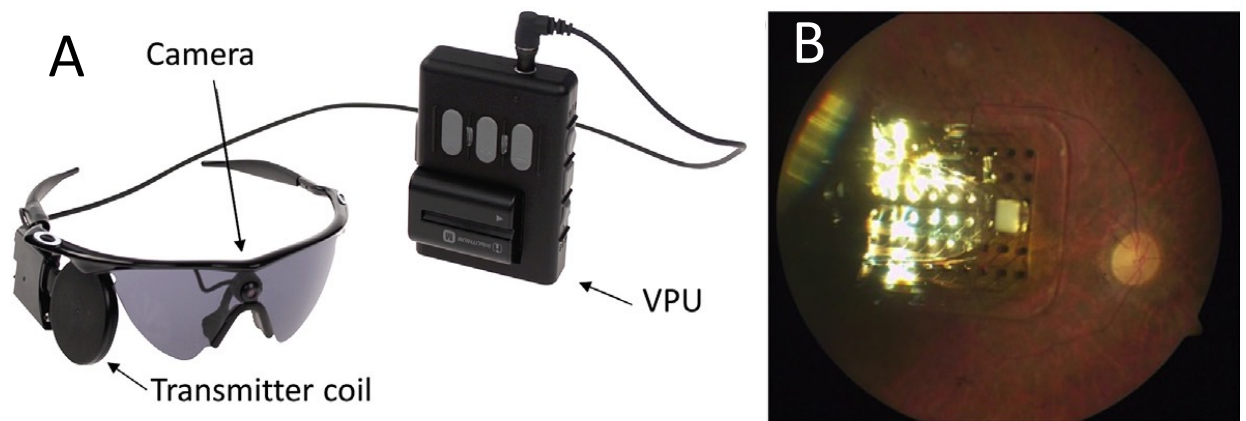


Figure 1-12 The Argus II® system consists of an external spectacle mounted camera and processing unit which transmit to an electrode array implanted in the epiretinal plane. (adapted from (74))

Therefore, the therapeutic hypothesis would be that if these cells are artificially introduced into diseased retinæ, they can differentiate and replace the function of lost cells (such as photoreceptors) (118).

One difficulty in using this approach to replace photoreceptors is the poor integration of transplanted cells into the retinal neural network (119-121). Cells previously thought to integrate neuronally by synaptic communication(122) have been shown rather to represent host cells cytoplasmically *fusing* with the delivered stem cells(121). Despite this, alternative uses for stem cells in the retina (such as to support the retinal pigment epithelium) have proven safe in phase I human trials(118, 123-125).

Gene replacement therapy

Rather than replacing the stimulation provided by photoreceptors, gene replacement therapy aims to preserve and restore responses of those surviving photoreceptors by delivering a replacement “good” copy of the causative mutated gene. This approach is conceptually attractive – it corrects the underlying genetic error in order to prevent further photoreceptor loss. In counterpoise, it could be said to be clinically unattractive: intervention is required early in the disease course, before photoreceptor death, when patients still have good vision and is not applicable to the 30 to 50% of patients with an IRD where the specific genetic mutation has not been determined(68). In addition, each treatment developed - at considerable effort and expense – is only applicable to a single recessive (or loss of function) genetic diagnosis(126). Dominant disease can lead to a “gain of function” mutation producing deleterious protein which would require to be additionally silenced for example by

siRNA(127). Therefore, the central strength of gene replacement therapy – specificity - is ultimately its central weakness.

Despite these potential disadvantages, the attractiveness of the retina as a model for developing gene therapies has proven phenomenally successful with an adenoassociated virus delivered gene replacement therapy now available for RPE65(128) associated disease. While there has been discussion as to the persistence of effect with one group in particular noting reduced functional efficacy at 3 years(129), even if the benefit is short lived, Voretigene® has successfully proven the principle of gene delivery to the degenerate human retina using adenoassociated viruses. Indeed AAV based treatments for other inherited retinal degenerations are now in clinical trial, including for Choroideraemia (REP1) (130), X-linked retinoschisis (RS1)(131), Retinitis Pigmentosa (MERTK)(132) and Achromotopsia (CNGA3) (NCT02610582).

Adenoassociated virus

The adenoassociated virus (AAV) is a non-human pathogen of the Parvoviridae family incorporating a 4.7kbp single strand DNA genome consisting of two open reading frames: replication (*rep*) & capsid (*cap*) along with two symmetric inverted terminal repeats (ITRs)(133). Their ability to transfect non-dividing cells and relative ease of recombinant manipulation have led to their extensive use for retinal gene transfer. In recombinant production(134), the native *rep* and *cap* are replaced by a payload gene. For effective transduction of target cells, this cannot exceed the size of the native genome c. 4kbp (proving an additional challenge for larger genes such as ABCA4 (7.4kbp), causative in Stargardt's disease(135)).

Twelve serotypes of AAV have been isolated(136); with affinities for different target cells varying depending on the capsid and viral genome properties(137). Capsids of one serotype can be used with genomes of another – for example AAV2.6 uses a serotype 2 genome and a type 6 capsid(136). This flexibility, along with approaches such as directed evolution and rational mutation design(101, 138-140) have led to the development of over 100 viral capsid variants(136) with useful affinities for specific cell types. For example, an AAV2.2 with four point mutations (Y272,444,500,730F(134, 138, 141)) has a demonstrated affinity for retinal cells – including bipolar cells and photoreceptors following intravitreal injection – and is used during this study (see Chapters 5,6 & 7).

Specificity for a particular cell type can be additionally influenced by the choice of promotor included with the payload gene: high efficiency, generic neuronal promoters (e.g. CBA, hSyn) can be included to encourage robust expression in as many types of cell as possible; alternatively, promoters with cell specific activity (such as L7 – see Chapter 3) can be incorporated to restrict expression to a single cell group. In general, there is often a trade-off between specificity and efficiency – increasingly specific promoters can be decreasingly efficient in driving expression in target cells compared to generic alternatives.

In addition to the properties of the viral vector used, the location and properties of target cells themselves can affect the ease with which AAVs can enter particular retinal cell types. For example, physical barriers (such as the glial seal described above), or route of injection - sub retinal or intravitreal can alter markedly the concentration of virus reaching target cells (such as bipolar cells deep within the retina). This is a consideration beyond proximity: intravitreal injections, although able

to spread and transfect the whole retina, can have increased rates of immune reaction(142). There can also be difficulty in translating – particularly from mouse models where the relative volume of the vitreous is much smaller than in larger mammals (e.g. humans). In order to enter cells, AAVs become attached to heparin sulphate proteoglycans (HSPGs) on the cells surface(143-147), before binding to the AAV receptor and entering the cell(148). This can create biochemical obstacles to transfection of the degenerate retina: the inner limiting membrane is known to be rich in HSPGs and so may sequester AAV before it can reach the target cells(149) and indeed, as described in Chapter 4, there may be changes to HSPG expression in the context of retinal degeneration.

Optogenetics

The adenoassociated viruses can be used not only to deliver replacement genetic material, but also *supplementary* material – such as optogenetic tools. *Optogenetics* refers to the light dependant modulation of cell properties (such as membrane potential) by expression of light sensitive proteins (“optogenetic tools”) within them(150). The field has rapidly developed since initial descriptions of light gated ion channels in the early 2000s(151-153)(see Chapter 6) with the approach now extensively used in basic neuroscience as a powerful tool to manipulate neurons *in vivo*(151, 154, 155) as well is in other areas where excitable tissue is investigated such as cardiology(156, 157).

Optogenetics are therefore particularly attractive for application to the IRDs - where the primary insult is a loss of light sensitive cells, with relative preservation of their associated neural processing architecture. By stimulating surviving cells, optogenetics could provide a mutation independent gene therapy technique, suitable

for use in the 40% of clinical IRD cases without a clinical diagnosis(68) and even, unlike gene replacement therapy, replacing lost function in later stages of the disease after photoreceptors have been lost.

Another advantage of AAV delivered optogenetics is likely to be the achievable resolution – as discussed, retinal chips are limited in the size and spacing of their stimulating electrodes: optogenetics in theory allows stimulation of independent cells. In combination with serotype and promoter selection, it could also be preferentially deployed to specific cell types (for example ON-bipolar cells, the most upstream surviving cell in retinal degeneration) in order to preserve residual intraretinal processing and with the prospect of more useful visual information being communicated to the brain – however this is only beginning to be investigated (see Chapter 5).

The variety of optogenetic tools now available also brings opportunity for more useful responses with tools of varying sensitivities, spectral peaks and kinetics(107) available. Many have been deployed in the degenerate retina in vivo: from native and recombinantly microbial light gated ion channels(152, 153) to native vertebrate opsins (rhodopsin, cone opsins and melanopsin(103, 106, 158-160)) and modified variants(105) – a full review of which is presented in Chapter 6.

However, the resultant responses of such tools have not been described in retinæ devoid of native photoresponses – most previous studies make use of retinal degenerate models retaining their own native melanopsin. While this may be closer to the clinical situation in IRDs: at the preclinical stage, a description of the character of the restored light responses from each approach, in isolation from any residual native responses is paramount. This is especially important with melanopsin based therapies,

given a likely contribution from the ipRGC to image forming vision, in order to differentiate exogenous from (potentiated) native responses (See Chapter 3).

Questions

Several unresolved questions are therefore presented when we consider potential optogenetic approaches to visual restoration in the IRDs.

- Do ON-bipolar cells, the most upstream survivors during degeneration, maintain a gene expression profile conducive to the function of optogenetic tools during retinal degeneration?
- If so, is targeting these cells specifically likely to provide improved response characteristics compared to a non-specific, high efficiency expression of optogenetic tools in the degenerate retina?
- How will responses compare when different optogenetic tools are specifically targeted to ON-bipolar or other cell types?
- Can novel cell specific promoters be used to target ON-bipolar cells with optogenetic tools using adenoassociated viral vectors?

Thesis Aims

These questions inform the overall aim of this research project: to compare different optogenetic approaches to visual restoration in a model of inherited retinal degeneration by:

1. Selecting, preparing and validating a suitable retinal degenerate animal models, additionally devoid of native melanopsin and allowing cell specific expression of optogenetic tools.
2. Determine if ON-bipolar cells are likely to maintain a gene expression profile permissive for use as optogenetic targets in the context of retinal degeneration.
3. Compare the efficacy of cell population specific delivery of melanopsin as an optogenetic tool to a non-specific approach in restoring light responses in an IRD model.
4. Directly compare the relative properties of three candidate optogenetic tools, targeted to specific retinal cell populations, in an IRD model
5. Develop a directly translatable method of optogenetic tool delivery specifically to ON-bipolar cells of the degenerate retina.

Chapter summary

In order to address these aims:

1. Experiments in Chapter 3 describe the development and validation of mouse models to allow expression of optogenetic tools in specific sub populations of cells in the context of a degenerate retina without native melanopsin.
2. Experiments are described in Chapter 4 which determine the gene expression profiles of retinal ON-bipolar cells in the context of retinal degeneration. The changes are explored over the course of degeneration and related to these cells' suitability for optogenetic targeting.
3. Experiments in Chapter 5 demonstrate a comparison between cell-specific targeting and non-specific delivery of an optogenetic tool to a degenerate retina devoid of all native photoresponses.
4. Chapter 6 describes the comparison of different optogenetic tools targeted to specific cell populations, again in degenerate retina without native melanopsin responses.
5. Chapter 7 investigates the potential of compressed AAV compatible cell specific promoters to allow ON-bipolar expression of an optogenetic tool – a requirement for clinical translation of targeted optogenetics.

Chapter 2 – Material and Methods

Animals

Mice

Mice were housed by the biomedical services section of Oxford University in accordance with institutional, local and national protocol. All mouse work was carried out under the Animals (for scientific procedures) Act (1986). The project licence holder was Prof. S. Peirson of the Nuffield Department of Clinical Neuroscience, University of Oxford (Project licence No. 30/3371). I hold a personal licence (No. I3C8B2FA6) under the aforementioned Act. The above was in accordance with the Association of Research in Vision in Ophthalmology (ARVO) statement on the use of laboratory animals.

Practically, mice were group housed (up to 6) in individually ventilated cages with *ad libitum* food and water at a temperature of 21°C, 60% humidity, on a 12:12 hour light-dark cycle, lights on 0700h. Mice were sourced from our own colonies and those of other groups as detailed (Table 2-1). Standard cage enrichment was provided (cardboard tube, sawdust, bedding & cardboard “house”). Cages were cleaned weekly. Detailed description of the strains used, breeding strategies and validation is discussed in Chapter 3. Mice were routinely ear clipped for identification purposes, the notches of skin tissue from the ears being preserved for genotyping.

Line	Genetic Background Strain	Source
<i>Pde6b</i> ^{rd1/rd1}	C3H/HeN	Kind gift from group of R Foster, Oxford University
<i>Opn4</i> ^{tm1Yau}	C57BL/6J	Kind gift from group of R Foster, Oxford University
Tg(L7-Cre)	FVB	Kind gift from group of R MacLaren, Oxford University
Tg(grk4-Cre)	C57BL/6J	Kind gift from group of P Oliver Oxford University

Table 2-1 Sources of mouse lines used in breeding program – please see Chapter 3 for further detail(77, 78, 161-164).

Tissue Preparation for genotyping

In order to extract DNA for genotyping assays, mouse ear clips were incubated with 300µl of sodium hydroxide solution (50 mmol/l) at 100°C for 45 minutes before being vortexed, and spun 13,500g for 6 minutes. 25µl of Tris buffer, pH8 (500mmol/l TRIZMA base, 500mmol/l TRIZMAS HCL pH was adjusted with 13 mol/l HCL (All Sigma Aldrich, Gillingham, UK)) was added to neutralise before a further centrifugation at 13,500g for 6 minutes.

Polymerase chain reaction for genotyping

DNA samples were then incubated with a PCR master mix and primers as described in Table 2-2, Table 2-3 and Table 2-4 and cycled as described in table Table 2-5. Protocols using Immored® (Bioline, London, UK) were then run on a 2% agarose gel incorporating 4 µmol/l ethidium bromide (Sigma Aldrich, Gillingham, UK) in Tris-acetate-EDTA (TEA) buffer (Severn Biotech, Kidderminster, UK) at 125volts for 25 minutes. SYBR Green® protocols underwent a melt curve protocol to determine the melting temperature of the product (Table 2-11).

Reaction component	Quantity (μl)
ImmoRed®	6.25
H2O	4
Primer 1	0.3125
Primer 2	0.3125
Primer 3 (optional)	0.3125
Primer 3 (optional)	0.3125
DNA	1

Table 2-2 Polymerase Chain Reaction (PCR) components used for genotyping *Pde6b^{rd1/rd1}*, *Tg(Cre)*, *Tg(YFP)*, *Oxr^d* and *Gpr179^{nob}*

Reaction component	Quantity (μl)
SYBR Green	10
H2O	7.5
Primer 1	0.25
Primer 2	0.25
DNA	2

Table 2-3 Polymerase Chain Reaction (PCR) components used for genotyping *Opn4^{tm1Yau}* (*LacZ*) & *Opn4⁺*

Allele	Primer	Sequence	Product size or product melt temperature Tm (bp or °C)	
<i>Pde6b^{rd1}</i>	1	TGACAATTACTCCTTTCCCTCAGTCT		
	2	GTAAACAGCAAGAGGCTTTATTGGGAA	<i>Pde6b^{rd1}</i>	550
	3	GCATTAATTCTGGGGCGCATG	<i>Pde6b⁺</i> (wildtype)	400
<i>Tg(Cre)</i>	F	GCGGTCTGGCAGTAAAACTATC		
	R	GTGAAACAGCATTGCTGCTCACTT		
	F (control)	CGTAGGCCACAGAATTGAAAGATCT	<i>Tg(Cre)</i>	101
	R (control)	GTAGGTGGAAATTCTAGCATCATCC	Control	324
<i>Tg(YFP)</i>	1	GGAGCGGGAGAAATGGATATG		
	2	AAGACCGCGAAGAGTTTGTC	<i>Tg(YFP)</i>	320
	3	AAAGTCGCTCTGAGTTGTTAT	Wildtype	603
<i>Gpr179^{nob}</i>	1	TGTGCCTGGGTATCTGTTGA		
	2	GCATGTGCCAAGGGTATCTT	<i>Gpr179^{nob}</i>	400
	3	GCTTACACACTTACACAGATAGATG	<i>Gpr179⁺</i> (wildtype)	113
<i>Opn4^{tm1Yau}</i> (<i>LacZ</i>)	F	AAGCAGTCAGCAGCCCAAAG		
	R	TGTCACCCTCTGGTCTTGC	<i>Opn4^{tm1Yau}</i> (<i>LacZ</i>)	86°C
<i>Opn4⁺</i>	F	AGAAGTGGCTTTGGGGA		
	R	GCAGAAGGCATAGAACTCG	<i>Opn4⁺</i>	80.2°C
<i>Oxr^d</i>	F	TGGAATATCCAGTCTCACC	<i>Oxr^d</i>	500
	R	CAGAGCACAGAAGCGTCTCA	<i>Oxr⁺</i> (wildtype)	300

Table 2-4 Oligonucleotide primers used in genotyping PCR protocols and their properties, all were custom synthesised by Sigma Aldrich, Gillingham, UK. bp = base pairs.

Step	<i>Pde6b^{rd1}</i>			<i>Tg(Cre)</i>			<i>Tg(YFP)</i>			<i>Oxr^d</i>		
	Temperature (°C)	Time (mins:secs)		Temperature (°C)	Time (mins:secs)		Temperature (°C)	Time (mins:secs)		Temperature (°C)	Time (mins:secs)	
1	95	10:00	Activation	95	10:00		95	10:00		95	10:00	
2	94	00:30	Denature	96	00:20		94	00:30		94	00:20	
3	60	00:30	Anneal	65	00:15		55	00:30		64	00:15	
4	72	01:00	Extend	72	00:10		72	00:30		72	00:10	
5	repeat from step 2 x40			repeat from step 2 x40			repeat from step 2 x40			repeat from step 2 x40		
6	72	10:00	Repeat	72	10:00		72	10:00		72	10:00	
7	21	∞	Final Extension	21	∞		21	∞		21	∞	
			Hold									

Step	<i>Gpr179^{nab}</i>			<i>Opn4^{tm1Yau} (LacZ)</i>			<i>Opn4⁺</i>		
	Temperature (°C)	Time (mins:secs)		Temperature (°C)	Time (mins:secs)		Temperature (°C)	Time (mins:secs)	
1	95	10:00	Activation	95	10:00		95	10:00	
2	94	00:45	Denature	95	00:20		95	00:20	
3	56	00:30	Anneal	60	01:00		66	01:00	
4	72	00:20	Extend	72	00:20		72	00:20	
5	repeat from step 2 x40			repeat from step 2 x40			repeat from step 2 x40		
6	72	10:00	Repeat						
7	21	∞	Final Extension	21	∞		21	∞	
			Hold						

Table 2-5 Polymerase Chain Reaction protocols used for genotyping.

Gene array

The isolation and processing of RNA from ON-bipolar cells, as well as the subsequent gene array was carried out by D Hickey as previously described(165), the results of this work were included in Chapter 4 for context.

Systematic literature review and prioritisation of candidate genes

The list of differentially expressed genes from this gene array experiment was processed as described in Chapter 4. Genes were grouped by ontological label based on a search of the NCBI “Gene” database:

(Gene name[gene]) AND (Mus musculus[orgn])

Following this, a systematic literature review of the literature was carried out and used to prioritise candidate genes for further investigation. The Medline® database was searched:

*((((Gene name[Title/Abstract]) AND retina[Title/Abstract]) AND
("1900"[Date - Publication] : "3000"[Date - Publication])) AND
"english"[Language])*

Initially titles were reviewed and genes with no relevant literature identified and removed from further investigation. Review of abstracts then allowed primary literature relevant to each gene’s retinal expression to be obtained and reviewed. Within each group of related ontology annotations, primary literature was used to

guide prioritisation of a genes for initial further investigation. This ranking was based on a subjective assessment of the primary literature to give a score from five in each of three domains:

1. The likelihood of involvement with the primary light signal transduction cascade in bipolar cells
2. Potential clinical implications
3. Actions in retinal remodelling

The gene with the highest rank score out of 15 in each group was taken forward for further investigation.

Adeno-associated Virus Vector Production

Cloning

Two compressed L7 promotor constructs (L7-6(166) and ple155(167)) were kindly received from Prof. Hirai, Gunma university (L7-6) and Prof. Simpson, University of British Columbia (pEMS1986, Addgene plasmid # 49117; <http://n2t.net/addgene:49117> ; RRID:Addgene_49117). These constructs were cloned onto our own AAV insert backbone containing hOPN4 (see Chapter 7 for full discussion) by the Genesys commercial laboratory (Genescript, Hong Kong). Promoters were cloned into the backbone by amplifying the inserts (Ple155 & L7-6) using PCR, digesting the backbone using MluI and KpnI restriction enzymes and ligating the two together. Final plasmids were validated by restriction digests and Sanger sequencing. This sequencing was repeated inhouse with sequencing primers as described in the Appendix. Full details of methodology and validation reports are included in the Appendix.

Plasmid Preparation

Bulk preparations of plasmids were required for large scale transfections. In all cases, a glycerol stock stab was cultured overnight at 37°C in 1.5 litres of Ampicillin (1:1000) Luria broth (Invitrogen, Carlsbad, USA) on an orbital shaker (Inova 42, New Brunswick Scientific, New Brunswick, USA). Plasmid was then purified from this culture following the manufacturer's instructions in a proprietary "Mega-prep®" kit (Invitrogen, Carlsbad, USA). Resultant plasmids were then sequenced using Sanger sequencing (primers listed in Appendix) by an outside company (Source Bioscience, Oxford, UK) to ensure faithful replication.

Cell culture

General protocols

All cell culture was carried out using aseptic no touch technique (ANTT)(168) in a HEPA filtered cell culture laminar flow hood. HEK293T were cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with L-glutamine (2nmol/l), penicillin (100unitsml⁻¹), streptomycin (110µgml⁻¹) (all Sigma Aldrich, Gillingham, UK) and 10% Fetal Bovine Serum (Invitrogen, Carlsbad, USA), passaged before confluence and split to reseed at a 1:10 ratio.

AAV production

Adenoassociated virus is produced by co-transfecting HEK293T cells with three plasmids:

1. The insert plasmid - containing the gene of interest along with promotor and inverted terminal repeats (ITRs)
2. The capsid plasmid - contains the rep and cap (capsid) genes
3. The helper plasmid – contains viral genes necessary for replication

The co-transfected cells are then lysed and virus purified and concentrated from the lysate using a protocol based on that of de Silva *et al* (134).

Co-transfection

Two multi-layered “hyper-flasks” (Corning inc, Corning, USA), representing approximately 3,440cm² of culture surface were seeded with HEK293T cells such that they were 80% confluent after 72h of culture with enriched DMEM (as above). The molar ratios of helper:capsid:insert plasmids was constant (detailed in Table 2-6 and Table 2-7).

The appropriate masses of plasmid were combined in a 50ml plastic conical bottom centrifuge tube (Falcon tube) and made up to 20ml with sodium chloride solution (150mmol/l). Similarly, 2.25ml of 10mmol/l Polyethylenimine (PEI) was made up to 20ml in a similar tube using sodium chloride solution for use as a transfection reagent. This PEI solution was then added dropwise to the plasmid mixture with constant agitation and the whole mixture allowed to rest for 20 minutes at 21°C with periodic agitation.

The transfection mixture was then added to 1 litre of enriched DMEM (described above) and mixed gently which was used to replace the culture media in the two “hyper-flasks.” These flasks were then incubated for three days at 37°C in an incubator.

	Plasmid Size (kbp)	Formula
Helper	18.7	18.7 x R
Capsid	7	7 x R
Insert	t	t x R
Total	K	1000μg
Ratio "R"	1000/K	

Table 2-6 The formula used to calculate the molar ratio of each plasmid needed to transfect cells for AAV production

	EF1α-DIO-hOpn4	CBA-hOpn4	hSyn-DIO-ReaChR	EF1α-DIO-Rhodopsin
	μgrams			
Helper	574.9	566	578.4	525.6
Capsid	215.2	214	216.5	249.1
Insert	209.8	220	205.1	225.3
	kbp			
t=	6.825	7.401	6.630	6.651

Table 2-7 The quantity of each plasmid used in this investigation. The number of base pairs (t) in each plasmid is also supplied in kilo base pairs (kbp).

Lysate		Iodixanol	NaCl	PBS-MK	H2O	Phenol Red
15%	Fraction	mlitre			μlitre	
25%	15%	2	1.6	1.6	2.8	x
40%	25%	2.5	x	1.2	2.3	10
	40%	3.35	x	1	0.65	x
	60%	5	x	x	x	10

Table 2-8 Iodixanol gradients were constructed in polypropylene ultracentrifuge tubes by layering fractions of increasing density below lighter fractions. Lysate was pipetted atop the fraction, the tube capped and spun at 350,000g for 90 minutes. After this, the 40% fraction (containing AAV) was extracted by piercing the tube with a needle as marked with the arrow. NaCl – sodium chloride 5 mol/l; PBS-MK – 5x strength PBS with 10mmol/l MgCl₂, 25mmol/l

Cell lysis

After the three-day incubation, “hyper-flasks” were manually agitated to loosen cells into the media which was then centrifuged at 4,000g for ten minutes at room temperature. The supernatant was discarded and 30ml of lysis buffer (150mmol/l NaCl, 1mol/l Tris(hydroxymethyl)aminomethane (both Sigma Aldrich, Gillingham, UK)) added to the cell pellet along with 2ml of protease inhibitor cocktail (Roche, Basel, Switzerland). Four freeze thaw cycles were then carried out.

Virus Purification

Defrosted lysate was incubated with 50units ml⁻¹ Pearce universal nuclease (ThermoFisher, Loughborough) to break down residual free DNA and reduce solution viscosity. The lysate was then centrifuged for 20 minutes at 3,700g. The supernatant was then added to the top of layered iodixanol gradients pre-layered in disposable polypropylene ultracentrifuge tubes (Beckman Coulter Ltd, Brea, USA) (Table 2-8).

Gradients were then centrifuged at 350,000g for 90minutes at 4°C. Following this, tubes were removed and a 19G needle and 1ml syringe were used to puncture each tube at the interface between the 40 and 60% fraction. The 40% fraction, containing the virus, was removed. This isolate was then subjected to centrifugal filtration concentration.

The isolate was added to an Amicon Ultra-15® tube (Millipore, Burlington, USA), initially spun for 30 minutes at 3,000g to reduce volume, then topped up with PBS and spun for 2 hours at 3,000g. This PBS wash was repeated twice more and on the final wash, the isolate was spun at 3,000g until the volume of concentrate reduced to 500µlitre (around 3 hours, varying with each preparation). After removal and

aliquot of the concentrate to -80°C storage, the filter was washed with 500µlitre of fresh PBS, and this wash also stored.

As indicated in the text, some of the preparations used in this thesis were made using a similar protocol by an outside commercial laboratory (University of North Carolina at Chapel Hill).

Adeno-associated virus validation

Virus was validated in terms of concentration of viral genomes, purity of protein content and ability to transfect cultured retinal cells and successfully produce protein. Viruses that passed these tests, were taken on for intravitreal injection with subsequent immunohistochemical studies to assess their *in vivo* efficacy.

Titre

Quantitative polymerase chain reaction was performed to determine viral genome concentrations in reference to a standard serial dilution of known plasmid concentration, using primers directed against the inverted terminal repeat (ITRs) of the AAV (see Table 2-9).

1µl of viral concentrate was incubated with 1µl of DNase, 1µl of buffer and 7µl of molecular grade water before gentle mixing and incubation for 1 hour at 37°C. The mixture was then diluted with a further 90µlitre of molecular grade water and heated to 95°C for 15 minutes to denature the enzyme. This provided a 1:100 sample stock solution from which further serial dilutions could be made(1:100, 1:1000, 1:10000, 1:100,000) in triplicate in a qPCR plate along with an appropriate quantity of master mix (Table 2-9, Table 2-10). In addition, a sample of insert plasmid was measured using a spectrophotometer (nanodrop®, Thermo Fisher, Loughborough, UK) to determine its molar concentration, and similar triplicate serial dilutions (1:10, 1:100, 1:1,000,

1:10,000, 1:100,000) were set up on the same qPCR plate as the sample to be tested, with the same master mix, forming a reference range.

This combined plate was then run, using standard PCR protocols (Table 2-11) and recorded C_T values for each well were exported to an MS Excel spreadsheet template for further processing. For each triplicate, if any C_T value was more than 0.5 divergent from the others, it was excluded and a mean taken of the remaining values. Values from the reference wells could be used to produce a line of best fit using linear regression, the equation of which could be used to calculate the molar concentration, and thence converted to viral genome concentrations, based on the C_T values from the sample stock dilution wells (Figure 2-1).

Purity

Presence of the expected three viral proteins (VP1, VP2 and VP3) without other protein contaminants was confirmed using denaturing SDS-PAGE electrophoresis(169). Viral concentrate was diluted 1:10 with Laemmli's buffer (Sigma, Gillingham, UK) and heated to 95°C for 10 minutes before being run on a 10% Tris-HCl gel (Hercules, USA) at 125 Volts and stained with EZBlue® (Sigma, Gillingham, UK). Pure samples demonstrated only three bands after staining corresponding to 87, 72 & 62 kDa – the size of VP1, VP2 & VP3 (Chapter 5).

Primer	Sequence
ITR F	GGAACCCCTAGTGATGGAG
ITR R	CGGCCTGAGTGAGCGA

Table 2-9 Oligonucleotide primers used in qPCR based viral titre

Reaction component	Quantity (μl)
SYBR Green	10
H2O	7.5
ITR F	0.25
ITR R	0.25
DNA	2

Table 2-10 Reaction components used in qPCR based viral titre

		AAV titre	
		Temperature (oC)	Time (mins:secs)
1	Activation	98	03:00
2	Denature	98	00:15
3	Anneal	60	00:30
4	Extend	72	00:10
5	Repeat	repeat from step 2 x40	
6	Denature	98	00:15
7	Anneal	60	01:00
8	Melt Curve	Increase in 0.3°C steps to 98°C	
9	Hold	21	∞

Table 2-11 Reaction protocol used in qPCR based viral titre

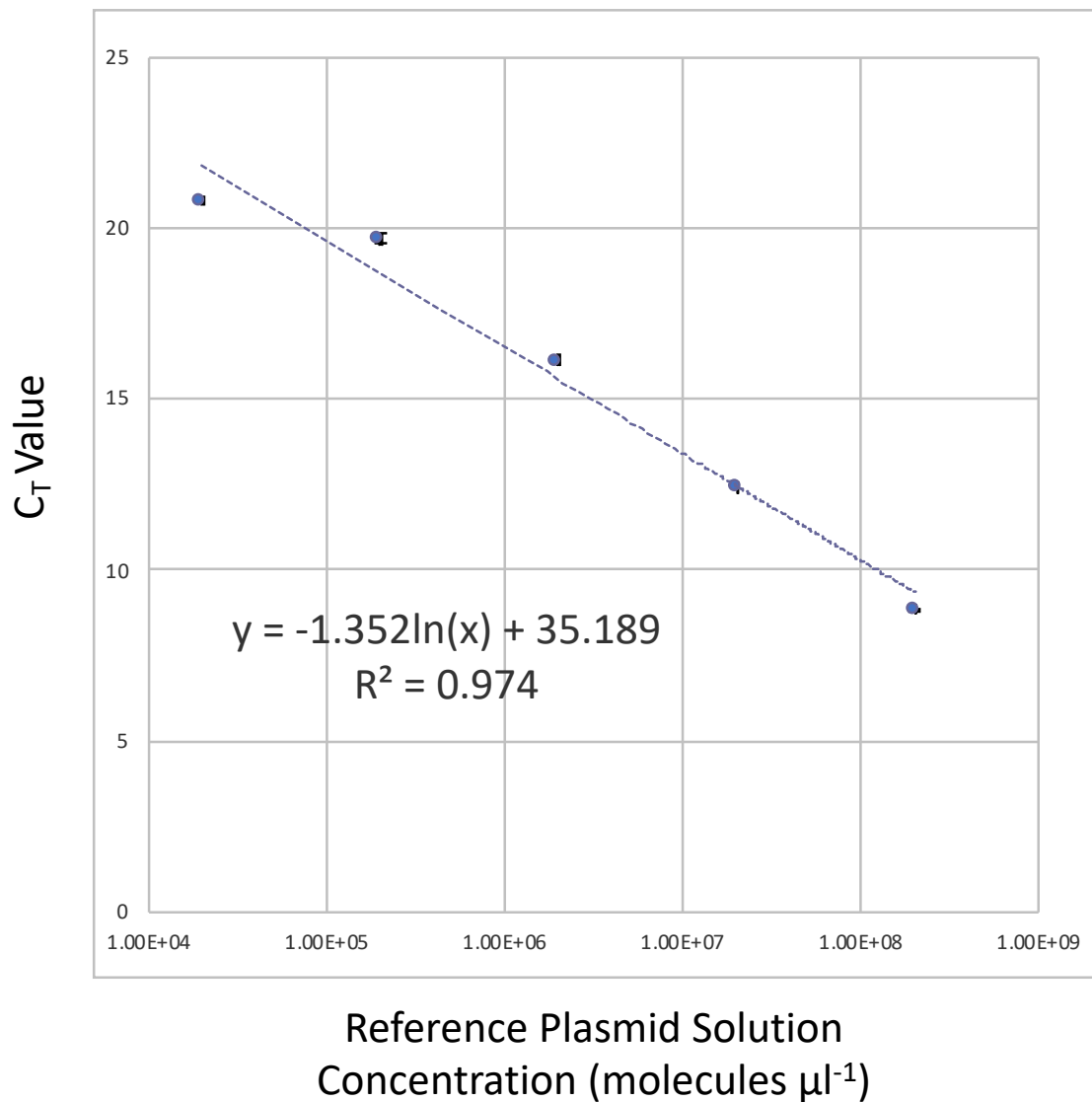


Figure 2-1 A standard curve used to estimate viral genome concentrations (in this case for the CBA-hOPN4 virus) using serial plasmid dilutions of known concentration. A line of best fit is drawn through the points and the equation of this line allows unknown viral concentrations to be calculated based on their C_T values on qPCR.

In vitro transduction ability

A dissociated retinal cell culture was prepared and then incubated with the candidate AAV; ICC of the culture was performed to verify production of the appropriate protein.

Dissociated retinal cell culture: preparation

Retinae were dissected from mouse eye cups immediately following culling and incubated with PBS on ice until a batch of 8 retinae had been collected. A papain digestion system (Worthington Biochemical Corporation, Lakewood, USA) was used according to the manufacturer's instructions. The collected retinae were incubated with 500µl of papain solution (20units ml⁻¹ papain and 0.005% DNase) at 37°C for 45 minutes in an agitating incubator. The sample was then triturated 12 times using a 1000µl pipette before centrifugation (300g for five minutes). Supernatant was discarded and the cell pellet resuspended in DNase/albumin inhibitor solution (from the manufacturer's kit). The cell concentration was then determined using a haemocytometer to guide the seeding of circa 400,000 cells into each well of a 6 well plate, containing four sterile poly-lysine cover slips (Corning inc., Corning, USA) immersed in enriched neurobasal media. Cells were cultured thus for two weeks (with half the volume of media replaced every third day) before inoculation with adenoassociated virus.

Dissociated retinal cells were cultured in Neuralbasal media (ThermoFisher, Loughborough UK) supplemented in the same fashion as DMEM described above and additionally with 2% "B27™-plus" supplement (containing vitamin A) (ThermoFisher, Loughborough, UK). Cells were cultured in an incubator at 37°C, 5% CO₂, 95% humidity.

Dissociated retinal cell culture transfection

A dissociated retinal cell culture was prepared as above using degenerate retina, and after two weeks of initial culture, cover slips were placed on a UV sterilised piece of Parafilm® (Bemis inc., Neenah, USA) inside a petri dish. 200µlitre of media was pipetted onto each slip, the hydrophobic Parafilm® keeping it in place. To this was added 1µlitre of neat viral concentrate, giving an approximate multiplicity of infection (assuming 10^5 cells per cover slip and a viral concentration of 10^{12} vg ml⁻¹) of circa 1×10^4 . After twenty-four hours culturing at 37°C, the coverslips were returned to their six well plates with half media being exchanged every 3rd day. After 21 days, cover slips were removed from their media, fixed and the appropriate ICC protocol performed as described below in the immunohistochemistry section.

Intravitreal Injections

Anaesthesia was induced and maintained in mice via inhalation of 1 to 5% isoflurane ("Isoflo"®, Zoetis, Walton Oaks, UK), carried in 100% oxygen (British Oxygen Company, Leicester, UK). After induction of anaesthesia in a gas box, mice were transferred to a face mask for maintenance of anaesthesia (Walker Anaesthetic Services, Stone, UK) and guttatae (gt.) 0.5% proxymethocaine & gt. 1% tropicamide (Minnims brand, Bausch & Lomb U.K Limited, Kingston-Upon-Thames) applied for topical anaesthesia and to dilate the pupil.

The mouse was then positioned laterally, initially with the right eye facing upward under an operating microscope (M620 TTS, Leica GmbH, Wetzlar, Germany). Gt. Povidone Iodine 5% (Minims brand, Bausch & Lomb U.K Limited, Kingston-Upon-Thames) was added, before Viscotears® (Bausch & Lomb U.K Limited, Kingston-Upon-

Thames) was applied to the cornea and a 2mm round cover slip positioned atop this to allow a view of the posterior segment.

The eye was stabilised by gripping the superior rectus with toothed forceps and a 35 gauge, bevelled needle (NF35BV), on a 10µl nanofil® syringe (both World Precision Instruments, Hitchin, UK) was introduced anterior to the gripping forceps. The needle was advanced into the vitreous, careful to avoid the lens, under direct visualisation. 1.5µl of undiluted virus or 0.9% saline was then injected into the vitreous cavity. Maintaining visualisation, the needle was held in place for 5 to 10 seconds before a slow withdrawal in order to minimise reflux. The mouse was then repositioned and the same process repeated for the fellow eye. Gt. poviodone iodine was again applied to both eyes and the mouse moved to a heated cage (27°C) to allow recovery from anaesthetic before return to their home cage. An operation note proforma (see appendix) was filled for each injection.

Immunohistochemistry

Cryosections

Directly after enucleation, eyes were transferred to 4% paraformaldehyde (PFA, Alfa Aesar, Ward Hill, USA) for twenty-four hours before incubation in 30% sucrose phosphate buffered saline (PBS) for a further forty-eight hours to cryoprotect. The eyes were then embedded in optimal cutting temperature (OCT) solution (Finetek, Torrance, Canada), frozen overnight at -80°C and 20µm sections cut on a 'FSE' cryotome (Thermo Fisher, Loughborough) at 25°C and captured onto poly-lysine coated microscopy slides (VWR, Leuven, Belgium).

Defrosted slides, with samples drawn around with a hydrophobic barrier pen ("Immedge," Vector labs, Burlingham, USA), were allowed to air dry for thirty minutes.

An additional antigen retrieval step (incubated at 95°C for twenty minutes with 10mmol/l Sodium Citrate pH 6.0, 0.05% Tween® (both Sigma Aldrich, Gillingham, UK)) was performed for staining in Chapter 4 only. Permeabilization was with Triton X® (Sigma Aldrich, Gillingham, UK) 0.2% in phosphate buffered saline (PBS). A blocking step followed with 10% serum from the animal in which the secondary antibody was raised (Table 2-13), diluted in 0.2% Triton X® for sixty minutes. Slides were then incubated overnight with a primary antibody solution at the stated concentration (see Table 2-12) diluted in 0.2% Triton X® with 2.5% serum from the animal in which the secondary antibody was raised (Table 2-13).

Four washes of ten minutes in 0.1% tween® were undertaken before incubation for two hours with a secondary antibody solution at a concentration of 1:250 in 0.2% Triton X®/PBS (Table 2-13). Three washes of ten minutes in 0.1% tween® were undertaken, followed by 10 minute incubation with 1:30,000 (circa. 300nmol/l) 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (ThermoFisher, Loughborough,UK) and a final wash for 10 minutes in ddH₂O. Slides were then dried and mounted with ProLong® Diamond mounting media (ThermoFisher, Loughborough, UK).

Retinal flat mounts

Directly after enucleation, eyes were transferred to 4% PFA for twenty-four hours before dissection under a microscope (EZ4D, Leica, Wetzlar, Germany) with short term storage in PBS. Permeabilization was with Triton X® 1% in PBS, followed by 3 ten minute washes in PBS. A blocking step followed with 10% serum from the animal in which the secondary antibody was raised (Table 2-13), diluted in 1% Triton X® for sixty minutes. Slides were then incubated for three days with a primary antibody solution at

the stated concentration (Table 2-12) diluted in 1% Triton X[®] with 2.5% serum from the animal in which the secondary antibody was raised (Table 2-13). Three washes of thirty minutes in 0.1% tween[®] were undertaken before incubation overnight with a secondary antibody solution at a concentration of 1:250 in 1% Triton X[®] - PBS (Table 2-13). Three washes of thirty minutes in 0.1% Tween[®] were undertaken, followed by a 30 minute incubation with 1:30,000 (circa. 300nmol/l) 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (ThermoFisher, Loughborough,UK) and a final wash for 30 minutes in ddH₂O. Slides were then dried and mounted with ProLong[®] Diamond mounting media (ThermoFisher, Loughborough, UK).

Validation of antibodies using transfected HEK293T cells

HEK293T cells (ATCC, Manassas, USA) were cultured to 70% confluence on four UV sterilised glass cover slips coated with poly-lysine in a 35mm petri dish (Both Corning inc., Corning, USA). Culture media was removed and replaced with RPMI media (Sigma Aldrich, Gillingham, UK) enriched with L-glutamine (2nmol/l), and 10% Fetal Bovine Serum (Invitrogen, Carlsbad, USA). Simultaneously, 200µlitres of serum free RPMI containing 6µl of Genejuice[®] transfection reagent (Merck Millipore, Burlington, US) was vortexed and allowed to rest at room temperature for 5 minutes.

Subsequently, 2µg of plasmid DNA was added to the transfection reagent and mixed gently before resting for 15 minutes at room temperature. The complexed transfection mixture was then added step wise to the 35mm well with gentle mixing to ensure even spread and incubated for six hours after which the media was replaced with fresh enriched DMEM culture media (as described above). Cells were fixed and subjected to the ICC protocol above to validate antibodies circa 48 hours after transfection.

Target	Target species	Manufacturer	Product Code	Host Animal	Dilution used
Annexin a7	Mouse	Abcam	ab197586	Rabbit	1:250
Contactin 1	Mouse	Abcam	ab66265	Rabbit	1:250
Shroom 2	Mouse	Atlas	hpa051646	Rabbit	1:250
Sulfatase 2	Mouse	Abcam	ab113405	Mouse	1:500
Melanopsin	Mouse	Advanced Targeting Systems	uf006 - UF006	Rabbit	1:1,000
Melanopsin	Human	Santa Cruz	sc32870	Rabbit	1:1,000
Green Fluorescent Protein	n/a	Aves	gfp1020	Chicken	1:1000
1D4 (Rhodopsin)	Human	NIH	-	Mouse	1:5000
β -galactasidase	n/a	Abcam	ab9361	Chicken	1:500
L7	Mouse	Santa Cruz	sc137064	Mouse	1:1,000

Table 2-12 Primary Antibodies used for immunohistochemistry and immunocytochemistry

Name	Target species	Host Animal	Excitation λ (nm)	Emission λ (nm)	Manufacturer	Product Code	Dilution used
Alexa [®] -568	Rabbit	Donkey	578	603	invitrogen	a10042	1:250
Alexa [®] -568	Chicken	Goat	578	603	invitrogen	ag175472	1:250
Alexa [®] -568	Mouse	Donkey	578	603	invitrogen	a10037	1:250
Alexa [®] -488	Rabbit	Donkey	495	519	invitrogen	a21206	1:250
Alexa [®] -488	Mouse	Donkey	495	519	invitrogen	a2102	1:250
Alexa [®] -488	Chicken	Donkey	495	519	Jackson Immunoresearch	T03-545-155	1:250
DAPI	n/a	n/a	358	461	ThermoFisher Scientific	D1306	300nmol ⁻¹

Table 2-13 Secondary Antibodies and nuclear stains used in immunohistochemistry and immunocytochemistry

Immunocytochemistry

Cells cultured on coverslips were fixed with 4% PFA for 10 minutes before three washes in ice-cold PBS of 5 minutes each. Coverslips were then incubated at 95°C for twenty minutes with 10mmol/l Sodium Citrate (pH 6.0, 0.05% Tween®) as an antigen retrieval step. Three washes in ice-cold PBS followed. Permeabilization was with Triton X® 0.2% in PBS, blocking was for sixty minutes with 10% serum from the animal in which the secondary antibody was raised (Table 2-13), diluted in 0.2% Triton X®. Slides were then incubated for two hours with a primary antibody solution at the stated concentration (Table 2-12), diluted in 0.2% Triton X® with 2.5% serum from the animal in which the secondary antibody was raised (Table 2-13).

Three washes in ice cold PBS of 5 minutes each followed, before incubation for one hour with a secondary antibody solution at a concentration of 1:250 in 0.2% Triton X®/PBS. Two further washes with ice cold PBS were performed before a 5 minute incubation with 1:30,000 (circa. 300nmol/l) 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (ThermoFisher, Loughborough,UK) and a final wash for 10 minutes in ddH₂O. Coverslips were then apposed, cell side down onto a drop of ProLong® Diamond mounting media on a poly-lysine coated coverslip.

Fluorescence light microscopy

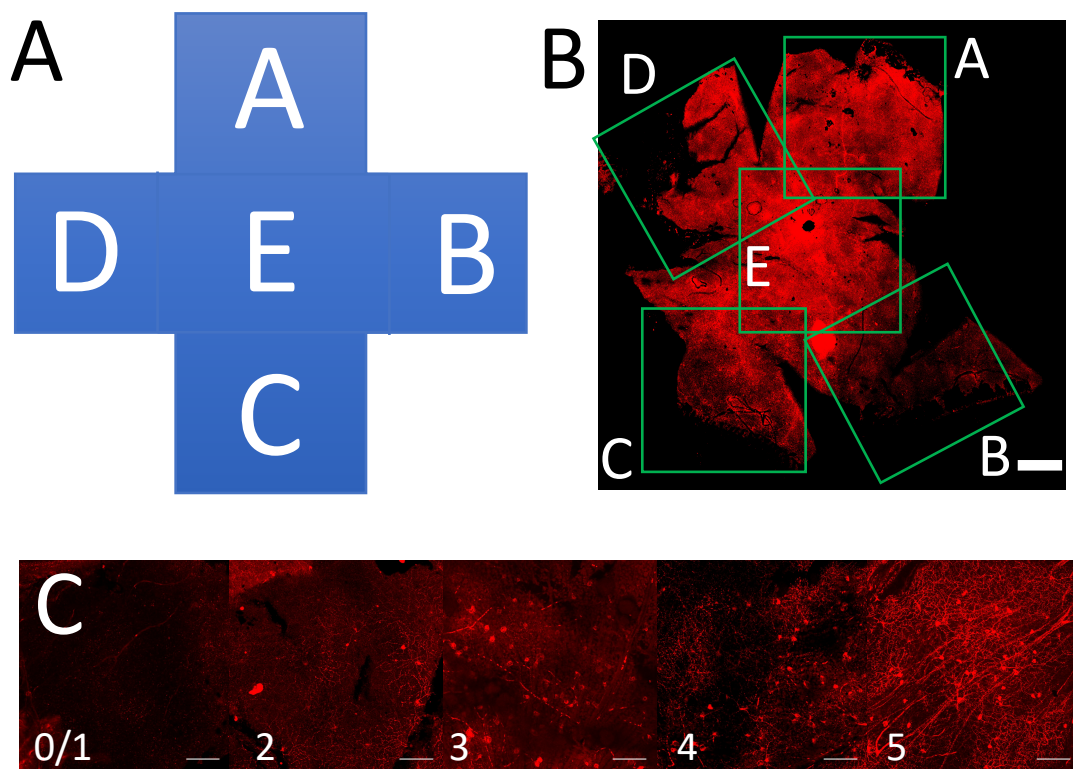
For initial examination of immunohistological sections, flat mounts and cells, an inverted fluorescence microscope (IX71, Olympus, Shinjuku, Japan) was used using filter combinations appropriate to the dyes associated with the secondary antibodies or DAPI (see Table 2-13)

Scoring retinal flatmounts

In order to give a semi-quantitative index of the area of coverage of retinal transfection, each stained flat mount retina was scored for spread of transfection. Each flat mount was split into 5 areas corresponding to the area around the peripapillary area and four areas of peripheral retina (Figure 2-2). Areas were each assigned a score based on the prevailing intensity of staining seen on fluorescence light microscopy (Figure 2-2). The mean of these five scores gave a composite for staining over the whole retina.

Confocal Microscopy

Immunohistological sections, flat mounts and cells were additionally visualised on a confocal microscope (LSM710; Zeiss, Oberkochen, Germany). Lasers of appropriate wavelengths (Table 2-13) were used to excite the relative dyes sequentially to allow an image to be built up. In general, images were taken at 40x magnification, with a pinhole aperture equal to 1 Airy unit on the 568nm channel. Laser and detector settings were adjusted to minimise pixel saturation and kept constant between images that were to be directly compared. In the XY plane, a resolution of 1024x1024 pixels was used in general with Z stacks of 16 images to visualise the depth of the retina in flat mounts, or 10 images to visualise the depth through cryosections. Whole retina views of flat mounts were achieved by stitching smaller, overlapping images. Images were then processed to using Fiji-ImageJ®(170).



Score	Description
u	Sector missing / damaged
0	No staining at all
1	Small areas of staining
2	More of sector blank than stained
3	More of sector stained than blank
4	Reasonable staining over whole sector
5	Good staining throughout sector with large numbers of cell bodies and processes throughout the whole area

Figure 2-2 Scoring of retinal flat mount coverage. A&B – each retina was divided into 5 sectors of roughly equal size. Scale bar 100µm C&D. A score was assigned to each sector using a 20X fluorescence microscopy objective. Examples of an area with such a score on fluorescence microscopy are provided; as is a description, in the table of what is expected at each score. Scale bars 50µm.

Transfection quantification index

To quantify the number of cells transfected in each retinal layer of each transfected retina, x40 confocal images focused on the appropriate layer were taken at four locations with staining evident on fluorescence microscopy review. The proportion of DAPI stained cells also staining for the protein of interest in an area of approximately 100µm x 100µm was recorded to give a semi-quantative fraction of transfected cells in that layer.

Co-localisation index

For analysis of colocalization of candidate genes in Chapter 4, a 10 frame Z-stack image spanning the depth of the entire retinal section was taken using a x40 objective at a point three fields of view from the *ora serrata* with consistent gain and power settings. These images were then analysed using Image J® (170) and the plugin JACOP(171) to give an intensity correlation quotient (ICQ)(172). In addition, the number of pixels demonstrating co-localisation above threshold in each image was normalised to the number of non-zero pixels in the image to give an index of colocalization that could be compared between images(173).

Behavioural Assays

In testing extra retinal responses to light, three assays were selected to assess behaviours mediated by different parts of the brain (see Chapter 5 for discussion). Pupillometry, an objective and quantifiable measure of the function of a light dependant reflex arc (174), the optokinetic tracking assay to assess subcortical responses to light and finally the novel object and the visual context recognition task to assess cortical processing of vision. The method for each assay is described first,

followed by the battery protocol (including habituation) as applied to each individual mouse.

Pupillometry Assay: the post illumination pupil response

Pupillometry was performed, following closely the published protocol of C Pothecary (55) (Figure 2-3). Animals were tested between zeitgeber time (ZT) 4 and 8 and were dark adapted for 3 hours before recordings were made. A narrow band Light Emitting Diode (Radio Spares Ltd, Watford, UK) $480\pm 10\text{nm}$ was used to produce light stimuli of $10^{14.83}$ photons $\text{cm}^{-2} \text{s}^{-1}$ and neutral density filters (L.o.t.-Oriel GmbH, Darmstadt, Germany) used to produce lower intensity stimuli of $10^{13.93}$, $10^{13.03}$ and $10^{11.98}$ photons $\text{cm}^{-2} \text{s}^{-1}$ (for brevity, referred to later in the text as circa 10^{15} , 10^{14} , 10^{13} and 10^{12} photons $\text{cm}^{-2} \text{s}^{-1}$ respectively). The spectral composition of the stimuli was measured using a radiometrically calibrated spectrophotometer (Ocean Optics, UK) (Figure 2-4). A 5cm diameter ganzfeld bowl (Pro-lite Technology, UK) was used to ensure even illumination of the mouse's left eye. Pupillary movements were recorded from the contralateral eye using a near infrared charge coupled camera ("Prosilica," Allied Vision, Stadtroda GmbH, Germany) at a rate of 10 frames s^{-1} , infra-red light emitting diodes (850 nm, 10 nm half-bandwidth) were used for ambient illumination of the (right) efferent pupil. Stimulus onset and recording was co-ordinated by custom software written by Carina Pothecary in the LabView programming environment (National Instruments, Austin, USA).

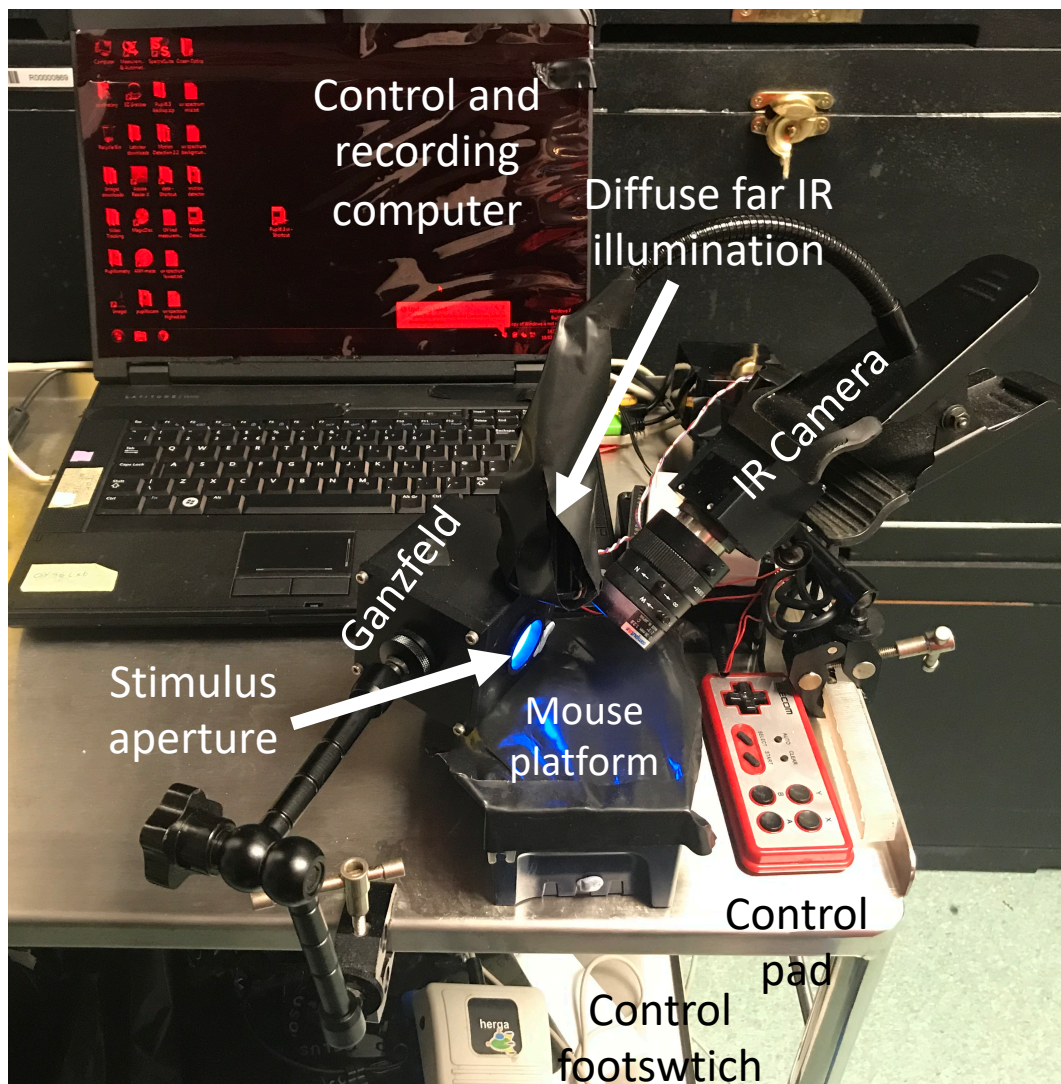


Figure 2-3 The pupillometry apparatus, as described in the text.

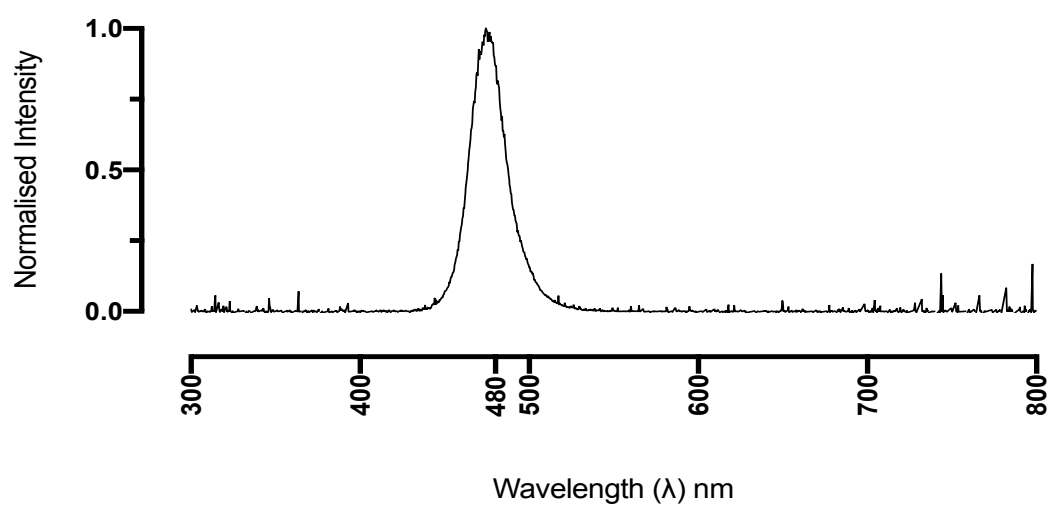


Figure 2-4 The spectral composition of light from the 480nm led used to stimulate pupillary constriction.

Recordings were made on unanaesthetised, appropriately habituated mice (see below), held using normal animal handling techniques (dorsal scruffing). Gt. Tropicamide 1% (Minims brand, Bausch & Lomb U.K Limited, Kingston-Upon-Thames) was instilled into the left eye only, 20 minutes prior to recording in order to maximise light intensity at the retina. The mouse was introduced to the pupil recording apparatus (Figure 2-3), briefly positioned (with feet on a flat surface) with his left eye in the centre of the ganzfeld bowl and the right positioned, in good focus in the view of the infrared camera. Recording then began in the dark for 2 seconds to determine baseline before a 10 second pulse of light at the appropriate intensity. After the end of the stimulus, recording continued for a further 18 seconds to give an overall recording length of 30 seconds. After a delay of at least 30 minutes, this recording was repeated.

All images were analysed using the Fiji-Image J(170) program. Images were imported as stacks; contrast enhanced, and threshold manually adjusted to highlight the pupil. The “*analyse particles*” function was then used to quantify the size of the pupil in each frame of the recording (Figure 2-5).

The maximum pupil size recorded in the first two second (dark) phase was used to normalise pupil size in all other frames which could be used to construct a response graph (Figure 2-5 D); in keeping with previously published studies (159), the pupillary size at two seconds post illumination was extracted for further analysis and comparisons. During both recording and analysis, the observer was masked as to the treatment group of the mice.

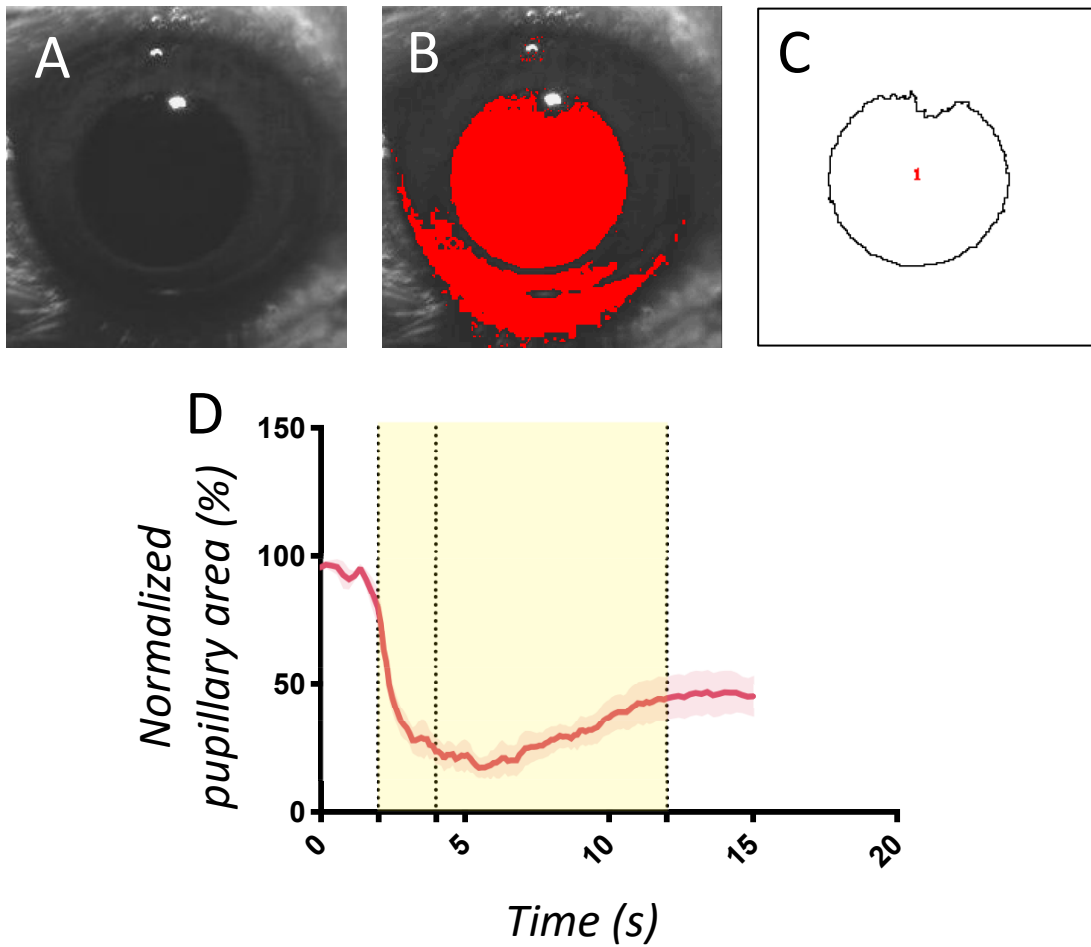


Figure 2-5 Processing pupil recordings to determine the post illumination pupil response.

- A. Images series are imported from the recording computer to image J (170) and contrast enhanced by 25%.
- B. Threshold values for images are set such that the circle of the pupil is clearly highlighted on the majority of images in the series, but there is no “touch” with artifactual areas such as the arc of threshold seen inferiorly in this image.
- C. The “analyze particles” function of image J is used to extract the size and shape of the pupil in each frame, which can be outputted from the program as a comma separated file and imported into an MS Excel processing template (written by C Potheary (55)).
- D. Pupillary area at each timepoint is normalized as a percentage of the largest pupil area in that trial and plotted against time. The yellow bar represents the period in which the light stimulus is turned on.

Optokinetic response assay

The optokinetic response assay followed closely the protocols described previously by TS Watson, based on the work of Douglas *et al* (175-177). A custom-built drum arena (Figure 2-6) with a radius of 15cm, 55cm tall made of Perspex was mounted on a similarly sized metallic turntable controlled by an inbuilt stepper motor. The stepper motor was controlled by computer using specialised software Auto-Step® (PC-Control Ltd, Kettering, UK). A stationary, raised metallic platform, 25cm from the arena floor, was present in the middle of the turntable for the subject mouse to sit on during trials. The arena was illuminated by an incandescent bulb and a digital camera (ePath® 6mm Epath, Kowloon, Hong Kong) was suspended 10cm above the arena.

Prior to beginning a trial, illumination was checked with a lux meter to be $1,000 \pm 50$ lux. Between ZT4 and ZT8, the mouse was introduced to the arena, with a pause of 30 seconds to allow acclimatisation before the drum began to rotate: first for 30 seconds in a clockwise direction at 2 revolutions per minute, then anticlockwise at the same speed for 30 seconds, the process was then repeated once to make up a trial. Four trials were performed on each mouse: the first using a white screen as a control, followed by striped screens with a spatial frequency of 0.1 cycles per degree (cpd), 0.2cpd and 0.4cpd and contrast of 100%.

Videos were replayed of each trial and scored manually by an observer masked to the animal's treatment group. A head tracking movement was defined as a movement in the same direction as and at the same speed as the drum through an arc of at least 45° . The number of tracks per trial was scored and averaged for each mouse over the separate observations made of it (see behavioural battery protocol below).

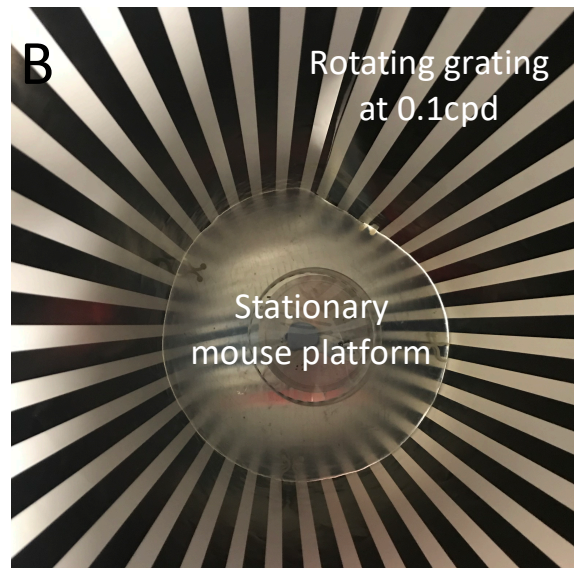


Figure 2-6 The optokinetic response assay. A. side view (no grating stimulus in situ). B. View from above with grating stimulus (0.1cpd) present

Novel object, visual context recognition assay

The novel object, visual context recognition assay followed closely the published and validated technique of Tam *et al* (178, 179). The area was constructed of a 20cm Perspex® cube, with a deficient roof, with each wall coated with a stimulus pattern as per Figure 2-6A (178, 179). The chevron was always placed to the left. Illumination was provided by a diffuse white fluorescent tube 10cm above the arena. Light levels were checked before each trial to be 50 ± 5 lux at the base of the arena. A digital camera (ePath® 6mm Epath, Kowloon, Hong Kong) was suspended 13cm above the arena and used to record the test phase.

The mouse, under a normal light cycle, between ZT4 and ZT8, underwent the sample phase where they were introduced to the arena with two identical novel objects (candle holder, Figure 2-7 D&E) in the top and bottom left corners of the arena for ten minutes. They then rested in their home cage for 5 minutes (the retention phase), before returning to the same arena for 3 minutes, where either of the objects had been moved to the other side of the arena (top or bottom right corner) - the test phase. In order to minimise place preference, treatment groups were counter balanced as to which object moved. To minimise odour cues, arena and objects were cleaned thoroughly with 70% Ethanol (Sigma, Gillingham, UK) between both sample and test phases.

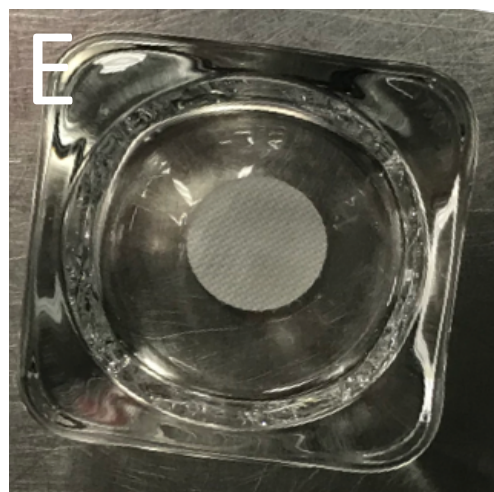
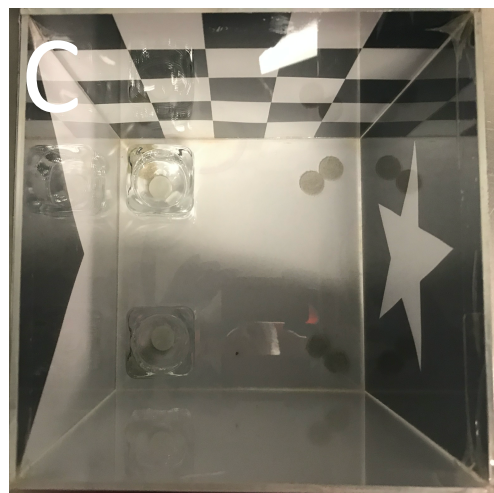
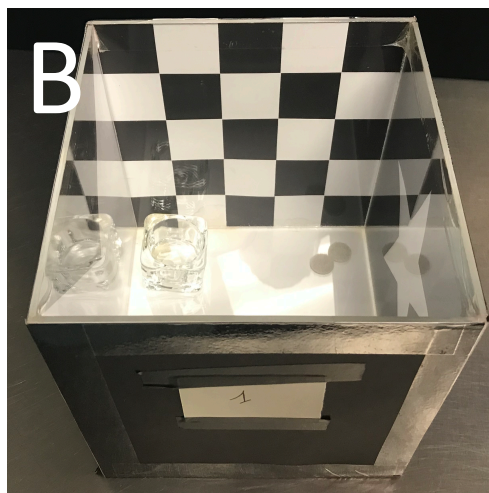
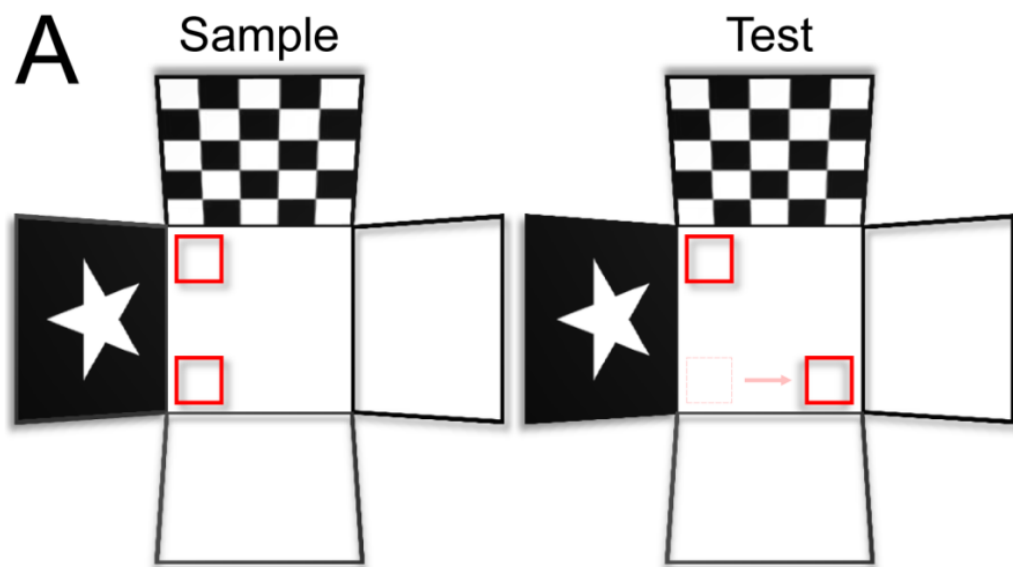


Figure 2-7 The novel object, visual context recognition task.

A. Modified from (179). Red boxes indicate the position of objects in sample and test phases (NB. In 50% of cases, the upper object was moved, rather than the lower to counterbalance any place preference).

B&C. Side and top views of arenas with objects set up for the sample phase.

D&E. Side and top views of the actual object used: a candle holder (as described in (179)).

Recordings of the test phase were then analysed using Anymaze® software (Stoelting, Dublin, Éire) to track the animal's head and record how long it spent exploring each of the objects. This was then expressed as a ratio of "time spent with novel object" / "time spent with familiar objects." Therefore, a ratio greater than one represents preference for the novel, a ratio less than one a preference for the familiar and a ratio of 1 equipoise.

Behavioural Battery Protocol

Day 1

Mice for behavioural testing were moved from their previous cages, to new home cages, mice were housed in groups of up to six with enrichment (cardboard tube, ample bedding and cardboard 'house') with *ad libitum* standard diet and water. They were housed under a constant 12hour light-dark cycle at 21°C. All mouse handling was by MJG (male, 32 years old).

Optokinetic response (OKR) assay habituation

1. Each mouse spent 3 minutes on the OKR arena platform with a stationary 0.6cpd high contrast grating.
2. This was followed by a habituation run: the grating was moved clockwise at 2 revolutions per minute for 30 seconds, then anticlockwise at the same speed for a further 30 seconds. This was then repeated
3. Each mouse was allowed to rest in the home cage for at least 30 minutes following this

Pupillometry & scruffing habituation

1. Each mouse was scruffed twice on the cage lid

2. On the third scruffing, the mouse was held in the pupillometer for 30 seconds and a trial pupillometry run (below) carried out – data from this was not recorded.
3. Mice were returned to their home cage and allowed to rest for 3 days

Day 4

1. OKR and Pupillometry habituation runs were repeated as on day 1

Day 5

1. Each mouse was habituated to the Novel Object Recognition (NOR) arena for 10 minutes, without any objects in the arena
2. Mice were allowed to rest for at least 30 minutes
3. The OKR assay was performed and results recorded

Day 6

1. NOR arena habituation was repeated as on day 5
2. Mice were dark adapted for 3 hours
3. The pupillometry assay was performed and results recorded

Day 7

1. NOR assay was performed and results recorded

Day 8

1. The pupillometry assay was performed and results recorded

Day 11

1. The pupillometry assay was performed and results recorded

Running Wheel Activity Monitoring

Mice were transferred to new, singly housed, home cages at the age of 6 weeks. The same light dark cycle as described above was maintained (lights on 0700h, lights off 1900h Intensity= 200lux). Cage running activity was then recorded for a period of two weeks before intravitreal injections were performed as described above. Running activity data was then monitored for a further eight weeks, before being collected and analysed using the Clocklab® system (Actimetrics, Wilmette, IL, USA).

Multiple Electrode Arrays

Mice were culled by cervical dislocation (in accordance with schedule 1 of the Animals (Scientific Procedures) Act, 1986)) in dim red light (>610nm) and subsequently enucleated and retinæ dissected. Dissection, mounting and recordings were performed under the same lighting conditions. Dissected retinæ were immediately transferred to a glass coated metal harp (Figure 2-8-F) (ALA Scientific Instruments, Farmingdale, USA) and thence, ganglion cell side down, onto a glass bottomed multiple electrode array (MEA) chamber (Multi Channel Systems, Reutlingen, Germany) (Figure 2-8 B-E). MEAs used had 60, 30µm recording electrodes arranged in a grid pattern 200µm apart (Figure 2-8 E). A gas permeable perfusion manifold (Figure 2-8 G) was attached to the MEA and the preparation continuously perfused with Aimes' media (Sigma Aldrich, Gillingham, UK) bubbled with 5% CO₂, to pH 7.3 and heated to 33°C.

The MEA was then mounted on a heated (33°C) head stage (Multichannel Systems, Reutlingen, Germany) which had capacity for two MEAs to be recorded from at once (Figure 2-8 A). Therefore, one MEA was used for each eye and both retinæ were left, perfusing, for 40 minutes to settle in the dark before recordings were made.

The retina to be recorded from was positioned in the light path of a Zeiss IX71 (Olympus, Shinjuku, Japan). Light stimuli were produced by an X-cite 120W metal halide light source (Thorlabs, Ely, UK) and conditioned by passing through spectral filters (480 ± 10 or 510 ± 10 nm) and neutral density filters ($0-6 \log_{10}$ units) (all filters, ThorLabs, Ely, UK) before being delivered to the sample via a 10x objective microscope lens positioned below the sample. Stimuli onset and off set were controlled by manual button pressing and effected by a high-speed shutter (Prior, Cambridge, UK).

Before each recording session began, the power of the light stimuli (with a 2 $\log_{10}$ unit neutral density filter and spectral filter of appropriate wavelength in place) was measured using an in-line power meter, set to the relevant wavelength (Thor Labs, Ely, UK). All light power and intensity measurements were converted where necessary using a validated irradiance conversion toolbox(180).

Recordings began with 30 seconds of baseline recording before the onset of a 10 second light pulse, recording was then continued until 90 seconds had elapsed. After each recording, an interval of 10 minutes was allowed for dark adaptation before the next trial. Recordings were made using the “MC-rack” software suite (Multichannel Systems, Reutlingen, Germany).

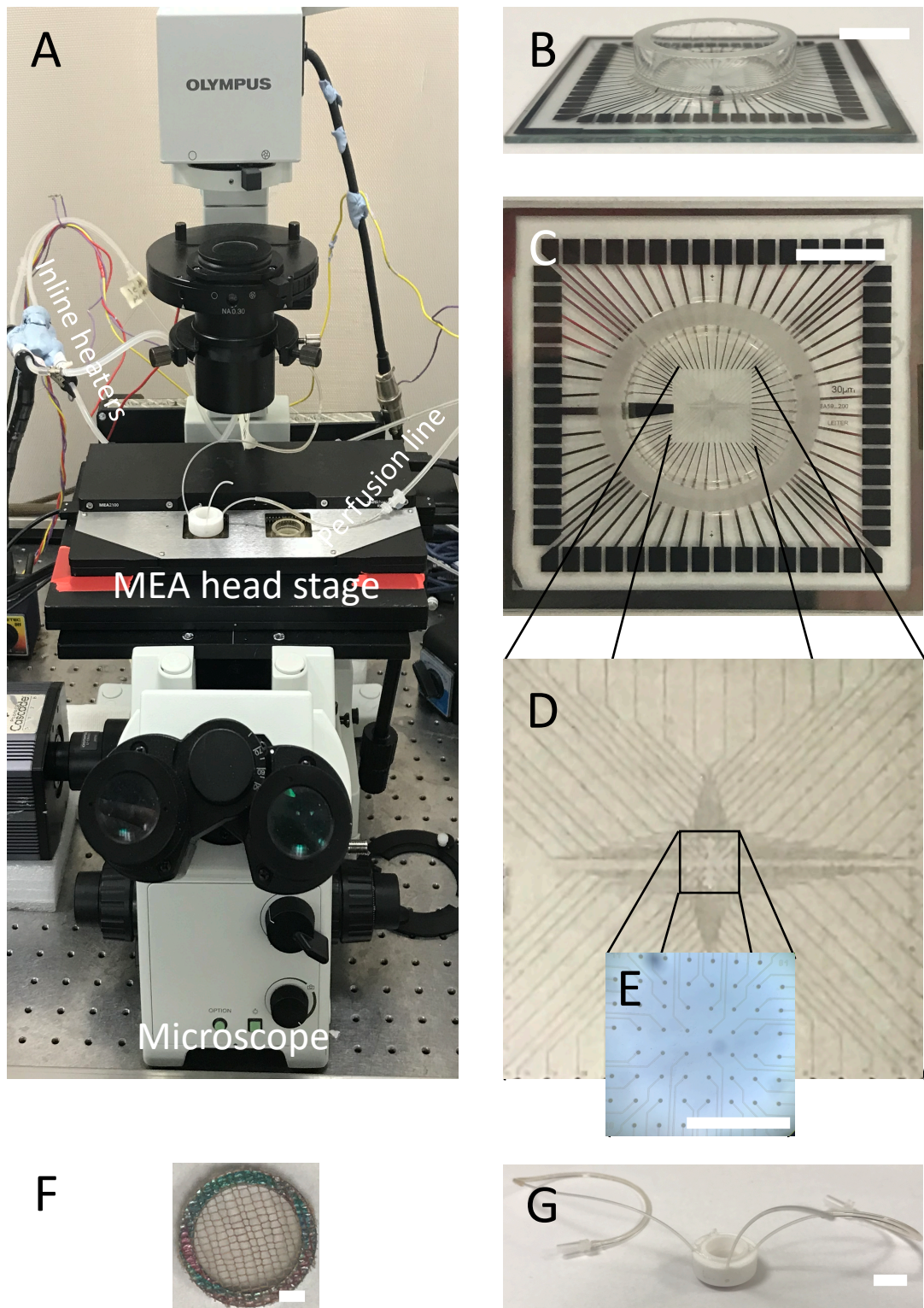


Figure 2-8 A. MEA recording set up with two MEAs in situ on the MEA headstage – the left hand MEA has the perfusion manifold (F) in situ with perfusion lines from the Aimes' solution bottle and to the waste trough in place. MEAs (B&C) are made of coated glass with 60 microelectrodes arranged in an array (D&E). Retina were floated onto a glass thread "harp" to allow easy apposition onto MEAs. Scale bars all = 1cm, except E&F where =1mm

Data Analysis

Retinal ganglion cell action potential firing rate was extracted in 1 second bins using the MC-rack software from filtered data, with an action potential spike defined as a deviation greater than three standard deviations from the mean baseline noise. This data was then transferred to an Excel spreadsheet template (Modified from the work of S Hughes(181)) for further processing (Figure 2-9 and Figure 2-10). For the majority of analysis, electrodes were classified as responsive if there was at least a 10Hz increase in firing rate in the 10 seconds following a stimulus passing through a 2 log₁₀ units neutral density filter (circa 10¹⁴ photons cm⁻² s⁻¹), compared with firing in the ten seconds preceding stimulus onset. Unresponsive electrodes were excluded from further analysis.

Data on the kinetics of responsive electrodes was determined first (as described in Chapters 5-7). Responses (maximum firing rate post stimulus) at each of 6 intensities (circa 10¹⁰ to 10¹⁵ photons cm⁻² s⁻¹) were normalised to the response at the intensity with the maximum response. A macro, written in visual basic and using MS[®] Excel's solver module (Modified from the work of S Hughes & J Rodgers(181-183)) using an iterative non-linear regression process, was used to fit a sigmoidal curve to this normalised data by minimising the sum of squares of the regression(184). This was trialled either by fixing the Hill Slope value to be 1(185), or allowing it to vary in order to try and achieve a better fit (see discussion in Chapters 5,6 &7). Starting values in both cases were defined as an EC₅₀ = 13 and hill slope = 1, as plausible values for photopigments(185). In both cases, only electrodes where a fit of $r^2 > 0.8$ were included in reported mean EC₅₀, or used in comparison of curves between groups.

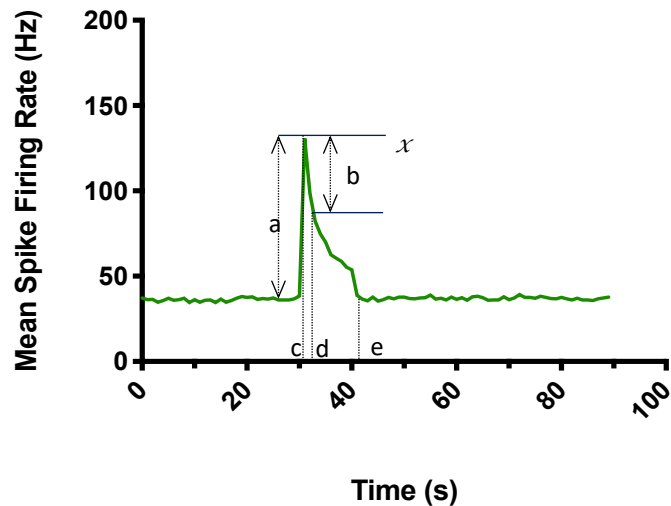


Figure 2-9 Metrics extracted from spike firing responses.

The maximum post stimulus rate (x)

- The highest rate recorded in the 10 seconds following the onset of light.

The time to maximum response (c)

- The time taken to reach the maximum post stimulus firing rate (x).

The half-life of the response ($t_{1/2}$)

- The time taken for the firing rate to fall to a rate half way between the maximum and the mean baseline (b or $a/2$). On this graph the interval between (c) and (d)

The time to onset

- The elapsed time after the start of the trial for the spike firing rate to be 10Hz above the mean baseline firing rate in the ten seconds before stimulus onset.

The duration of the response

- The time, after stimulus onset, for the firing rate to return to within 3 standard deviations of the mean baseline firing rate (e).

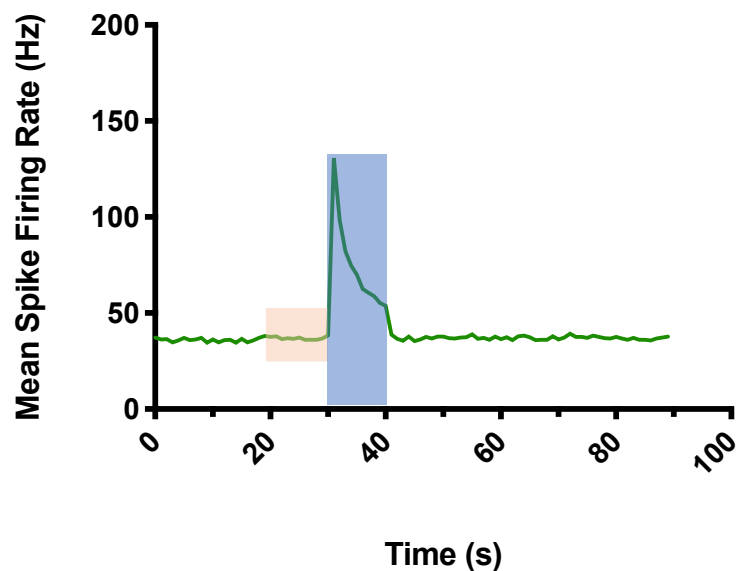


Figure 2-10 Metrics extracted from spike firing responses.

Mean baseline firing rate

- The mean firing rate in the 10 seconds preceding stimulus onset (red box in this diagram)

Maximum baseline firing rate

- The maximum firing rate in the 10 seconds preceding stimulus onset (red box in this diagram)

Mean post stimulus rate

- The mean rate of firing in the 10 seconds following stimulus onset (blue box in this diagram)

Statistical analysis

All statistical analysis was carried out using Prism® software version 7.0 or 8.0 (GraphPad, San Diego, USA). Significance was defined as $p < 0.05$. Normality was tested for using the Shapiro-Wilk and Kolmogorov-Smirnov tests. For data failing these tests, a Mann-Whitney or Kruskal-Wallis test was used as indicated. Otherwise, paired and unpaired t-tests, one or two way analysis of variance (ANOVA) were used as indicated. In general, data is expressed as $\bar{x} \pm$ (standard error of the mean).

Chapter 3 – Results: development and validation of mouse lines

Introduction

The development of a suitable model is central to answering these proposed research questions, therefore this Chapter will explore the background, production and validation the models used in later experimental work.

Choice of Species: practicality

The laboratory mouse is a practical model: they are easy to house, breed within a short timeframe, are reasonably genetically close to humans (with 79% $\alpha\alpha$ identity in RP genes)(186) and there is a large experience of genetic modification of the species. As a vertebrate, mouse retina has a trilaminar retinal hierarchy allowing image processing to begin within the retina – broadly analogous to humans

However, the limitations of the mouse eye as a model must also be remembered - there are several major differences to that of a human. As a nocturnal creature, the mouse has adapted to its scotopic environment – having no fovea or macular pigment and a lower density of cones, it is also a dichromat and has limited binocular overlap (30 degrees, compared to humans 120 degrees)(186) and visual acuity(187-189) – see Chapter four. Practically, murine eyes are small, with vastly different vitreous dynamics and so make difficult surgical models(133, 186).

These advantages and disadvantages must be carefully considered in the context of the research question being asked. In the case of this study, we are concerned particularly with protein expression within individual cells of the retina in the context of a disease (IRD) phenotype. With an unparalleled variety of tools available to do this easily(190), the advantages of the laboratory mouse far outweigh its disadvantages.

Models of retinal degeneration: a phenotype analogous to the human disease

The laboratory mouse could be said to have a propensity to inherited retinal degenerations, in one study a screen of a thousand lines at the Jackson Laboratory found over two hundred to harbour mutations in one of three causative genes(191). This has meant, that in addition to the hundreds of models of degeneration developed with a reverse genetics approach during the recent genetic age(192), there is a large literature describing spontaneously occurring (forward genetic(193)) models stretching back to the 1920s(78). Several such lines have been extensively characterised over the course of the last century(194, 195): genetically and phenotypically over the whole course of the disease – therefore in choosing a model of inherited retinal degeneration these lines were considered first.

Around sixteen such models are available commercially(196) of which five suffer from syndromic Retinitis Pigmentosa (and so discounted, given that Retinitis Pigmentosa simplex alternatives were available)(195). In addition, several (labelled rd2, rd6-8 & rd10) had relatively slow rates of degeneration which (given the short healthy lifespan of a mouse) were less practical given the interventional nature of the proposed work. Of the remaining lines, the *rd1* is by far the best characterised, as well as being a direct analogue of a form of human recessive retinitis pigmentosa (Figure 3-1).

The *rd1* line was described phenotypically with a recessive inheritance in the 1920s(77, 78) and later genetically as being caused by a truncating nonsense mutation (C→A transversion, codon 347) of the *Pde6b* gene. This removes the catalytic domain of the beta-subunit of rod cGMP-phosphodiesterase for which the gene codes(161, 197) - hence the model is referred to as *Pde6b^{rd1}* in this thesis.

In *Pde6b^{rd1/rd1}* homozygotes, poor function of this photoreceptor specific enzyme leads to build up of cGMP, cell toxicity and eventually cell death(198), reflected phenotypically with vessel attenuation, pigmentation (Figure 3-1) and a rapid loss of photoreceptors seen from around postnatal day (P) 8 until around four weeks of age by which rods are no longer apparent (Figure 3-1). This is followed by a slower secondary loss of cones, with 75% remaining at P17, and even 1.5% remain at 18 months (albeit with grossly dysmorphic morphology)(81, 199).

While *Pde6b^{rd1/+}* heterozygotes appear similar histologically and behaviourally to wild types,(200, 201) they have been shown to have elevated [cGMP] and altered electroretinograms(202-204) – given this, heterozygotes were not used as experimental animals.

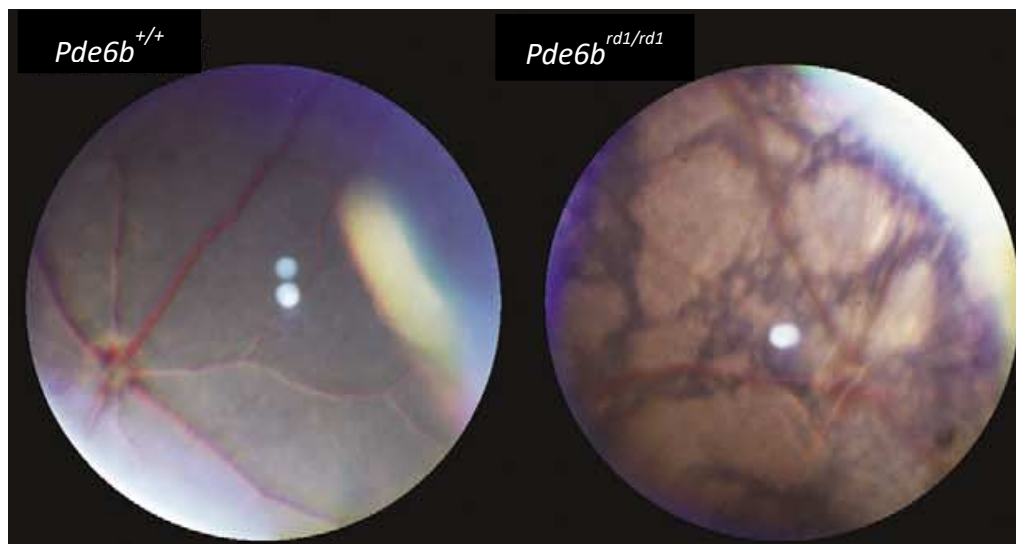


Figure 3-1 Retinal Phenotype of the wild type and *Pde6b*^{rd1/rd1} mice in late degeneration. Note bone spicule pigmentation in *Pde6b*^{rd1/rd1}, analogous to human Retinitis Pigmentosa. Modified from(193).

A model without native melanopsin: a retina with no native light responses

Even in late stage degeneration in *Pde6b*^{rd1/rd1} homozygotes, devoid of all rods and cones, retinal light responses remain due to the melanopsin system(205). While these mice, like the patients they model, will have no useful image forming vision, they retain non-visual responses to light such as circadian entrainment and pupillary responses(162, 206). While this has been effectively controlled for in previous demonstrations of optogenetic visual restoration in models of retinal degeneration(106, 158, 159, 207) by comparing to untreated controls, this is not ideal. When using an exogenous opsin (especially melanopsin itself) as an optogenetic tool, it can be difficult to differentiate responses that are as a result of treatment, from those that are a potentiation of residual native responses. Therefore, a model without any residual responses (combining rod/cone dysfunction and a lack of melanopsin function) would be preferable in increasing signal to noise ratios and easing analysis - especially of electrophysiological data such as multiple electrode arrays (MEAs).

Ganjawala et al(208) have recently attempted to overcome this problem by making use of the TKO (triple knock out) model which combines a knock out of the native melanopsin gene (*Opn4*) and two genes essential to rod (*Gnat1*) and cone (*Cnga3*) phototransduction. This model however does not have a retinal degeneration phenotype and so is unlikely to well model the situation in human retinitis pigmentosa. There is also considerable evidence that the knockout of *Gnat1* and *Cnga3* does not expunge photoreceptor responses completely due to compensating alternative transduction pathways and these mice have been noted to have a residual electroretinogram(209).

To overcome these limitations, we elected to cross breed our selected degeneration line (*Pde6b^{rd1/rd1}*) with the melanopsin knock out mouse (*Opn4^{tm1Yau}*). *Opn4^{tm1Yau}* was originally created by inserting a construct containing β -galactase (*LacZ*) into the melanopsin gene(162), replacing melanopsin expression with *LacZ*. Therefore, our new line would not only have a well characterised retinal degeneration phenotype, but be additionally devoid of native melanopsin response.

Expressing proteins of interest in required cell populations

During retinal degeneration, vision is lost due to the preferential loss of photoreceptors, with the neural retina surviving. This neural tissue is where much of our early, hierarchical, visual processing begins and targeting, albeit easier to reach, retinal ganglion cells bypasses key elements of this such as parallel ON & OFF pathway processing. In attempts to preserve this processing, the targeting of bipolar cells has been evaluated in order to maintain appropriate ON and OFF responses (102, 106, 210).

This has been complicated by practical difficulties in achieving reliable, specific and high efficiency AAV mediated transfection of these cells. While improvements to viral capsids and cell specific promotor have been made(105, 165, 211) (see Chapter 7) others have advocated dispensing with specific targeting in order to achieve generalised high efficiency transduction(159): specificity and efficiency are often said to be dichotomous in this context. While work continues to improve AAV mediated delivery, an alternative approach will be necessary to fairly compare targeted delivery.

Inducible delivery systems: *Cre.LoxP*

Inducible transgenic expression systems have provided great insight in many areas of biology by allowing expression of genes of interest be modulated in specific cell

populations within an organism. The Cre.loxP system is one such that is particularly relevant due to its ability to be combined with AAV vectors(212) and ready availability of retina cell type specific models(213).

Cre recombinase (*Cre* = “*Can recombine*”) is a 38kD tyrosine site specific recombinase from the P₁ bacteriophage that recognises a specific 34bp sequence called LoxP (*LoxP* = *locus of crossover in P₁*)(214). If two such sequences are found within a stretch of DNA in the same orientation, the DNA between them will be excised (Figure 3-2a), if they are in opposite orientations, it will be inverted (Figure 3-2b)(212): the intervening DNA is said to be “*floxed*”. Gene expression can therefore be restricted to cells where the Cre enzyme by either adding a “floxed” stop codon before the gene (Figure 3-3), or placing a whole inverted gene within two opposite flox sequences (Figure 3-2)(214).

The Cre.LoxP system is particularly attractive as LoxP sites are short and so can be easily integrated into AAV insert sequences incorporating high efficiency promoters, so that although the AAV vector may enter many different cell types, protein will be expressed (at high efficiency) only in cells where Cre enzyme is present. In addition, with transgenic mouse lines, Cre can be expressed under promoters selected for the cell type specificity(213, 215, 216) which would otherwise be unsuitable for use within an AAV.

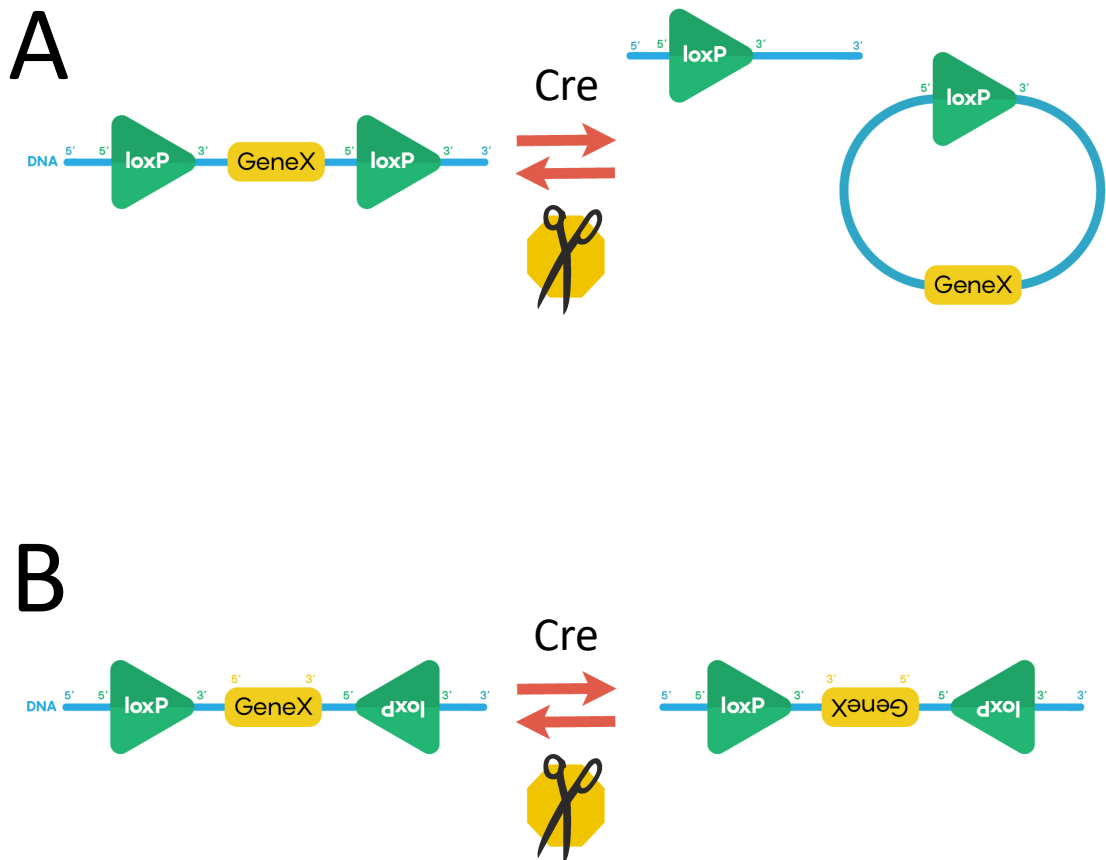


Figure 3-2

- A. When loxP sequences are arranged in the same direction, the Cre enzyme catalyses excision (deletion) of the stretch of DNA between the two markers. Modified from(217).
- B. When loxP sequences are arranged in opposite directional orientation, the Cre enzyme catalyses the inversion of the intervening DNA. Modified from(217).

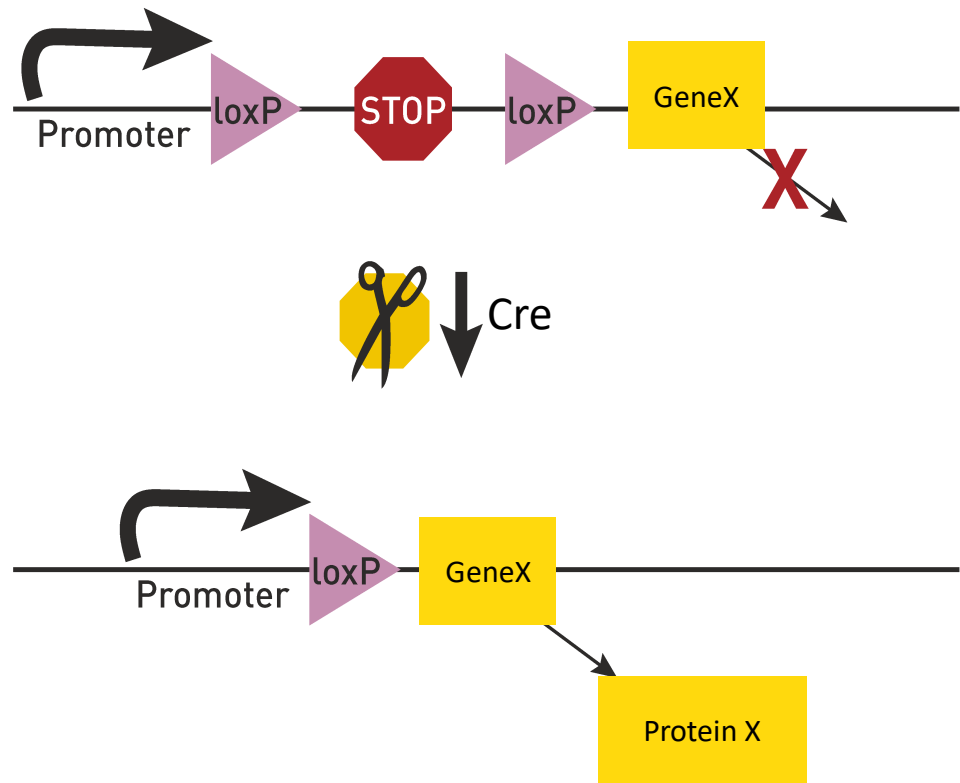


Figure 3-3 When loxP sequences are arranged in the same direction, around a stop codon downstream of a promoter, they can be used to control expression of genes of interest. In this example, in cells without the Cre enzyme, GeneX will not be expressed due to the preceding stop codon. With the addition of Cre however, the stop codon is excised and expression permitted – but only in those cells. Modified from(196)

Inducible Cre.LoxP systems for retinal bipolar cells

A number of Cre mouse lines are available with expression driven by promoters with varying specificity for retinal bipolar cells including those associated with (among others) the *L7*(163), *Htr2a*(218), *Chx10*(219) and *Grm6*(35) promoters. The first two of these had retinal expression characterised by crossing with a line carrying floxed EYFP: mainly rod ON-bipolar cells (75%) expressed the indicator in *L7* with some type 6 cone ON-bipolar cells(213, 220) while in the *Htr2a* mouse type 3b and 4 cone bipolar cells were highlighted(220). This Cre expression was also assessed functionally with intravitreal delivery of a modified capsid AAV vector containing a similar indicator under the control of a *floxed* promoter to the *L7*.Cre mouse resulting in a similar expression of mainly Rod ON-bipolar cells with some cone types 2 & 6 bipolar cells and a small population of retinal ganglion cells(221)(see Chapters 7).

As these approaches of targeting ON-type bipolar cells with proteins driven by high efficiency *floxed* promoters via modified capsid AAV and by crossing with *floxed* indicator lines were central to our experimental plan and had been well demonstrated in the *L7* line, it was selected for incorporation into our model. The *L7*.Cre or C57/BL6^{Tg(L7-Cre)} line was originally generated by Marino et al(163) by integrating a DNA fragment containing the *Cre* gene within exon 4 of the *L7* gene (see also Chapter 7).

Inducible Cre.LoxP systems for retinal ganglion cells

Similar to *L7*, many Cre lines with expression in the retinal ganglion cells are available with varying subpopulation specificities. As may be expected with the large numbers of RGC subtypes (approaching thirty(222)) the vast majority of these label multiple RGC subtypes types with a large overlap with amacrine cells(222, 223). A small number of lines however are more specific in their Cre expression and have been characterised

in the mouse retina such as the *Grik4.Cre*(164) mouse with expression in ON type direction selective retinal ganglion cells (213, 222).

The *Grik4* gene codes for a subunit of the kainite glutamate receptor and its promotor was first used to drive Cre expression in the C57/BL6^{Tg(Grik4-Cre)} line by Nakazawa et al(164). A variant of this line was available from a local collaborator(224) however with the gene *Oxr1* Oxidation resistance 1 additionally knocked out (*Oxr^{d/d}*)(225). This resulted in an extra breeding round to return the *Oxr* locus to a homozygous wild type state.

Transgenic indicators of Cre expression

As we planned to use Cre lines to label cells transgenically (Chapter 4), a line carrying a *floxed* indicator protein was also required. As it had been successfully applied previously in the Cre lines we wished to use(213, 220), the C57/B6Gt(*ROSA*)26Sor^{tm1(EYFP)Cos} mouse(226) was selected which carries a copy of EYFP preceded by *floxed* stop codon (Figure 3-3) at the Rosa26 locus.

Experimental design and results

Description of breeding approach

In order to answer the proposed research questions, three separate mouse lines, based on crossbreeding of the above founder lines were required:

1. “LMAC” – that would express L7.Cre in bipolar cells, have a retinal degeneration phenotype and carry a *floxed* EYFP indicator to label Cre + retinal bipolar cells in order to allow their isolation by FACS.
 - a. See Figure 3-4 for details of breeding strategy
 - b. See Chapter 4 for experimental work using this line
2. “LMAF” - that would express L7.Cre in bipolar cells, have a retinal degeneration phenotype and additionally be devoid of native melanopsin in order to allow comparison of different methods of optogenetic restoration of light responses in the retina without residual native light responses.
 - a. See Figure 3-5 for details of breeding strategy
 - b. See Chapters 5 to 7 experimental work using this line
3. “CGRK” - that would express grik4.Cre in bipolar cells, have a retinal degeneration phenotype and additionally be devoid of native melanopsin in order to allow comparison of different methods of optogenetic restoration of light responses in the retina without residual native light responses.
 - a. See Figure 3-6 for details of breeding strategy
 - b. See Chapters 5 and 6 for experimental work using this line

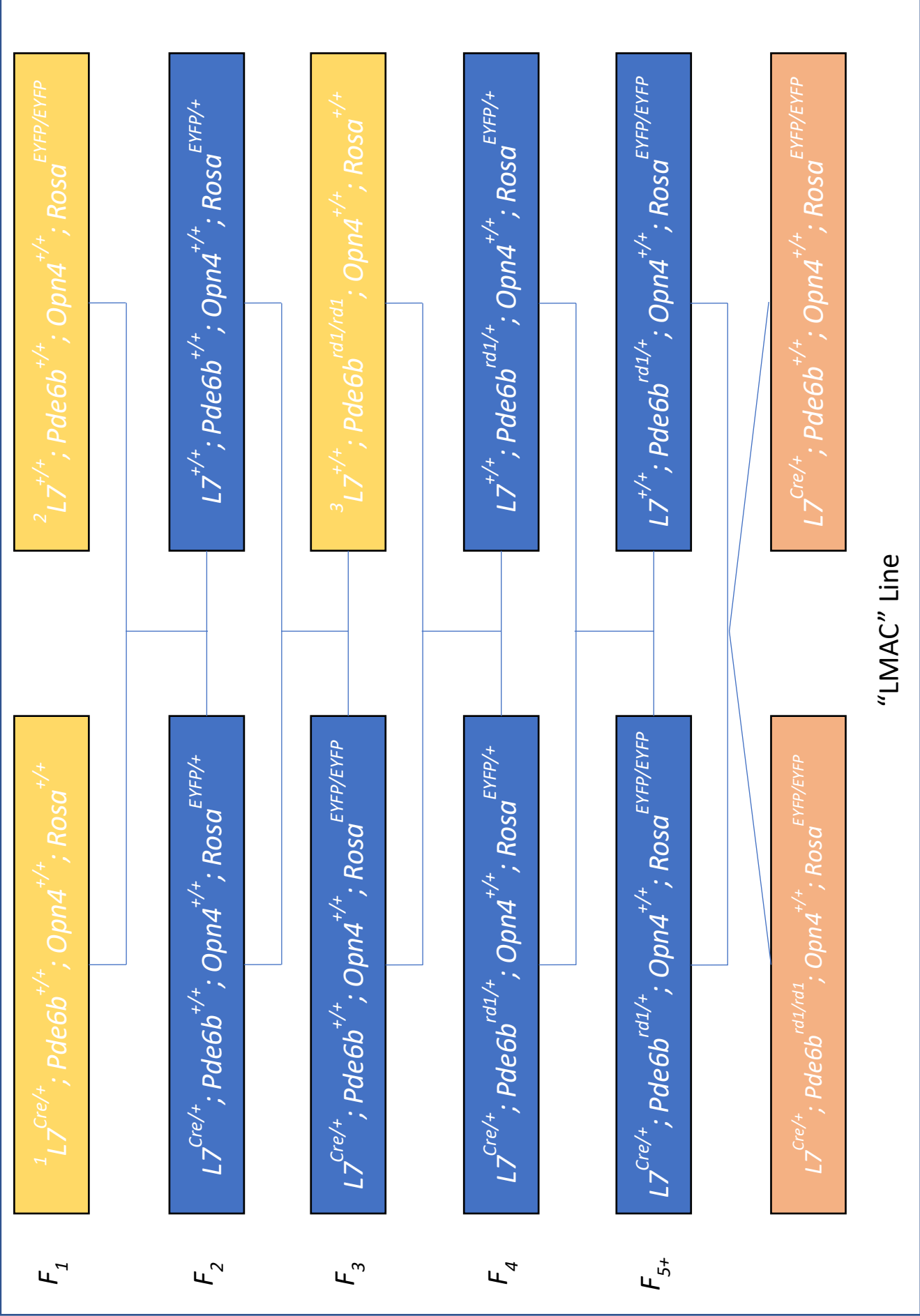


Figure 3-4 Breeding strategy used to produce the (“LMAC” – an arbitrary name) L7.Cre, retinally degenerate, floxed EYFP indicator line.

Yellow box – new lines introduced into the breeding strategy

Blue box – intermediate breeders, drawn from the progeny of the previous generation

Orange box - offspring genotypes used in experimental work

For brevity, gene names and symbols have been abbreviated:

L7^{Cre/+} refers to the Tg(L7-Cre) locus(163).

Pde6b^{rd1/rd1} refers to the retina degeneration causing mutation (rd1) in the Pde6b gene

Opn4^{LacZ/LacZ} refers to the Opn4^{tm1you} melanopsin knock out line(162)

Rosa^{EYFP/EYFP} refers to the Gt(ROSA)26Sor^{tm1(EYFP)Cos} (226).

^{1.} The L7.Cre line described in the text(163);

^{2.} The rd1 mouse described in the text (161, 197)

^{3.} The floxed EYFP Gt(ROSA)26Sor^{tm1(EYFP)Cos} (226).

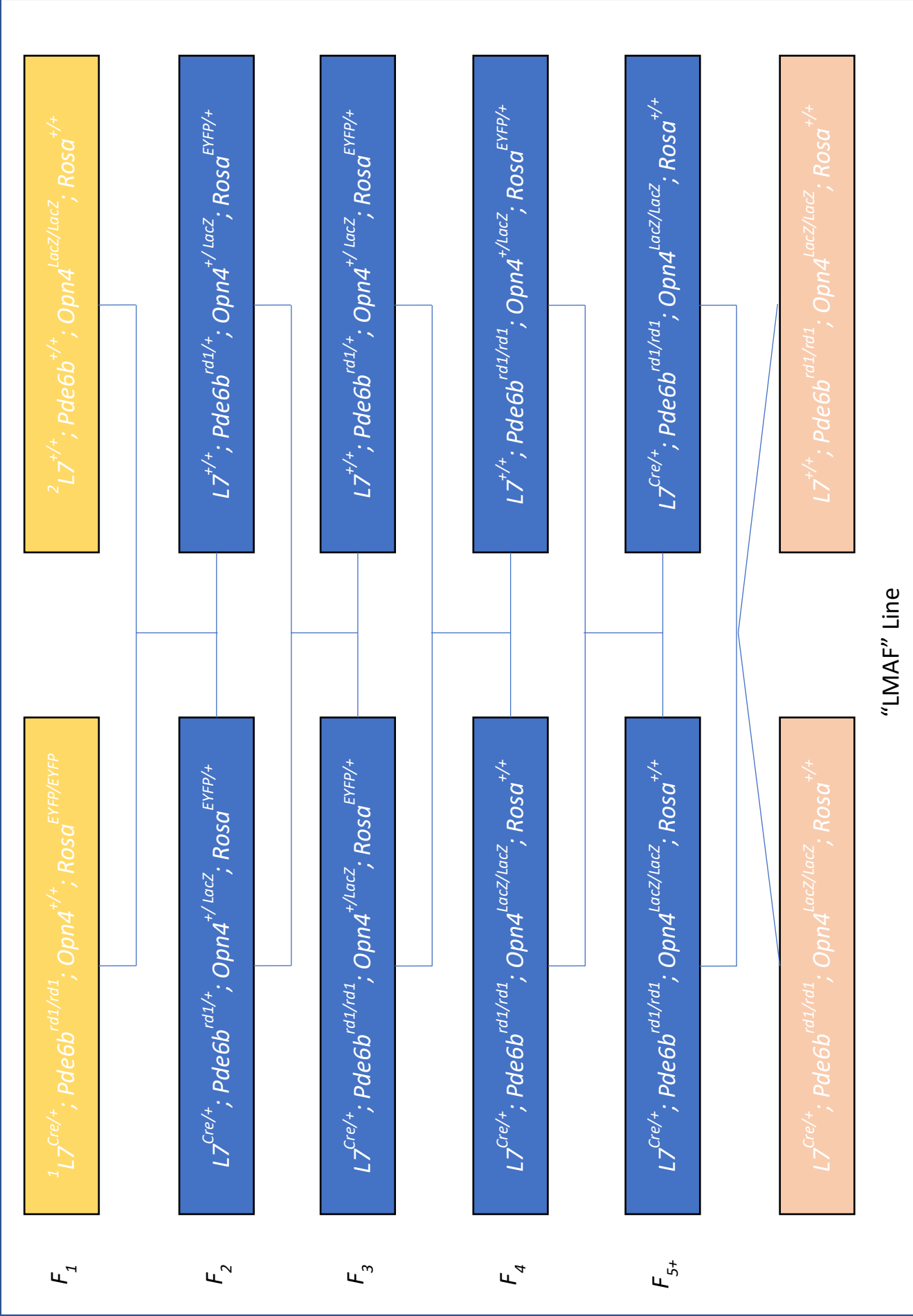


Figure 3-5 The breeding strategy used to produce the ("LMAF" – an arbitrary name) L7.Cre, retinally degenerate, melanopsin knock out line.

Yellow box – new lines introduced into the breeding strategy

Blue box – intermediate breeders, drawn from the progeny of the previous generation

Orange box - offspring genotypes used in experimental work

For brevity, gene names and symbols have been abbreviated:

L7^{Cre/+} refers to the Tg(L7-Cre) locus(163).

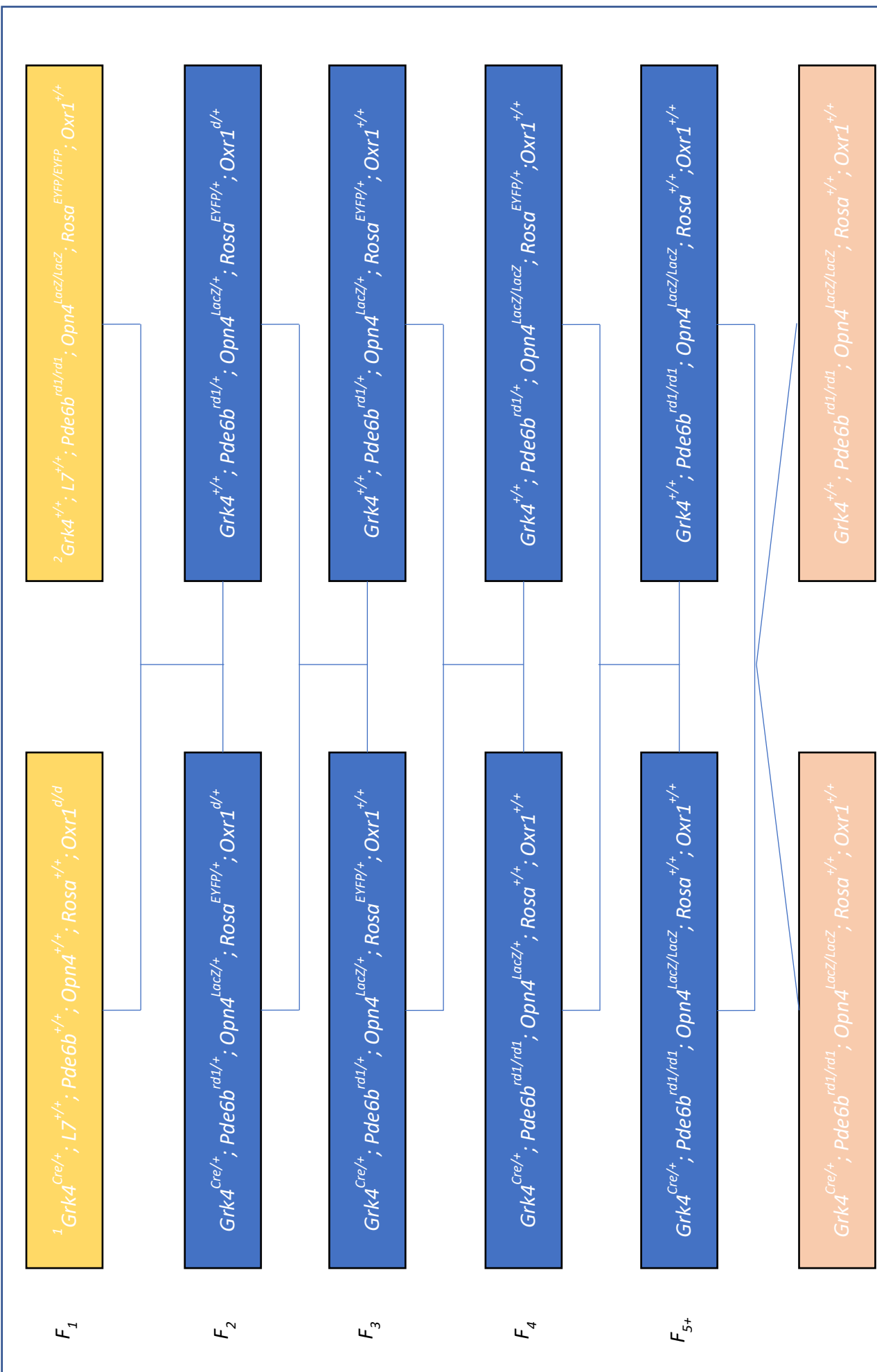
Pde6b^{rd1/rd1} refers to the retina degeneration causing mutation (rd1) in the *Pde6b* gene

Opn4^{LacZ/LacZ} refers to the *Opn4^{tm1you}* melanopsin knock out line(162)

Rosa^{EYFP/EYFP} refers to the *Gt(ROSA)26Sor^{tm1(EYFP)Cos}*(226).

1. Progeny from the "LMAF" line described in Figure 3-4

2. the *Opn4^{tm1you}* melanopsin knock out line(162)



“CGRK” Line

Figure 3-6 Breeding strategy used to produce the ("CGRK" – an arbitrary name) *grk4.Cre*, retinally degenerate, melanopsin knock out line.

Yellow box – new lines introduced into the breeding strategy

Blue box – intermediate breeders, drawn from the progeny of the previous generation

Orange box - offspring genotypes used in experimental work

For brevity, gene names and symbols have been abbreviated:

Grk4^{Cre/+} refers to the *Tg(Grk4-Cre)* locus(164, 224).

Pde6b^{rd1/rd1} refers to the retina degeneration causing mutation (*rd1*) in the *Pde6b* gene

Opn4^{LacZ/LacZ} refers to the *Opn4^{tm1you}* melanopsin knock out line(162)

Rosa^{EYFP/EYFP} refers to the *Gt(ROSA)26Sor^{tm1(EYFP)Cos}* (226).

Oxr1^{d/d} refers to the oxidation resistance 1 knock out allele (see text)(224, 225)

1. The *Grk4^{Cre/+} Oxr1^{d/d}* cross received from the Oliver lab (164, 224, 225)

2. Progeny from the "LMAF" line (Figure 3-5) that do not carry *Tg(L7-Cre)*

During the breeding phase of each of the three lines, validation of genotype (for each gene of interest) was required to guide selection of breeding pairs for the subsequent generation; additionally, validation of phenotypes was also required of the resulting mice that were to be used as experimental animals.

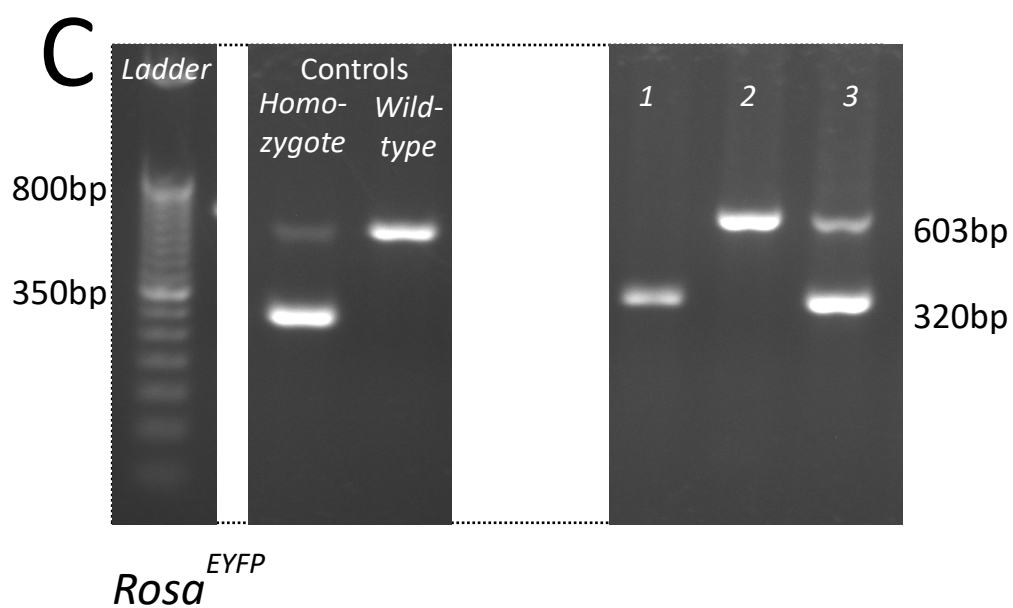
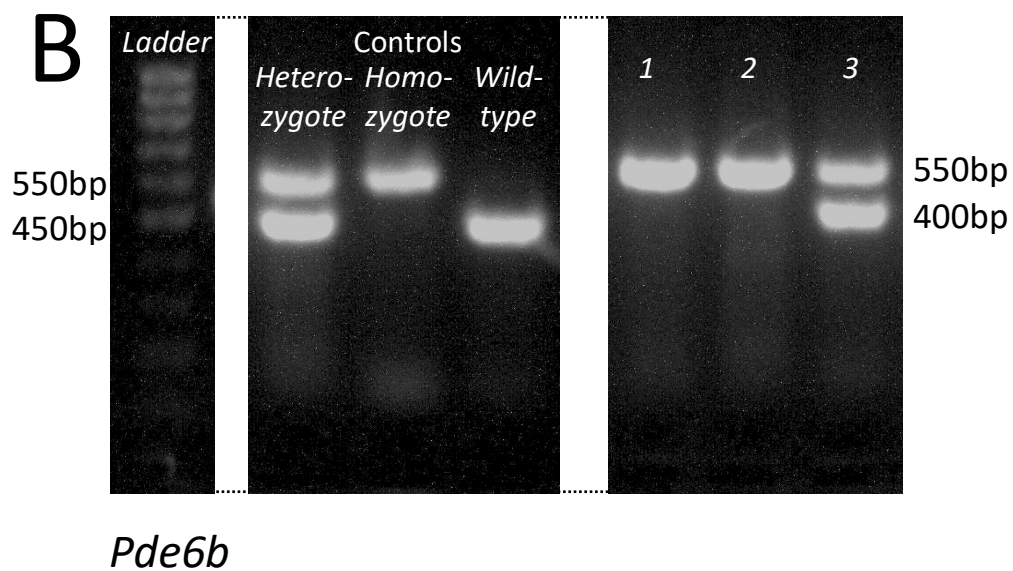
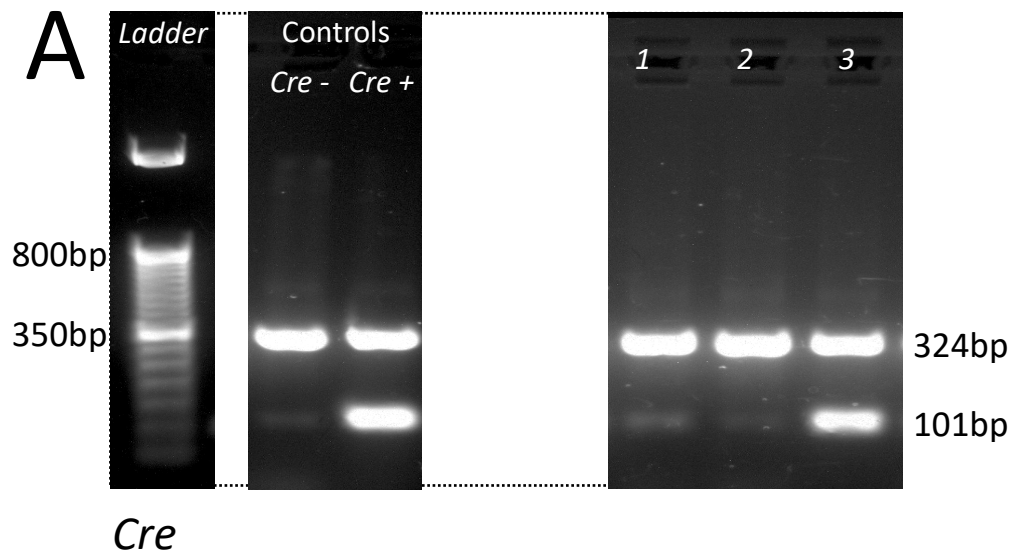
Validation of the transgenic mouse lines

PCR genotyping

The mainstay of genetic validation during breeding of the three lines was the genotyping of each locus of interest using either traditional PCR protocols followed by agarose gel electrophoresis (*Tg(L7-Cre)*, *Tg(Grk4-Cre)*, *(ROSA)26Sor^{tm1(EYFP)Cos}*, *Oxr1*, *Gpr179* - Figure 3-7) or quantitative PCR (*Opn4*, *LacZ* – Figure 3-8). Mutations in *Gpr179* are associated with a form of congenital stationary night blindness in humans(227), mutations of which are endemic in laboratory mice(228) and can result in dysfunction of the native bipolar light signalling cascade. Therefore, the first generation of all breeding strategies were genotyped and found to be homozygous wildtype for this mutation.

Immunohistochemical phenotype validation

To ensure that retinal phenotypes continue to correspond to those expected given the determined genotype, immunohistochemical staining of retinal cryosections was undertaken. IHC was performed on a genotyped progeny of every new breeding pair set up in all lines. Mice were not used for breeding or experimental work until this phenotype had been confirmed. Staining was performed for: YFP, LacZ and OPN4, the phenotype of retinal degeneration was visible without immune staining as the photoreceptor layer was missing in *Pde6b^{rd1/rd1}* animals (Figure 3-9, Figure 3-10, Figure 3-11).



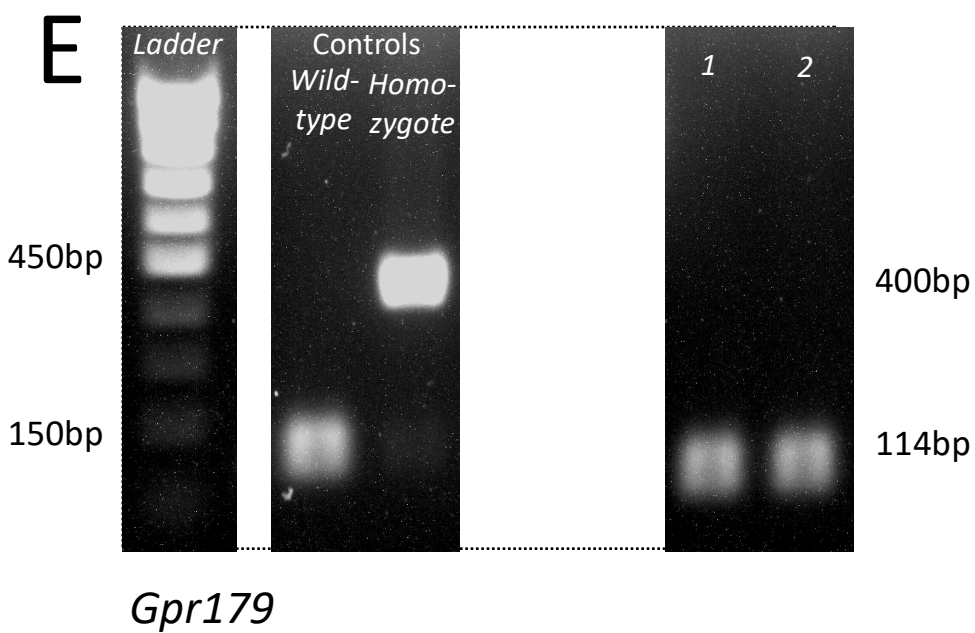
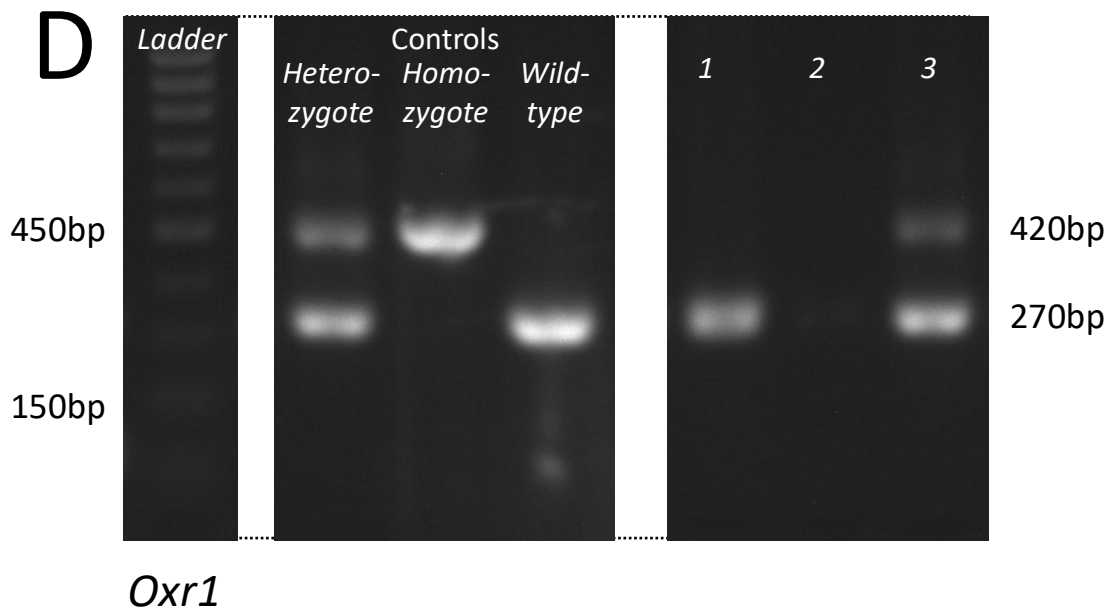


Figure 3-7 Example appearances of genotyping polymerase chain reaction products run on 2% agarose gel. Labels on left hand side of images refer to corresponding bands on the ladder. Labels on the right give expected sizes of PCR reaction products.

A – Cre recombinase. Note the band at 324bp representing a control PCR reaction run in multiplex in the same samples as the genotype reaction which returns a 101bp product when a Cre gene is present anywhere in the sample (this reaction cannot differentiate hetero- from homozygotes)

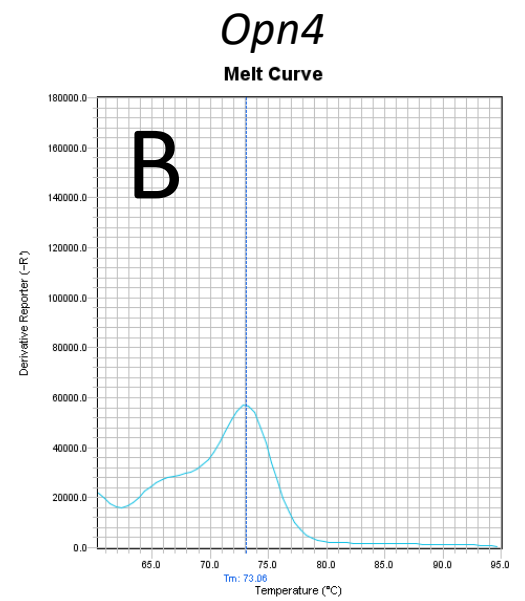
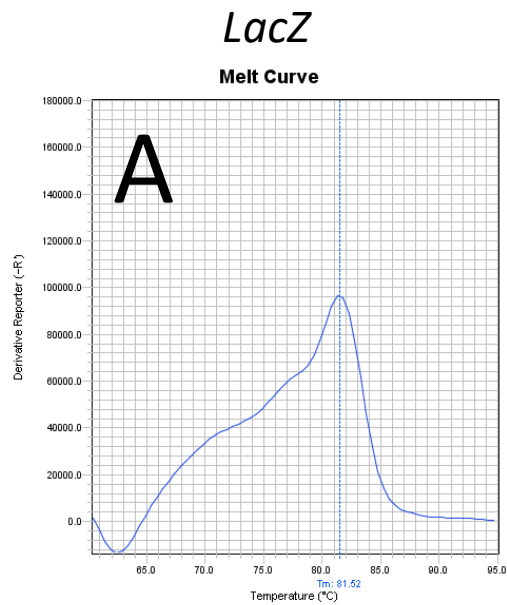
B – *Pde6b^{rd1}* mutation

C – *Rosa26^{EYFP}*

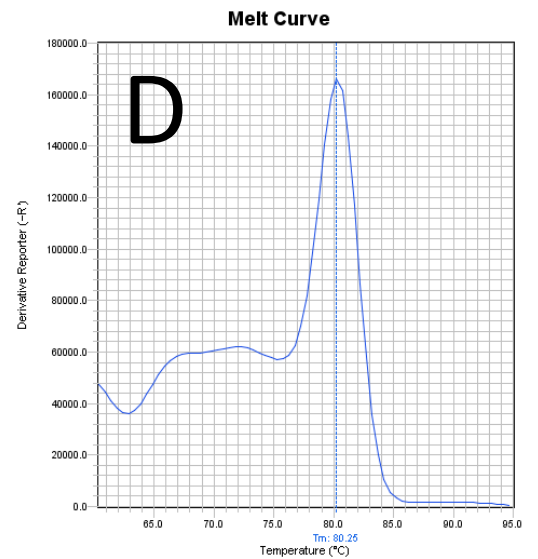
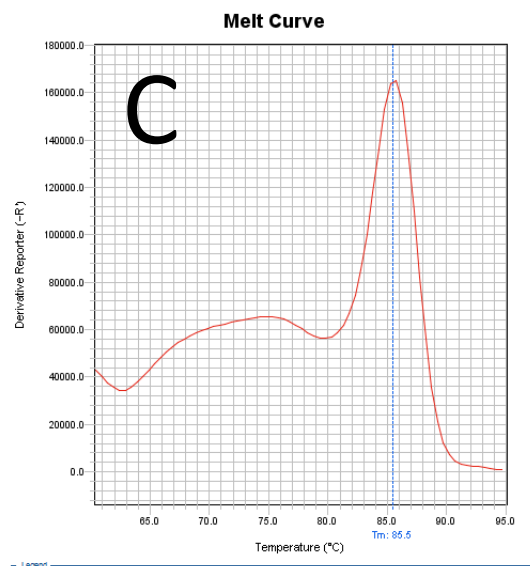
D – *Oxr^d* Note lane 2 in this example, where the reaction failed and there are no bands

E – *Gpr179*

Negative control



Positive control



Amplification trace

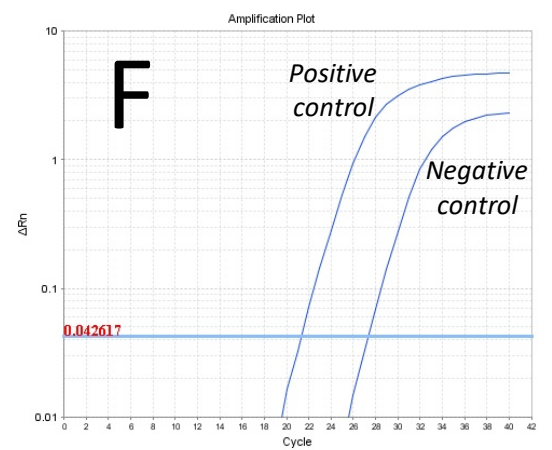
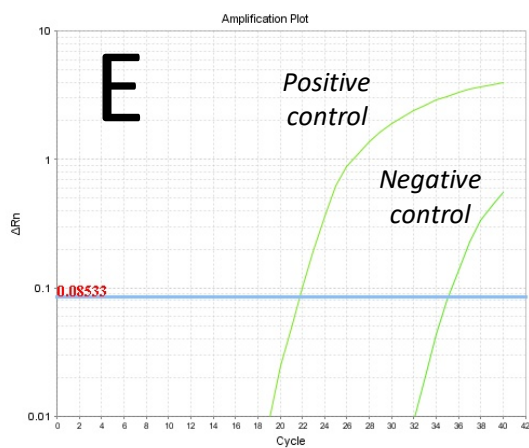


Figure 3-8 Genotyping for $Opn4^{LacZ}$ (knock out) or $Opn4^+$ (wild type)

A & B - Melt curves from negative control sample i.e. samples not containing the allele of interest (A - for $Opn4^{LacZ}$; B – $Opn4^+$ wild type melanopsin.

C & D - Melt curves from negative control sample i.e. samples not containing the allele of interest (A - for $Opn4^{LacZ}$; B – $Opn4^+$ wild type melanopsin.

E & F – Example amplification traces from positive or negative controls

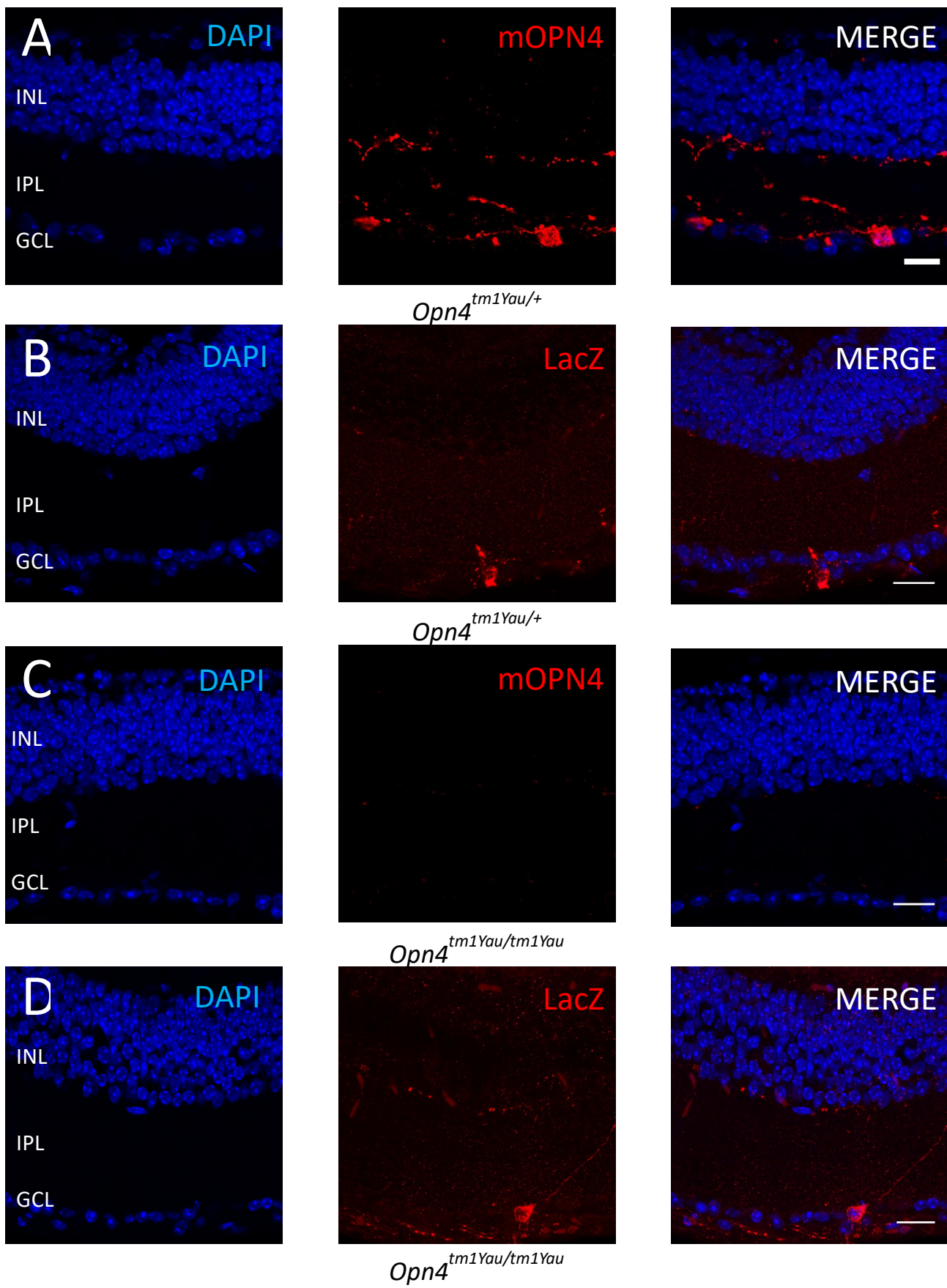


Figure 3-9 . IHC of retinal cross sections from two “LMAF” mice. Note the lack of photoreceptor layer as both mice were $Pde6b^{rd1/rd1}$ and therefore expected to display a retinal degeneration phenotype.

*A&B – This mouse was an intermediate breeder, heterozygous for the wildtype (mouse) *Opn4* gene $OPN4^{tm1Yau/+}$. Staining for both Lac Z & mOPN4 in the same mouse in the ipRGC s confirms the expected phenotype.*

*C&D - This mouse was a final breeder, homozygous for the *tm1Yau* LacZ construct $OPN4^{tm1Yau/tm1Yau}$, and therefore has no native melanopsin, hence the expected lack of staining in panel C. Confirming the expected phenotype.*

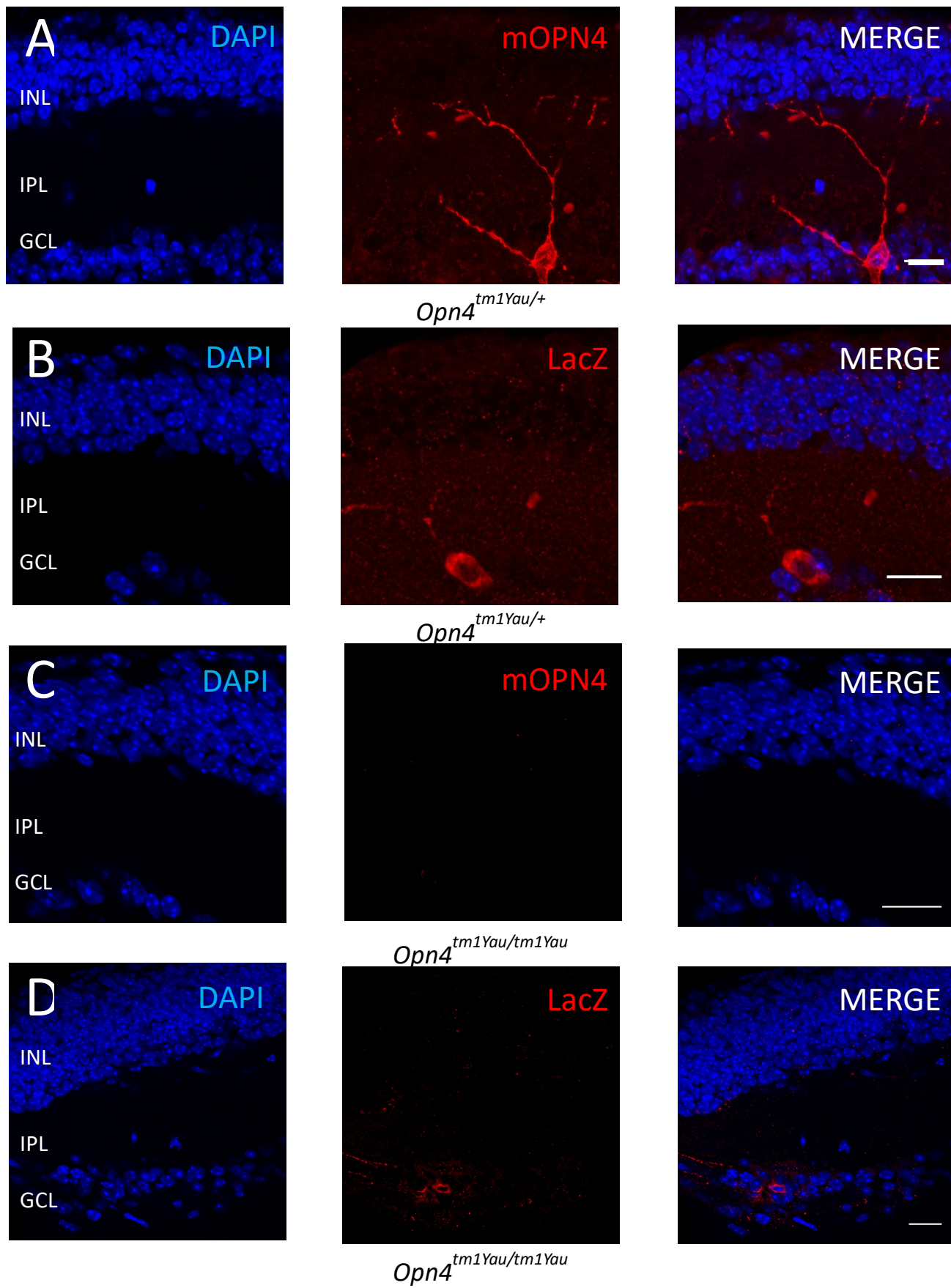


Figure 3-10 IHC of retinal cross sections from two “CGRK” mice. Note the lack of photoreceptor layer as both mice were $Pde6b^{rd1/rd1}$ and therefore expected to display a retinal degeneration phenotype.

*A&B – This mouse was an intermediate breeder, heterozygous for the wildtype (mouse) *Opn4* gene $OPN4^{tm1Yau/+}$. Staining for both Lac Z & mOPN4 in the same mouse in the ipRGC s confirms the expected phenotype.*

*C&D – This mouse was a final breeder, homozygous for the *tm1Yau* LacZ construct $OPN4^{tm1Yau/tm1Yau}$, and therefore has no native melanopsin, hence the expected lack of staining in panel C. Confirming the expected phenotype.*

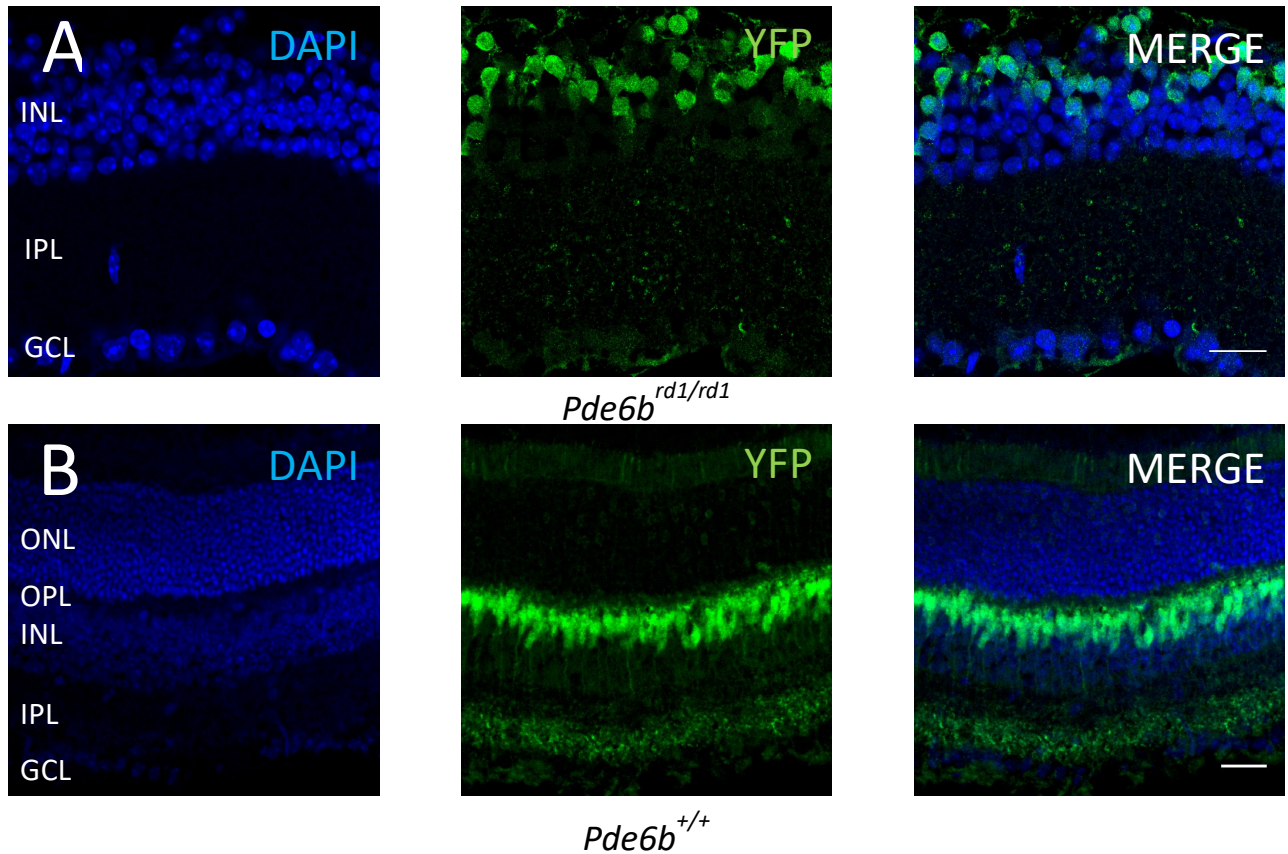


Figure 3-11 IHC of a retinal cryosection from an "LMAC" mouse. Note YFP staining in the inner nuclear layer, consistent with Cre expression in L7 expressing bipolar cells in turn allowing expression of floxed EYFP, confirming expected phenotype.

A - Also note loss of photoreceptor layer in A, in keeping with expected *rd1* mutation phenotype.

B - Note presence of photoreceptor layer in B in keeping with expected wild type phenotype. Image credit panel B – Doron Hickey(165)

Scale bars 20µm. INL – Inner nuclear Layer, IPL – Inner plexiform layer, GCL – Retinal ganglion cell layer, OPL - Outer plexiform layer, ONL – Outer Nuclear Layer

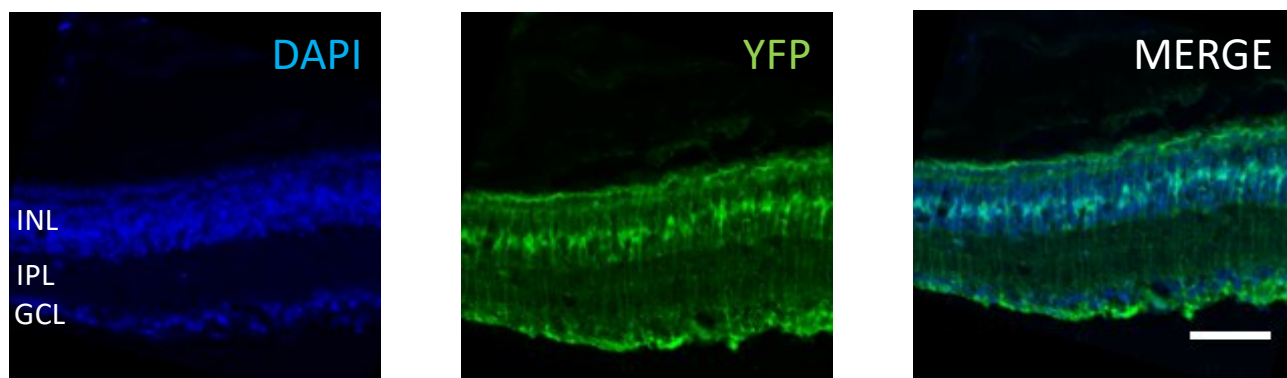


Figure 3-12 . IHC of a retinal cryosection from an "LMAC" mouse demonstrating "indicator read through." Note diffuse staining of whole retina with YFP, rather than restriction to bipolar cells. Also note loss of photoreceptor layer, in keeping with expected *rd1* mutation phenotype EYFP. Scale bar 50µm INL – Inner nuclear Layer, IPL – Inner plexiform layer, GCL – Retinal ganglion cell layer

The necessity of this approach was confirmed when after several generations of breeding in the “LMAC” line, in some animals EYFP began to be seen not only in bipolar cells, as expected, but in most cell types of the retina (including Müller cells - Figure 3-12 suggesting “read-through” of the floxed stop codon at the *Rosa26* locus in these mice.

Functional phenotype validation (Cre)

In addition, IHC validation of Cre protein expression would also have been useful.

However very few published studies have reported direct staining of Cre on retina IHC in transgenic Cre lines, instead relying on floxed reporters (and I also found IHC for Cre to be unreliable, having tried several antibodies). A factor in this may be the relatively low levels of Cre protein required for functionality and certainly the L7 promotor construct used here(229) has been reported to be of low efficiency(166, 230).

However, it was relatively easy to functionally phenotype our Cre expressing lines by intravitreally administering a control AAV containing a floxed protein(220) (human melanopsin) that in turn could be stained for with IHC (Figure 3-13 & Figure 3-14).

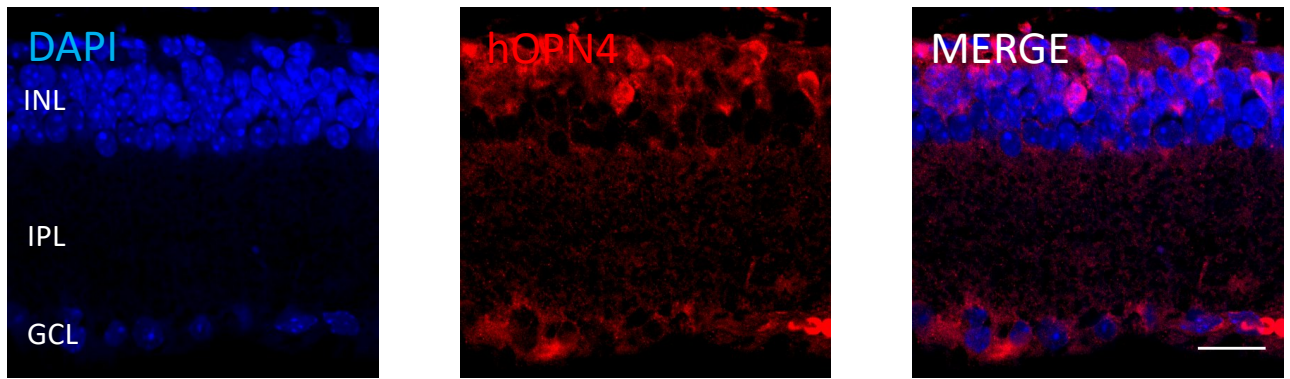


Figure 3-13 Functional assessment of Cre activity using floxed AAV in a "LMAF" mouse. Note expression of hOPN4 in inner nuclear cell layer. Scale bar 20 μ m, INL – Inner nuclear Layer, IPL – Inner plexiform layer, GCL – Retinal ganglion cell layer

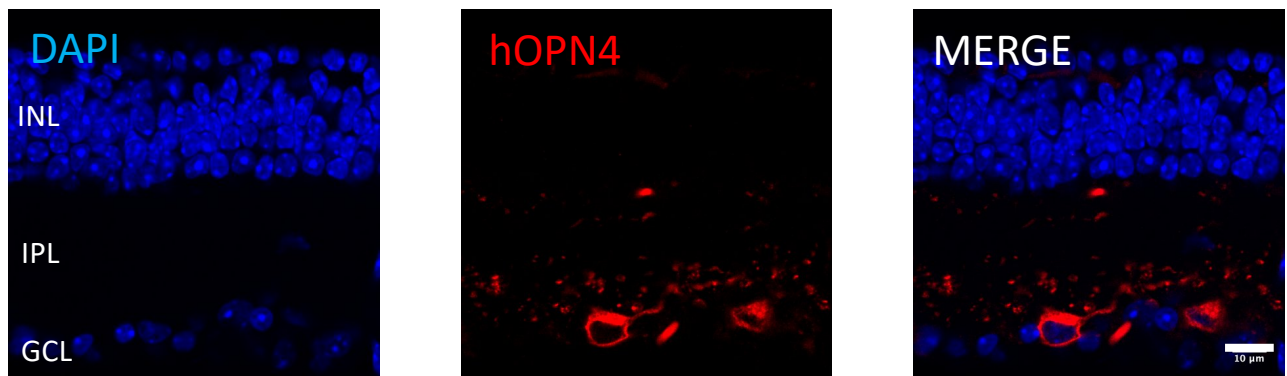


Figure 3-14 Functional assessment of Cre activity using floxed AAV in a "CGRK" mouse. Note expression of hOPN4 in RGC layer. Scale bar 10 μ m, INL – Inner nuclear Layer, IPL – Inner plexiform layer, GCL – Retinal ganglion cell layer

Discussion

In this Chapter the design of three mouse models essential to answer our scientific questions have been summarised: the choice of origin lines, the strategy to breed these together; the genotyping and phenotyping of the resulting progeny before use in experimental work. In summary all three lines produced mice of the required phenotype with functional Cre in the appropriate cells to allow cell specific expression of floxed proteins (such as optogenetic tools and indicators delivered by AAV).

Limitations of the lines produced

EYFP “read through”

As noted above, after several generations of breeding it was noted that occasional mice within the “LMAC” line would appear to express EYFP in almost all of their retinal cells. Initially, as this was sporadic (and did not appear to follow a specific pattern of inheritance), mice with such a phenotype could be discovered using screening with scanning laser ophthalmoscopy (SLO) and excluded from experimentation or breeding(165). Interestingly this effect had also been noted in similar mice at other units(231).

More robust indicator lines had become available since the original breeding of the “LMAC” line. For example, incorporating multiple floxed stop codons(232), rather than a single one in order to prevent such “read through” of the (presumably methylated) single floxed stop codon in the *Gt(ROSA)26Sor^{tm1(EYFP)Cos}* line. Practically however, as the indicator would not be specifically required in the “LMAF” and “CGRK” lines other than for phenotyping, it was felt prudent to simply breed out the EYFP, returning the Rosa26 locus to wildtype.

Consideration of background strain

Common inbred strains of laboratory mice are known to have varying ability in visual tasks(233) and visually disruptive mutations are often found in common strains of laboratory mice(234, 235) (see Table 3-1). Therefore, a consideration of the likely genetic background of our lines is warranted.

The majority of our origin mice were based on a C57/BL6/J background, a strain often noted to be relatively visually able and free from such inbred defects(233, 234), however the C3H/HeN & FVB backgrounds have been associated with endemic mutations *Pde6b*^{rd1/rd1}(191, 195) and *Gpr179*^{nob/nob}(228) which were specifically genotyped for.

Even with this, as other - as yet uncharacterised - strain differences may alter the innate electrophysiological and behavioural responses to light, careful consideration was given to backcrossing the described lines onto a known background strain (such as the C57/BL6/J) for reasons of comparability and consistency. However, with up to five genes to consider as well as the need to breed Cre transgenes as hemizygotes due to unknown loci of insertion(236) this process would be enormously laborious, lengthy and costly. Therefore, these colonies were not systematically backcrossed, but kept as self-sufficient inbred lines after the initial breeding phase for several reasons.

In the case of the “LMAC” colony (Chapter 4) a more varied genetic background could be said to allow more transferrable conclusions to be drawn regarding gene expression changes(165). Practically for all lines, the breeding of Cre transgenes as hemizygotes allowed for the production of sibling mice with and without Cre, but with an identical mixed genetic background, that could act as experimental controls (rather

than wildtype mice); similarly, in the “LMAC” allowing litter mates with $Pderb^{rd1/rd1}$ or $Pde6b^{+/+}$ for direct comparison.

Origin Line	Genetic Background Strain	Common visually significant mutations(234)
Pde6b ^{rd1/rd1} (77, 78, 161)	C3H/HeN	<i>Gpr179^{nob/nob}</i> , <i>Pde6b^{rd1/rd1}</i>
OPN4 ^{tm1Yau} (162)	C57BL/6J	Normal vision
Tg(L7-Cre)(163)	FVB	<i>Pde6b^{rd1/rd1}</i>
Tg(grk4-Cre)(164)	C57BL/6J	Normal vision
Gt(ROSA)26Sor ^{tm1(EYFP)Cos} (226)	C57BL/6J	Normal vision
Oxr1 ^{d/d} (225)	C57BL/6J	Normal vision

Table 3-1 Genetic background of the various origin lines contributing to the three multiple transgenic lines described in the text: “LMAC”, “LMAF” and “CGRK.” The righthand column refers to genes in which mutations are commonly found in the background strain – not necessarily the particular line used.

Conclusion

While these limitations must be remembered in interpreting experimental results, this Chapter has described the planning, production and validation of three powerful multiple transgenic mouse lines, eminently suited to investigating the proposed research questions. The following four Chapters will discuss in detail experiments and conclusion drawn from the use of these models.

Chapter 4 Results: Bipolar cell gene changes in degenerate retinae

Introduction^{1,2}

Adeno-associated virus (AAV) delivered optogenetic tools have been shown to restore electrophysiological and behavioural responses to light in animal models of IRDs(100, 106, 159) and the general principle of survivor-cell stimulation has also been demonstrated clinically with retinal prostheses (111, 237).

However, what remains less clear is if there is an advantage to optogenetic stimulation of specific cell populations higher in the retinal hierarchy - such as the bipolar cell - rather than the more accessible retinal ganglion cells. Preserving the early image processing carried out by bipolar cells is conceptually attractive, however, marked retinal remodelling does occur following the loss of the photoreceptor(69, 238), potentially compromising the suitability of these cells as targets for such stimulation. Indeed, changes in morphology, synaptic connections, electrophysiological responses and receptor expression(79, 80, 85, 89, 90, 239, 240) have been observed in human and animal studies (Chapter 1).

What is less well understood is how changes specifically within the bipolar cells during degeneration might affect their viability as optogenetic targets. Particularly, if they are free from pathophysiological metabolic stress, receptive to adeno-associated

¹ The first sections of this Chapter (Initial breeding of "LMAC" animals, FAC sorting of cells and preparation and performance of gene array) represent work carried out by D.G. Hickey. This is included here for clarity – that the entire project may be presented as a continuous narrative. The remainder of the Chapter (data analysis, candidate gene selection and immunohistochemistry) is my own work.

² This work has been submitted (in a revised form) for publication in the journal "Human Molecular Genetics".

viral vectors, suitable for opsin based optogenetic tools and able to propagate their light signal by releasing neurotransmitter in response to exogenous optogenetic stimulation. While studies of global gene expression changes in the degenerate retina exist in the literature(91, 241-245), none has considered the retinal bipolar cell in isolation.

Investigation of bipolar cells is particularly apposite for two reasons: first, the development of delivery tools to specifically target discrete retinal cell populations (such as cell specific promoters(246) and AAV capsid tropism(134)) are making clinical cell-specific delivery a realistic possibility (Chapters 1 and 7). Secondly, human opsins such as rhodopsin(106) and melanopsin(159, 160) are being described as sensitive optogenetic tools. These are thought to couple to endogenous G-protein signalling cascades(52) allowing greater signal amplification compared to microbial opsins, such as channelrhodopsin, which lack such coupling. However, this could be affected by changes in expression of constituents of these signalling cascades in bipolar cells during retinal degeneration.

Therefore, investigation of retinal bipolar cells specifically in the context of inherited retinal degeneration is paramount in determining if this conceptually attractive strategy of bipolar specific targeting is practically feasible. Do retinal bipolar cells in the degenerating retina continue to express genes necessary for AAV delivered opsins to effectively restore effective light responses in degenerate retina? To answer this question, gene expression patterns were compared in isolated bipolar cells from retinae with a degenerate phenotype and those from retinae from non-degenerate phenotype. A shortlist of differentially expressed genes was drawn up and four exemplar candidates highlighted for further functional investigation.

Experimental design and results

Summary of experimental approach

To examine gene expression in bipolar cells, it was first necessary to isolate these cells (Figure 4-1, Chapter 2), for this the “LMAC” mouse line (Chapter 3) was used. These mice express EYFP protein within their retinal ON-bipolar cells while also demonstrating either a wildtype or retinal degenerate phenotype due to the *Pde6b^{rd1}* mutation carried in the line. This presence of the EYFP protein in rod and cone type 2 & 6 ON-bipolar cells(220) allowed fluorescence assisted cell sorting (FACS) to isolate labelled (ON-bipolar) cells from suspensions of dissociated retinal cells from either degenerate or wildtype retinae.

RNA was then extracted from the isolates and used on an Illumina microarray®, which in turn could be used to compare expression in isolated bipolar cells from either degenerate or wildtype retinae. Genes differentially expressed were systematically reviewed and four candidate genes further characterised with IHC.

FACS isolation, validation and RNA extraction³

*As previously reported:(165), twelve “LMAC” mice (six *Pde6b^{+/+}* and six *Pde6b^{rd1/rd1}*)*

underwent cervical dislocation at P89 to P91, six hours into their light phase with

immediate enucleation. All mice were littermates, three of each group were female.

Retinae were dissected with special care to remove retinal pigment epithelium before

cell dissociation using a papain dissociation protocol.

³ NB - This section “FACS isolation, validation and RNA extraction” represents work carried out by a colleague (Hickey 2016) (165 Hickey, D.G. (2016), In *NDCN*. University of Oxford, Oxford, Vol. D. Phil. and I provide it here for context.

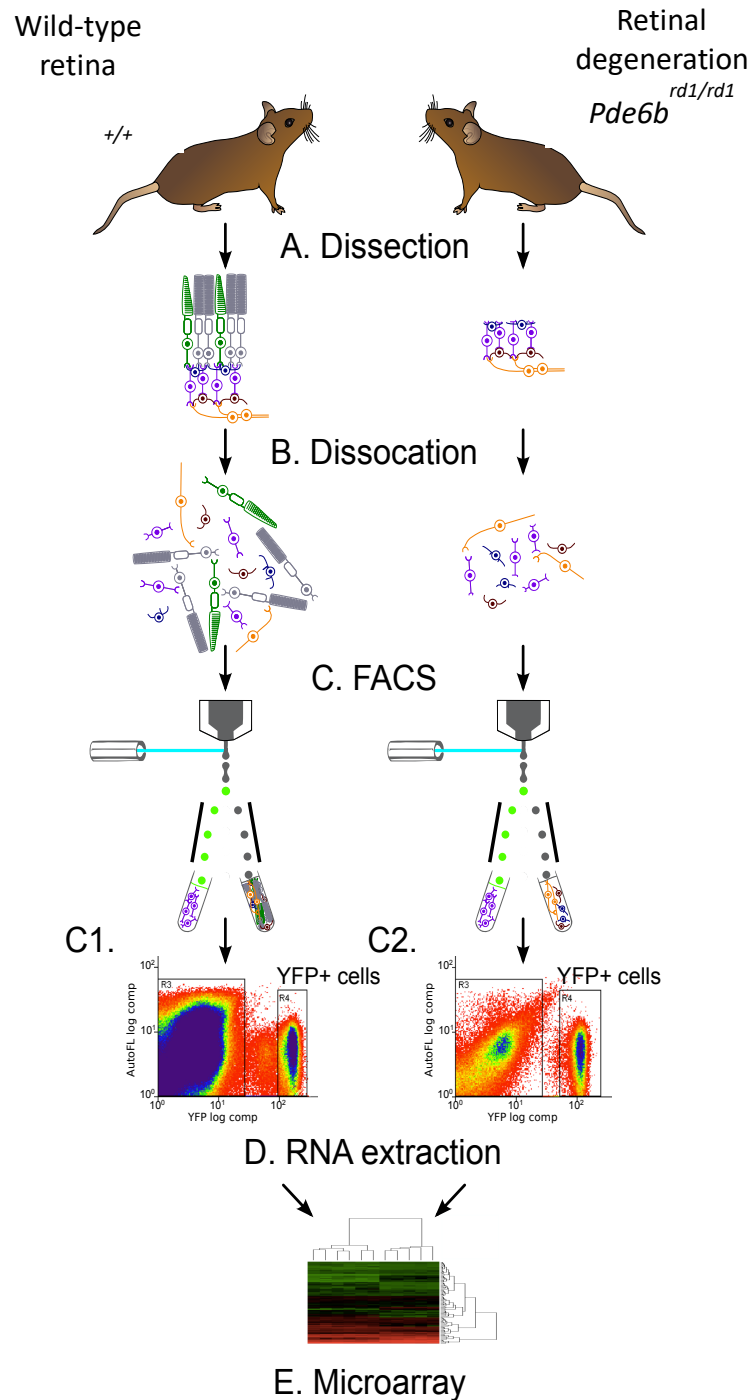


Figure 4-1 Fluorescence-activated cell sorting for a microarray study of retinal bipolar cells from degenerate retinas. A – “LMAC” mice expressing floxed EYFP and Cre under the control of a bipolar cell specific promoter (L7, see Chapter 3) additionally with either a wildtype (*Pde6b*^{+/+}) or degenerate (*Pde6b*^{rd1/rd1}) retinal phenotype were culled. B - Retinae were immediately dissected with careful removal of the RPE before dissociation C - FACS was then used to isolate EYFP positive cells, D - RNA was extracted from the EYFP+ cell isolates and E – This RNA was used for a microarray study to compare gene expression between wildtype (*Pde6b*^{+/+}) and degenerate (*Pde6b*^{rd1/rd1}) mice NB. C1 represents an illustrative FACS dot plot of a single dissociated, non-degenerate L7.Cre.EYFP retina and C2 the same from a degenerate retina. Figure modified from(165)

The resulting dissociated cells were subjected to FACS (Figure 4-1). The identity of the sorted samples was confirmed by immunocytochemistry (ICC) with EYFP marker positive cells seen to co-stain with the bipolar cell marker PKC α . Quantitative polymerase chain reaction (qPCR) using standard methods(247) demonstrated a fifteen-fold increase in the EYFP+ isolate compared to the EYFP- for bipolar cell specific genes (Cabp5, Grm6, Pcp2 and Prkca) when normalised to housekeeping genes (Gapdh, Actb, Rplp0). Rod specific gene (Rho & Cnga1) expression in the EYFP+ isolate less than 1% of that in the EYFP- isolate.

RNA was extracted from these cell isolates with a mean RNA concentration of 364 ± 143 pg/ul; RIN 9.5 ± 0.1 in Pbe6b^{rd1/rd1} retinæ and 608 ± 263 pg/ul; RIN 9.4 ± 0.2 in Pbe6b^{+/+}. It was then processed for use on a MouseWG-6 v2 Expression Bead Chip (Illumina). Expression levels were compared using Lumi(248) and Limma(249) packages for R(250). Probes with fluorescence values not significantly different ($P > 0.01$) from control were excluded and quantile normalization was used to remove technical variation between arrays. Results were corrected for multiple testing using False Discovery Rate (FDR) testing(251)(Table 4-1). Probes were considered to be differentially expressed when adjusted $p < 0.05$. Quality controls integrated into the arrays included hybridization controls; mismatch and perfect match probes for stringency; labelling controls and negative controls.

Sixty-six such genes were significantly differentially expressed between Pde6b^{+/+} and Pde6b^{rd1/rd1} and are listed in Table 4-1.

Symbol	Probe/gene name	log ₂ fold Δ	p value
A - Probes upregulated in degeneration			
Igfn1	Immunoglobulin-like and fibronectin type III domain containing 1	4.10	0.0181
Cgn	Cingulin	3.52	0.0138
MsrB2	Methionine sulfoxide reductase B2	3.03	0.0344
Cabyr	Calcium binding tyrosine-(Y)-phosphorylation regulated	2.25	0.0380
Efnb1	Ephrin-B1	2.15	0.0312
Ccdc171	Coiled-coil domain containing 171	1.99	0.0458
Phc1	Polyhomeotic homolog 1	1.93	0.0276
Sfxn1	Sideroflexin 1	1.92	0.0195
Fam19a3	Family with sequence similarity 19 (chemokine (C-C motif)-like) A3	1.87	0.0458
Shroom2	Shroom family member 2	1.81	0.0416
Sfrs1	Serine/arginine-rich splicing factor 1	1.72	0.0417
Sept4	Septin 4	1.54	0.0324
Sulf2	Sulfatase 2	1.43	0.0344
B - Probes downregulated in degeneration			
Nrm	Nurim (nuclear envelope membrane protein)	-1.40	0.0416
Gm362	Predicted gene 362	-1.46	0.0138
Pde9a	Phosphodiesterase 9A	-1.55	0.0416
Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	-1.55	0.0414
Gabrg2	Gamma-aminobutyric acid (GABA) A receptor, gamma 2	-1.56	0.0494
Anxa7	Annexin A7	-1.56	0.0244
Fam195a	Family with sequence similarity 195, member A	-1.61	0.0053
Atox1	Antioxidant 1 copper chaperone	-1.62	0.0414
Socs5	Suppressor of cytokine signaling 5	-1.64	0.0149
Csrnp2	Cysteine-serine-rich nuclear protein 2	-1.65	0.0276
Rnf11	Ring finger protein 11	-1.75	0.0244
Cntn1	Contactin 1	-1.79	0.0494
COXIII	Mitochondrial cytochrome oxidase II subunit	-1.83	0.0244
Ap3m2	Adapter-related protein complex 3, mu 2 subunit	-1.85	0.0231
Rnf11	Ring finger protein 11	-1.88	0.0060
Mif	Macrophage migration inhibitory factor	-1.91	0.0337
Pde1c	Phosphodiesterase 1C, calmodulin-dependent 70kDa	-1.96	0.0053
Pcdha7	Protocadherin alpha 7	-1.98	0.0264
Extl3	Exostosin-like glycosyltransferase 3	-2.05	0.0291
Unc13a	Unc-13 homolog A	-2.11	0.0060
Gm5478	Predicted pseudogene 5478	-2.13	0.0103
Tmsb10	Thymosin beta 10	-2.15	0.0494
Pgk1	Phosphoglycerate kinase 1	-2.30	0.0414
Stmnd1	Stathmin domain containing 1	-2.42	0.0034
Mt2	Metallothionein 2	-2.50	0.0126
Yaf2	YY1 associated factor 2	-2.54	0.0473
Clip1	CAP-GLY domain containing linker protein 1	-2.75	0.0060
Slc7a3	Solute carrier family 7 (cationic amino acid transporter, Y+ system), 3	-2.87	0.0103
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-3.10	0.0171
Them6	Thioesterase superfamily member 6	-3.24	0.0011
Hdac9	Histone deacetylase 9	-3.69	0.0029
Ptpr	Protein tyrosine phosphatase, receptor type, R	-3.72	0.0135
Naa11	N(alpha)-acetyltransferase 11, NatA catalytic subunit	-3.83	0.0004
Lynx1	Ly6/neurotoxin 1	-8.26	0.0135
Mt1	Metallothionein 1	-10.73	0.0015

Table 4-1 Differentially expressed genes between cells isolated from degenerate and non-degenerate mouse retinae. Statistically significant, differentially expressed, probes are listed according to log₂ fold change. A positive log fold change represents upregulation in the degenerate samples. “p value” represents the adjusted p-value following False Detection Rate (FDR) testing.

Comparison of gene expression

In order to determine if bipolar cells in the degenerating retina continue to express genes necessary for AAV delivered opsins to effectively restore light responses in degenerate retina, we first considered the native light signalling cascade in these cells. Probes for genes specifically annotated as: “*g-protein coupled receptor subunits*,” “*g-protein associated kinases*,” “*arrestins*,” “*transient receptor potential M channels*” and “*neurotransmitter secretion*” (including *Grm6*(17), *Gnao1*(252), *Gnb5*(253), *Gng13*(254) and *Trpm1* (36) and others – see Table 4-2) were specifically interrogated. The aim was to identify any differentially expressed gene that may be impacting on the known native light signal through bipolar cells however, none were significantly differentially expressed. Nor were genes more generally implicated in cell signalling (*e.g. glutamate, glycine and GABA receptors*, Table 4-3). In fact the only “intracellular effector protein” probe found to be unequivocally differentially expressed was *Pde9a*, which is known not to contribute to the scotopic light response and is so less likely to be central to the rod bipolar cell signalling cascade(255).

Photoreceptor “carry over”

With no major changes in pathways that may have been expected to alter in the context of retinal degeneration, alternative methods were sought to identify candidate genes for further characterisation. However, when using more unbiased approaches such as pathway analysis, a particular feature of our FACS approach had to be considered: that of photoreceptor “carry over.” When sorting dissociated retinal cells by FACS, the large number of rod photoreceptor cells in the wildtype retina and their invaginated bipolar cell synapses can potentially lead to rods being carried over

Probe	Log ₂ fold Δ	p value	Probe	Log ₂ fold Δ	p value
Receptor			Intracellular effectors		
Grm6	1.054	0.8573	Adcy1	-2.97	0.0004
Gα subunits			Adcy1	1.05	0.8044
Gna11	1.04	0.7896	Adcy2	1.77	0.5721
Gna11	-1.07	0.8574	Adcy3	1.01	0.9480
Gna14	-1.08	0.7764	Adcy8	1.06	0.8086
Gnai1	-1.09	0.9360	Adcy9	1.05	0.8035
Gnai2	-1.04	0.8810	Pde1b	-1.37	0.5764
Gnai3	1.01	0.9863	Pde1b	-1.41	0.6178
Gnao1	-1.23	0.7900	Pde1c	-1.96	0.0053
Gnao1	1.02	0.9835	Pde1c	-1.22	0.1861
Gnaq	-1.40	0.0539	Pde1c	-1.09	0.7629
Gnaq	-1.03	0.8832	Pde1c	1.19	0.7932
Gβγ subunits			Pde1c	-1.10	0.8337
Gnb3	1.91	0.1685	Pde1c	1.00	0.9986
Gnb4	-1.05	0.9335	Pde3a	-1.03	0.8542
Gnb5	-1.01	0.9670	Pde3a	1.01	0.9441
Gng2	-1.10	0.8044	Pde3a	1.00	1.0000
Gng3	1.34	0.8414	Pde3b	-1.02	0.9259
Gng5	1.04	0.8175	Pde4a	-1.12	0.5721
Gng7	1.64	0.6041	Pde4b	-1.26	0.4073
Gng7	1.07	0.7679	Pde4b	-1.20	0.7671
Gng10	-1.08	0.7839	Pde4b	1.27	0.8311
Gng11	-1.05	0.8035	Pde4b	-1.02	0.9409
Gng13	-1.13	0.7109	Pde4d	-2.05	0.5154
Bipolar cell-specific			Pde4d	-1.00	0.9918
Cabp5	1.48	0.5394	Pde7a	-1.06	0.7887
Pcp2	-1.12	0.8044	Pde7b	-1.16	0.6154
Prkca	-1.09	0.7514	Pde8b	-1.02	0.8908
Scgn	1.40	0.8044	Pde9a	-1.55	0.0416
Scgn	-1.02	0.9746	Plcb3	1.07	0.7536
Vsx1	1.04	0.9102	Plcb4	1.24	0.8044
Vsx2	1.21	0.8056	TRP channels		
Vsx2	1.10	0.9021	Trpc6	-1.05	0.7749
Kinases			Trpc7	-1.29	0.7887
Grk4	1.13	0.7241	Trpm1	1.19	0.7847
Grk4	-1.07	0.7896	Trpm1	-1.13	0.8035
Grk5	1.09	0.7918	Trpm1	1.09	0.8220
Grk5	1.04	0.8642	Trpm1	1.19	0.8401
Housekeeping genes			Arrestin		
Actb	1.45	0.6114	Arrb2	-1.11	0.8673
Actb	-1.31	0.7128	Gapdh		
Actb	-1.03	0.9801	Rplp0	-1.51	0.1950
Actb	-1.03	0.9802	Rplp0	-1.15	0.8035
			Rplp0	1.02	0.9900

Table 4-2 In a gene array study comparing FACS-isolated EYFP-positive cells from degenerate and non-degenerate L7-Cre EYFP mouse retinas, genes related to intracellular communication, the native on-bipolar signal transduction cascade. A selection of “housekeeping” genes are included for comparison. A positive log fold change represents upregulation in degenerate samples. “p value” represents the adjusted p-value following False Detection Rate (FDR) testing.

Probe	Log ₂ fold Δ	p value	Probe	Log ₂ fold Δ	p value
Kainate type			AMPA type		
Grik1	1.57	0.6786	Gria1	-1.39	0.7587
Grik1	1.07	0.7896	Gria1	1.05	0.8440
Grik1	1.13	0.7934	Gria1	1.04	0.8681
Grik1	1.09	0.8785	Gria1	1.04	0.8708
Grik1	-1.03	0.9705	Gria2	1.27	0.6526
Grik4	-1.01	0.9784	Gria2	1.21	0.7415
Vglut transporter			Gria3	-1.04	0.8035
Slc17a6	-1.05	0.8679	Gria3	-1.06	0.9259
Slc17a6	-1.01	0.9865	Gria4	1.07	0.7587
Slc17a7	-1.05	0.8592	NMDA type		
Slc17a8	-1.08	0.7415	Grin2c	-1.02	0.9094
GABA and Glycine receptor subunits					
GABA			Glycine		
Gabra1	-1.05	0.8824	Glr2	-1.06	0.8015
Gabra3	1.07	0.8194	Glr4	1.09	0.7753
Gabrb1	1.01	0.9632	Glr2	1.07	0.7969
Gabrb2	1.11	0.6142			
Gabrb2	1.01	0.9870			
Gabrb3	1.12	0.7500			
Gabrb3	-1.11	0.8013			
Gabrb3	-1.03	0.9382			
Gabrg2	-1.56	0.0494			
Gabrg2	-1.48	0.6302			
Gabrg2	-1.36	0.6308			
Gabrg2	1.06	0.7420			
Gabrg2	-1.06	0.8721			
Gabrg2	-1.10	0.9477			
Gabrr1	-1.56	0.5706			
Gabrr1	-1.04	0.8718			
Gabrr2	-1.16	0.7896			

Table 4-3 In a gene array study comparing FACS-isolated EYFP-positive cells from degenerate and non-degenerate L7-Cre EYFP mouse retinas, genes relating to glutamate, glycine and GABA receptors. A positive log fold change represents upregulation in degenerate samples. "p value" represents the adjusted p-value following False Detection Rate (FDR) testing.

with labelled bipolar cells into the wrong isolate. One method of reducing this risk would be to extend the papain dissociation protocol to ensure less residual cell-cell adhesion, even with this, the nature of the invaginated photoreceptor synapse may make eliminating this carryover completely impossible. In addition, this must be weighed against the associated increased risk of damage to the isolated cells or RNA itself with more aggressive dissociation protocols.

This balance is especially important in studies such as ours where a comparison is being made to an isolate from degenerate retina - where there are no such rods - and a wild type - where they are one of the most numerous cells. Previous studies have attempted to control for this in different ways - for example, Siegert et al(256) normalise all gene expression levels based on expression of known rod specific genes whereas Punzo et al(243), exclude any known rod specific genes from further analysis while Berg et al(257) examine changes in array expression of known PR genes to quantify levels of contamination. qPCR for two rod specific genes (*Cnga1*, *Rho*, chosen *a priori*) were not significant – giving an indication of low rates of rod carry over in our samples. However, our gene array data did present differential expression of a small number of genes with either a rod ontological annotation or where previous literature reported low or absent protein expression in bipolar cells compared to photoreceptors (Table 4-4).

Recognising this potential limitation and to avoid difficulties in interpretation, we excluded these genes from further investigation(243, 257) but did not systematically alter our data otherwise(256). Like all of the approaches previously described, this relies on an *a priori* knowledge of gene photoreceptor specificity, but given the relative purity of our samples and the likely small number of rod specific

genes, this must be balanced against the much larger drawbacks of whole retina (unsorted) samples.

Probe/gene name	log ₂ fold change	p value
-----------------	------------------------------	---------

Rod Specific		
Gnat1	-21.14	0.0002
Pdc	-1.83	0.0421
Pde6b	-3.47	0.0004
Nrl	-2.79	0.0380
Sag	-6.01	0.0009
Slc24a1	-5.27	0.0231
Pde6g	-3.02	0.0473
Reep6	-2.56	0.0380
Esrrb	-2.00	0.0153

Probe/gene name	log ₂ fold change	p value
-----------------	------------------------------	---------

Rod contentious		
Gpnmb	-4.14	0.0035
Ldha	-4.61	0.0014
Car2	-2.94	0.0004
Adcy1	-2.97	0.0004

Table 4-4 Left - Genes with rod specific ontological annotation; Right - Genes with literature suggesting contentiousness due to low bipolar compared to photoreceptor protein expression. A positive log fold change represents upregulation in degenerate samples. "p value" represents the adjusted p-value following False Detection Rate (FDR) testing.

Identifying candidate genes for further characterisation: database approaches

Following the removal of these genes, a systematic sequence of methods (Figure 4-2) was initially applied in an attempt to highlight interesting candidate genes for further characterisation. The Retinal Network (RetNet™) database(258) is a repository of known gene–disease relationships in human ophthalmology and was used to identify any differentially expressed genes that may already have been linked to a disease. Only one candidate was identified from this search: Shroom 2 - *Srm2*.

Protein-protein interaction prediction software ((259)) was used to highlight groups of potential candidate genes linked by a common interaction. The benefit of this approach is that it is less dependent on our prior knowledge of specific gene function and so therefore can reduce bias to highlight candidate genes with potentially little previous implication in our disease of interest (retinal degeneration). One common function ("*transition metal ion binding*") was identified linking four of our differentially expressed genes (*Atox1* coding for a copper metallochaperone protein of the same name(260), *MsrB2* coding for Methionine sulfoxide reductase B2(261) and *Mt1* & *Mt2* coding for the metallothioneins(262)): all involved in preventing and repairing oxidative damage.

This association was reinforced by use of a pathway analysis database (Reactome®(263, 264)) to highlight genes linked by associations with particular biochemical pathways. When our list of differentially expressed genes was queried for mouse (*Mus musculus*), no pathways were over represented. When the gene list was extrapolated to human (*Homo Sapiens*) analogues however, two pathways were highlighted as being overrepresented ("*Metallothioneins bind metals*" $p=0.009$ and

“Response to metal ions” $p=0.013$, Figure 4-3) involving similar genes (*Msrb2*, *Mt1* & *Mt2*) as the protein interaction prediction.

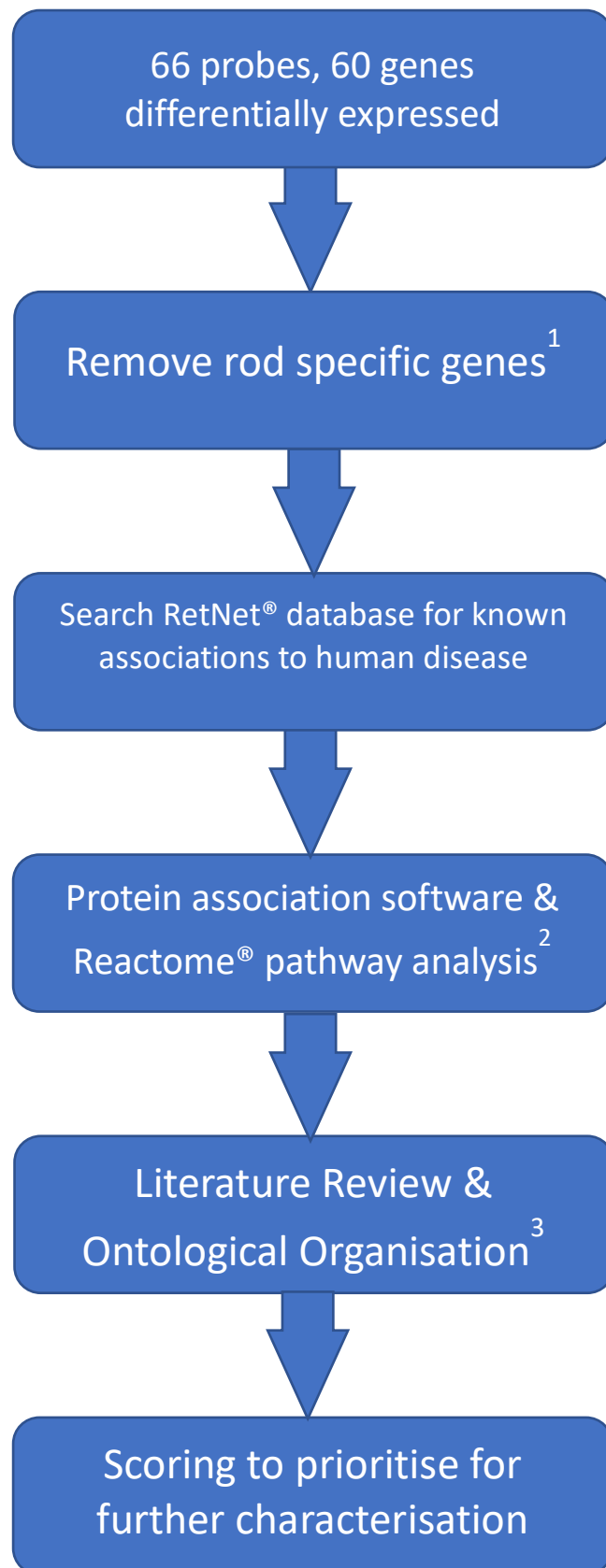


Figure 4-2 Process of prioritisation of candidate genes for further characterisation using immunohistochemistry.

¹ – see Table 4-4; ² – see Figure 4-3; ³ – see Figure 4-4

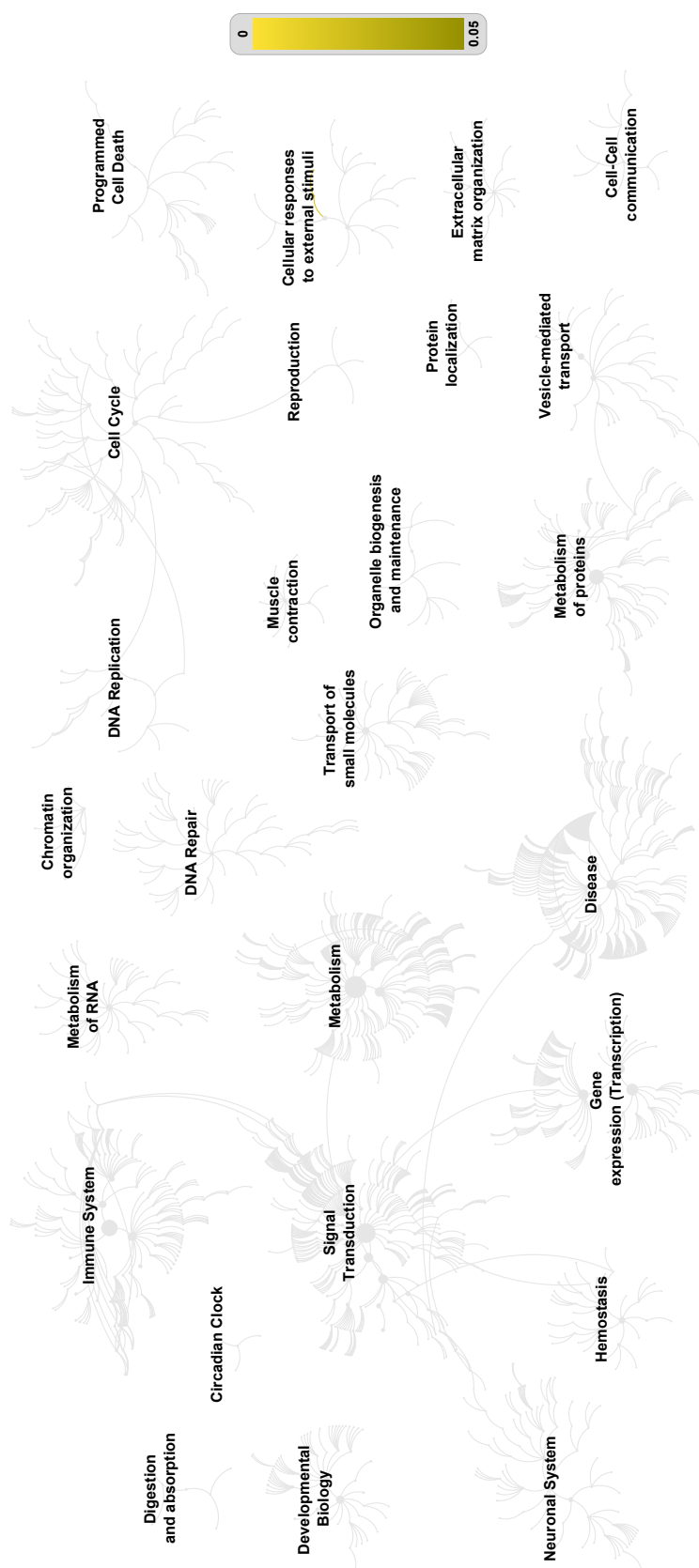


Figure 4-3 Reactome® pathway analysis. Graphical representation of all possible pathways. Note there are only two overrepresented pathways (“Metallothioneins bind metals” $p=0.009$ and “Response to metal ions” $p=0.013$). Yellow lines represent more significantly, grey lines less significantly overrepresented pathways in the list of differentially expressed genes.

Identifying candidate genes for further characterisation: literature review

These database approaches, while relatively unbiased had identified only a small number of genes for potential further characterisation. This, coupled with the relatively short list of differentially expressed genes (60 out of c. 20,000 probes) meant that a systematic, but manual, evaluation was possible for each of the genes in turn.

A search of the gene ontology consortium annotation database(265) and a literature review (Figure 4-4) was performed for each gene. This was used to group the potential candidates by function(265) (Table 4-5) and score them for relevance to our research question (see Chapter 2 - methods). The highest scoring potential candidate in each of the first four groups (bold typeface in Table 4-5) was selected as an exemplar for further investigation at the protein level using IHC (*Srm2*, *Slf2*, *Anxa7*, *Cntn1*). A candidate from the fifth group ("Aerobic and anaerobic respiration, cellular responses to stress") was not taken forward for initial further characterisation (see discussion).

Further characterising candidate genes: Immunohistochemistry

Staining for corresponding proteins on retinal immunohistochemistry was used to further characterise the four candidate genes (*Srm2*, *Slf2*, *Anxa7*, *Cntn1*) identified using the above approaches. This was performed on retinal cryo-sections from *Pde6b*^{rd1/rd1} and *Pde6b*^{+/+} mice both at P90 (to correspond with the gene array work) but also in early (P40) and late (P120 and P150) timepoints in degeneration (85) to give an impression of how staining may change over time.

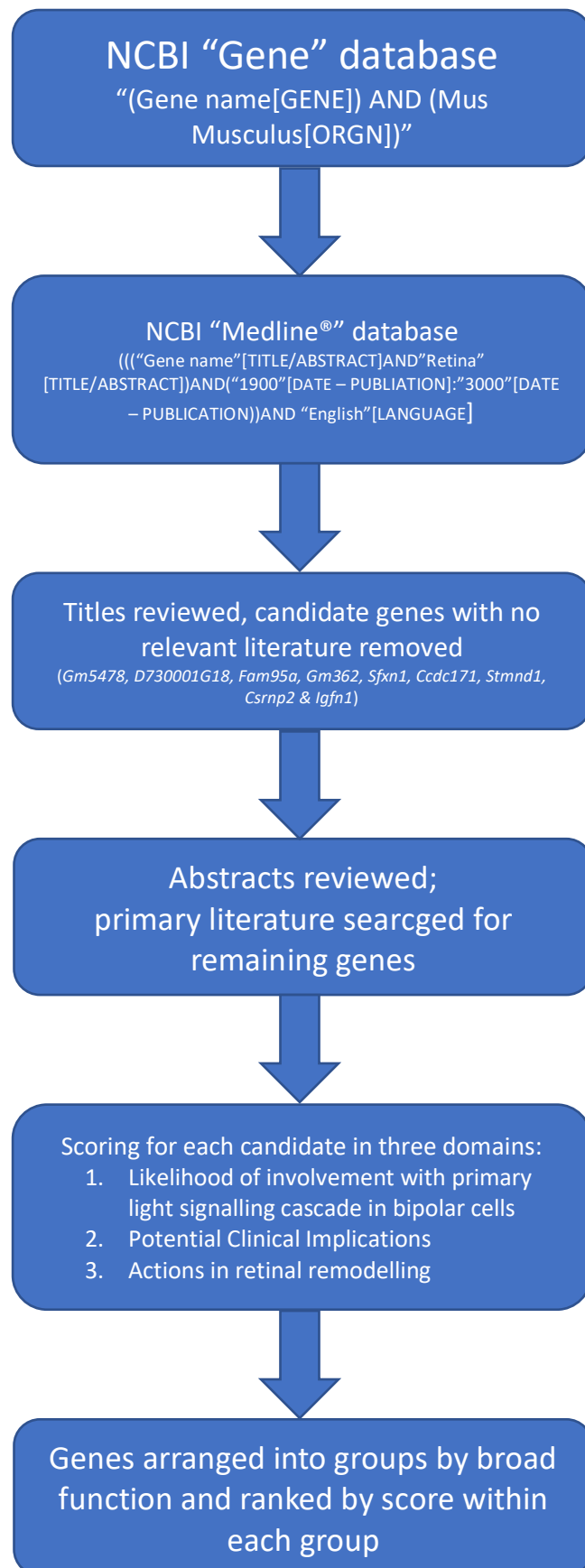


Figure 4-4 Process for literature review, ontological organisation and systematic prioritisation of candidate genes for further characterisation. Search terms are included in the box referring to the relevant database.

Symbol	$\Delta \log_2$	Ontology term 1	Ontology term 2
A - Actin cytoskeleton, microtubules and intracellular transport			
ap3m2	-1.85	anterograde synaptic vesicle transport	intracellular protein transport
Cng	3.52	microtubule cytoskeleton organization	microtubule binding
sept4	1.54	cilium assembly	mitotic cytokinesis
srn2	1.81	actin cytoskeleton organization	melanosome organization
tmsb10	-2.15	actin cytoskeleton organization	regulation of cell migration
B - Heparin sulphate proteoglycan metabolism			
extl3	-2.05	heparan sulphate proteoglycan biosynthesis	protein glycosylation
sulf2	1.43	heparan sulphate proteoglycan metabolic process	arylsulfatase activity
C - Cell signalling, calcium homeostasis			
anxa7	-1.56	calcium ion binding	membrane fusion
cabyr	2.25	sperm capacitation	calcium-mediated signalling
gabrg2	-1.56	gamma-aminobutyric acid signalling pathway	synaptic transmission, GABAergic
pde1c	-1.96	signal transduction	response to calcium ion
slc7a3	-2.87	amino acid transport	arginine transport
unc13a	-2.11	neurotransmitter secretion	SNARE binding
D - Neural cell growth, survival & remodeling			
cntn1	-1.79	neuron projection development	nervous system development
efnb1	2.15	axon guidance	presynapse assembly
hdac9	-3.69	DNA repair	chromatin organization
lynx1	-8.26	synaptic transmission, cholinergic	acetylcholine receptor binding
mif	-1.91	regulation of cell proliferation	positive regulation of axon regeneration
nrm	-1.40	nuclear membrane	membrane
pcdha7	-1.98	cell adhesion	cell-cell recognition
phc1	1.93	cellular response to retinoic acid	histone ubiquitination
ptpr	-3.72	negative regulation of ERK1 and ERK2 cascade	ERBB2 signalling pathway
sfrs1	1.72	mRNA 5'-splice site recognition	regulation of transcription, DNA-templated
socs5	-1.64	regulation of growth	JAK-STAT cascade
yaf2	-2.54	regulation of transcription, DNA-templated	transcription, DNA-templated
E - Aerobic and anaerobic respiration, cellular response to stress			
atox1	-1.62	transition metal ion binding	response to oxidative stress
clip1	-2.75	transition metal ion binding	microtubule bundle formation
cox3	-1.83	aerobic electron transport chain	aerobic respiration
msrb2	3.03	transition metal ion binding	cellular response to stress
mt1	-10.73	transition metal ion binding	cellular metal ion homeostasis
ndufb2	-1.55	mitochondrial respiratory chain complex I	oxidation-reduction process
pgk1	-2.30	glycolytic process	carbohydrate metabolic process
F - Miscellaneous			
abca1	-3.10	cholesterol homeostasis	cellular response to retinoic acid
Fam19a3	1.87	positive regulation of microglial cell activation	negative regulation of microglial cell activation
naa11	-3.83	n-terminal protein amino acid acetylation	n-terminal peptidyl-glutamic acid acetylation
mf11	-1.75	protein autoubiquitination	ubiquitin-dependent protein catabolic process

Table 4-5 Genes differentially expressed between cells isolated from degenerate and non-degenerate mouse retinae grouped by broad function based on gene ontology (two listed for each gene)(265).

Candidate genes are arranged alphabetically within their groups. Those selected for further characterisation are in bold typeface. A positive log fold change represents upregulation in degenerate samples. "p value" represents the adjusted p-value following False Detection Rate (FDR) testing.

Selected antibodies (see Chapter 2) for each of the respective proteins were first validated by performing immunocytochemistry on cultured human embryonic kidney cells (HEK293T) transfected with the appropriate plasmid (Figure 4-5). Each of the four candidate genes selected (*Srm2*, *Sulf2*, *Anxa7*, *Cntn1*) showed staining for the corresponding protein co-localising with bipolar markers (CHX10 or PKC α) at postnatal day 90 (P90) in our IHC study (Table 4-6, Figure 4-6 to Figure 4-9). This was reinforced by image analysis with all stained sections having an Intensity Correlation Quotient ICQ value(172) between 0 and 5: indicating colocalization of staining for candidate and bipolar cell markers. Mean colocalised pixels above threshold at each timepoint (P40, P90, P120, P150), for each genotype gave a semi-quantitative index of protein levels. These were compared using a 2-way ANOVA with Sidak's method to correct for multiple comparisons between groups and Tukey's for comparisons within groups (summarised in Table 4-6).

For *Srm2* (Shroom 2) this showed a difference between genotypes ($F_{(1,8)} = 23.85$; $p=0.0012$), and with time ($F_{(3,8)} = 4.865$; $p=0.0327$) and also an interaction between time and genotype ($F_{(3,8)} = 0.09$; $p=0.0059$). Sidak's method showed significantly higher staining in degenerate retinæ at P90 ($p=0.0136$) and P120 ($p=0.0026$) see Figure 4-6. In the *Pde6b*^{rd1/rd1} group, Tukey's method demonstrated differences between P150 and both P40 ($p=0.0307$) and P80 ($p=0.0157$) with a drop in expression as degeneration progresses. In the *Pde6b*^{+/+} group P120 differed from P40 ($p=0.0126$) and P150 ($p=0.0199$): a trough in expression at P120 (Figure 4-6).

For *Slf2* (Sulphatase 2), there was no difference between genotypes ($F_{(1,8)} = 3.775$; $p=0.0879$), there was a change with time ($F_{(3,8)} = 19.9$; $p=0.005$), but no interaction between time and genotype ($F_{(3,8)} = 2.718$; $p=0.1148$). In the *Pde6b*^{rd1/rd1}

group, Tukey's method demonstrated differences between P80 and both P40 ($p=0.0083$) and P150 ($p=0.0028$) as well as between P120 and P150 ($p=0.0248$) summarised as a drop in staining mid degeneration. In the *Pde6b*^{+/+} group, P150 differed from P40 ($p=0.0146$), P80 ($p=0.0052$) and P120 ($p=0.0116$) – showing increasing expression with age (Figure 4-7).

For *Anxa7* (Annexin a7), there was a difference between genotypes, ($F_{(1,9)} = 5.31$; $p=0.0467$), but not with time ($F_{(3,9)} = 0.1829$; $p=0.9053$), nor was there an interaction between time and genotype ($F_{(3,9)} = 0.6712$; $p=0.5909$). Sidak's method did not show any significant difference between genotypes at any individual time point (Figure 4-8) suggesting that Annexin a7 staining is constant over time in both groups.

With *Cntn1* (Contactin 1), there was a difference between genotypes ($F_{(1,13)} = 8.272$; $p=0.0130$), but not with time ($F_{(3,13)} = 0.168$; $p=0.9161$), there was an interaction between time and genotype ($F_{(3,13)} = 7.366$; $p=0.0039$). Sidak's method showed a significant difference between genotypes at P90 only ($p=0.0026$) with reduced expression in degenerate retinæ at this timepoint (Figure 4-9).

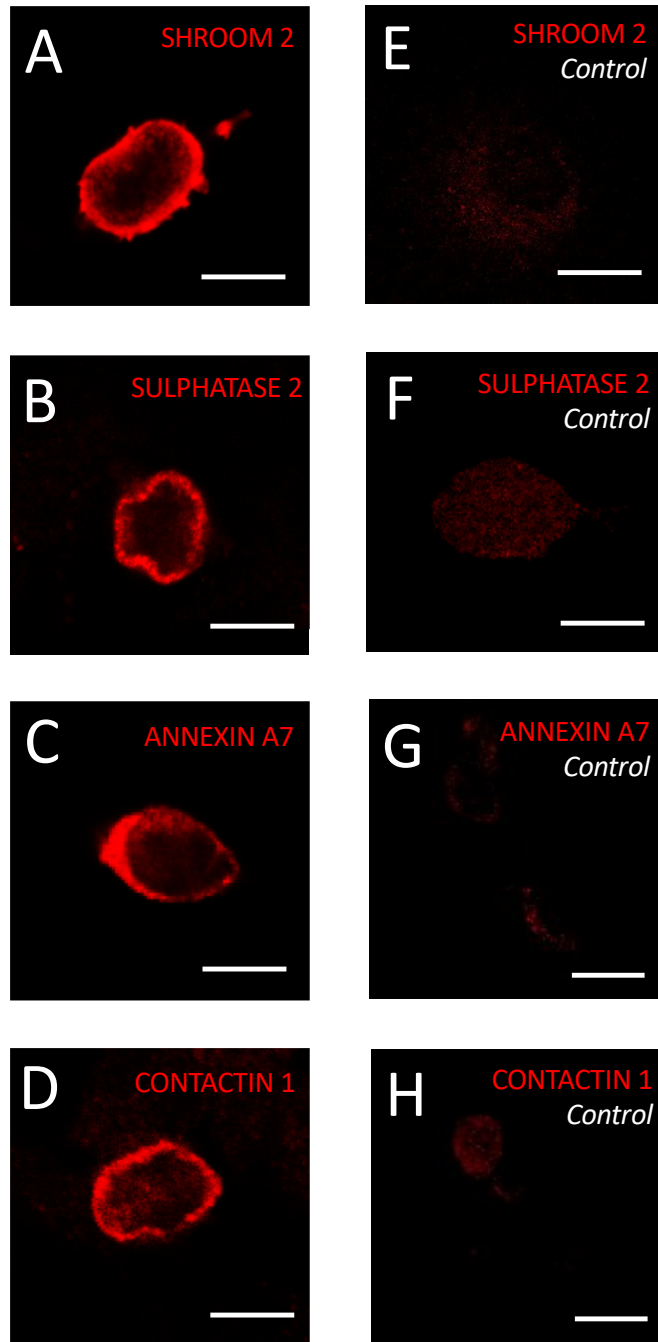


Figure 4-5 Validation of antibodies. Immunocytochemistry images of HEK293T cells transfected to express the protein corresponding to the labelled antibody. All four stain at a level far above that of a non-transfected control. Note staining pattern of all four antibodies is consistent with protein in or around the cell membrane as expected.

A - Antibody against Shroom 2

B – Antibody against Sulphastase 2

C – Antibody against Annexin a7

D – Antibody against Contactin 1

E to H – corresponding controls

Scale bars - 10 μ m

<i>Gene</i>	<i>Srm2</i>	<i>Suf2</i>	<i>Anxa7</i>	<i>Ctnn1</i>
<i>Protein</i>	Shroom 2	Sulphatase 2	Annexin a7	Contactin 1
<i>Function</i>	Actin cytoskeleton, axon guidance	HSPg metabolism	Membrane fusion, neurotransmitter release	Neuron projection development, synaptic remodelling
<i>Comments</i>	Regulates actin cytoskeleton and therefore cell shape, axon sprouting and organelle location (essential for viral transduction, second messenger systems and neurotransmitter release) (266).	Extracellular endosulphatase. Removes sulphate residues from cell surface HSPg residues(267) - important in the entry of AAV into cells(145) as well as retinal synaptic plasticity	Calcium dependant phospholipid binding protein implicated in synaptic neurotransmitter release and the bipolar cell light response(268-270). PKC α (important in activation and termination of bipolar cell light response phosphorylates annexin a7 promoting membrane fusion and so neurotransmitter release(270). PKC α expression was not altered in our gene array comparison.	A cell surface glycoprotein implicated in synaptic plasticity(271)
<i>Human disease caused defect in gene</i>	Implicated in retinal degeneration with deafness(266)	Not reported	Not reported	Congenital Myopathy(271)
<i>Mouse knock outs</i>	Knock down - failure of retinal lamination, full knock out - no retinal phenotype. Other <i>Srm</i> family members may compensate(271).	Deficiencies in neural remodelling, no major developmental flaws or retinal defects reported(272)	No gross neurological phenotype, retina not examined(269)	Impaired synaptic long-term depression (LTD)(273). Over-expression improves long term potentiation (LTP)(274). No retinal phenotype at P14(275)
<i>Expression at P90 (gene array)in Pde6b^{rd1/rd1}vs Pde6b^{wf/wf} (FDR adj' p value)</i>	Up ($p=0.042$)	Up ($p=0.034$)	Down ($p=0.024$)	Down ($p=0.049$)
<i>Expected location of protein within cell</i>	Cell membrane(276) confirmed in cell culture (Figure 4-5) & IHC (Figure 4-6)	Cell surface, extracellular(267) Confirmed in cell culture (Figure 4-5) & IHC (Figure 4-7)	Cell membrane(277) confirmed in cell culture (Figure 4-5) & IHC (Figure 4-8)	Cell surface(271), confirmed in cell culture (Figure 4-5) & IHC (Figure 4-9)
<i>Previous IHC of Pde6b^{wf/wf} retinae</i>	Retinal pigment epithelium, bipolar cell bodies, inner plexiform layer(276)	Bipolar cell bodies, outer plexiform layer photoreceptors synapses(278)	Not described previously in retina	Bipolar cell and outer plexiform layer(271)
<i>2-way ANOVA – difference by genotype (Pde6b^{rd1/rd1}vs Pde6b^{wf/wf})</i>	Yes F(1, 8) = 23.85; $p=0.0012$	No F(1, 8) = 3.775; $p=0.0879$	Yes F(1, 9) = 5.31; $p=0.0467$	Yes F(1, 13) = 8.272; $p=0.0130$
<i>2-way ANOVA – difference by time</i>	Yes F(3, 8) = 4.865; $p=0.0327$	Yes F(3, 8) = 19.9; $p=0.005$	No F(3, 9) = 0.1829; $p=0.9053$	No F(3, 13) = 0.168; $p=0.9161$
<i>2-way ANOVA - interaction genotype x time</i>	Yes F(3, 8) = 9.09; $p=0.0059$	No F(3, 8) = 2.718; $p=0.1148$	No F(3, 9) = 0.6712; $p=0.5909$	Yes F(3, 13) = 7.366; $p=0.0039$
<i>Significant Post Hoc Tests</i>	Sidak P80 – $p=0.0136$; P120 - $p=0.0026$ Tukey Pde6b ^{rd1/rd1} ;P40 vs P150 $p=0.0307$; P80 vs P150 $p=0.0157$; Pde6b ^{wf/wf} ;P40 vs P120 $p=0.0126$; P120 vs P150 $p=0.0199$	Tukey Pde6b ^{rd1/rd1} ;P40v s P80 $p=0.0083$; P80 vs P150 $p=0.0028$; P120 to P150 $p=0.0248$; Pde6b ^{wf/wf} ; P40 vs P150 $p=0.0146$; P80 vs P150 $p=0.0052$; P120 vs P150 $p=0.0116$	Sidak Not significant at any individual timepoint	Sidak P80 $p=0.0026$

Table 4-6 A summary of previously published information regarding four candidate genes selected for further characterisation. The bottom rows summarise our findings on semiquantitative co-localisation analysis of immunohistochemistry as described in the text and Figure 4-6 to Figure 4-9. A two-way ANOVA was used with post hoc Sidak and Tukey approaches to control for multiple comparisons. For clarity - post hoc tests where $p>0.05$ are not shown are omitted.

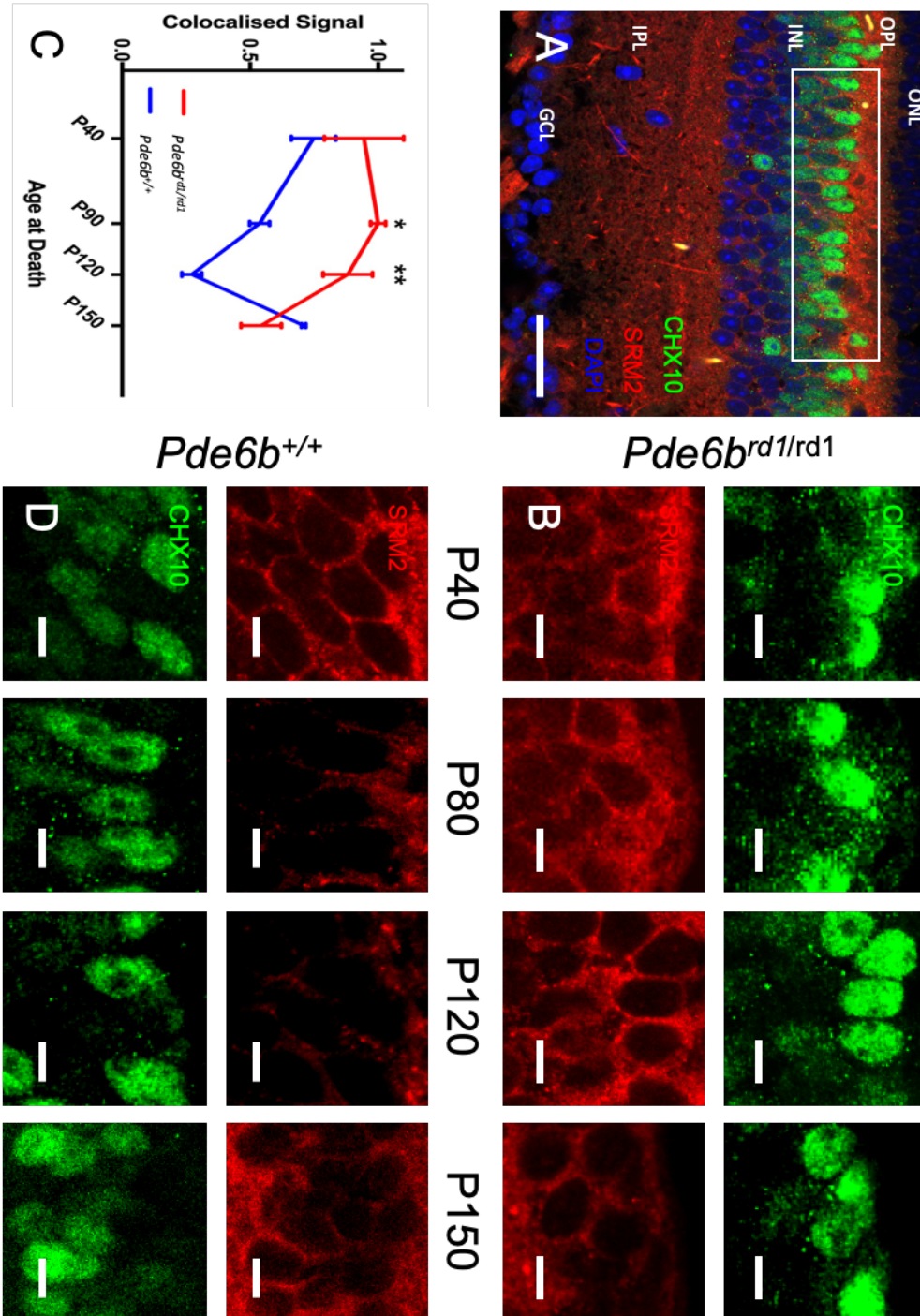


Figure 4-6 Shroom 2 IHC (Srm2 upregulated on gene array at P90)

A - A retinal cryo-section stained for DAPI (blue), CHX10 (green) and Shroom2 (red). ONL – Outer nuclear layer, OPL – Outer Plexiform layer, INL- Inner nuclear layer, IPL – inner plexiform layer, GCL – Ganglion cell layer. Scale bar = 20µm. White box indicates area from which close up images in panels B & D are taken.

B & D - Close up images of bipolar cell bodies in *Pde6b^{rd1/rd1}* retina (B) and *Pde6b^{+/+}* (D) at four time points during degeneration (P40, P80, P120, P150). CHX10 (green) and shroom 2 (red). Scale bars 5µm.

C - Colocalised pixels above threshold - a semiquantitative index of protein expression. Normalised to highest value over all retinas stained for Shroom 2. Comparison between groups by two-way ANOVA with Sidak's method to account for multiple comparisons: P90 adjusted $p = 0.0136$, P120 adjusted $p = 0.0026$. red line = *Pde6b^{rd1/rd1}*; blue line = *Pde6b^{+/+}*.

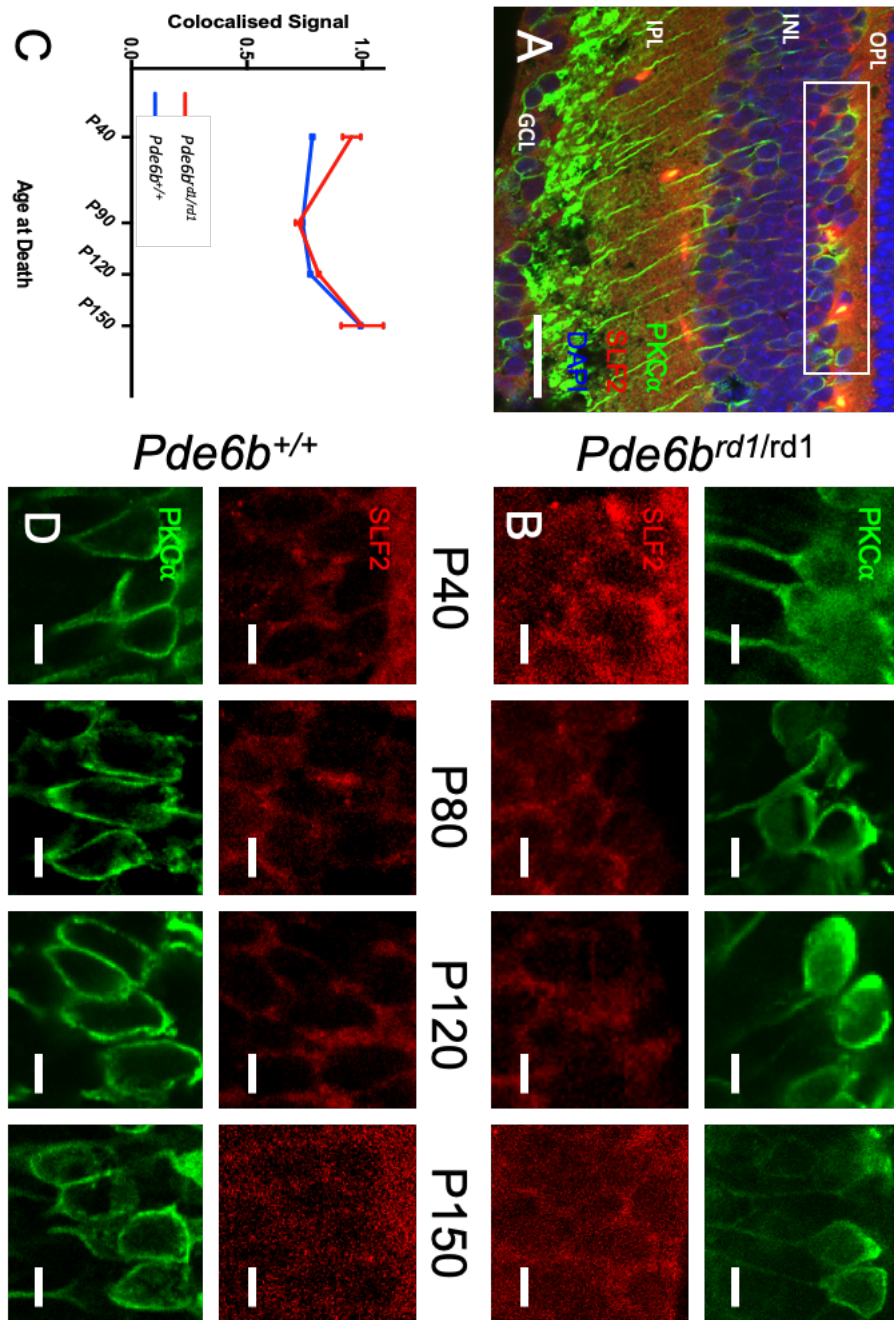


Figure 4-7 Sulphatase 2 IHC (upregulated on gene array at P90)

A - A retinal cryo-section stained for DAPI (blue), PKC α (green) and sulphatase 2 (red). ONL – Outer nuclear layer, OPL – Outer Plexiform layer, INL- Inner nuclear layer, IPL–inner plexiform layer, GCL – Ganglion cell layer. Scale bar = 20 μ m. White box indicates area from which close up images in panels B & D are taken.

B & D - Close up images of bipolar cell bodies in *Pde6b^{rd1/rd1}* retina (B) and *Pde6b^{+/+}* (D) at four time points during degeneration (P40, P80, P120, P150). PKC α (green) and Sulphatase 2 (red). Scale bars 5 μ m

C - Colocalised pixels above threshold - a semiquantitative index of protein expression. Normalised to highest value over all retinæ stained for Sulphatase 2. Star markers on graph represent adjusted p values, Sidak's method for multiple comparison (ie. None reaching significance, see results section for details). red line = *Pde6b^{rd1/rd1}*; blue line = *Pde6b^{+/+}*

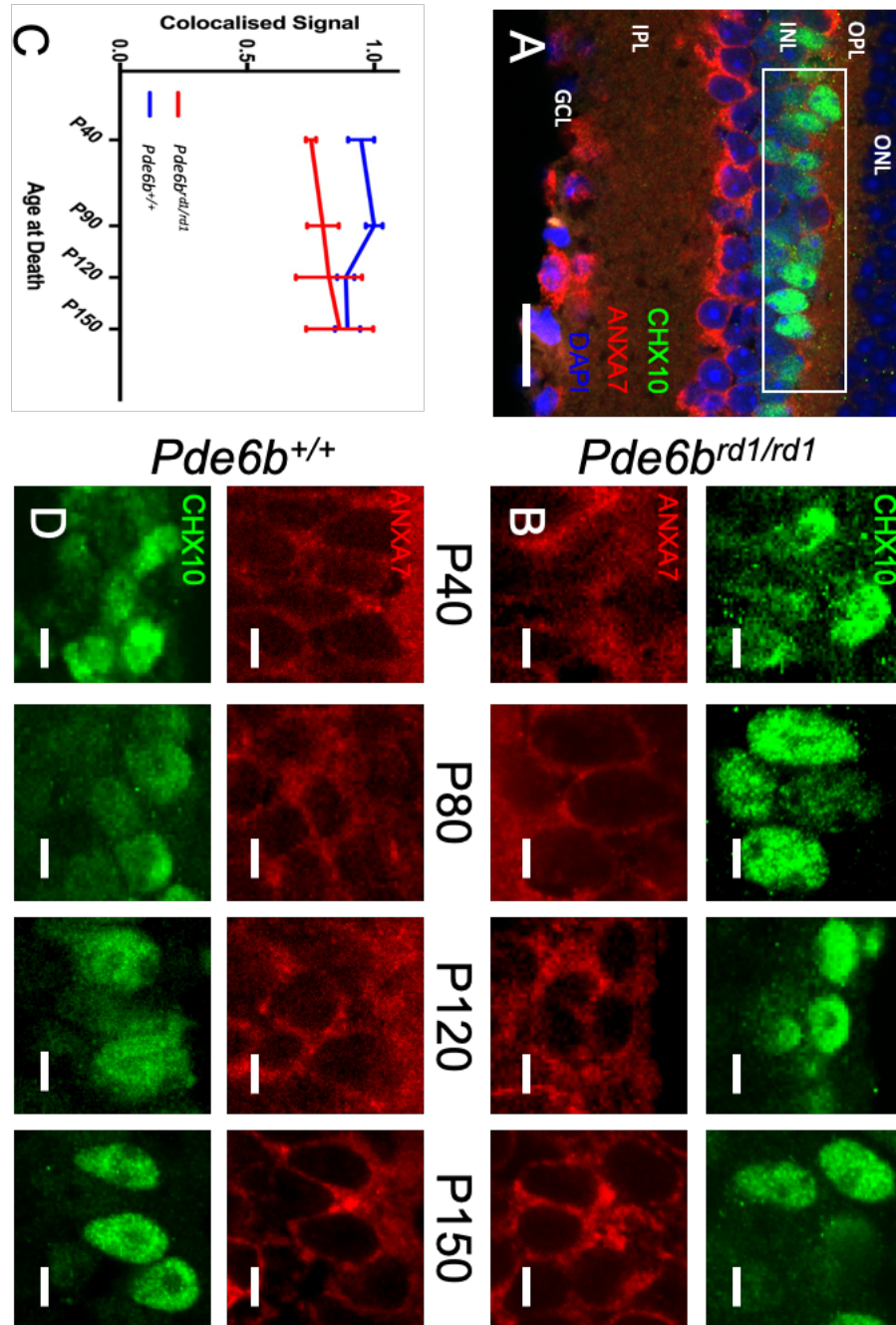


Figure 4-8 Annexin a7 IHC (Anxa7 downregulated on gene array at P90).

A – A retinal cryo-section stained for DAPI (blue), CHX10 (green) and annexin a7 (red). ONL – Outer nuclear layer, OPL – Outer Plexiform layer, INL- Inner nuclear layer, IPL –inner plexiform layer, GCL – Ganglion cell layer. Scale bar = 20μm. White box indicates area from which close up images in panels B & D are taken.

B & D - Close up images of bipolar cell bodies in *Pde6b^{rd1/rd1}* retinæ (B) and *Pde6b^{+/+}* (D) at four time points during degeneration (P40, P80, P120, P150). CHX10 (green) and annexin a7 (red). Scale bars 5μm.

C - Colocalised pixels above threshold - a semiquantitative index of protein staining. Normalised to highest value over all retinæ stained for Annexin a7. Star markers on graph represent adjusted p values, Sidak's method for multiple comparison (ie. None reaching significance, see results section for details). red line = *Pde6b^{rd1/rd1}*; blue line = *Pde6b^{+/+}*.

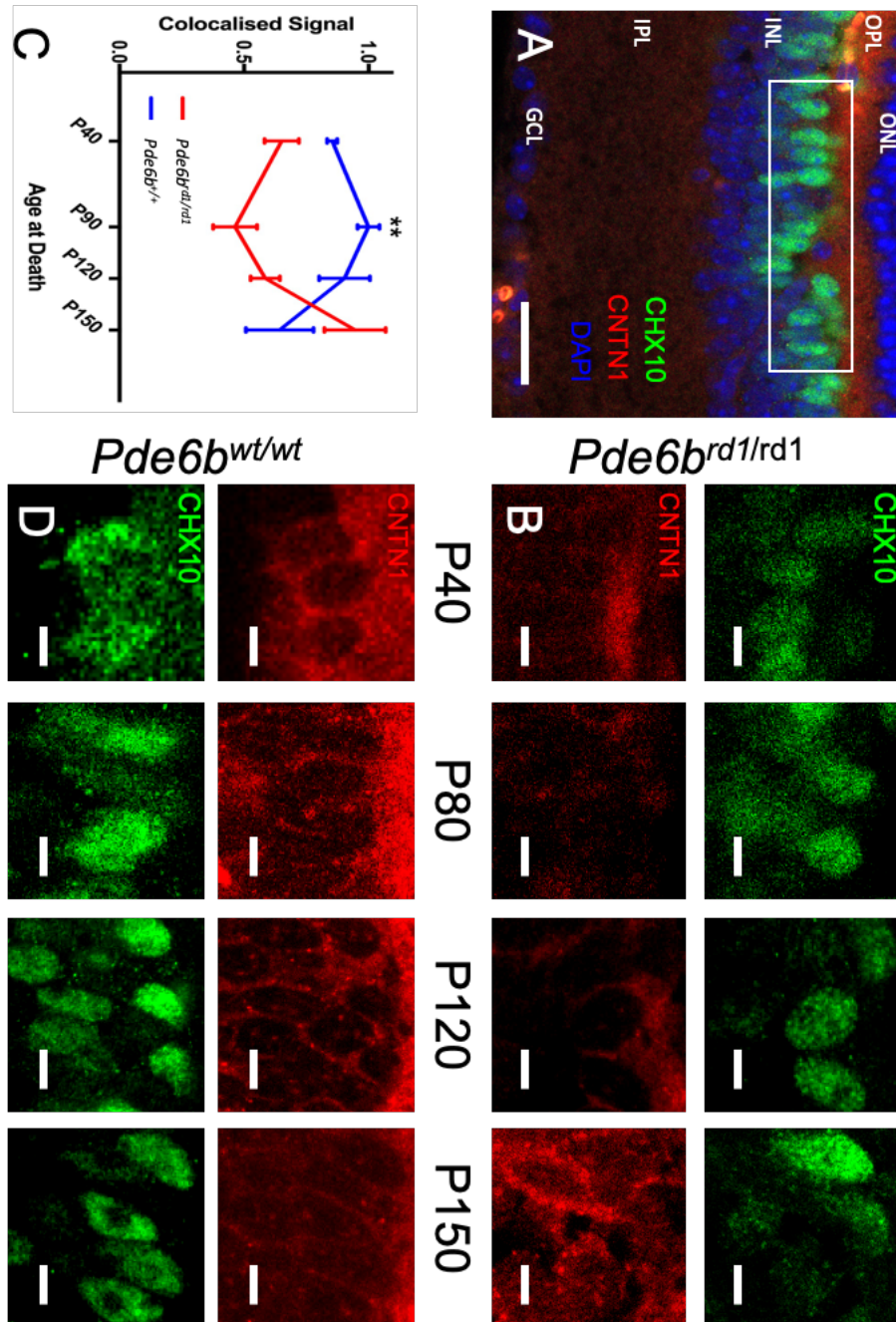


Figure 4-9 Contactin 1 IHC (Cntn1 downregulated on gene array at P90).

A – A retinal cryo-section stained for DAPI (blue), CHX10 (green) and Contactin 1 (red). ONL – Outer nuclear layer, OPL – Outer Plexiform layer, INL- Inner nuclear layer, IPL – inner plexiform layer, GCL – Ganglion cell layer. Scale bar = 20 μ m. White box indicates area from which close up images in panels B & D are taken.

B & D - Close up images of bipolar cell bodies in *Pde6b^{rd1/rd1}* retina (B) and *Pde6b^{+/+}* (D) at four time points during degeneration (P40, P80, P120, P150). CHX10 (green) and Contactin 1 (red). Scale bars 5 μ m.

C - Colocalised pixels above threshold - a semiquantitative index of protein expression. Normalised to highest value over all retinæ stained for Contactin 1. Star markers on graph represent adjusted p values, Sidak's method for multiple comparison at P80 $p=0.0026$ (see results section for details). red line = *Pde6b^{rd1/rd1}*; blue line = *Pde6b^{+/+}*

Discussion

Much of mammalian basic image processing is initiated within the neural retina before signals reach the brain. As vision is lost in the IRDs through preferential photoreceptor death, the retinal bipolar cells become the highest order surviving cells of this neural hierarchy. As such, their stimulation may - in principle - enable more intra-retinal processing to be preserved, presenting them as particularly attractive targets for optogenetic visual restoration.

Targeting bipolar cells assumes that, within a degenerate retina, these cells retain expression of the second messengers required for light signalling. It also assumes such cells survive in a stable form without metabolic stress to allow effective propagation of this signal by synaptic neurotransmitter release and, importantly, are able to be transfected by AAV. Bipolar cells need to maintain a selection of proteins to support glutamate neurotransmitter release, and a key question is whether these are maintained following the loss of light input during degeneration. Increasing evidence, confirms that bipolar cells do undergo remodelling in the later stages of retinal degeneration with changes in morphology, synaptic connections, electrophysiological responses and receptor expression(79, 82, 85, 86, 89-91, 240).

Gene expression in context

Here we show that, despite this remodelling, bipolar cells undergo remarkably limited transcriptomic changes in response to the loss of synaptic inputs from photoreceptors. While gene array methods may not have statistical power to detect very small changes in expression of single genes, the approach described here of limiting our comparison as far as possible to a single cell population aims to accentuate those changes that are biologically most significant.

However, given the marked changes seen at the anatomical level over the whole retina during the neural remodelling of degeneration, it is perhaps surprising that we found such a small number of genes differentially expressed in bipolar cells (66 out of a total of c. 20,000 probes, Table 4-1). The absence of differential expression of genes related to the known native bipolar cell light signalling cascade via the mGluR6 receptor, G_o pathway and TRPM1 channel or indeed in second messaging or glutaminergic transmission more generally (Table 4-2 & Table 4-3) is particularly interesting given reports of functional loss of sensitivity to glutamate even early in degeneration(239). We see no significant alteration in expression of genes associated with glutamate receptor subunits (nor GABAergic, nor glycinergic ones, Table 4-3) which is intriguing given the switch from metabotropic to ionotropic glutamate transmission in ON-bipolar seen at a functional and anatomic level in bipolar cells during degeneration. ON-bipolar cells can be seen to undergo “class switching” from metabotropic mediated (sign inverting) ON-responses to ionotropic mediated (sign maintaining) OFF-responses during degeneration (89, 90, 239).

Our observation could be accounted for, in part, by time course – such functional changes in glutamate transmission occur within a matter of hours after removal of photoreceptor input(90) and we are comparing expression at ninety days. Previous reports have also emphasised the redistribution of mGluR6 staining from synapses to axons – perhaps suggesting that functional changes may be secondary to the location of receptors within the cell, rather than secondary to expression levels alone(79, 90).

The finding of such stability at a gene expression level is particularly informative when seen in the light of studies where opsin based optogenetic

tools(106, 159, 160) are targeted to ON-bipolar cells to functionally restore light responses in degenerate retina. Despite marked functional and anatomical remodelling, the parts of the bipolar cell signalling cascade necessary for optogenetic restoration appear to persist both at a gene expression and functional level during retinal degeneration.

Commonalities in differentially expressed genes: metabolic stress

Despite this stability, differences in expression were evident in sixty-six probes and so consideration must be given to any function connecting these. Initial searches of a gene-phenotype database(70, 258) revealed only one (*Srm2*) to have been previously associated with human retinal disease, and even this may have been due to its proximity on the X chromosome to another gene known to be pathogenic in ocular albinism (Table 4-6)(276, 279). Therefore, to identify possible candidate genes from our list linked by a common function or interaction, with minimum bias, pathway and protein-protein interaction analysis approaches were used.

This process returned a small number of common functions, all relating to metabolism and oxidative stress. As photoreceptors are lost during retinal degeneration, and these cells have high aerobic metabolic demand, it is possible that the oxidative stress on surrounding tissue falls, reducing the need for energy and antioxidant proteins(79). Therefore, by P90 in the *Pde6b*^{rd1/rd1} retina, bipolar cells should no longer show signs of oxidative stress if they are to avoid activating the apoptotic pathways(86) and survive to act as optogenetic targets. In keeping with this, we see significant downregulation of genes associated with the respiratory chain and prevention of acute oxidative damage ((*Atox1*, *Clip1*, *Mt1*, *Mt2*(260, 262) Table 4-5) with upregulation of *Msrb2* (Methionine Reductase B2) - involved in repairing oxidised

methionine residues (261). As changes in these genes were as expected, they were not immediately prioritised for further characterisation.

Identifying Candidate Genes

With less biased approaches (such as database searches) not identifying immediately promising collections of candidate genes grouped by function, an alternative approach to attempt to determine why these sixty genes in particular were differentially expressed was required. As their number was relatively small, a systematic review of a gene ontology database and literature review for each gene was feasible (Figure 4-2). This involved the necessary exclusion of nine genes (Figure 4-3) where there was little literature available, however none of these genes were highlighted in unbiased interaction or pathway analysis approaches either – so would not have been highlighted in such a strictly unbiased approach either.

This collation of data on each potential gene allowed them to be grouped by function (albeit more broadly than would occur if classifying strictly ontology term alone) and ranked by relevance to the research questions. This also allowed review of previously reported immunohistochemistry studies for each of the four candidate genes selected and, in some cases, an indication of expected function given previous knock out studies (Table 4-6).

In selecting candidate genes for further characterisation, the advantages and disadvantages, that accompany all approaches, become apparent. Initially striving to be as unbiased as possible by using pathway analysis and database searches, it became apparent these approaches did not have the power to highlight pathways with such a small group of genes. In turn, the size of the group became an advantage with later approaches, allowing a manual, but systematic consideration of previous

knowledge on each of the genes and a the prioritisation of candidate genes to use as exemplars for further characterisation.

Further characterisation of selected candidate genes

Four such candidate genes were highlighted for further consideration: *Srm2*, *Slf2*, *Anxa7* and *Cntn1* and collated prior knowledge of these genes is presented in Table 4-6.

Shroom 2

The pattern of increased Shroom 2 staining and expression (Figure 4-6) that we see in degeneration, with a maximum in mid-degeneration (where neural modelling is at its highest), is consistent with its described role in cytoskeleton remodelling, cell shape regulation and membrane blebbing(266) given the retraction of bipolar cells axons and change in shape seen in histological studies of degeneration(82, 238). The absence of any retinal phenotype in a recent full *srm2* knock-out model(280) is interesting: previous knock down models had predicted a failure of retinal lamination in development – secondary to its role in melanosome migration (essential to normal retinal development) (266). Redundancy of other members of the Shroom family could account for this, nonetheless assessment of the knock-out's visual function and resilience to retinal insults (such as degeneration) would be most informative in further eliciting *Srm2*'s role in degeneration.

Sulphatase 2

The Heparin Sulphate Proteoglycans (HSPG), from which Sulphatase 2 removes sulphate residues, are involved in (but not essential to) the binding of AAV in advance of its entry to the cell(145). Importantly, AAVs are less able to bind HSPGs that are less sulphonated (for example due to increased sulphatase activity – *Slf2* was upregulated

in gene array work)(145, 146). Interestingly, our semiquantitative IHC (Figure 4-7), unlike gene array data, suggests no difference in Sulphatase 2 staining compared to wildtype during degeneration, but a gradual increase in staining over time in both genotypes. However, changes in distribution of Sulfatase 2 protein between its secreted and cell surface forms (267) could mask underlying changes in expression as found on our gene array study.

This potential role in AAV cell surface binding is potentially highly relevant, especially given documented difficulties in transfecting bipolar cells with AAV compared to other retinal cell types in degeneration (102, 149). In addition, *Extl3* (involved in HSPG production(272)) is downregulated in bipolar cells of *Pde6b^{rd1/rd1}* retinae in our study (Table 4-5) and HSPGs are known to be present in large numbers on the inner limiting membrane – possibly acting to sequester injected AAV (143). Together, this would suggest further investigation of AAV transduction with reference to *slf2* is warranted: for example, by investigating if bipolar AAV transfection were to be easier in the absence of *sulf2* function in a sulf-2 knock out model and if so by investigating *sulf2* inhibition as an adjunct to bipolar cell AAV transfection.

Annexin a7

Effective optogenetic therapy requires effective neurotransmitter release from targeted cells and two genes central to this process appeared downregulated in degenerate retinae (*Anxa7* and *Unc13a*, Table 4-5). While expressed at bipolar cell “conventional synapses(281),” UNC13A does not appear at the main ribbon synapses bipolar cells use to propagate the light signal with great speed and efficiency, so is less likely to have a direct role in the primary glutamate output from these cells.

However, Annexin a7 has been shown to be phosphorylated by the ON-bipolar cell specific kinase PKC α (270), which is central to the activation and termination of bipolar cells' light responses(282, 283) (Table 4-6) and not downregulated in degeneration (Table 4-2). This phosphorylation can increase Annexin a7's ability to promote vesicle fusion at the membrane(268). Therefore, lower Annexin a7 levels may be implicated in the negative regulation of the exocytic membrane fusion process leading to glutamate from bipolar cells in response to light(268), so was prioritised for further characterisation.

Annexin a7 showed staining just significantly lower than wildtype during degeneration (but with no individual timepoint identified as significant on post hoc analysis, and no change with time, Table 4-6), perhaps suggesting that this aspect of the bipolar signalling cascade is indeed still functional, but perhaps with a reduced rate of protein turnover when light signalling is lost during retinal degeneration.

A more complete understanding of the role of Annexin a7 in the wild type bipolar light response will need to be determined if any downregulation is likely to directly impair bipolar cells ability to act as optogenetic targets, or indeed represent an opportunity to manipulate response kinetics.

Contactin 1

To act as optogenetic targets, bipolar cells must not only be able to release neurotransmitter, but to form useful synapses to communicate the transduced light response. Contactin 1 has a role in regulating synaptic plasticity in the nervous system, being especially important in long-term potentiation (LTP) (274, 284) and long-term depression (LTD) (273, 285). Therefore our finding that *Cntn1* was *downregulated* in

retinal degeneration – a process defined by neural remodelling(238) - was perhaps unexpected.

This lowered expression - reflected in a transient drop in IHC staining (at P90 only) (Figure 4-9, Table 4-6) is however congruent with the findings of Haenisch et al(286) who noted an initial marked decrease of neural *Cntn1* mRNA expression followed by an increase back to baseline when investigating nerve crush (deafferentation) in zebra fish(286). In isolation, this could suggest a benefit to increasing *Cntn1* expression, or indeed early optogenetic intervention in the retina: restoring afferent signal input before any drop in expression or maladaptive synaptic remodelling can be induced. Certainly, investigation of retinal degeneration in both *Cntn1* over and under expression models would be informative.

Conclusion

This Chapter describes the first study of bipolar cell gene expression in the degenerating retina. Despite the marked morphological remodelling of these cells described in previous studies, we unexpectedly find bipolar cells to have a relatively stable gene expression profile in degeneration. Notably absent are gene changes suggesting major oxidative stress, loss of the second messenger or neurotransmitter release systems essential to normal (or optogenetic) light signalling in these cells.

Several candidate genes, potentially implicated in bipolar cells' interactions with other cells, were seen to be altered. Four of these were selected for further characterisation over the course of degeneration pointing towards potentially fruitful future research areas to enhance the efficiency of therapeutic retinal optogenetics. Taken together, these findings suggest that despite remodelling, retinal bipolar cells

maintain the broad cellular machinery necessary to act as good optogenetic targets for visual restoration.

Chapter 5 – Results: Comparing non-specific and targeted delivery of melanopsin in the degenerate retina

Introduction

As demonstrated in the preceding Chapter, retinal ON-bipolar cells in degenerate retinæ continue to express genes associated with the second messenger systems important for opsin based optogenetic therapy. As discussed in Chapter 1, these cells represent the highest surviving cells of the neural-retinal hierarchy and are therefore attractive targets in attempts to retain intra-retinal image processing. In addition, many optogenetic tools act to depolarise cells in response to light – as do ON-bipolar cells - adding to their particular appeal as targets. Targeting optogenetic tools to multiple cell types in the retina with a non-specific approach could conceivably activate multiple retinal processing pathways (e.g. ON and OFF systems) at the same time, leading to output signals incompatible with higher visual processing - requiring remodelling. In counterpoise to this, non-targeted delivery of melanopsin has been used in previous *in vivo* studies(159, 160, 287), showing promise, despite the potential loss of this intraretinal processing. However, this work has not extended to a direct comparison with cell population specific targeting of melanopsin.

Melanopsin is a particularly attractive optogenetic tool as it is a native human retinal opsin protein with high sensitivity on account of amplification from second messenger coupling ($G_{q/11}$). In addition, unlike cone and rod opsins, melanopsin does not require an exogenous supply of retinal chromophore from the visual cycle (it can regenerate its own chromophore, or “self-isomerise” (54, 62)). A frequently proposed disadvantage of melanopsin is its slower kinetics (with response onsets and offsets in the order of hundreds of milliseconds or seconds (159, 160, 288)) – suited to its

contribution to encoding irradiance. However, there are suggestions that this is not an intrinsic property of the opsin, but of the transduction cascade and the cell in which it is expressed. For example, light induced current latencies of exogenously expressed mouse melanopsin were to 130 times shorter when expressed in *Drosophila* rhabdomeres compared to native expression in ipRGC s (60).

Therefore, in order to elucidate if these theoretical advantages of ON-bipolar cells are borne out in practice, it is necessary to functionally assess human melanopsin when expression is restricted to these cells and compare this to both non-specific targeting and restriction to other cell types (e.g. retinal ganglion cells)

While melanopsin has not been specifically targeted to bipolar cells, a melanopsin based chimeric optogenetic tool(105) has been selectively delivered to ON-bipolar cells using AAV and a specific promoter (*grm6*-SV40)(105). However, due to poor levels of expression with this approach, this group changed to a transgenic mouse approach with the chimera driven by a full length *Grm6* promoter. With both methods, less than specific targeting was noted(289) and no comparison was made between either virally or transgenic targeted delivery to non-specific expression. Therefore, there is a need to demonstrate both if melanopsin can be successfully specifically targeted to bipolar cells using viral delivery and if so, to compare this to previously demonstrated non-specific delivery (159) (160).

In addition, all of these studies made use of models still expressing native melanopsin in ipRGC s. This may add to difficulty in interpretation when reconstituted responses are so similar to residual, native melanopsin responses which are known to contribute to both visual(45, 48, 290) and non-visual light responses(205).

The aim of the following Chapter will be to directly compare restricted expression with non-specific expression of melanopsin via viral delivery in a retina completely without intrinsic residual light responses. AAVs containing melanopsin (either without or with loxP sequence to restrict expression to Cre expressing cells (ie. “floxed”) were used with two animal models. The “LMAF” and “CGRK” mouse lines, described in Chapter 3, are both melanopsin knock outs with retinal degeneration (*Pde6b^{rd1/rd1}*). The “LMAF” line additionally expresses Cre recombinase under the promoter L7 which, in the retina, is specific for ON-bipolar cells (and a small subset of L7 expressing bistratified RGCs(221)). The “CGRK” line alternatively expresses Cre under the Grik4 promoter, specific to a well circumscribed subset of mono-stratified, direction sensitive, ON-RGCs(213, 222).

I hypothesised that L7 targeted delivery of melanopsin would provide superior restoration of light sensitive responses in this degenerate model as it would preserve intraretinal image processing. Conversely, I hypothesised that ganglion cell specific targeting would be less advantageous than bipolar targeting without this preservation of processing, but superior to non-specific delivery as it would not carry the risk of activating inhibitory as well as excitatory pathways in response to the same stimulus.

Note on nomenclature

During this Chapter, for ease of labelling, mouse lines and treatment groups are abbreviated,

1. “LMAF” is used in place of *Tg(L7-Cre)^{+/-} Pde6b^{rd1/rd} Opn4^{tm1yau/tm1yau}*
2. “CGRK” is used in place of *Tg(grk4-Cre)^{+/-} Pde6b^{rd1/rd} Opn4^{tm1yau/tm1yau}*
3. “Floxed hOPN4” in place of *EF1 α -hOPN4 (floxed) virus* – see Figure 5-1A
4. “Non-Floxed hOPN4” in place of *CBA-hOPN4 (non-floxed) virus* – see Figure 5-1B

Experimental design and results

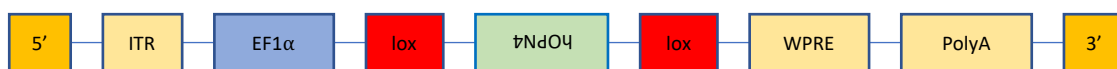
In order to test these hypotheses, two AAV vectors were produced containing hOPN4 under the control of high efficiency promoters: EF1 α (elongation factor 1 α) in the “*floxed*” and CBA (Chicken Beta Actin) in the *non-floxed* construct (Figure 5-1). These viral preparations were then administered to “*LMAF*” or “*CGRK*” mice with subsequent histology to confirm protein expression in the expected cell groups, *ex vivo* multiple electrode array recordings to assess cellular responses to light at the retinal level and behavioural assays to assess light responses at a whole organism level. Detailed methodology for all techniques is described in Chapter 2.

Production and *in vitro* validation of hOPN4 viral vectors

Two insert plasmids(165, 175) were used to construct two viral vectors (Figure 5-1). Both vectors incorporated hOPN4 driven by a high efficiency promoter (EF1 α or CBA), however the former (Figure 5-1a) also contained loxP sequences flanking the inverted human melanopsin gene sequence in order to restrict expression to those cells expressing the Cre recombinase enzyme (hence *floxed*). Pilot work had previously established that this construct restricts melanopsin expression to bipolar cells in an “*LMAC*” mouse after injection of such AAVs(165) (see Chapter 3). The *non-floxed* construct (Figure 5-1b) was identical to that used in a previously published *in vivo* study showing long term restoration of light responses when expressed in a degenerate retina(159) (Figure 5-1).

Viral vectors were produced from these constructs with an AAV 2/2 quad-mutant capsid (Chapter 2) at the viral vector core at the University of North Carolina. Quantitative polymerase chain reaction (qPCR) was used to determine the viral titre of each: 1.72x10¹²vg/ml (viral genomes per millilitre) for the *floxed* virus and

A



B



Figure 5-1 Linearized representation of insert plasmids used to make (A) floxed and (B) non-floxed AAV vectors to deliver the hOPN4 gene. Inverted sequences are shown upside down. Elements required for effective AAV transfection are in yellow: ITR – inverted terminal repeat, WPRE – Woodchuck hepatitis virus Post-transcriptional Regulatory Element, PolyA – PolyAdenosine tail. Promoters are in blue. EF1 α – Elongation factor 1 α , CBA – Chicken Beta Actin. LoxP sequences are in red. Payload (translated in target cells) is in green: hOPN4 – Human Melanopsin, dsRed – discosoma Red

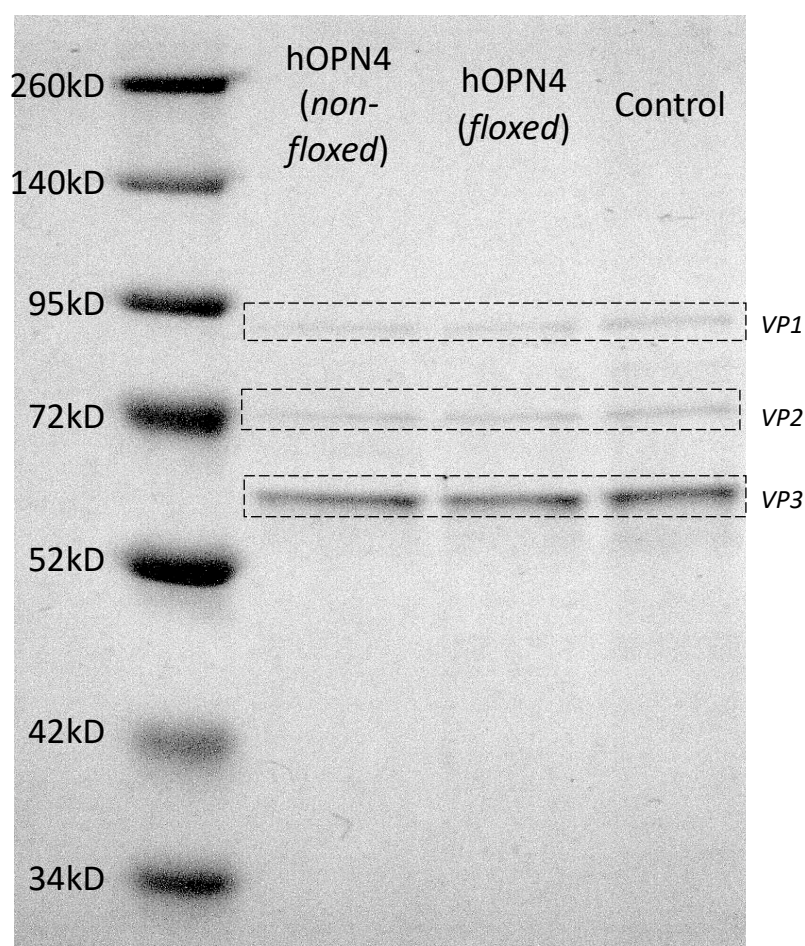


Figure 5-2 Denaturing SDS-PAGE page gel of samples of AAV containing either floxed or non-floxed melanopsin (subsequently used for in vivo validation). Three discrete bands are visible at the expected sizes for viral proteins (62, 72 and 87kD) with no other prominent bands: indicating lack of contamination. VP1,2,3 – Viral protein 1,2,3. The control is AAV from a stock previously validated in vivo.

4.43x10¹²vg/ml for the *non-floxed* virus. Both preparations displayed only three clear bands on SDS-PAGE electrophoresis (Figure 5-2) demonstrating an absence of significant protein contamination.

With this evidence of relative purity, the vectors' ability to transfect cells *in vitro* was assessed prior to *in vivo* use. Dissociated retinal cell cultures were prepared from "LMAF" mice and allowed to culture on 1cm laminin coated coverslips for two weeks before inoculation for 24hours with a volume of AAV corresponding to 5x10⁹vg per coverslip (see Chapter 2 for methods). Immunocytochemistry (ICC) of the cultures was performed three weeks later. This demonstrated hOPN4 staining in 17.8±4.3% of DAPI stained cells (n=177) in the *non-floxed* group and 11.8±2.1% (n=204) in the *floxed* group (Figure 5-3). This demonstrates that both preparations contain intact AAV particles and all the elements necessary to enter retinal cells and produce melanopsin protein. While this finding of successful expression in cultured retinal cells is an important validation of AAV preparations, it cannot be regarded as a direct metric of efficacy: these cells are unlikely to be in their native state given the artificial nature of both dissociation and culture, however it did signify that progression to *in vivo* testing was indicated.

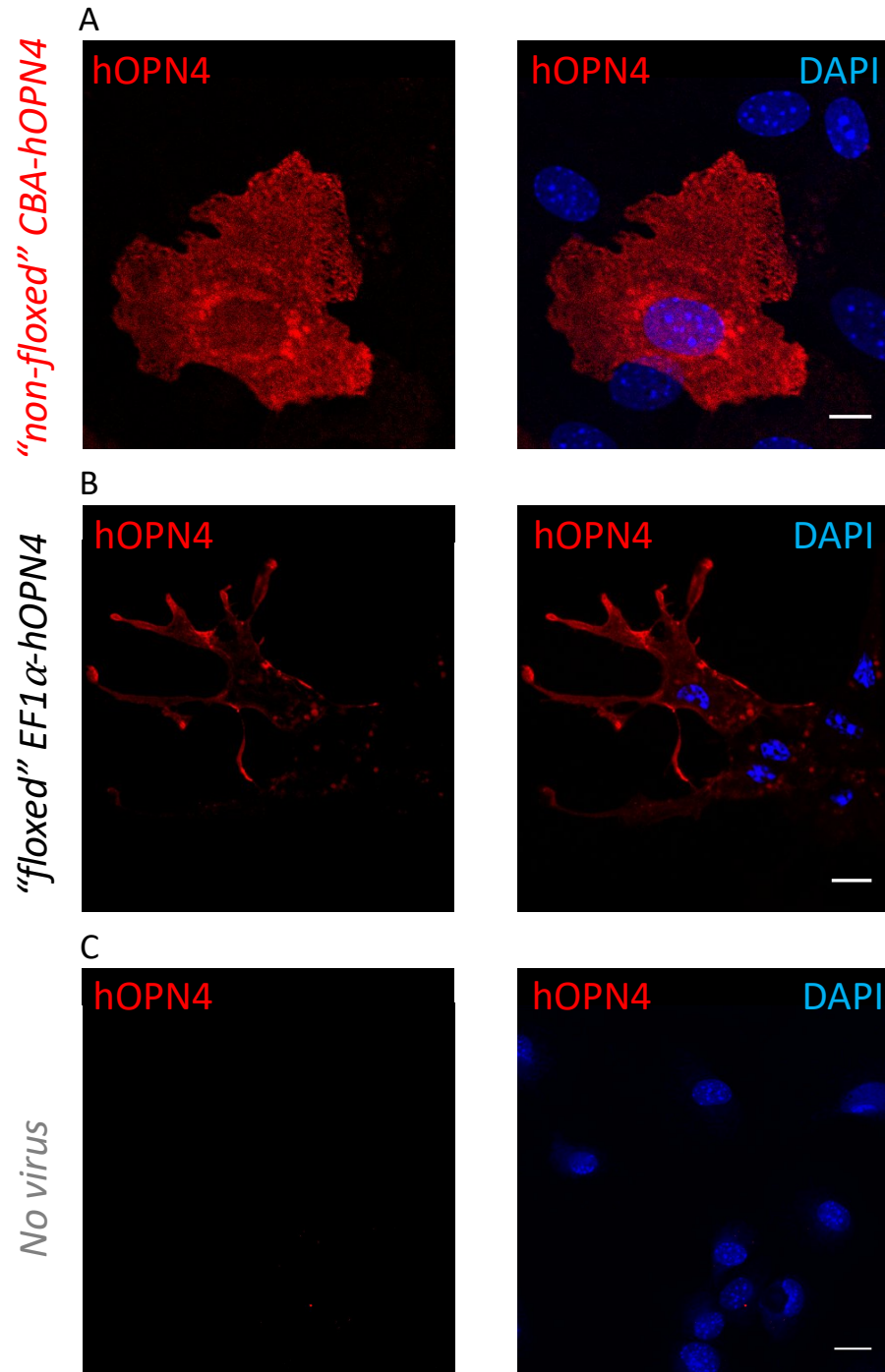


Figure 5-3

- Immunocytochemistry of dissociated retinal cell culture from "LMAF" mice incubated with an AAV containing non-floxed hOPN4. DAPI - nuclear stain; scale bar 10 μ m
- Immunocytochemistry of dissociated retinal cell culture from "LMAF" mice incubated with an AAV containing "floxed" hOPN4. DAPI - nuclear stain; scale bar 10 μ m
- Immunocytochemistry of dissociated retinal cell culture from "LMAF" mice incubated without AAV. DAPI - nuclear stain; scale bar 10 μ m

In vivo validation of hOPN4 viral vectors

Given this evidence for efficacy *in vitro*, *in vivo* validation was required, not only to ensure quantitative efficacy of AAV induced protein production, but to qualitatively assess cell-specificity of the vectors. Do these viruses drive expression (in the case of the *floxed* construct) solely within the expected cells or (in the case of the *non-floxed* construct) in a variety of cell types in the degenerate retina?

Three previous studies have delivered melanopsin to the degenerate retina. One used exactly the same (*non-floxed*) construct described here, which was injected subretinally and showed long term melanopsin expression and function(159). While a further two studies used an AAV construct with melanopsin driven by an alternative, high efficiency promoter (CMV) were delivered intravitreally(160, 291).

While the subretinal method is more suitable for use in animals with large vitreous volumes (such as humans), it is technically more difficult, more prone to surgical complications and generally restricts transfection to the discrete area of the detached retinal bleb. The mouse however, has a small volume of vitreous distribution compared to the size of its eye, therefore intravitreal AAV delivery can be used to efficiently transfect retinal cell types over the whole retina(139, 149) and was therefore used in this study.

Both the *floxed* and *non-floxed* construct AAVs were administered intravitreally to seven-week-old “*LMAF*” or “*CGRK*” mice who were culled four weeks later to allow flat mount and cryo-section IHC for hOPN4. An overall summary of findings is presented in in Figure 5-4, followed by more detailed analysis of melanopsin staining in each treatment group in Figure 5-5 to Figure 5-13. Melanopsin had different staining patterns in different layers of the retina, depending on treatment group.

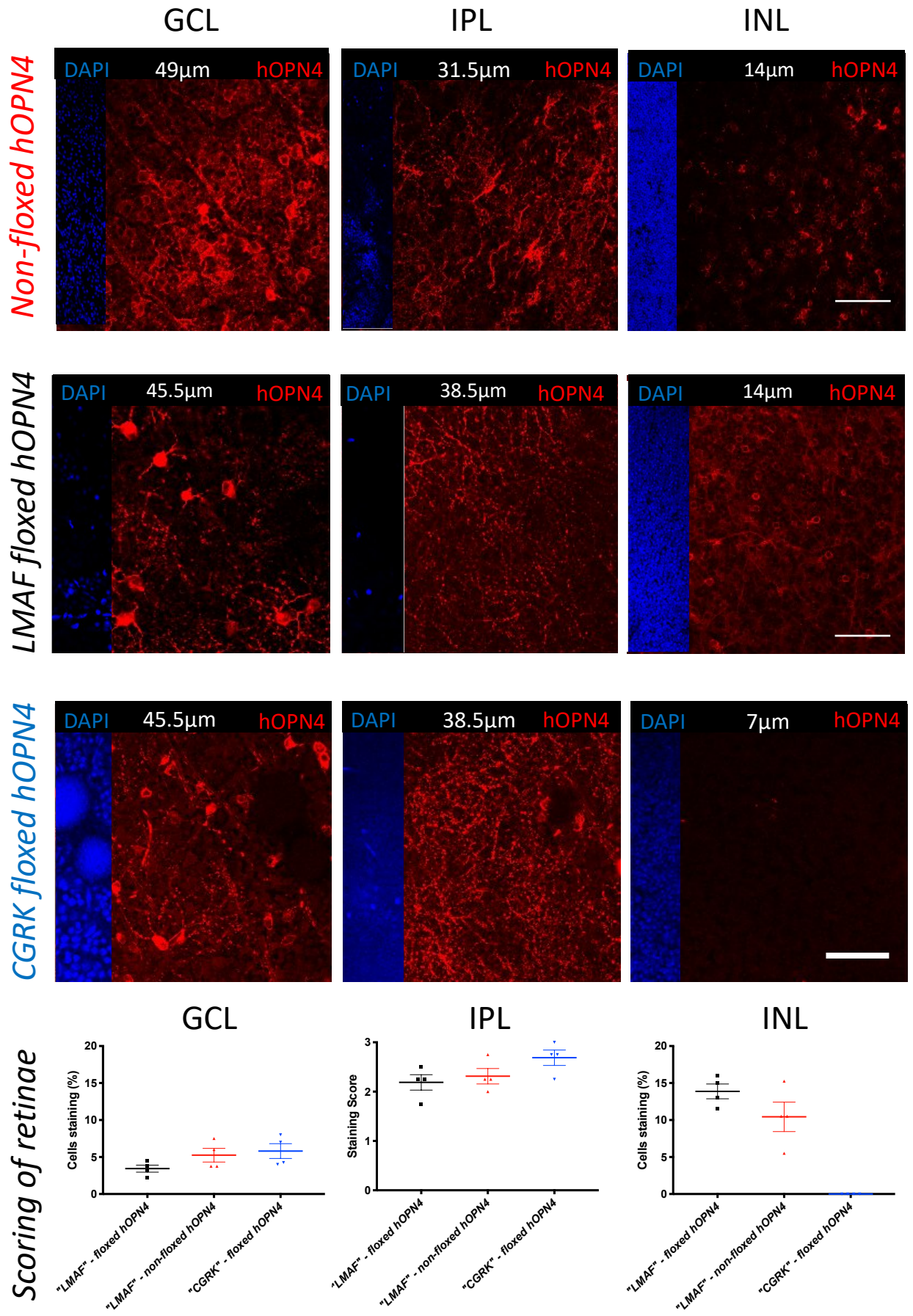


Figure 5-4 Summary of hOPN4 expression observed following delivery of floxed and non-floxed constructs to “LMAF” and “CGRK” mice. Note differences in patterns of expression between the two Cre models. The image panels give example staining at each of three planes (INL, IPL and GCL) and are drawn from Figure 5-6 Figure 5-9 and Figure 5-12 where further detail in each individual group can be found.

The graph panels represent the mean proportion of stained cells in 100 μ m x 100 μ m fields at that retinal layer (four fields per retina, four retinas per group). In the IPL, a score was given rather than a cell count (1= no stain, 2= moderate stain, 3= intense staining).

The depths here refer to this images position in the Z stacks Figure 5-6, Figure 5-9 & Figure 5-12. INL – Inner nuclear layer, IPL – inner plexiform layer, RGL – retinal ganglion cell layer. Scale bars 50 μ m

Intravitreal delivery of floxed hOPN4 to “LMAF” mice was seen to lead to staining in the inner nuclear layer (INL) ($13.88 \pm 1.01\%$ of cells, $n=422$) as well as in the retinal ganglion cell layer (GCL) ($3.438 \pm 0.47\%$ of cells, $n=196$) and Inner plexiform layer (Figure 5-4 to Figure 5-7). This would reflect the distribution of protein expression expected with a predominance in bipolar cells & a sub-population of retinal ganglion cells(213, 221). The co-staining of these cells for human melanopsin and L7 protein (which itself co-stains with PKC α , a widely used marker of ON-bipolar cells Figure 5-7) was in keeping with these cells representing the population previously described by Ivanova et al(221) and seen in previous use of this particular construct(165).

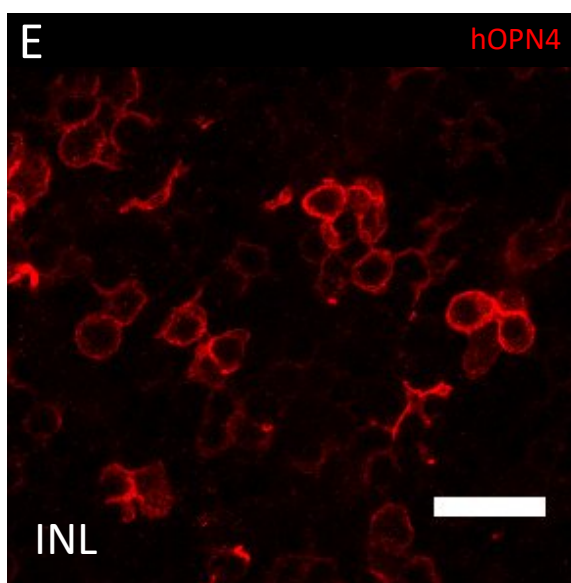
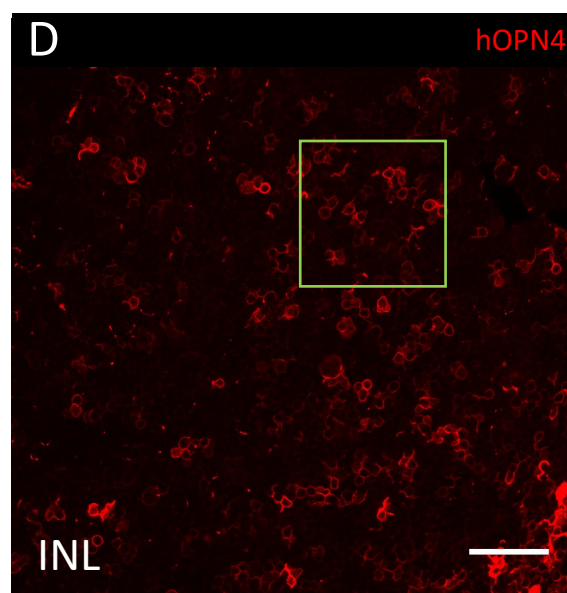
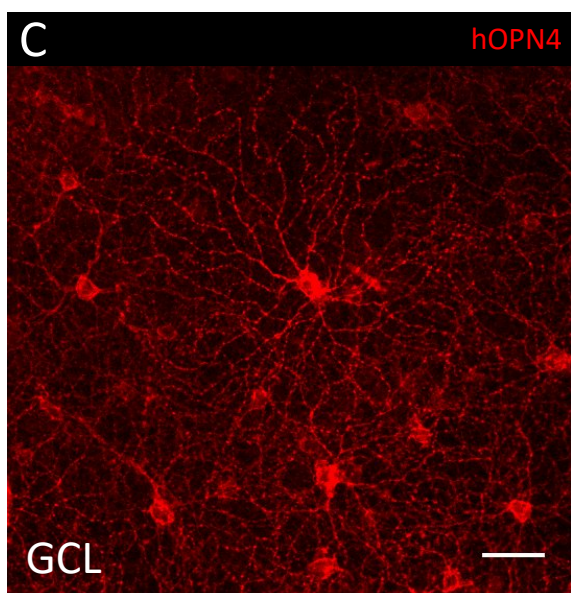
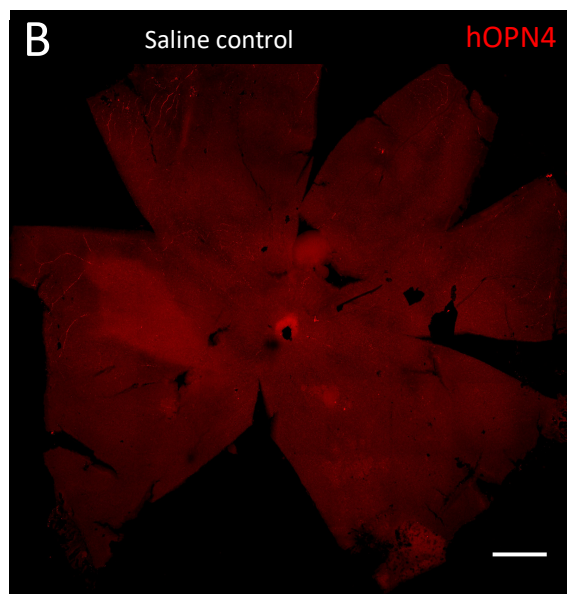
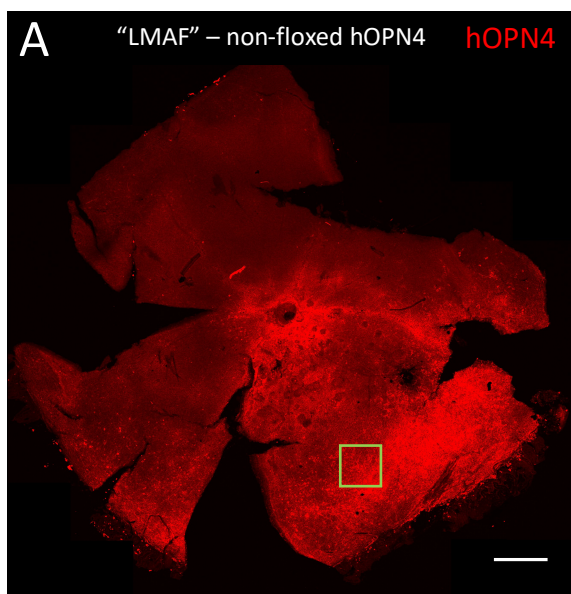
Intravitreal delivery of non-floxed-hOPN4 to “LMAF” mice resulted in staining observed in the inner nuclear layer ($10.44 \pm 1.99\%$ of cells, $n=376$, Figure 5-4), with locations and morphologies suggestive of bipolar and horizontal cells (see Figure 5-9), the identity of stained cells was confirmed by co-labelling with cell type specific markers (Figure 5-10). The prominence of horizontal cell staining with this construct is consistent with that previously described(159, 175) with subretinal delivery. However, unlike with subretinal delivery(159, 175), staining of cells in the GCL can also be seen with intravitreal delivery ($5.25 \pm 0.92\%$ of cells, $n=208$) (see Figure 5-9), as well as, ON-bipolar and to a lesser degree, GABAergic amacrine cells (Figure 5-10).

Intravitreal delivery of the floxed hOPN4 construct to “CGRK” mice demonstrated staining restricted to a subpopulation of cells in the ganglion cell layer ($5.81 \pm 0.99\%$ of cells $n=410$) and dendrites in the inner plexiform layer, with no staining ($0.00 \pm 0.00\%$ of cells $n=216$) in the inner nuclear layer (Figure 5-4, Figure 5-11 & Figure

5-12). Co-staining of these cells with Brn3a is in keeping with the expected restriction of expression by the Cre-lox system to a subpopulation of mono-stratified retinal ganglion cells(213, 222) (Figure 5-13).

Retinal coverage

One advantage of intravitreal delivery of AAV over sub retinal, is the possibility of achieving coverage of a wider area of retina. With subretinal delivery, transfection is limited to the vicinity of the bleb. Therefore, a semi-quantitative index of retinal protein expression over whole retinae was obtained by manually scoring retina flat mounts, stained for hOPN4, on a sectoral basis (see Chapter 2). As can be seen in Figure 5-14, in all three treatment groups, there was a wide range in the level of staining per quadrant – suggesting variable and patchy expression.



"LMAF" – non-floxed hOPN4

Figure 5-5 Confocal micrographs of a flat mount of an “LMAF” retina four weeks after injection with the “nonfloxed” CBA-hOPN4 construct.

- A. Low power tile of confocal images stitched to give view of whole retina – note areas of lower staining superiorly and on the left of the image. Green box gives approximation of location of the image in panel C. Scale bar 500 μ m*
- B. A saline injected control stained for hOPN4 and imaged as panel A.*
- C. Single low power (x20) image from figure A at layer of ganglion cell layer. Scale bar 50 μ m*
- D. High power (x40) image of the inner nuclear layer from same retina. Green box gives approximation of location of the image in panel E. Scale bar 30 μ m*
- E. Close up of high power (x40) image of the inner nuclear layer. Scale bar 10 μ m*

GCL – retinal ganglion cell layer; INL – inner nuclear layer

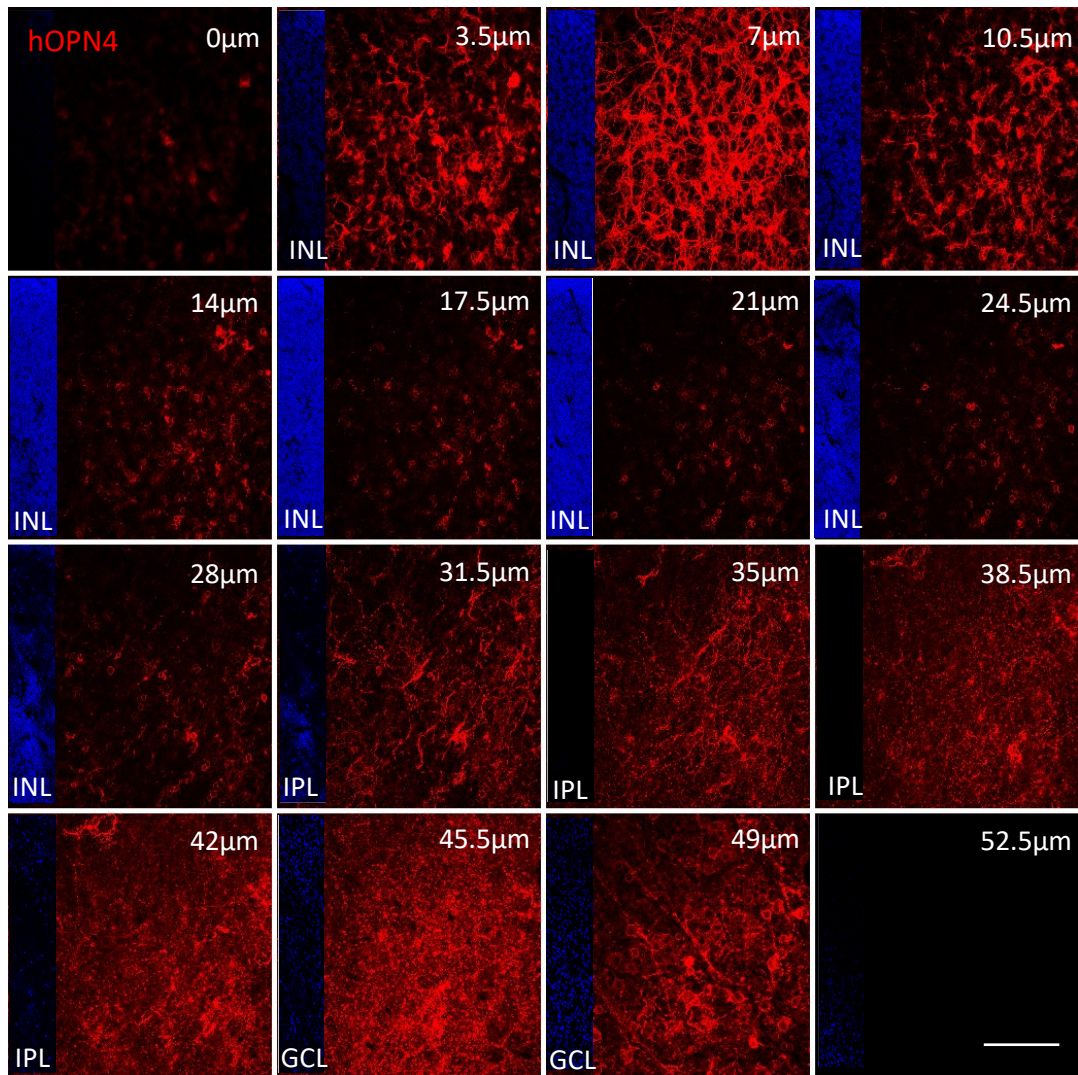
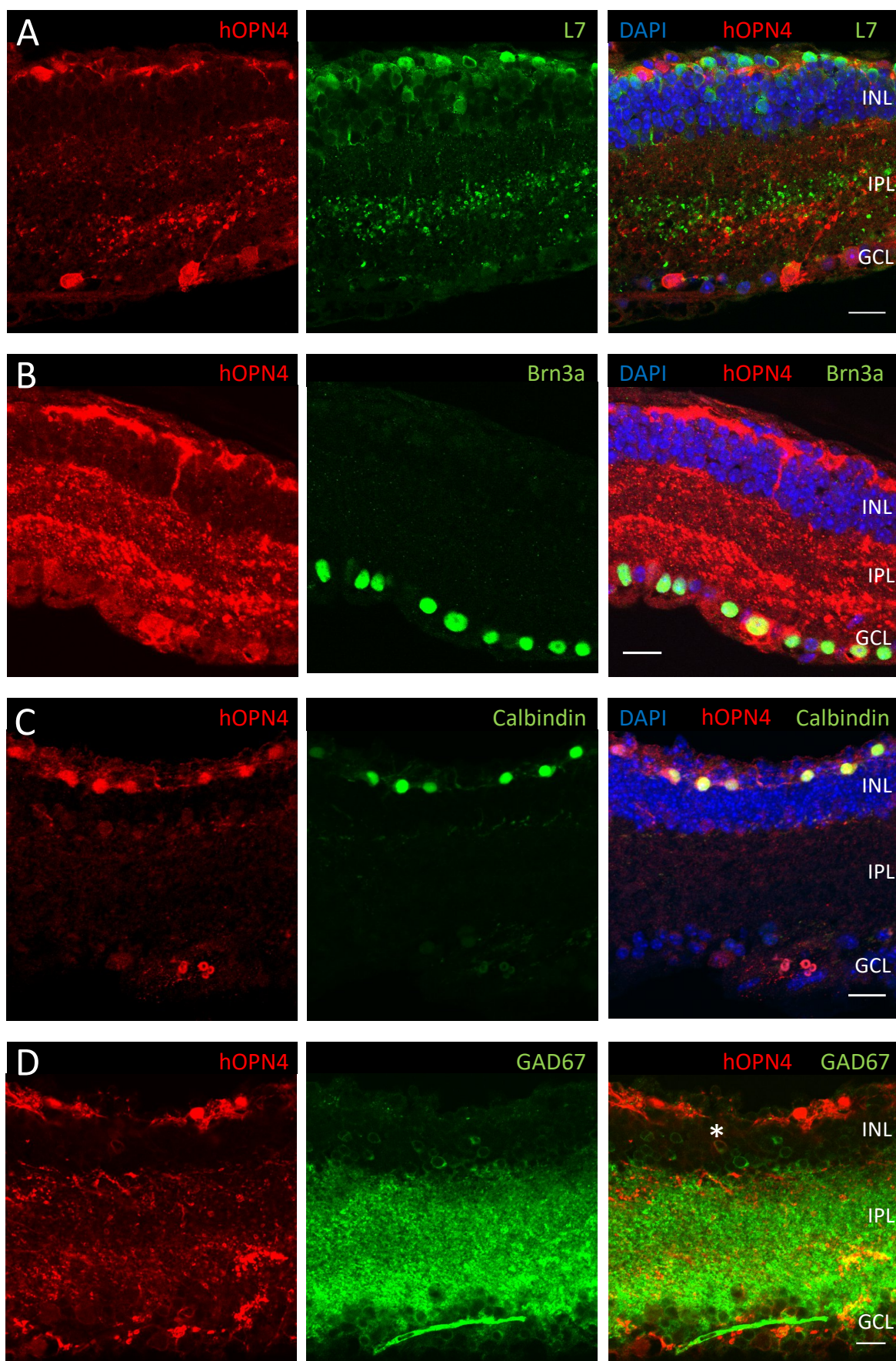


Figure 5-6 Montage of confocal micrographs of an “LMAF” retina four weeks after injection with the CBA-hOPN4 construct at x40 magnification. These represent slices of the same “Z stack” from the same retina described in Figure 5-4 & Figure 5-5. The depths described in each panel refer to the distance in the Z plane from the top left panel (just below the retina). Side panels show DAPI nuclear staining to orientate to depth. INL – inner nuclear layer. IPL – Inner plexiform layer. GCL – Retinal ganglion cell layer. Scale bar is 30μm

“LMAF” – non-floxed hOPN4



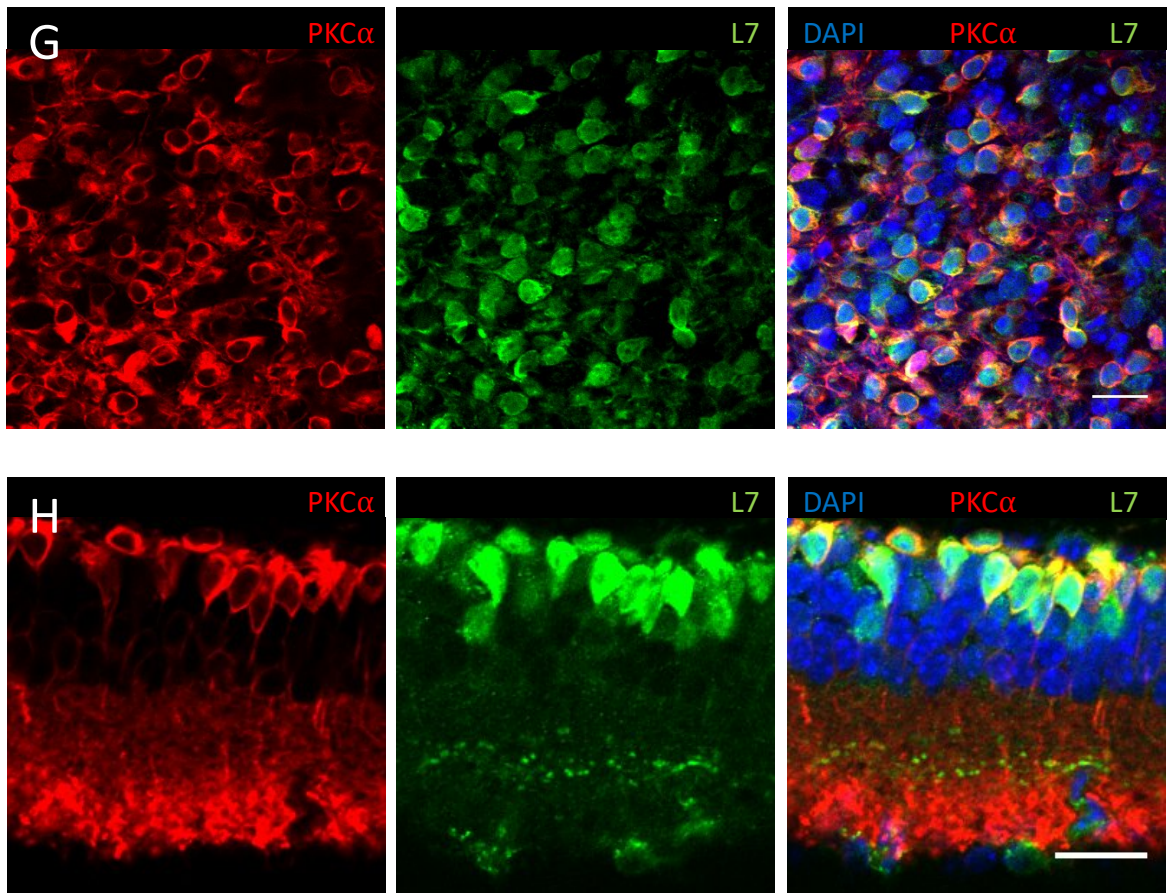


Figure 5-7 Cryosections from an "LMAF" retina four weeks after injection with the non-floxed CBA.hOPN4 construct. Co-stained with cell specific markers

- A. L7 – ON-bipolar cells. Note staining for hOPN4 mostly in Ganglion cell layer and inner nuclear layer.
- B. Brn3A – Subtypes of retinal ganglion cells – note co-staining
- C. Calbindin – Horizontal cells
- D. GAD67 – GABAergic Amacrine cells
- E. L7 co-localises with PKC α (a marker of ON-bipolar cells in flat mount IHC of the inner nuclear layer
- F. L7 co-localises with PKC α (a marker of ON-bipolar cells) in retinal cryosection IHC.

INL – inner nuclear layer, IPL – inner plexiform layer, GCL – Retinal ganglion cell layer. Scale bars 20 μ m.

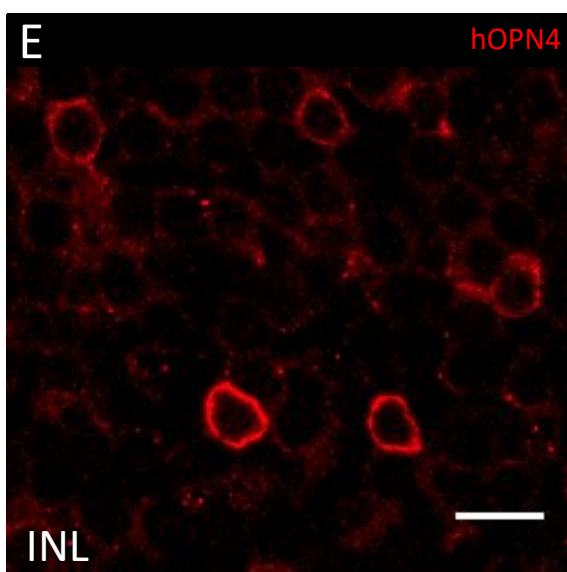
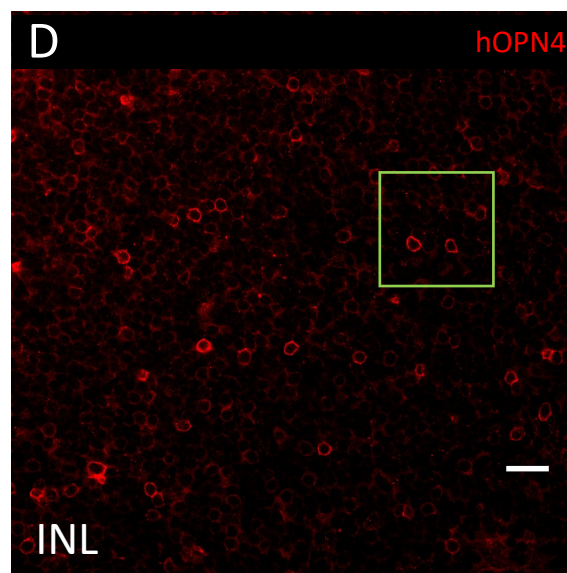
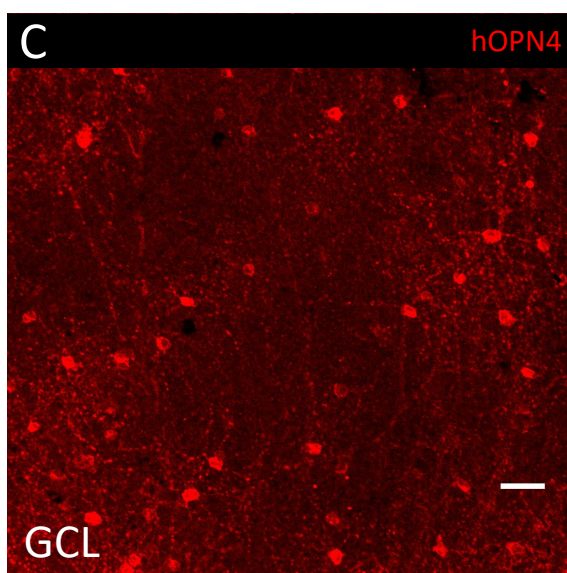
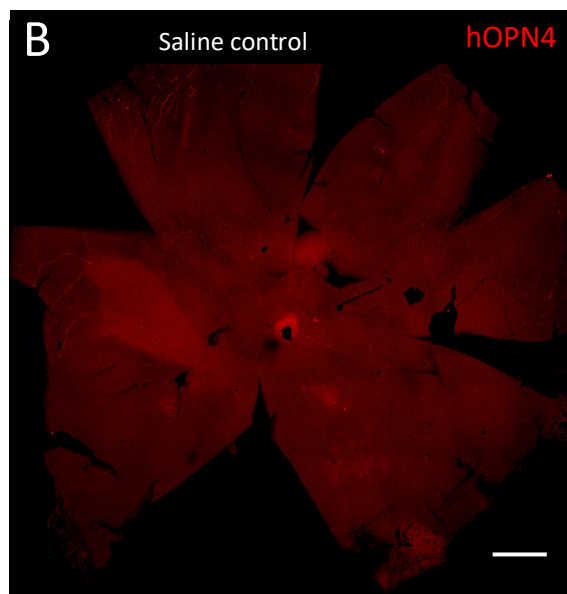
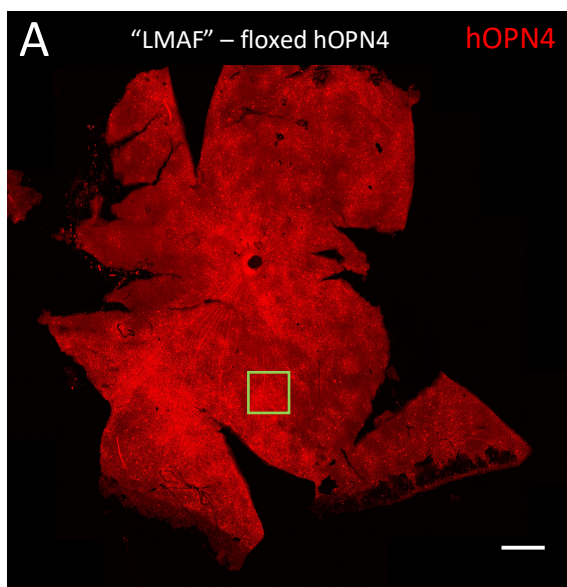
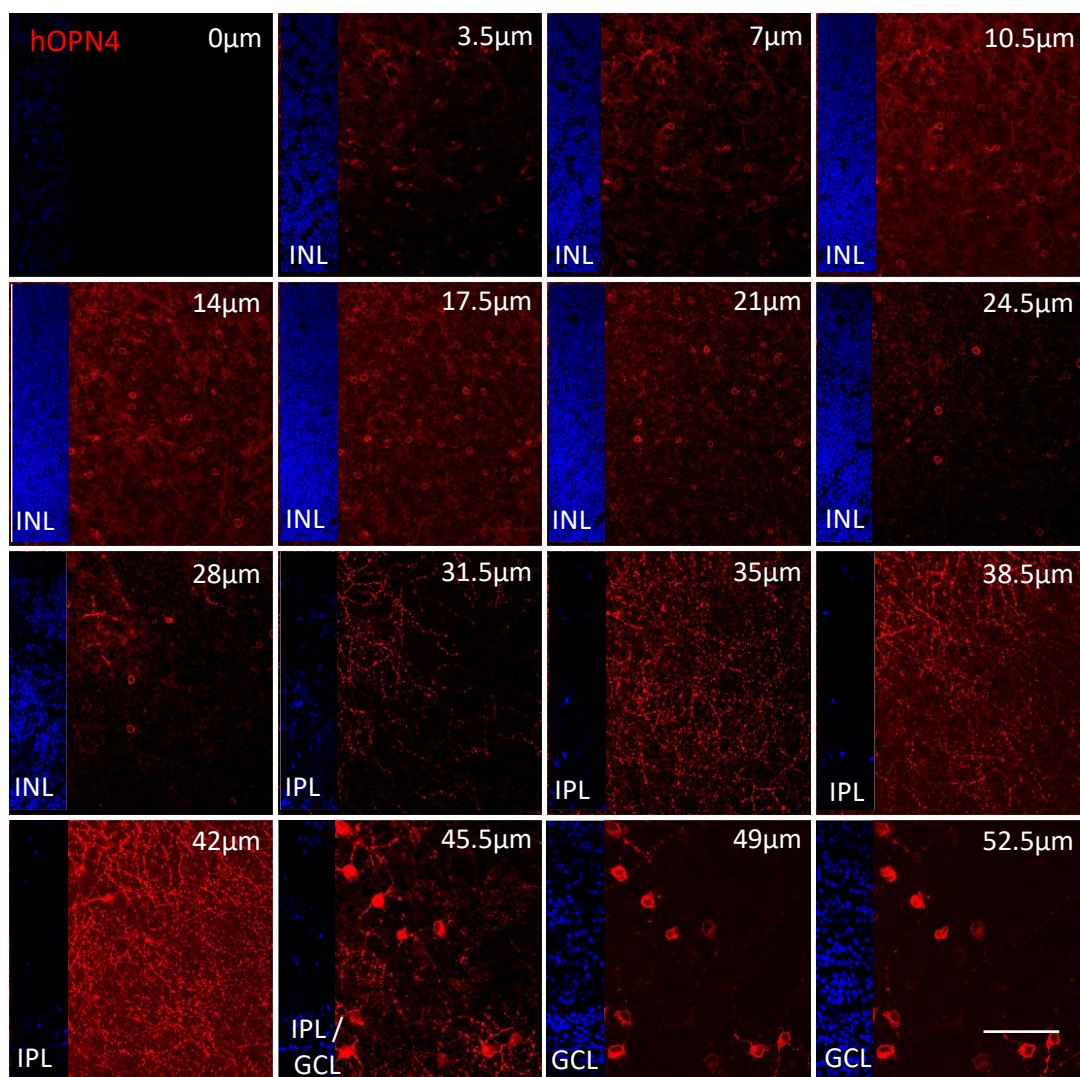


Figure 5-8 Confocal micrographs of a flat mount of an “LMAF” retina four weeks after injection with the floxed EF1 α hOPN4 construct.

- A. Low power tile of confocal images stitched to give view of whole retina – note areas of lower staining on the left and inferior part of the retina. Green box gives approximation of location of the image in panel B. Scale bar 500 μ m*
- B. A flatmounted retina from a saline injected control, stained for hOPN4, note absence of staining.*
- C. Single low power (x20) image from figure A. Scale bar 50 μ m*
- D. High power (x40) image of the inner nuclear layer from same retina (See Figure 5-9 depth 17.5 μ m). Green box gives approximation of location of the image in panel D. Scale bar 30 μ m*
- E. Close up of high power (x40) image of the inner nuclear layer from the same retina as panel panel A. Note staining concentrated to cell membrane, as expected for melanopsin expression. Scale bar 10 μ m*

INL -inner nuclear layer, GCL – Retinal ganglion cell layer



“LMAF” – floxed hOPN4

Figure 5-9 Montage of confocal micrographs of an “LMAF” retina four weeks after injection with the floxed EF1 α hOPN4 construct. These represent slices of the same “Z stack” from the same retina described in Figure 5-4 & Figure 5-8. For clarity, the red channel (representing hOPN4 staining) is presented. The depths described in each panel refer to the distance in the Z plane from the top left panel (just below the retina). Note staining of cell bodies in the GCL and the INL. Side panels show DAPI nuclear staining to orientate to depth INL – inner nuclear layer. IPL – Inner plexiform layer. GCL – Retinal ganglion cell layer. Scale bar is 30μm

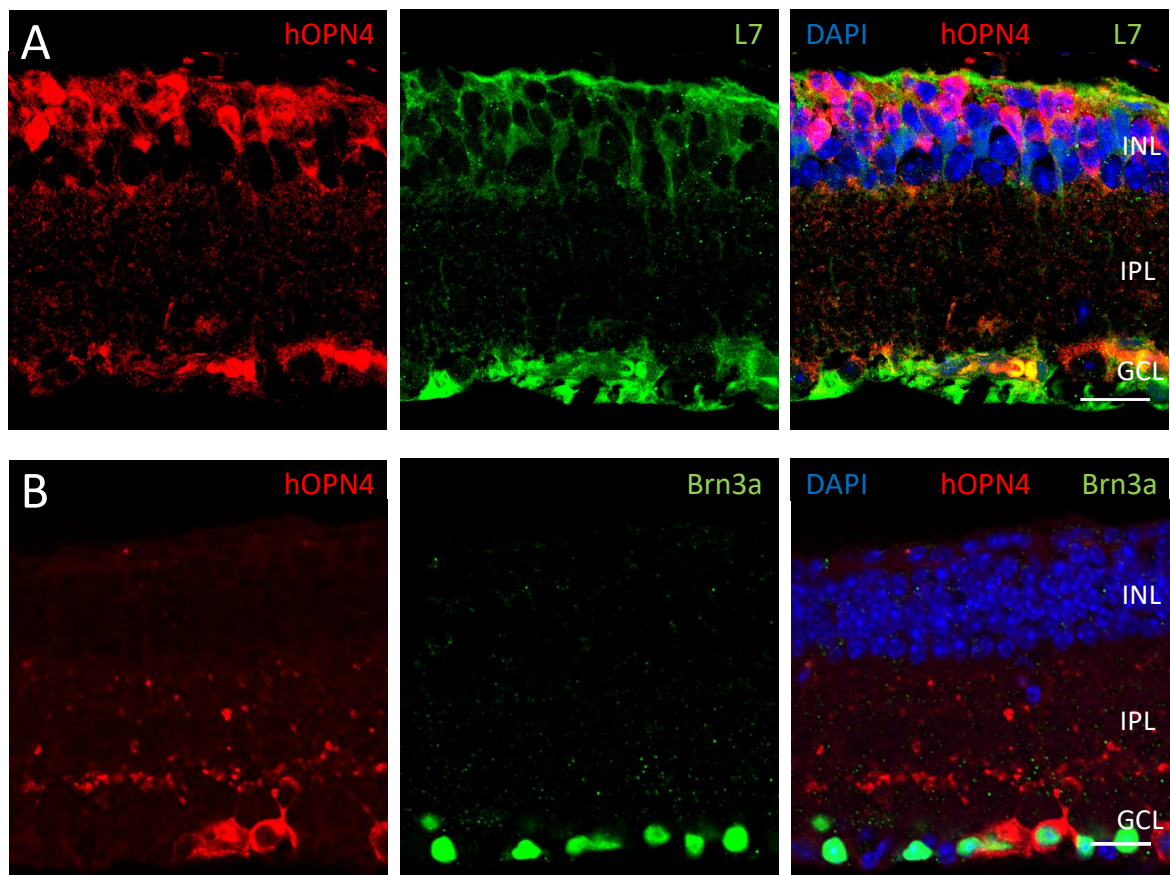
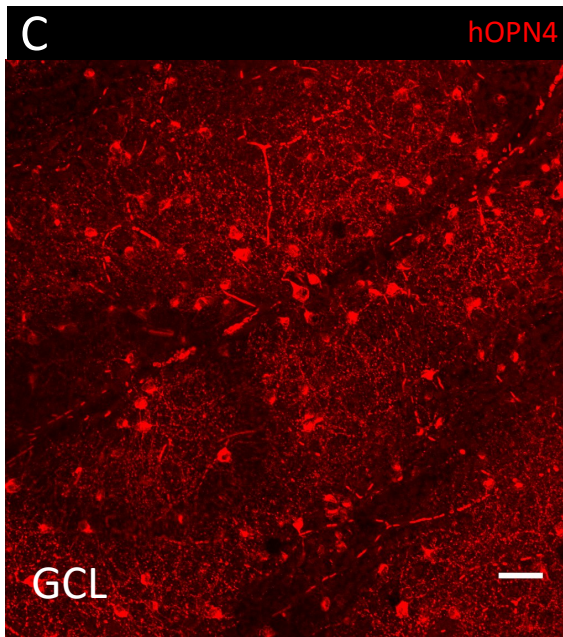
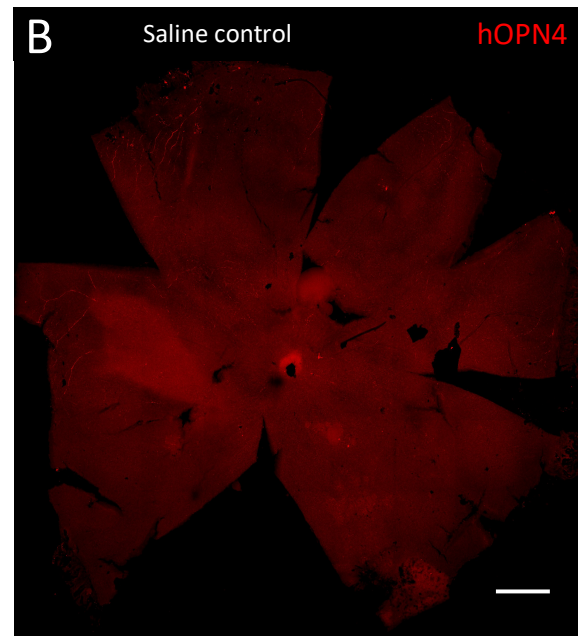
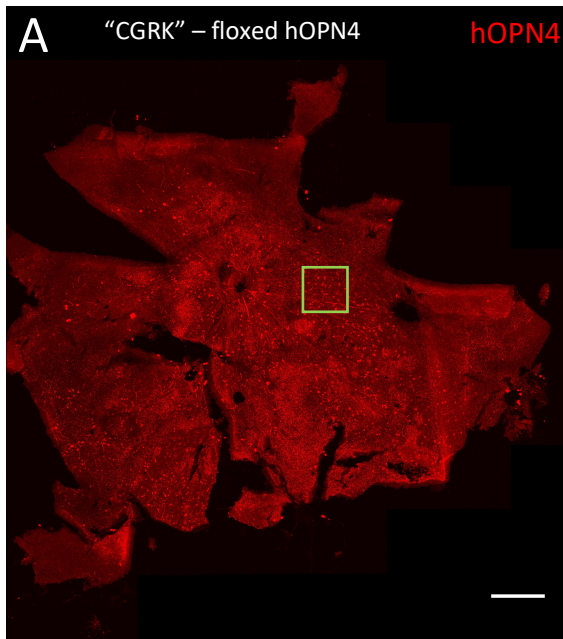


Figure 5-10 Cryosections from an "LMAF" retina four weeks after injection with the floxed EF1 α hOPN4 construct. Scale bar 50 μ m. INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer



"CGRK" – floxed hOPN4

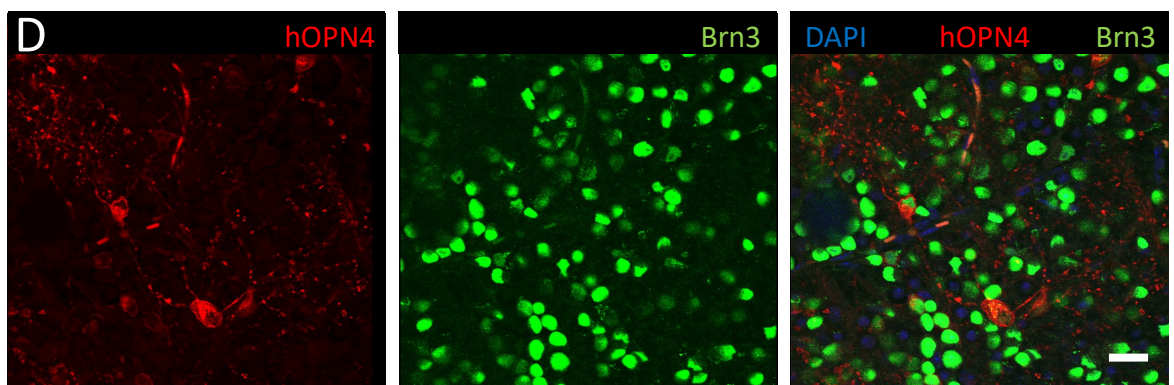


Figure 5-11 Confocal micrographs of a flat mount of a "CGRK" retina four weeks after injection with the floxed EF1 α -hOPN4 construct.

- A. Low power tile of confocal images stitched to give view of whole retina. Green box gives approximation of location of the image in panel B. Scale bar 500 μ m*
- B. A saline injected control, also stained for hOPN4, note lack of staining.*
- C. Single low power (x20) image from figure A. Scale bar 20 μ m*
- D. High power (x40) image of the ganglion cell layer from same retina. Scale bar 10 μ m*

GCL – retinal ganglion cell layer

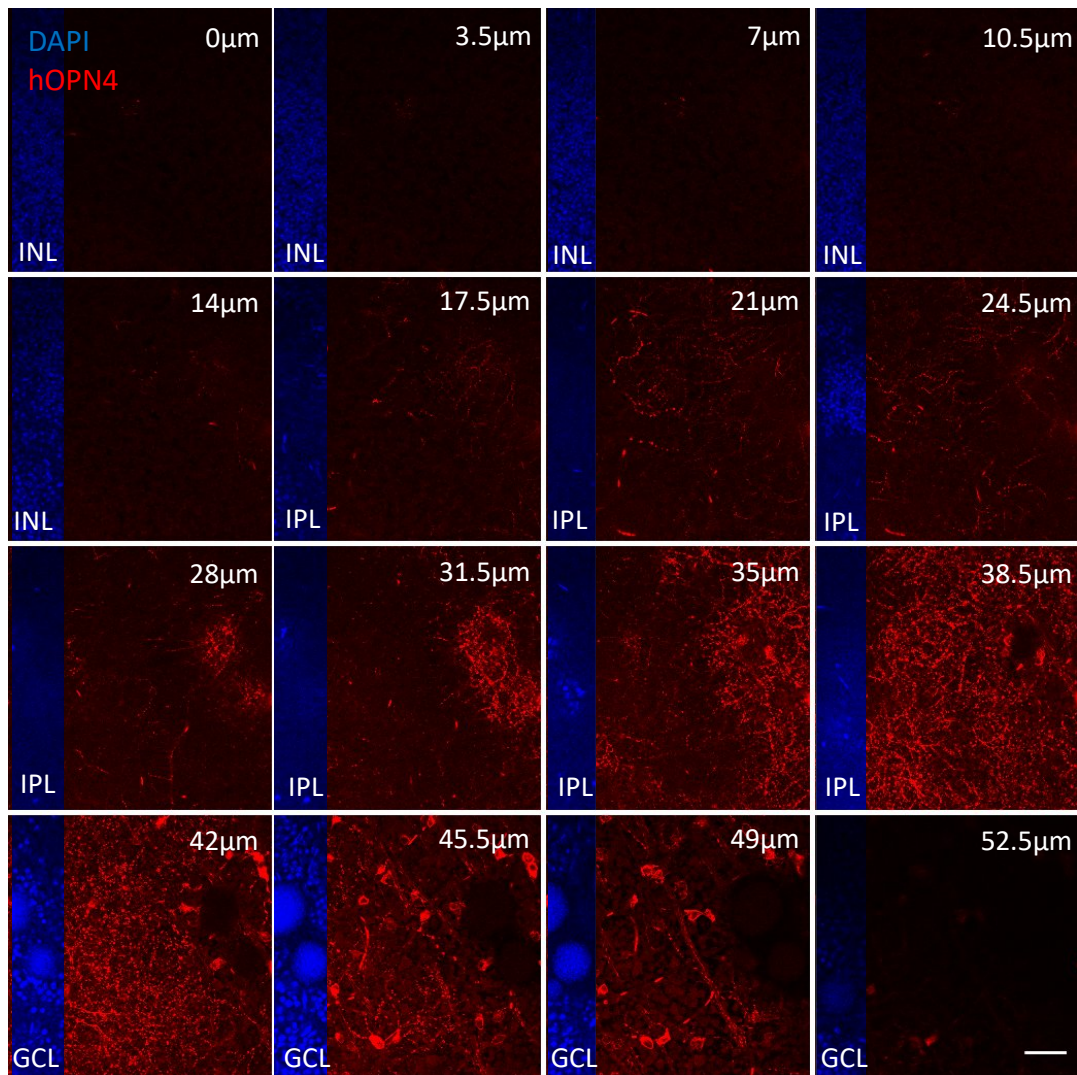


Figure 5-12 Montage of confocal micrographs of a "CGRK" retina four weeks after injection with the floxed $EF1\alpha$ hOPN4 construct. These represent slices of the same "Z stack" from the same retina described in Figure Y. For clarity, the red channel (representing hOPN4 staining) is presented in the main panel. The side panel in each pane represents the DAPI (nuclear) staining associated with that pane as an indication of location within the layers of the retina. Note staining in GCL, but not in the INL. The depths described in each panel refer to the distance in the Z plane from the top left panel (just below the retina). INL – inner nuclear layer. IPL – Inner plexiform layer. GCL – Retinal ganglion cell layer. Scale bar is 30μm

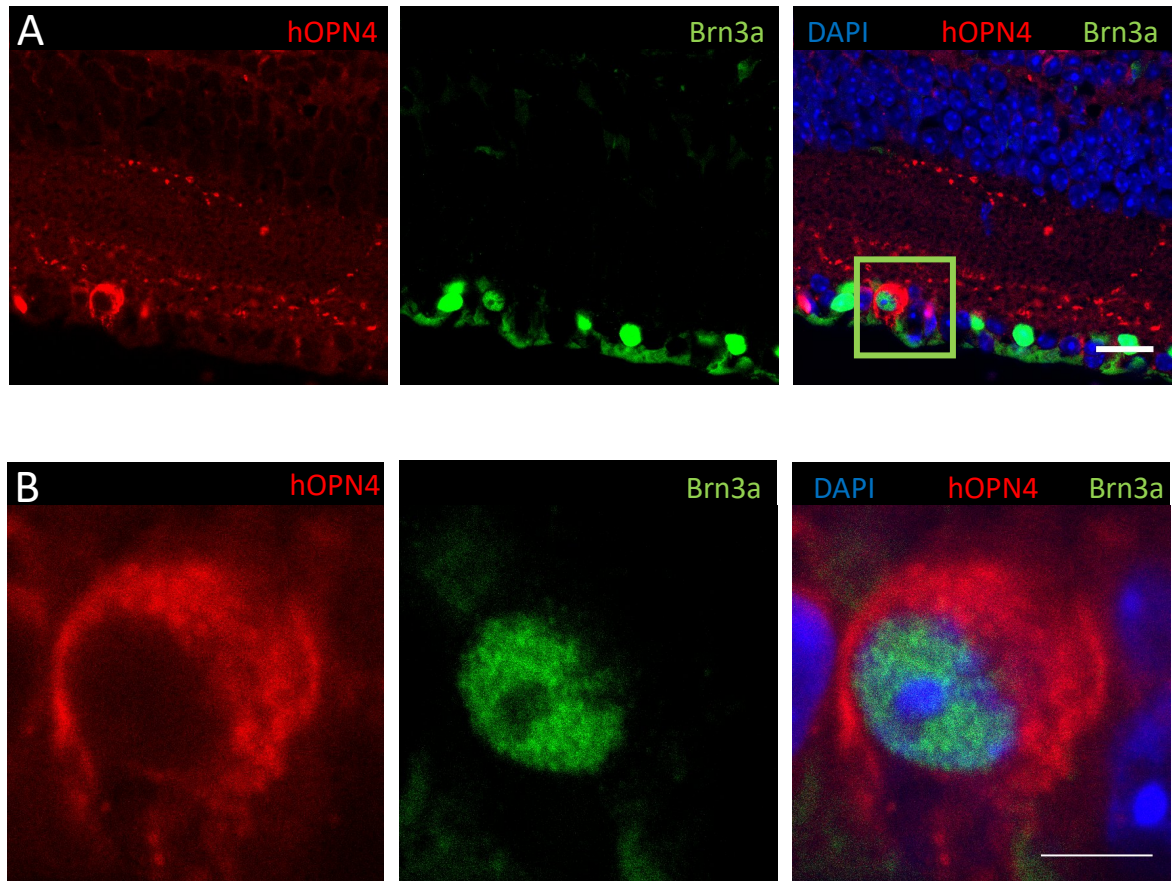


Figure 5-13 Cryosection from a "CGRK" retina four weeks after injection with the floxed EF1 α hOPN4 construct. Co-stained with Brn3. Green box indicates area of close up in B. Scale bar 20 μ m.

B. Close up of same image, scale bar 5 μ m.

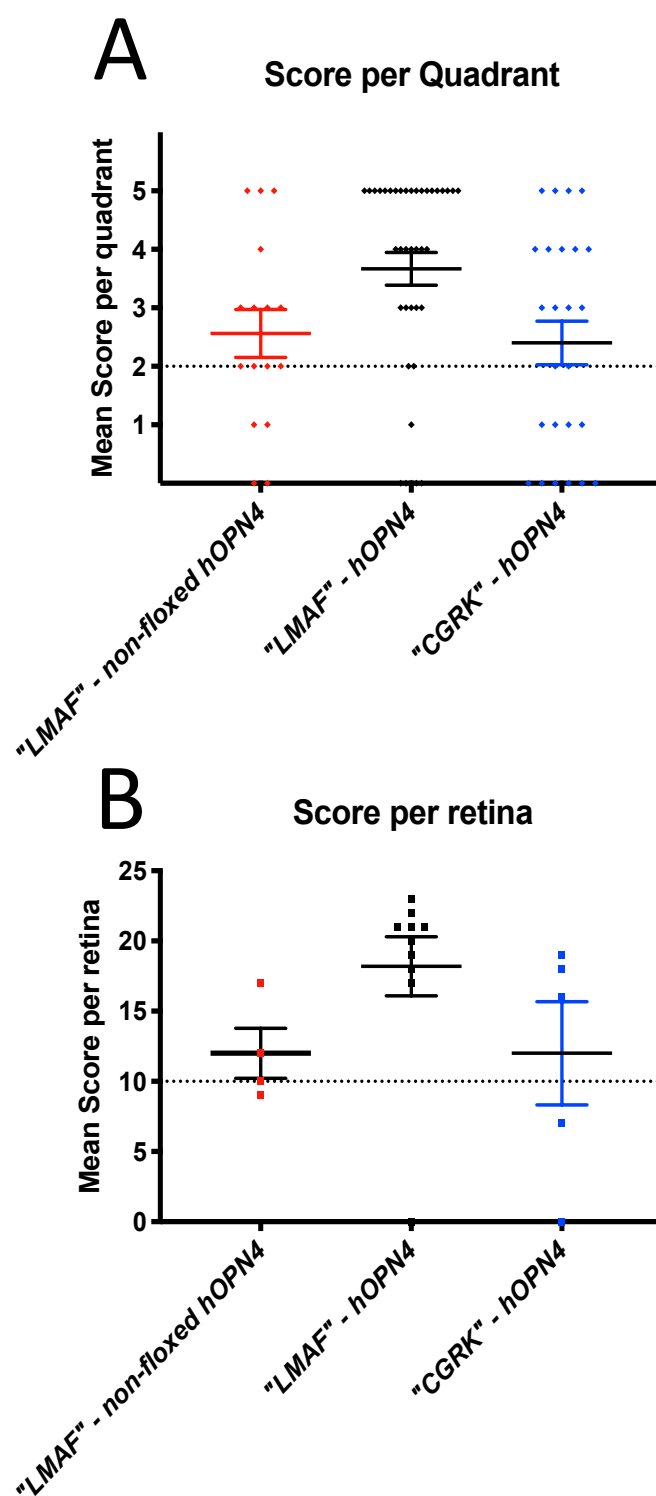


Figure 5-14 Semiquantitative indices of retinal flat mount staining of retina from either "LMAF" or "CGRK" mice treated with AAV containing either floxed or unfloxed hOPN4 and IHC stained for hOPN4 (see details of scoring method see Chapter 2).

A – Mean score per retinal quadrant

B – Mean score per retina

Reconstitution of electrophysiological responses to light

With evidence of AAV induced melanopsin expression in cells of the degenerate retina, further investigation at a functional level was indicated. Therefore, eight “LMAF” mice received bilateral intravitreal injections of the *non-floxed* virus, a further eight “LMAF” mice received the *floxed* virus; as did eight “CGRK” mice. Eight “LMAF” and eight “CGRK” mice were also given saline sham intravitreal injections and housed in the same manner as the treatment animals. Mice were between six and eight weeks of age at injection and were culled nine weeks later for *ex-vivo* electrophysiology using multiple electrode arrays (MEAs) following behavioural testing.

As neither “LMAF” nor “CGRK” retinæ have any residual retinal photo-responses (being both *Pde6b^{rd1/rd1}* and *Opn4^{-/-}* - Figure 5-17a & b) the presence of any response to light would be highly indicative of successful AAV delivery of an optogenetic tool in itself (Figure 5-17 C-E). Additionally, as the *non-floxed* construct contained a red fluorescent tag (dsRed) – it was possible to confirm visually that transfected cells were present in the area of retina that was apposed to the multiple electrode array (Figure 5-15).

Recordings of RGC spike firing were made from between five and eight retinæ in each group with light responses seen in all three groups with between 18.3 and 24.1% of all electrodes showing light responses (Figure 5-16 & Table 5-1). A light responsive electrode was defined as one with a mean rise of 10Hz in spike firing rate, maintained for 10 seconds (rolling 10 second bins) when compared to mean baseline firing rate in the 10 seconds preceding the stimulus. In the saline sham injection control group, 1% of electrodes were scored as responsive by this metric: all seven of

these individual records were manually reviewed (5 of them being adjacent electrodes on the same retina) and did not represent light responses, but spurious readings (noise), that were not present of repeat trials and were so excluded from further analysis. Similarly, six electrodes (five adjacent on the same retina) were scored responsive in the saline treated CGRK group, but seen on manual review to similarly represent noise.

There was a significant difference in the number of responsive electrodes between groups (One way ANOVA, $F_{(3,21)}=4.76$, $p=0.0110$), but on post hoc testing this was seen only to be between the saline controls and treatment groups, not between treatment groups (Tukey's correction, "*LMAF*"- floxed-hOPN4 $p=0.0258$, "*LMAF*" non-floxed hOPN4 $p=0.0128$, CGRK floxed-hOPN4 $p=0.0203$; Figure 5-16).

Not every electrode was responsive to light in the treatment groups, which is to be expected due not only to variable AAV coverage, but also technical limitations in the preparation and apposition of the retinal tissue to the array. Without residual native responses however, only cells apposed to an electrode with optogenetically transduced light signals would generate an increase in spike firing rate. As areas of transfection are variable across the retina and the MEA only covers a relatively small area of the retina (c.2mm²), the number of responsive electrodes on any individual trial will be stochastic.

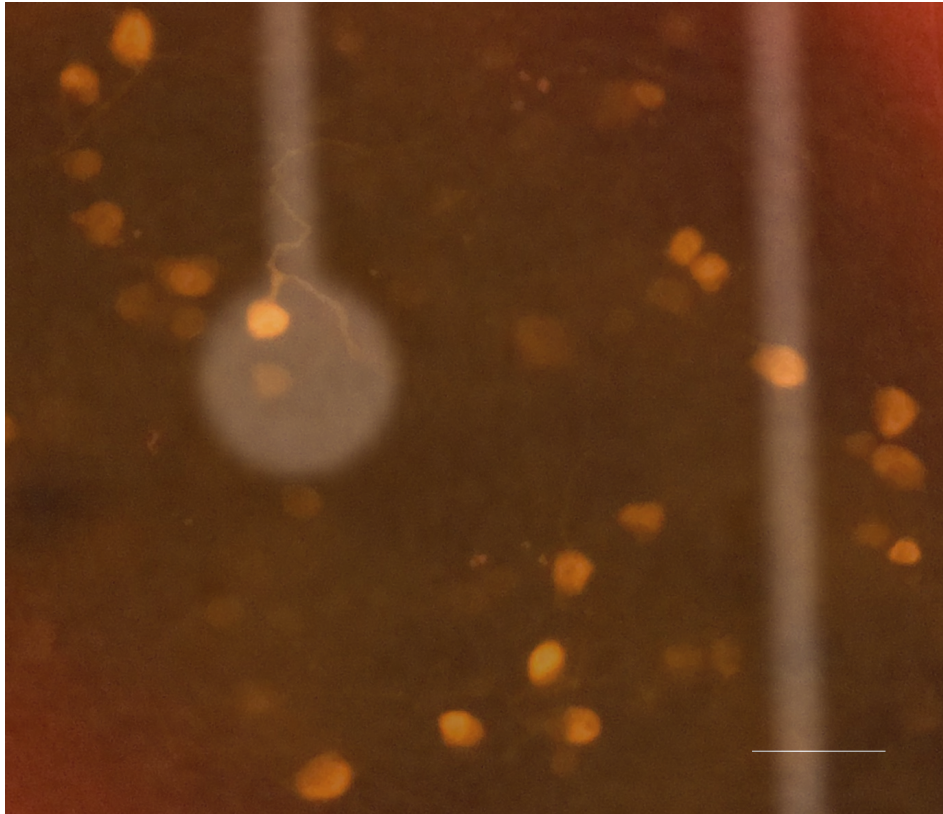


Figure 5-15 A Fluorescence photomicrograph taken of the area over the MEA recording electrodes from an LMAF retina treated with the “non-floxed” hOPN4 virus in the retinal plane. Note red fluorescence of transfected cells on account of the dsRed incorporated along with hOPN4 in this virus which concentrates in the cell bodies. A similar photomicrograph, taken in the electrode plane is superimposed to indicate relative scale. Scale bar 20 μ m.

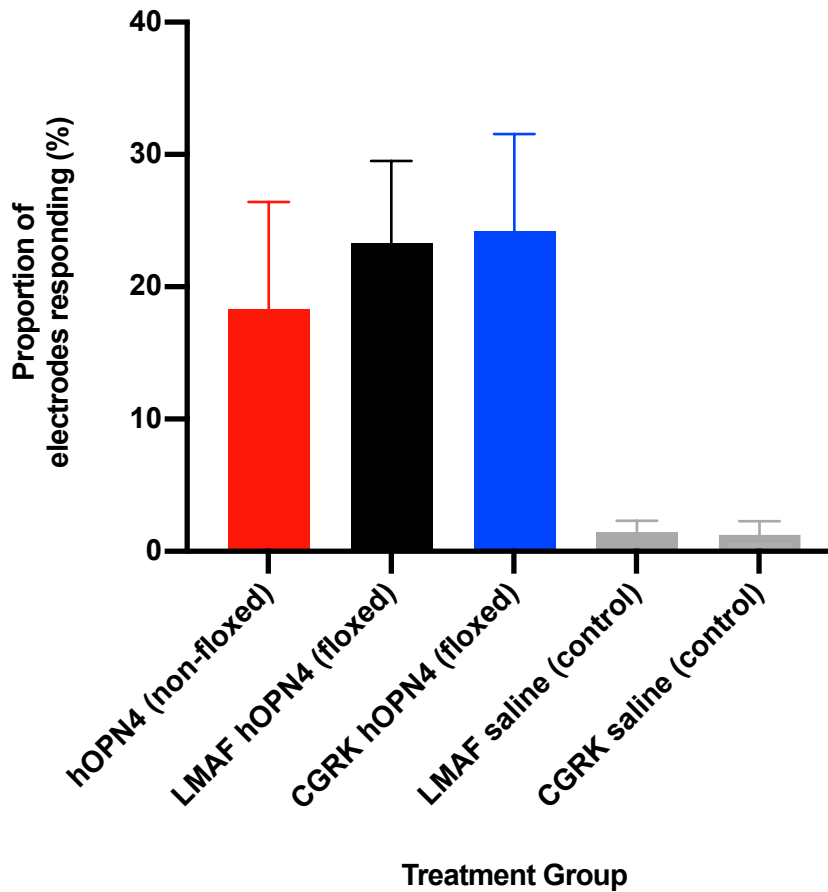


Figure 5-16 The proportion of electrodes in each group scored as responsive.

Treatment	hOPN4 (Non-Floxed) LMAF	hOPN4 (floxed) LMAF	hOPN4 (floxed) CGRK	Saline LMAF	Saline CGRK
Retinae (N=)	5	6	6	8	8
Total responsive electrodes (n=)	55	84	87	7*	6*
Total possible electrodes	300	360	360	480	480
Proportion	18.3%	23.3%	24.1%	1.4%	1.2%

Table 5-1 The proportion of electrodes in each group scored as responsive. Light responsiveness is defined as a rise in firing rate of 10Hz between mean baseline firing rate in the 10 seconds before stimulus onset and ten seconds after onset. *In both control groups, a small number of electrodes were scored as light responsive when the same criteria were applied. These traces were individually reviewed and found not to represent a light responses, but noise and were so excluded.

Electrophysiological Light Response

Raw recordings from an example electrode in each group are provided in Figure 5-17, these raw recordings were processed in real time to give an instantaneous rate of spike firing, per electrode, that could be plotted against time – this is shown in Figure 5-18 for all electrodes in a single retina as retinotopic arrays of graphs with a magnified example of a single electrode's trace. The comparison of spike firing rate traces in response to full field light pulses of increasing intensity can be seen in Figure 5-19 & Figure 5-20, with a superimposition in the lowermost panel to allow comparison.

In order to further investigate this, traces from light responsive electrodes were analysed in order to further characterise the sensitivity and kinetics of these reconstituted light responses (Figure 5-21 & Figure 5-22). Indices of firing rates before and in response to stimulus onset were obtained (see Chapter 2) and compared using two-way ANOVAs with post hoc testing between groups using Tukey's correction for multiple comparisons. Average baseline firing, as may be expected, did not vary with intensity ($F_{(6,1561)} = 0.06749$; $p=0.9988$) but did vary with group ($F_{(2, 1561)} = 14.54$; $p<0.0001$), albeit only with slightly lower baseline firing in the "CGRK" at two intensities and there was no interaction between intensity and group ($F_{(12, 1561)} = 0.1138$; $p>0.9999$) (see Figure 5-21a for post hoc test results).

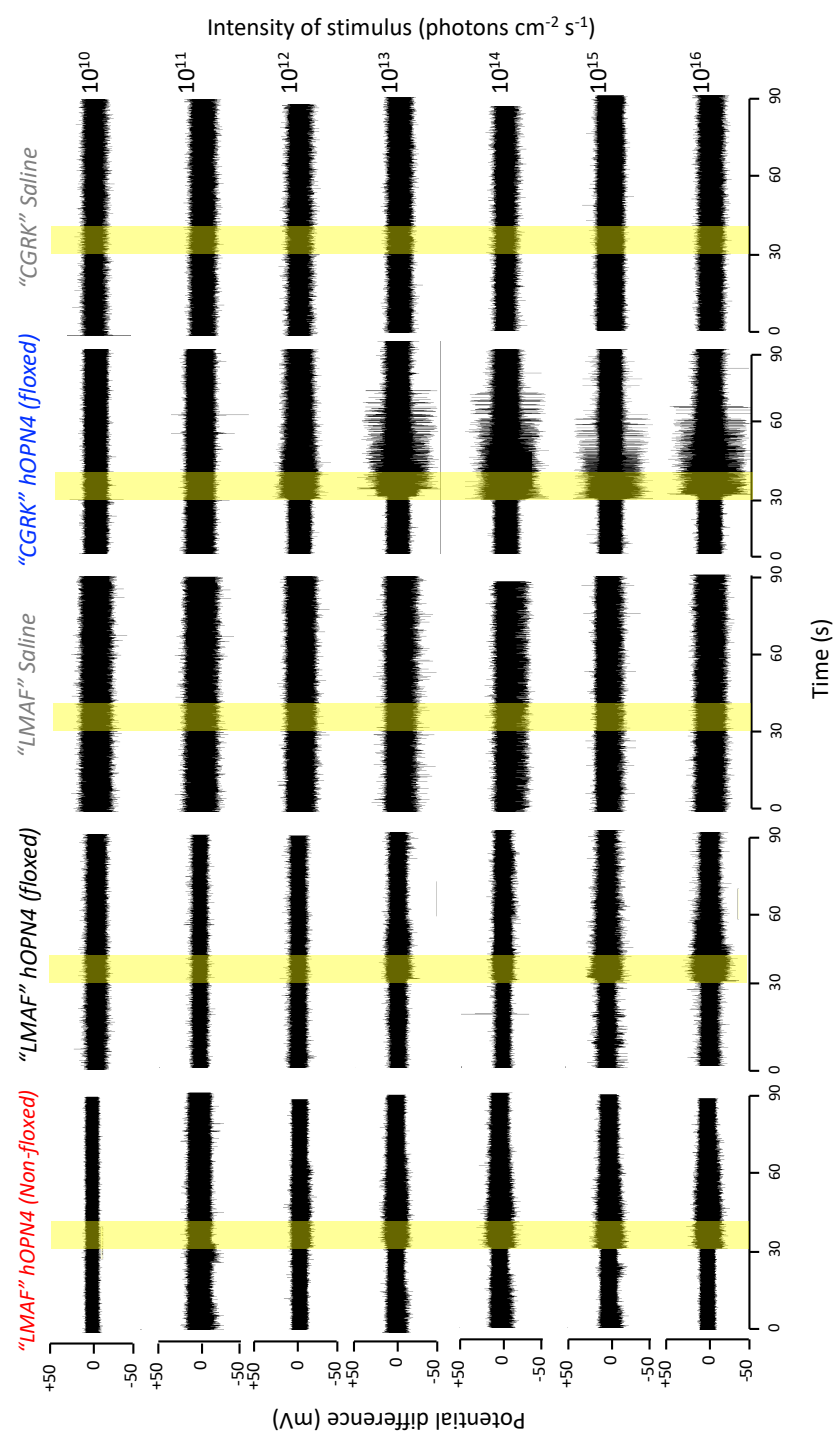


Figure 5-17 Example raw recording traces from a single electrode in each treatment group stimulated with a ten second pulse of 480nm light (yellow bar) at seven light intensities (starting with 10^{10} photons $\text{cm}^{-2} \text{s}^{-1}$, with a 10 minute gap between each recording).

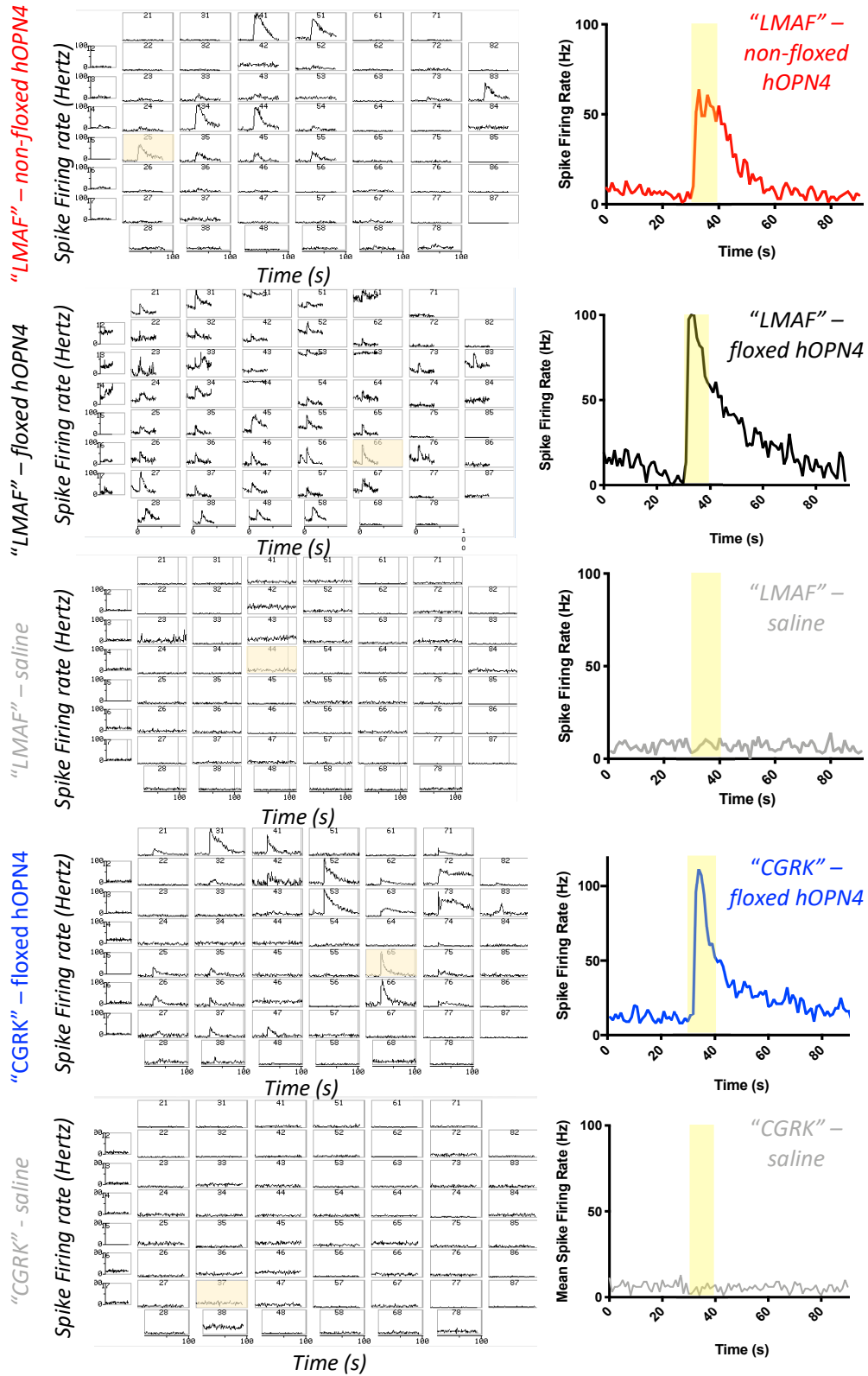


Figure 5-18 For each group, an array of spike firing rate traces from one retina is shown on the left.

On the right, one of the electrode traces (indicated by yellow shading on array) is redrawn at higher magnification. The yellow bar indicates the 10 second light stimulus (480nm , $10^{14.1}\text{ photons cm}^{-2}\text{ s}^{-1}$) after 30 seconds of recording.

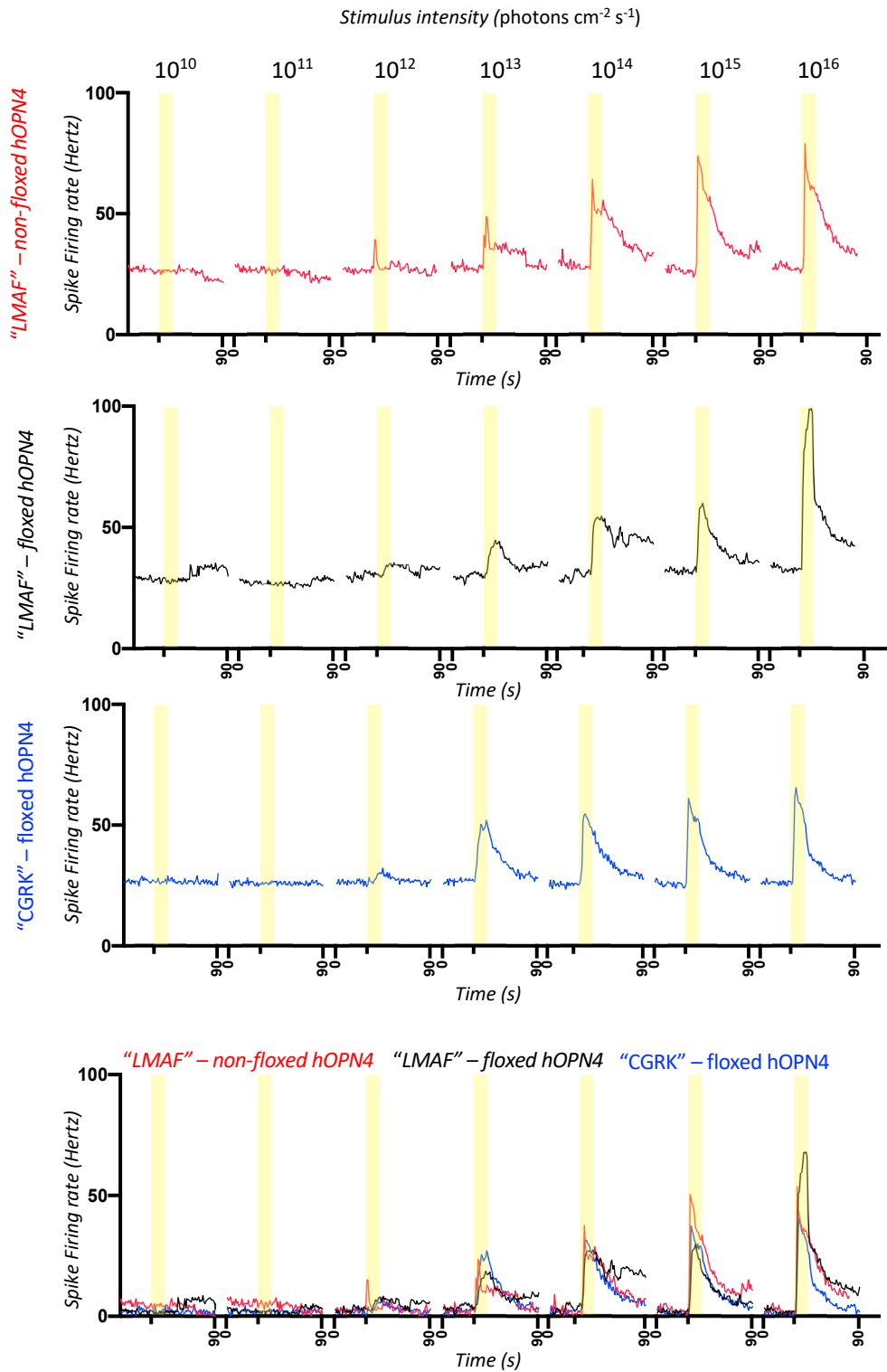


Figure 5-19 An averaged spike firing response obtained by taking the mean of responses from all responsive electrodes in each group in response to a 10 second light pulse after 30 seconds of recording at increasing intensities. Timing of light pulse is indicated by yellow bar. The inter-pulse interval was 10 minutes.

The bottom panel shows the same traces, each normalised to their lowest value and superimposed on a single graph to facilitate comparison

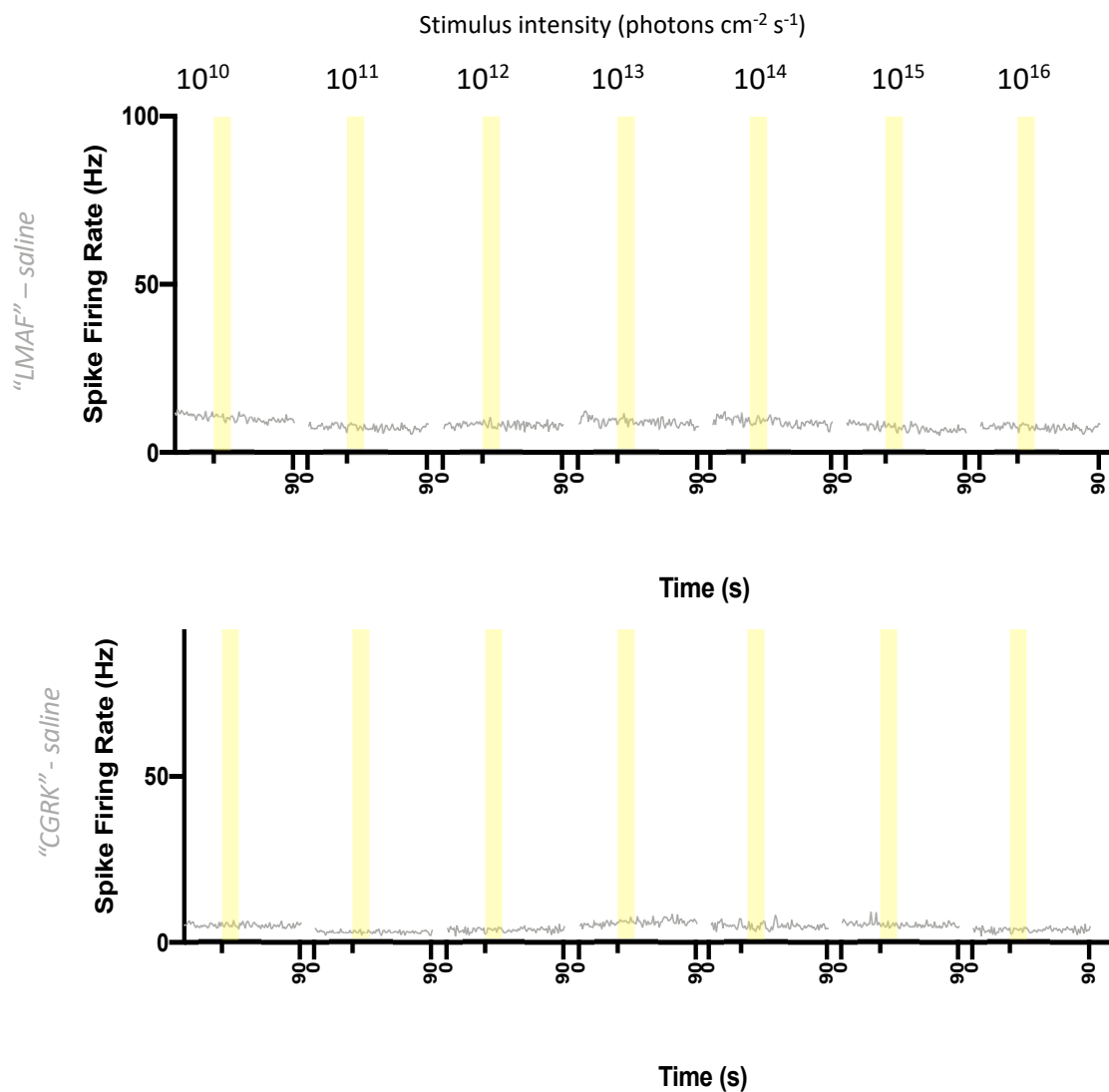


Figure 5-20 An averaged spike firing response obtained by taking the mean of responses from all electrodes showing spike firing in each group in response to a 10 second light pulse (480nm, $10^{14.1}$ photons $\text{cm}^{-2} \text{s}^{-1}$) after 30 seconds of recording – indicated by yellow bar. (for groups A to E respectively $n=60,59,27,25,59$).

Similarly, the maximum pre-stimulus firing rate did not vary with intensity ($F_{(6, 1561)} = 0.3051$; $p=0.9345$) but did with group ($F_{(2, 1561)} = 15.2$; $p<0.0001$), again with slightly lower rates in the “CGRK” group and no interaction between intensity and group ($F_{(12, 1561)} = 0.2136$; $p=0.9979$) (Figure 5-21b for post hoc test results). Together, this would suggest that while treated “CGRK” mice may have slightly lower baseline firing rates in the dark compared to other treatment groups, though this is not consistent (being present in only 5 out of 14 trials).

Mean firing rates after stimulus onset were also compared and did show significant difference: varying with both intensity ($F_{(6, 1561)} = 39.51$; $p < 0.0001$) and treatment group ($F_{(2, 1561)} = 11.61$; $p < 0.0001$), with an interaction between the two ($F_{(12, 1561)} = 1.991$; $p=0.0218$) (Figure 5-21c for post hoc test results). This demonstrates a relationship of higher firing rates after a brighter stimulus, and that this appears to be more pronounced in the *non-floxed* – “LMAF” group.

A similar relationship is seen in the maximum firing rate achieved after stimulation: varying with both intensity ($F_{(6, 1561)} = 67.32$; $p < 0.0001$) and treatment group ($F_{(2, 1561)} = 20.98$; $p < 0.0001$), with an interaction between the two ($F_{(12, 1561)} = 2.926$; $p=0.0005$) (see Figure 5-21d for post hoc test results). This again demonstrates a relationship of higher firing rates after brighter stimuli and also shows that this increase is more pronounced in “LMAF” mice than in “CGRK.”

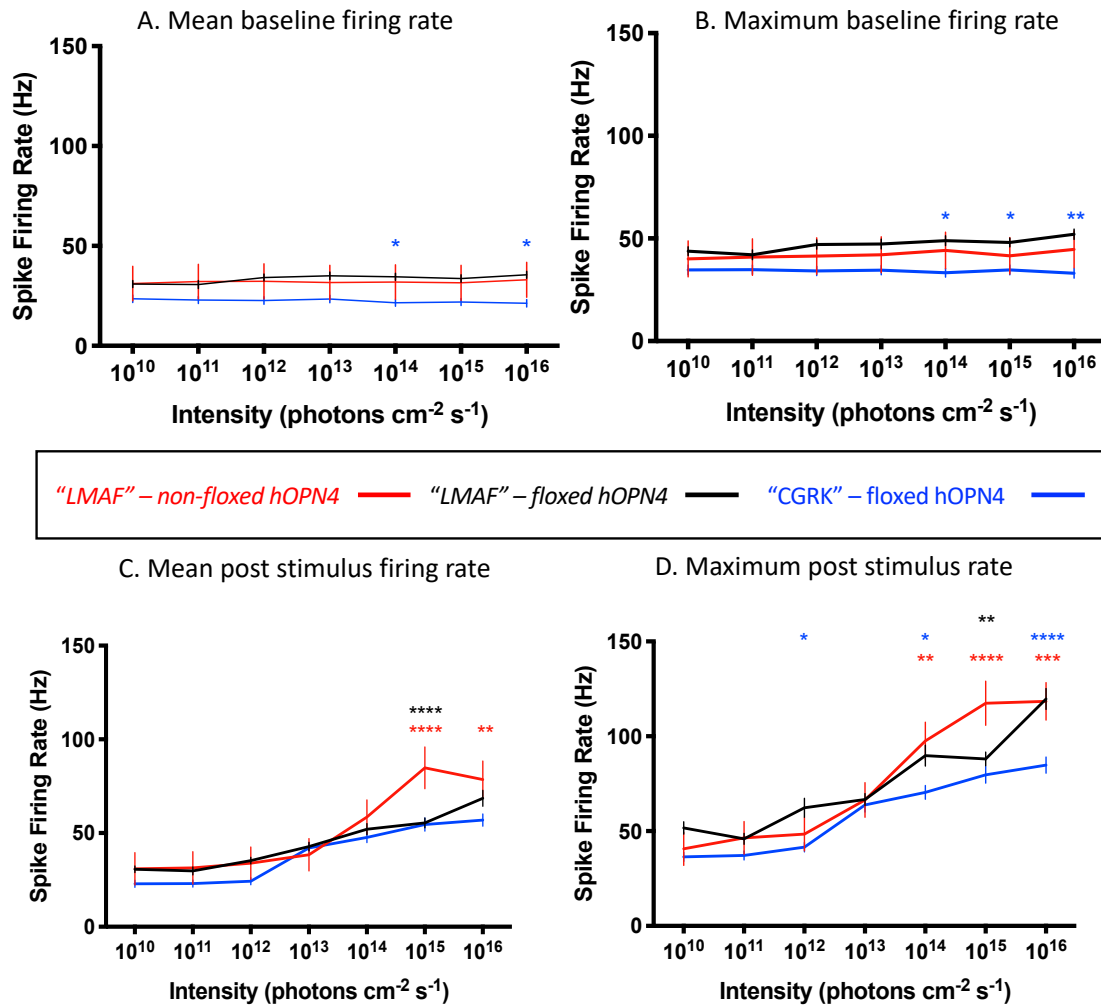


Figure 5-21 Base line and post stimulation firing rates by treatment group and intensity.

In all panels, a two way ANOVA was performed with Tukey's correction for multiple comparisons, asterix here refer to the p value of this post hoc test. Black stars refer to LMAF treated with floxed vs. non-floxed hOPN4 virus, blue stars to LMAF vs CGRK treated with floxed hOPN4, red stars to CGRK treated with floxed vs. non-floxed hOPN4 virus. Only significant differences are starred on the graph and included below. For ANOVA results, please see text.

"Floxed" - LMAF N=6 retinae; n=84 electrodes

"Floxed" – CGRK N=7 retinae; n=87 electrodes

"non-floxed" - LMAF N=5 retinae; n=55 electrodes

- Mean baseline firing rate – the mean rate of firing in the 10 seconds preceding stimulus onset, (1 second bins). Blue asterisk: 10^{14} $p=0.0409$, 10^{16} $p=0.0230$
- Maximum baseline firing rate – the highest rate of firing found in the 30second recording before stimulus onset (1 second bins). Blue astirix: 10^{14} $p=0.0181$, 10^{15} $p=0.0497$, 10^{16} $p=0.0026$.
- The mean rate in the 10 seconds after stimulus onset (1 second bins). Black astirix: 10^{15} $p<0.0001$. Red astirix: 10^{15} $p<0.0001$, 10^{16} $p=0.0051$
- The Maximum rate in the 10 seconds following stimulus onset (1 second bins). Black astirix: 10^{15} $p=0.001$ Blue astirix: 10^{12} $p=0.0111$, 10^{14} $p=0.0200$, 10^{16} $p<0.0001$. Red astirix: 10^{14} $p=0.00524$, 10^{15} $p<0.0001$, 10^{16} $p=0.0001$

Electrophysiological Light Response Kinetics

Similar indices of onset and offset response kinetics in response to stimuli of differing intensities were also obtained and compared using two-way ANOVAs with post hoc testing between groups with Tukey's correction for multiple comparisons.

Time from stimulus onset to response onset did reduce with increasing intensity ($F_{(6, 1621)} = 69.56; p < 0.0001$) and also demonstrated a variation with treatment group, however this only appear to be significant at one intensity (10^{14} photons $\text{cm}^{-2} \text{s}^{-1}$) ($F_{(2, 1621)} = 4.274; p = 0.0141$, Figure 5-22a for post hoc tests), there was no interaction between intensity and treatment group ($F_{(12, 1621)} = 1.434; p = 0.1433$). This suggests, as may be expected, that with brighter stimuli, responses are quicker to start, but this is not consistently different between treatment groups.

Similarly, the time to maximum response rate reduced significantly with increasing stimulus intensity ($F_{(6, 2117)} = 62.07; p < 0.0001$), it also varied with treatment group ($F_{(2, 2117)} = 15.35; p < 0.0001$, see Figure 5-22b for post hoc testing) and there was an interaction between the two ($F_{(12, 2117)} = 2.154; p = 0.0116$). This suggests again, brighter stimuli induce a shorter time to maximum response, but that this may be less pronounced in “LMAF” mice treated with *floxed* melanopsin.

Metrics of the offset of responses also showed differences. The overall duration of response increased with increasing stimulus intensity ($F_{(6, 1621)} = 153; p < 0.0001$) and varied with group ($F_{(2, 1621)} = 26.51; p < 0.0001$, Figure 5-22c for post hoc tests). There was also an interaction between intensity and treatment group ($F_{(12, 1621)} = 7.549; p < 0.0001$) suggesting that while response durations increased with brighter stimuli, this was less pronounced when melanopsin was targeted to ON-bipolar cells.

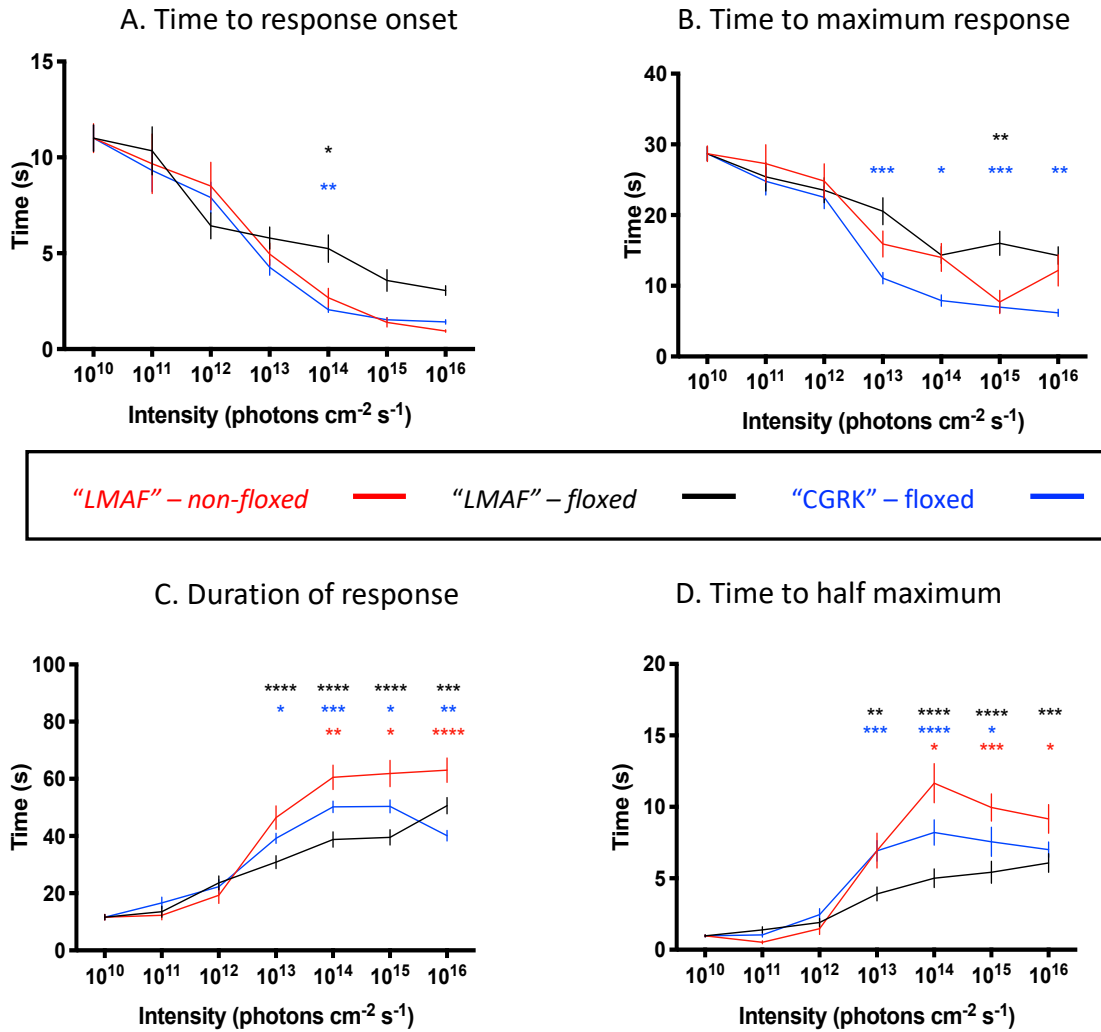


Figure 5-22 Onset (A&B) and offset (C&D) response kinetics by treatment group and intensity.

In all panels, a two-way ANOVA was performed with Tukey's correction for multiple comparisons, asterisk here refer to the p value of this post hoc test. Black stars refer to LMAF treated with floxed vs. non-floxed hOPN4 virus, blue stars to LMAF vs CGRK treated with floxed hOPN4, red stars to CGRK treated with floxed vs. non-floxed hOPN4 virus. Only significant differences are starred on the graph and included below. For ANOVA results, please see text.

"Floxed" - LMAF N=6 retinæ; n=84 electrodes

"Floxed" – CGRK N=7 retinæ; n=87 electrodes

"non-floxed" - LMAF N=5 retinæ; n=55 electrodes

- Time of response onset after beginning of recording, the 10second light stimulus is indicated by the horizontal yellow bar. Black Asterisk 10^{14} $p=0.0494$ Blue asterisk : 10^{14} $p=0.0026$
- Time after the beginning of the recording that the maximum firing rate was recorded. The 10 second light stimulus is indicated by the horizontal yellow bar. Black asterisk 10^{15} $p=0.0048$. Blue asterisk: 10^{13} $p=0.0159$, 10^{14} $p=0.0200$, 10^{15} $p=0.0001$ 10^{16} $p=0.0015$.
- The duration of the response. Maximum baseline firing rate – the highest rate of firing found in the 30 second recording before stimulus onset. Black asterisk 10^{13} $p<0.0001$, 10^{14} $p<0.0001$, 10^{15} $p<0.0001$ 10^{16} $p=0.0016$. Blue asterisk: 10^{13} $p=0.0253$, 10^{14} $p=0.007$, 10^{15} $p=0.016$, 10^{16} $p=0.0021$. Red asterisk: 10^{14} $p=0.0090$, 10^{15} $p=0.028$, 10^{16} $p<0.0001$
- The time from maximum response to half maximum response. ($t_{1/2}$). Black Asterisk 10^{13} $p=0.001$, 10^{14} $p<0.0001$, 10^{15} $p<0.0001$, 10^{16} $p=0.0009$. Blue asterisk: 10^{13} $p=0.0002$, 10^{14} $p<0.0001$, 10^{15} $p=0.0130$. Red asterisk: 10^{14} $p=0.0132$, 10^{15} $p=0.0002$, 10^{16} $p=0.0293$

Comparable findings were seen when comparing the half-life ($t_{1/2}$) of electrophysiological responses. $t_{1/2}$ increased with increasing stimulus intensity ($F_{(6, 2116)} = 108.6$; $p < 0.0001$) and varied with treatment group ($F_{(2, 2116)} = 30.68$; $p < 0.0001$, see Figure 5-22d for post hoc tests), there was an interaction between the two ($F_{(12, 2116)} = 7.156$; $p < 0.0001$). Again suggesting, that brighter stimuli led to longer $t_{1/2}$, but that this was not as pronounced when melanopsin was targeted to ON-bipolar cells specifically.

Electrophysiological Irradiance Response Curves

Using stimuli of varying intensity allowed a relationship between intensity and normalised response size to be modelled. A sigmoidal curve was fitted to the normalised response of each responsive electrode. Fits were made either with a constant Hill slope value of 1, or with the Hill slope value allowed to vary to achieve the best fit (Figure 5-23 & Figure 5-24). When Hill-slope values were allowed to vary, the mean slope for an electrode from the “LMAF” - *non-floxed*, “LMAF” - *floxed*, and “CGRK” - *floxed* groups was 0.8831 ± 0.06 , 0.5143 ± 0.03 and 0.9248 ± 0.07 (Figure 5-23, see histograms). These values were not significantly different between the “LMAF” - *non-floxed* and “CGRK” - *floxed* groups, however the “LMAF” - *floxed* was significantly shallower (ordinary one-way ANOVA; $F_{(2,109)} = 6.853$; $p = 0.0016$; Tukey’s correction $p = 0.0087$, 0.0013). Similarly, only in the “LMAF” - *floxed*, was the Hill slope value significantly different from 1 when it was allowed to vary (One sample t-test; $p < 0.0001$). Given this divergence, for consistency, a variable Hill slope approach was used in further comparisons, but fits using a fixed Hill slope value of 1 are provided for information in Figure 5-24.

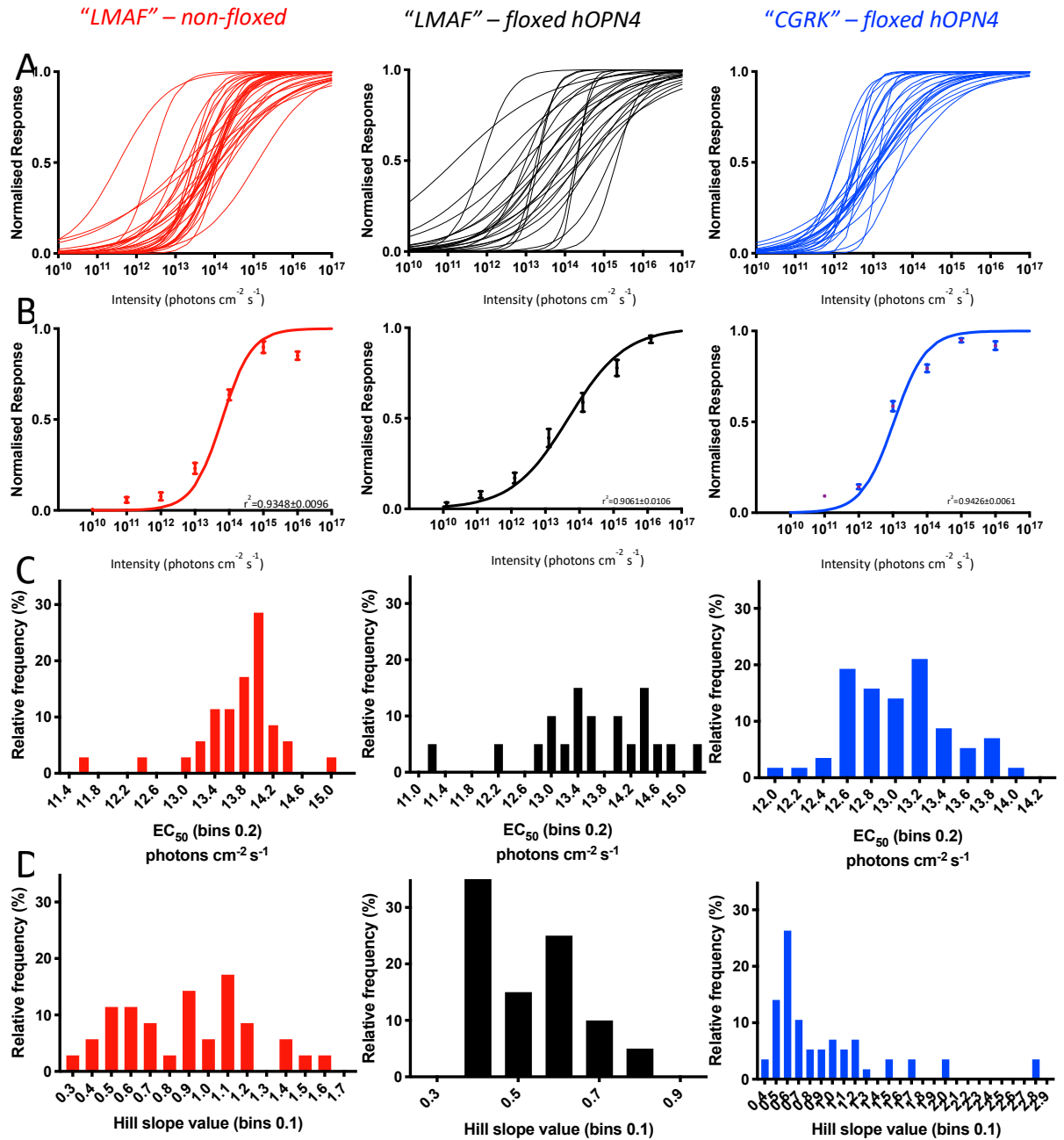


Figure 5-23 Curve fitting of Irradiance response curves (IRCs). IRC curves can be fitted either using a constant Hill slope =1, or by allowing the Hill slope to vary. In this figure, the Hill slope is allowed to vary to achieve the best fit.

A – All electrodes in each group, fitted using a variable Hill slope. Only electrodes with a good fit ($r^2 > 0.8$) are plotted.

B – The mean EC_{50} from all individual electrodes was used to plot a mean IRC curve; the mean $\pm$ SEM of r^2 values for the individual electrodes is shown. Also plotted on the graph (as round points) are the mean $\pm$ SEM of the normalised responses at each intensity tested. Note better fit in "LMAF" – floxed hOPN4 group.

"LMAF" – non-floxed hOPN4; $EC_{50} = 13.74 \pm 0.1081$; Hill slope = 0.8831 ± 0.06 , $n = 35$ of 55

"LMAF" – floxed hOPN4; $EC_{50} = 13.64 \pm 0.2100$; Hill slope 0.5143 ± 0.03 (mean $\pm$ SEM); $n = 20$ of 84 (electrodes)

"CGRK" – floxed hOPN4; $EC_{50} = 13.03 \pm 0.0556$; Hill slope 0.9248 ± 0.07 $n = 57$ of 87

C – Histograms demonstrating the distribution of individual electrodes EC_{50} values in each group.

D. Histograms demonstrating the distribution of individual electrode Hill slope values in each group

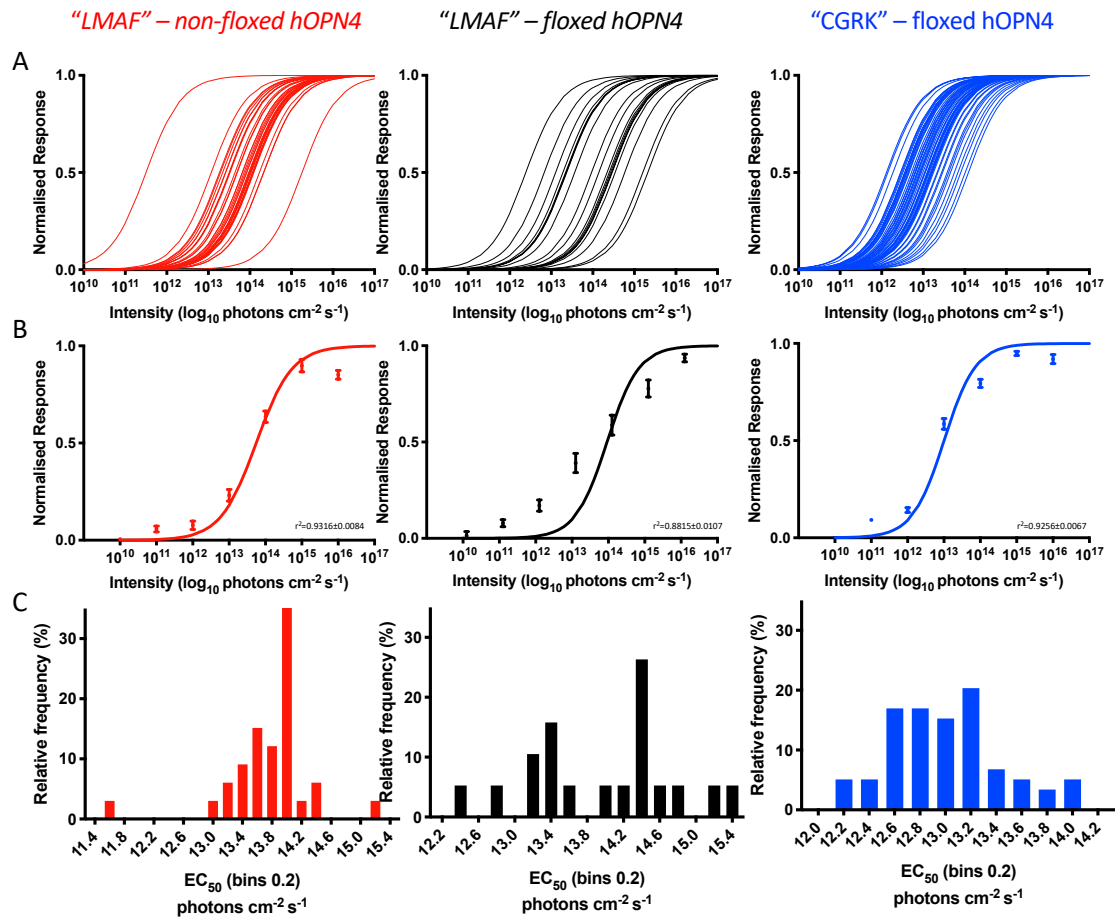


Figure 5-24 Curve fitting of Irradiance response curves (IRCs)

IRC curves can be fitted either using a constant Hill slope =1, or by allowing the Hill slope to vary. In this figure, the Hill slope is set at 1.

A – All electrodes in that group, fitted using a Hill slope = 1. Only electrodes with a good fit ($r^2 > 0.8$) are plotted.

B – The mean EC_{50} from all individual electrodes was used to plot a mean IRC curve fit; the mean r^2 is shown $\pm$ SEM. Also plotted on the graph are the mean $\pm$ SEM of the normalised responses at each intensity tested. Note poor fit in "LMAF" – floxed hOPN4 group.

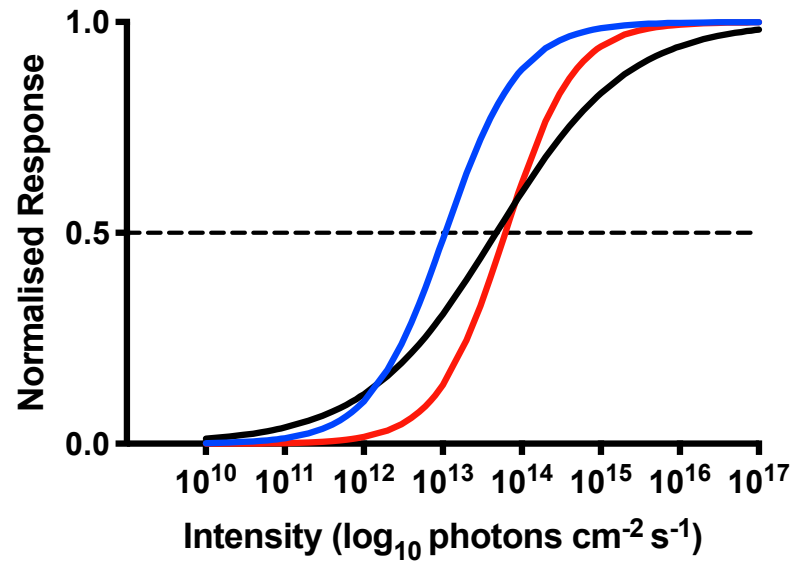
LMAF" – non-floxed hOPN4; $EC_{50} = 13.79 \pm 0.1001$; $n = 33$ of 55

"LMAF" – floxed hOPN4; $EC_{50} = 13.96 \pm 0.1846$ (mean $\pm$ SEM); $n = 19$ of 84 (responsive electrodes)

"CGRK" – floxed hOPN4; $EC_{50} = 13.00 \pm 0.05792$; $n = 59$ of 87

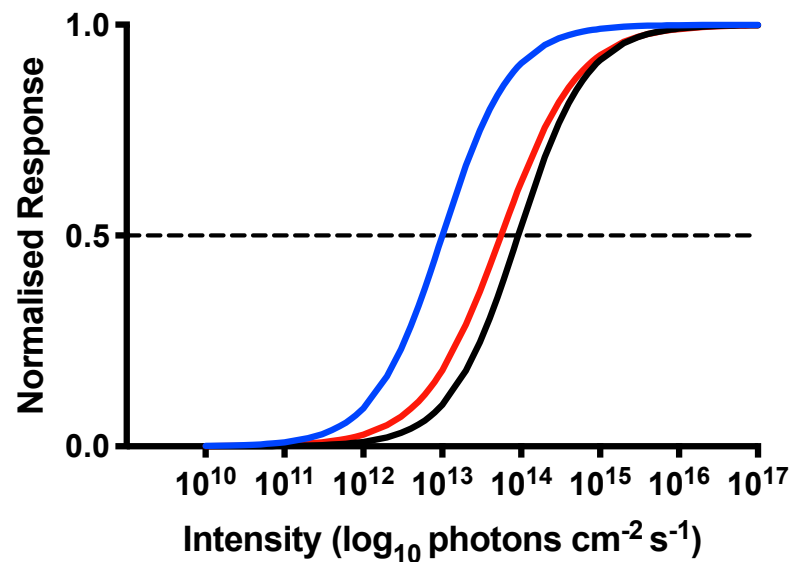
C – As in row A, all fitted electrodes in each group are plotted individually, however here the Hill-slope value is allowed to vary to achieve the best fit. Only electrodes with a good fit ($r^2 > 0.8$) are plotted.

C. Histograms demonstrating the distribution of individual electrodes EC_{50} values in each group.



"LMAF" – non-floxed hOPN4 — "LMAF" – floxed hOPN4 — "CGRK" – floxed hOPN4 —

Figure 5-25 All three curves from Figure 5-23. Drawn using the mean EC_{50} & Hill slope values from all fitted electrodes in each group.



"LMAF" – non-floxed hOPN4 — "LMAF" – floxed hOPN4 — "CGRK" – floxed hOPN4 —

Figure 5-26 All three curves from Figure 5-24 Drawn using the mean EC_{50} & Hill slope values from all fitted electrodes in each group.

The number of electrodes with a dose response relationship fitting well to the sigmoidal relationship ($r^2 > 0.8$) was – 35 out of 55 (64%) in the “LMAF” - *non-floxed* group,, 20 of the 84 (24%) in the “LMAF” – *floxed group* and 57 of 87 (66%) in the “CGRK” - *floxed*. The mean of the EC_{50} (excitatory concentration for 50% response) of best fit of all the electrodes in each treatment group was $EC_{50} = 13.74 \pm 0.11$, 13.64 ± 0.21 and $13.03 \pm 0.06 \log_{10} \text{ photons cm}^{-2} \text{ s}^{-1}$ respectively ((Figure 5-23 for histograms)). This was taken and used to produce a mean irradiance response curve (IRC) for each group (Figure 5-25 & Figure 5-26)

These mean values could also be used to compare groups, with an ordinary one way ANOVA showing a significant difference between the three treatment groups ($F_{(2, 109)} = 18.31$; $p < 0.0001$) (Figure 5-23). Post hoc testing using Tukey’s correction for multiple comparisons indicated no difference in sensitivity response between “LMAF” mice injected with *floxed* or *non-floxed* hOPN4 ($p = 0.8053$). In contrast to this, “CGRK” mice injected with *floxed* hOPN4 had significantly more sensitive responses than “LMAF” mice either injected with *floxed* ($p = 0.0004$) or *non-floxed* ($p = < 0.0001$) hOPN4. In all, this would suggest little advantage, in terms of sensitivity, in targeting bipolar cells over a non-specific delivery of melanopsin. In contrast, specific targeting of retinal ganglion cells would appear to provide more sensitive individual cell responses.

Behavioural Responses

Pupillometry

With such demonstration of individual cell responses to light at the level of retinal electrophysiology and with previous studies(159, 160, 291) suggesting that such responses can lead to reconstitution of whole animal responses – assessing behavioural responses to light in these animals is important. Is the light signal, transduced in the retina, transmitted to the brain in a form that can be used by the animal to alter its behaviour? A protocol of three behavioural assays was constructed making use of pupillometry(205), the optokinetic response assay(200) and a novel object visual context recognition task(179) in order to assess behaviour at both subcortical and cortical levels (see Chapter 2).

In response to a 10 second pulse of light (480nm, 10^{15} photons $\text{cm}^{-2}\text{s}^{-1}$) a wildtype mouse responds with a pupillary constriction (Figure 5-27). However, given their lack of functional rods, cones or melanopsin(205) saline injected control “*LMAF*” and “*CGRK*” mice do not have a detectable pupillary response to the same stimulus (Figure 5-27). When treatment groups were compared to such controls the only significant difference (at the highest intensity 10^{15} photons $\text{cm}^{-2}\text{s}^{-1}$) was between the two *Opn4*^{+/+} (wildtype control) groups and the treated groups (KW statistic = 32.83, df. = 45, $p < 0.0001$, Dunn’s correction for multiple comparison, Figure 5-28). Comparison of responses to varying intensities of stimulus within treatment groups showed no significant difference, except in the “*LMAF*” injected with *floxed* hOPN4 (Figure 5-29)

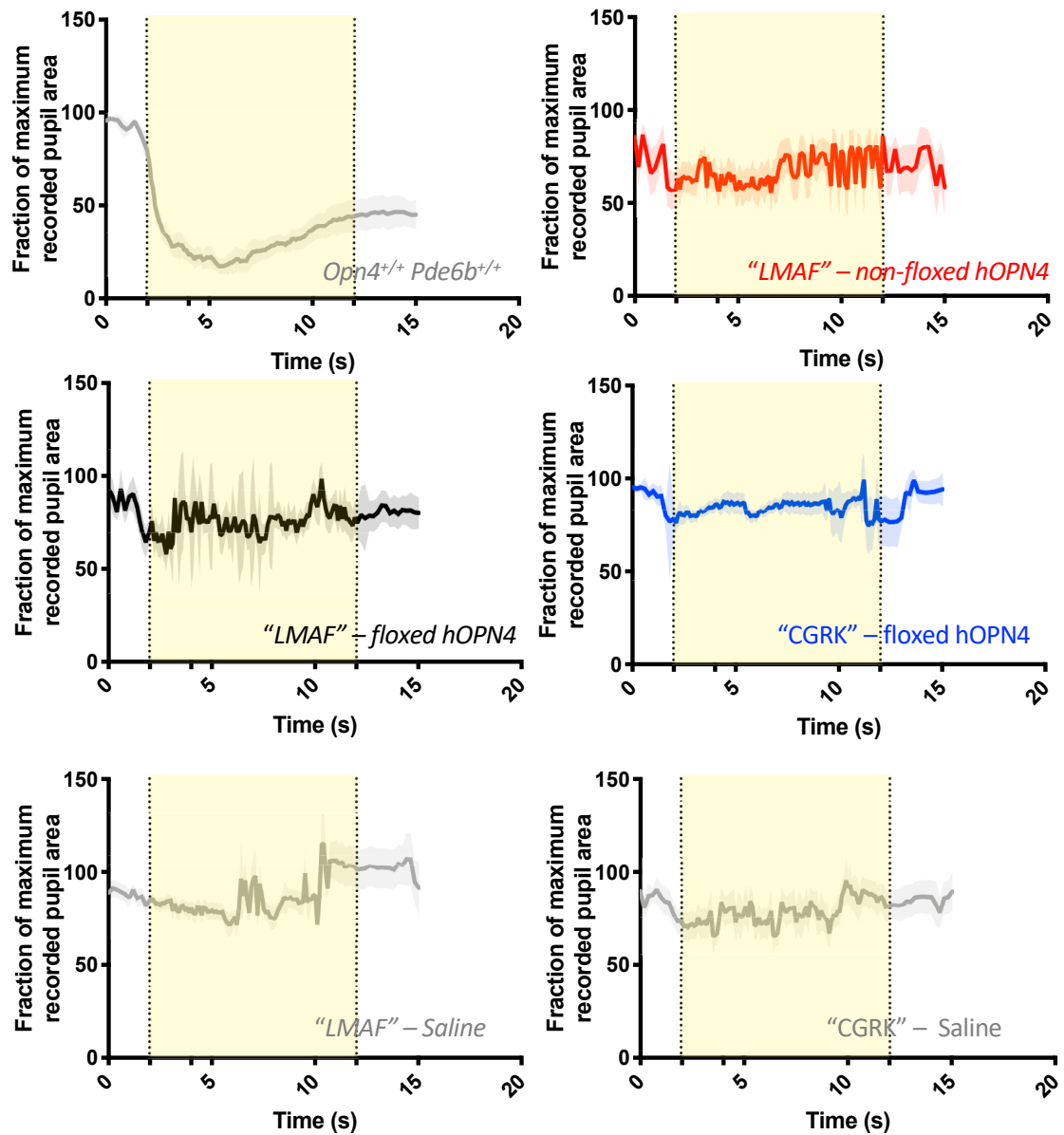


Figure 5-27 Mean pupillometry traces averaged over all trials and all animals in each group. mean $\pm$ SEM

Yellow bar represents onset of a 480nm, 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$ stimulus of 2 seconds duration.

The panel marked (*Opn4^{+/+} Pde6b^{+/+}*) represents un-injected wildtype controls.

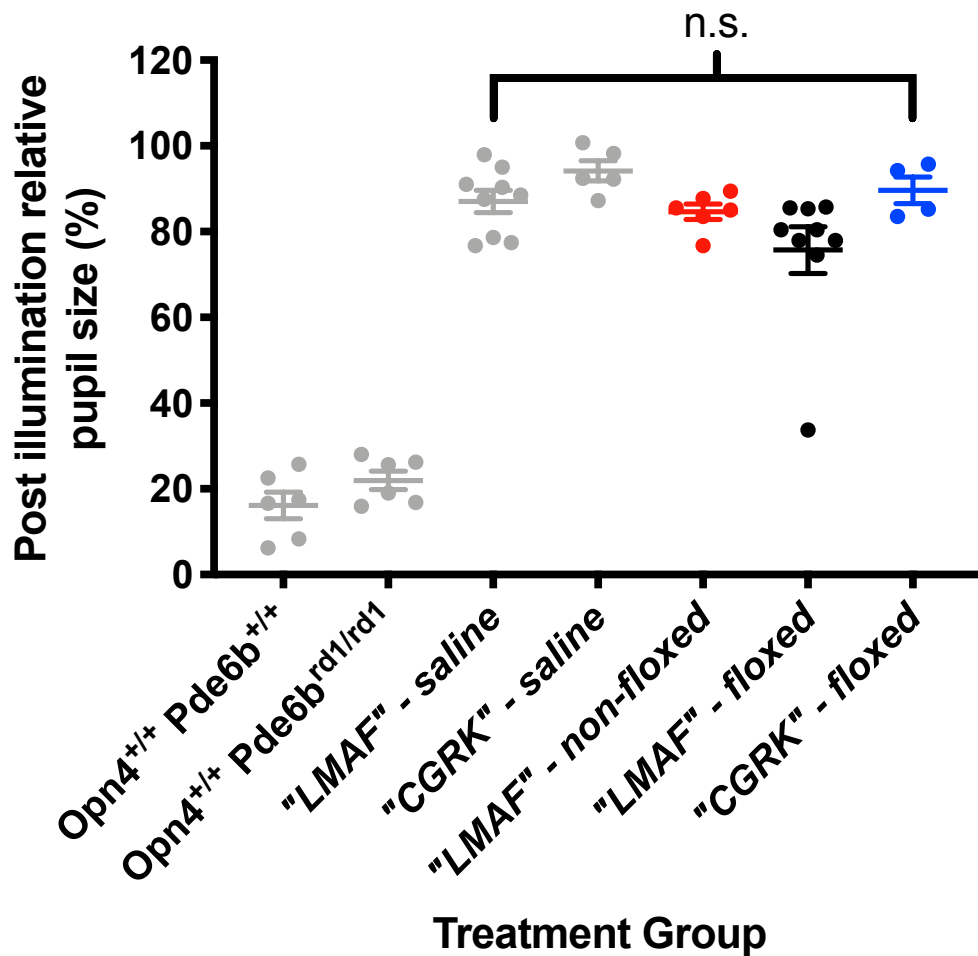


Figure 5-28 Pupillary responses at 2 seconds post illumination with a 480nm stimulus of 10^{15} photons $\text{cm}^{-2}\text{s}^{-1}$.

The post illumination relative pupil size is defined as the size of the pupil two seconds after the onset of illumination as a proportion of the largest pupil size recorded in the two seconds before stimulus onset.

One sample t-test (or Wilcoxon rank test for "LMAF" – floxed where distribution was not normal) demonstrated a significant difference from 100 in all groups apart from the CGRK groups ($p = <0.0001$, <0.0001 , 0.001 , $n.s.$, 0.0004 , 0.0039 , $n.s.$).

When comparing all groups, Kruskal-Wallis test showed a significant difference KW statistic = 32.83, $df. = 45$, $p < 0.0001$, Dunn's correction for multiple comparisons however that this difference was confined to between the two Opn4^{+/+} control groups and the injected groups. There were no significant differences between any on the injected groups.

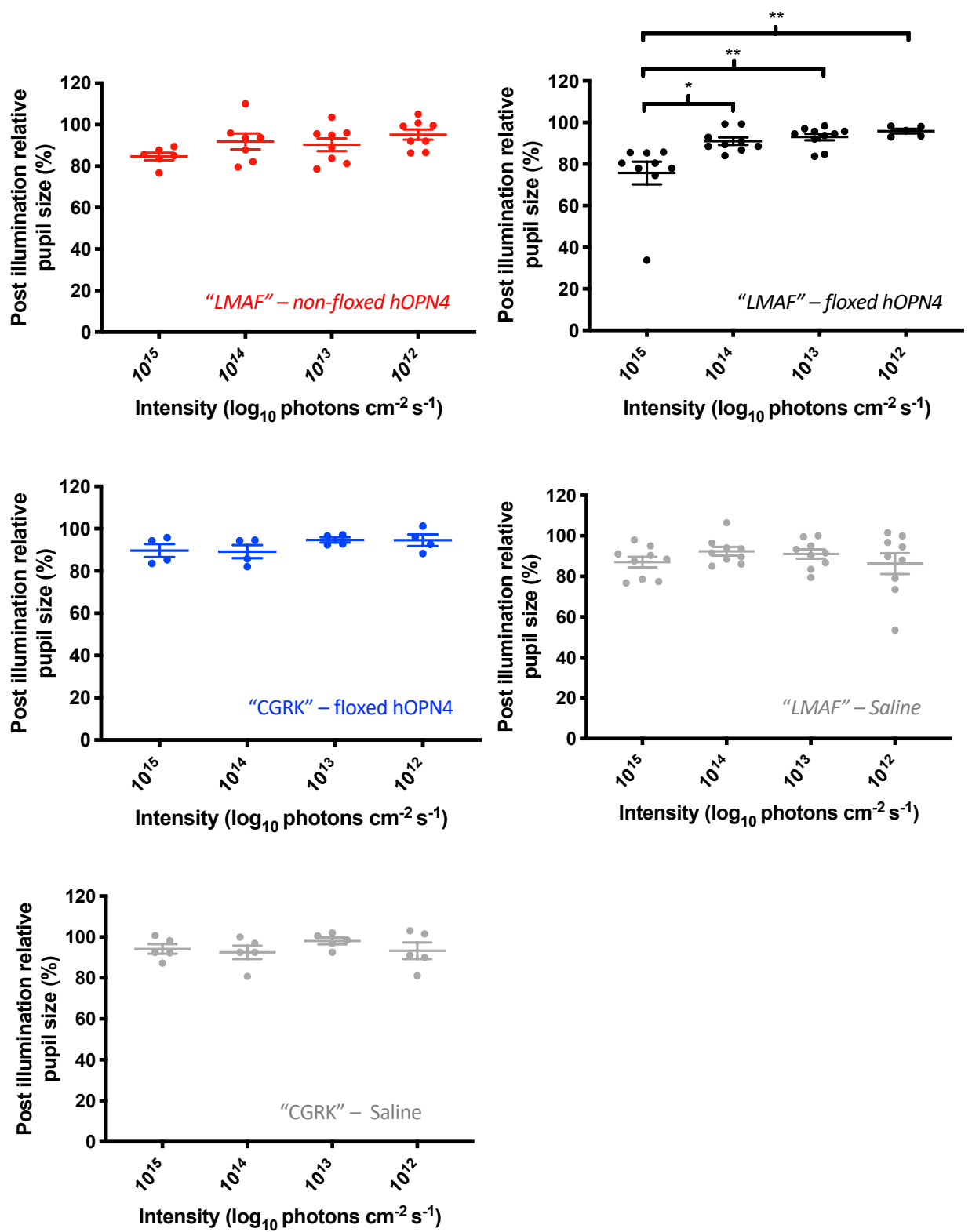


Figure 5-29 Pupillary responses at 2 seconds post illumination with a 480nm stimulus of varying intensity. See Chapter 2 for methods.

The distribution of the LMAF – floxed hOPN4 group at 10^{15} was not normal (Kolmogorov-Smirnov $p=0.0014$, Shapiro-Wilk $p=0.0001$); all other intensities in all groups passed these normality tests.

One sample t-test (or Wilcoxon rank test for “LMAF” – floxed) demonstrated a significant difference from 100% at all intensities, in groups, apart from the two CGRK groups ($p= <0.0001$, <0.0001 , 0.001 , $n.s.$; 0.0004 , 0.0039 , $n.s.$, $n.s.$ respectively)

One way ANOVA (or Kruskal- Wallis test for “LMAF”) showed no significant differences between any intensities in four of the groups:

A. $F(3, 32) = 0.8183$; $p = 0.4934$

B. $F(3, 16) = 0.678$; $p = 0.5776$

C. $F(3, 25) = 2.071$; $p = 0.1296$,

E. $F(3, 12) = 1.272$; $p = 0.3282$

D. LMAF – floxed hOPN4 the 10^{15} intensity stimulus induced a significantly larger constriction than any of the other intensities (KW statistic = 17.65; $df. 33$; $p=0.0005$), Dunn’s correction for multiple comparisons ($p=0.0351$, 0.0034 , 0.0019).

Optokinetic Response

As with pupillometry, both wild type and *Pde6b*^{rd1/rd1} mice with native melanopsin were used as positive controls to validate the assay. A two-way ANOVA demonstrated a significantly larger response with lower spatial frequencies ($F_{(2, 381)} = 77.73$; $p < 0.0001$), a difference between treatment groups ($F_{(6, 381)} = 114.3$; $p < 0.0001$) and an interaction between the two ($F_{(12, 381)} = 24.95$; $p < 0.0001$). However, post hoc testing (Tukey's correction for multiple comparisons) revealed this difference to be accounted for by differences between wild type controls and all other groups (all $p < 0.0001$) at 0.2 and 0.4Hz; there were no differences between treatment and saline control groups, there were no significant differences at all between groups at the 0.6Hz frequency (Figure 5-30 & Figure 5-31). In summary, this suggests that while the optokinetic assay is valid, but was not able to discern a reconstitution of the response in any treated mouse at any spatial frequency.

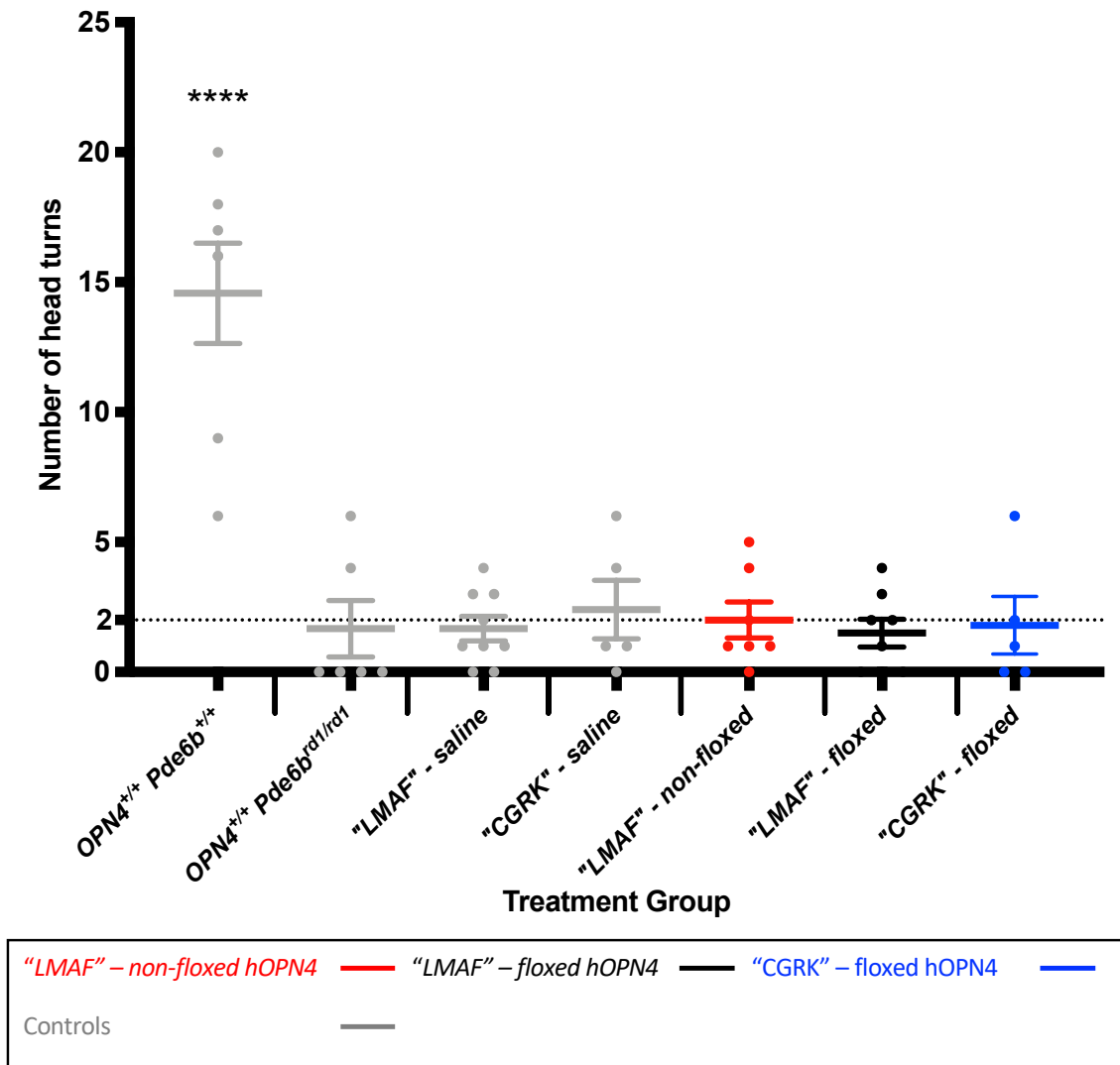


Figure 5-30 . The optokinetic tracking assay at 0.2Hz spatial frequency. Control mice (wild type and *Opn4*^{-/-} are provided along with experimental mice for comparison)

For each group a one sample t-test was used to test for significant difference from a rate of 2 turns min⁻¹, this was not significant in any treatment group. It was however significant in wild type mice at the special frequency of 0.2 (p=0.0006)

Stars on graph represent post hoc testing (Tukey's correction for multiple comparison) after two-way ANOVA (see text); P<0.0001 when compared to all other groups.

Novel object, visual context recognition task

This task was recently described as a useful metric of vision at the cortical level in the mouse(179) (Chapter 2). Again, an attempt was made to validate the assay in its current set up in our laboratory using wild type and *Pde6b^{rd1/rd1}* mice who retained their own native melanopsin. However, as can be seen in Figure 5-32, the test was unable to identify wild type (sighted) mice. A one sample t-test for each group revealed that no group (even wild type controls) had a preference ratio significantly above 0.0 (a positive value in this assay indicating object preference and therefore an ability to use visually encoded cues). One way ANOVA demonstrated no significant difference between treatment group mean ratios ($F_{(6,39)} = 0.8358, p=0.5498$). In summary, in the set up used here, this method is not a reliable assay of visual function as it could not discriminate positive (wild type) from negative (saline injected “*LMAF*” or “*CGRK*”) controls.

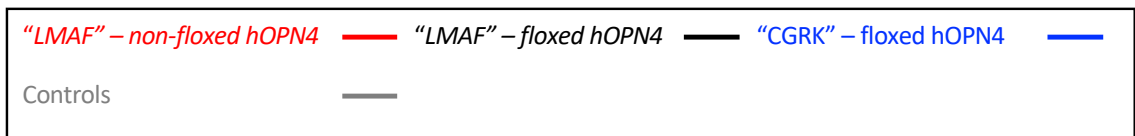
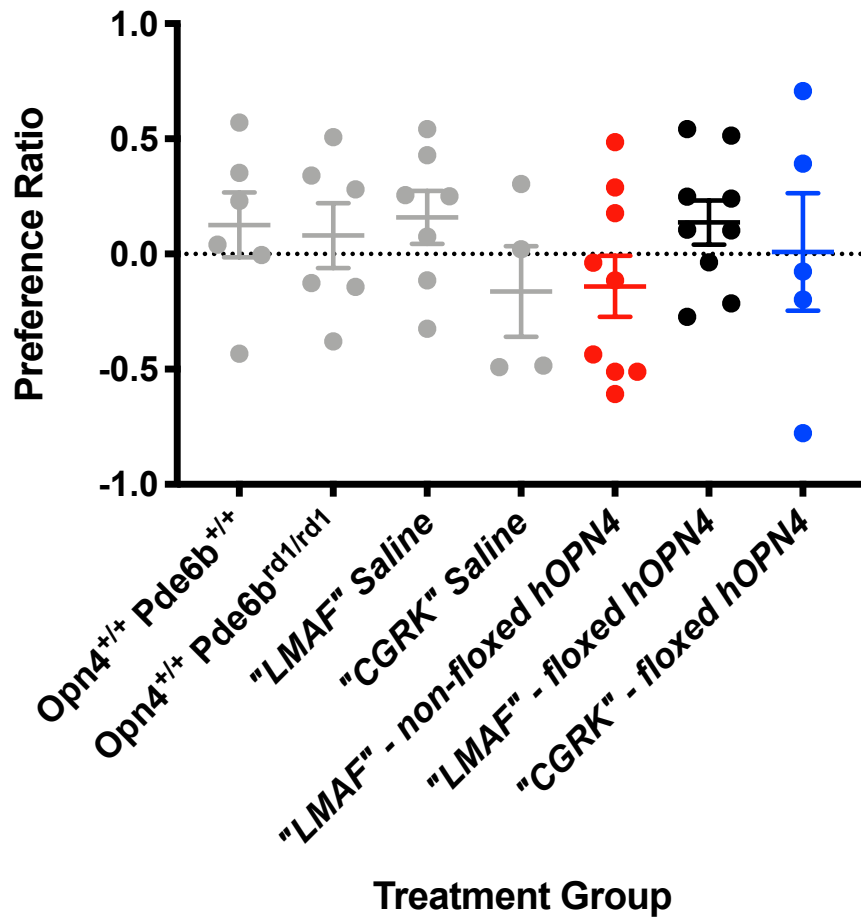


Figure 5-32 The novel object, visual context recognition task. Control mice (uninjected wild type and Opn4^{+/+} Pde6b^{rd1/rd1} controls are provided along with experimental mice for comparison).

A positive ratio indicates preference for the novel object. A negative ratio indicates a preference for the familiar object. A ratio of 0.0 indicates equipoise.

For each group a one sample t-test was used to test for significant difference from a ratio of 0.0, this was not significant in any group. A one-way ANOVA was used compare means between groups and no significant difference was found. $F_{(6,39)} = 0.8358$, $p=0.5498$.

Circadian entrainment – a pilot experiment

With a failure of AAV delivery of melanopsin in these mice to restore detectable

responses in the above assays, consideration was given to determining if the observed retinal electrophysiological results could drive another important process for which light is important: circadian entrainment. A pilot experiment was therefore set up with 12 “*LMAF*” mice being singly housed in cages under a 12:12h light:dark cycle with monitored running wheels. Wheel revolutions were taken as a surrogate marker of mouse activity.

These mice were monitored for period of two weeks, and as can be seen in Figure 5-33A & Figure 5-34A, their activity ran with a period 23.22 ± 0.06 hours in the *floxed – hOPN4* group and 23.37 ± 0.075 hours in the *non-floxed* group, these were not significantly different (two tailed t-test $p = 0.6662$). The period was defined as the time from the start of activity on one day, to the start of activity of the next. As these mice have no functional rod, cone or melanopsin systems, this behaviour was as expected, given previous reports(205).

After two weeks, 6 of the mice were injected intravitreally with the *non-floxed* hOPN4 AAV and another 6 with the *floxed-hOPN4* construct. Mice were returned to their cages, allowed to recover for 5 days before continuing to be monitored for a further 10 weeks (90 days in total). Mean periods in the post injection period compared to the pre-injection period (Figure 5-33B & Figure 5-34B) were only significantly different for one mouse (No. 20 - two tailed t-test, $p < 0.0001$) showing that, apart from in that case, there was no sustained change in period following injections (mouse No. 6 was excluded, as no retinal hOPN4 staining was seen on immunohistochemistry). Consideration of the individual actograms indicates one

mouse in the non-floxed group (No.2) entrains activity for approximately 14 days in the post injection period: one explanation for this, given the time course could be the restoration of an induced light signal from exogenous melanopsin to which the mouse can entrain, which is later lost indicating a need to proceed to a full experimental evaluation of this (see discussion).

Finally, mice were culled for retinal flat mount immunohistochemistry, hOPN4 staining coverage of the retina was scored Figure 5-33C & Figure 5-34C. While variable, all retina apart from those from mouse No. 6 had staining for melanopsin protein.

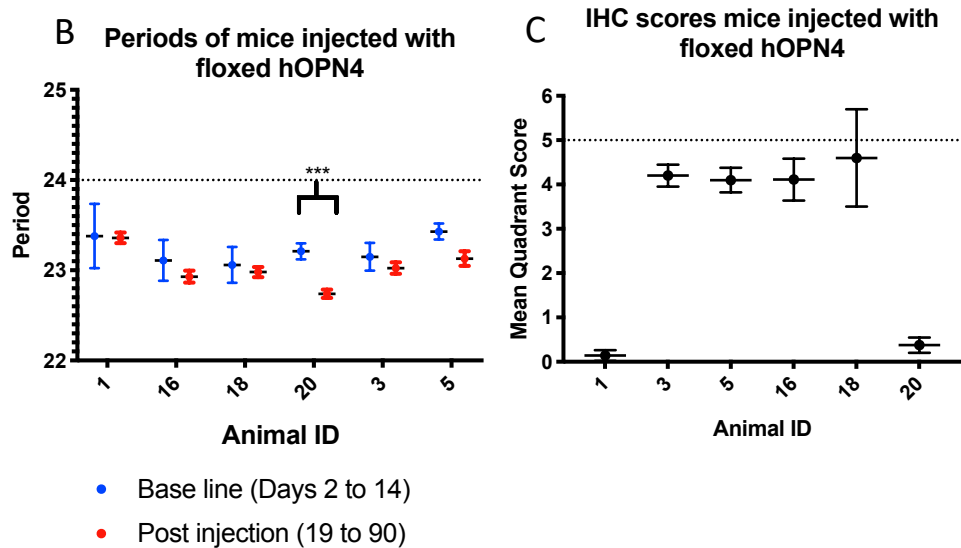
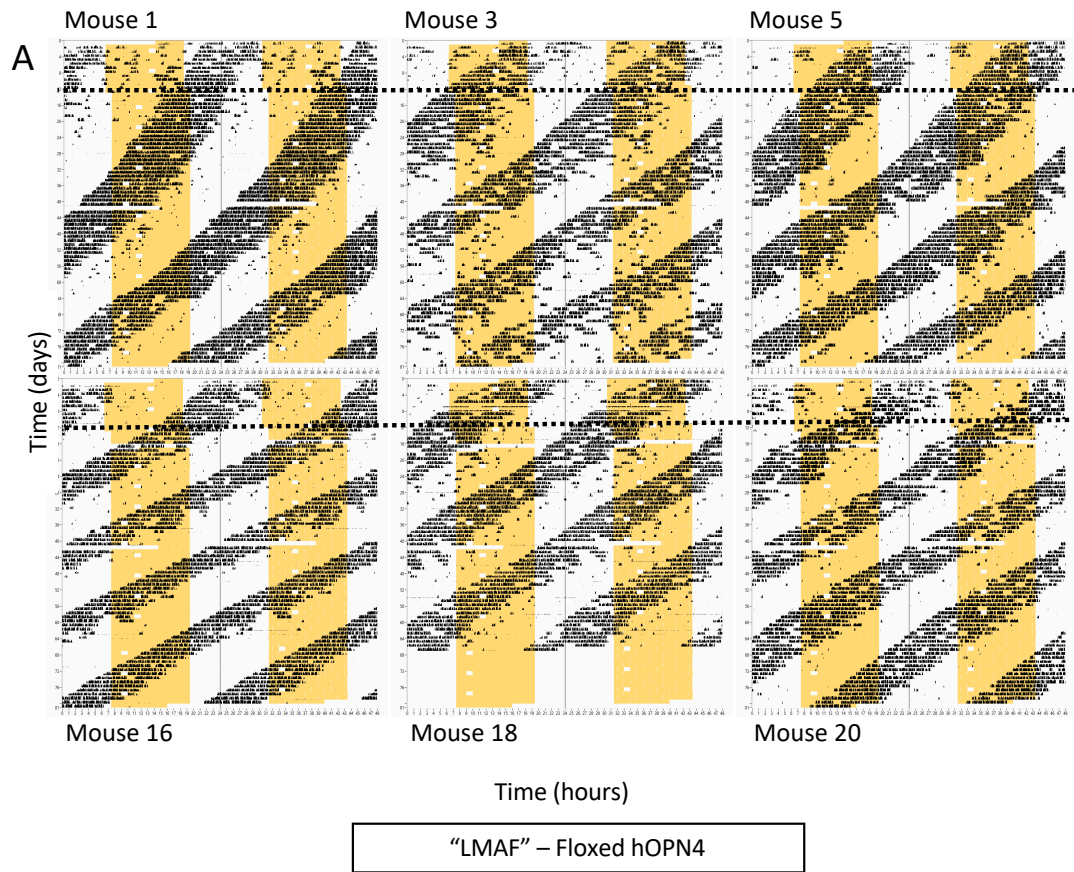


Figure 5-33 A. Actograms (double plotted) of mouse running wheel activity. "LMAF" – floxed hOPN4 group. The dashed line indicates the timing of the injection. The mouse number refers to the order of the cage in which it is housed (these mice were interspersed with mice from the "LMAF" – non-floxed group).

B. The mean periods for each mouse in the pre-injection (days 2 to 14) and post injection (days 19 to 90) periods.

C. Mean IHC scoring values of hOPN4 coverage in each of the mice. A mean of 5 would indicate high staining in all four quadrants, 0 no staining in any quadrant (see Chapter 2)

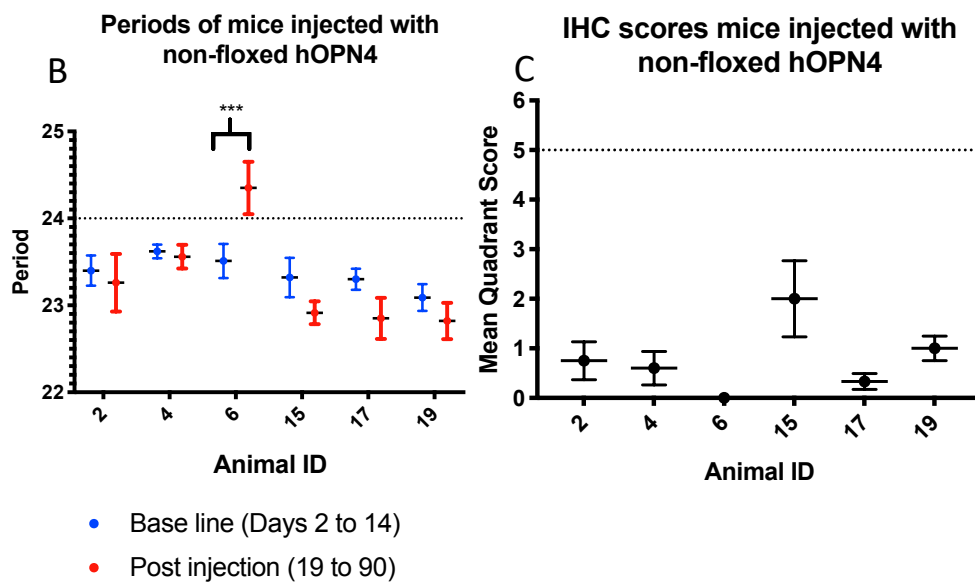
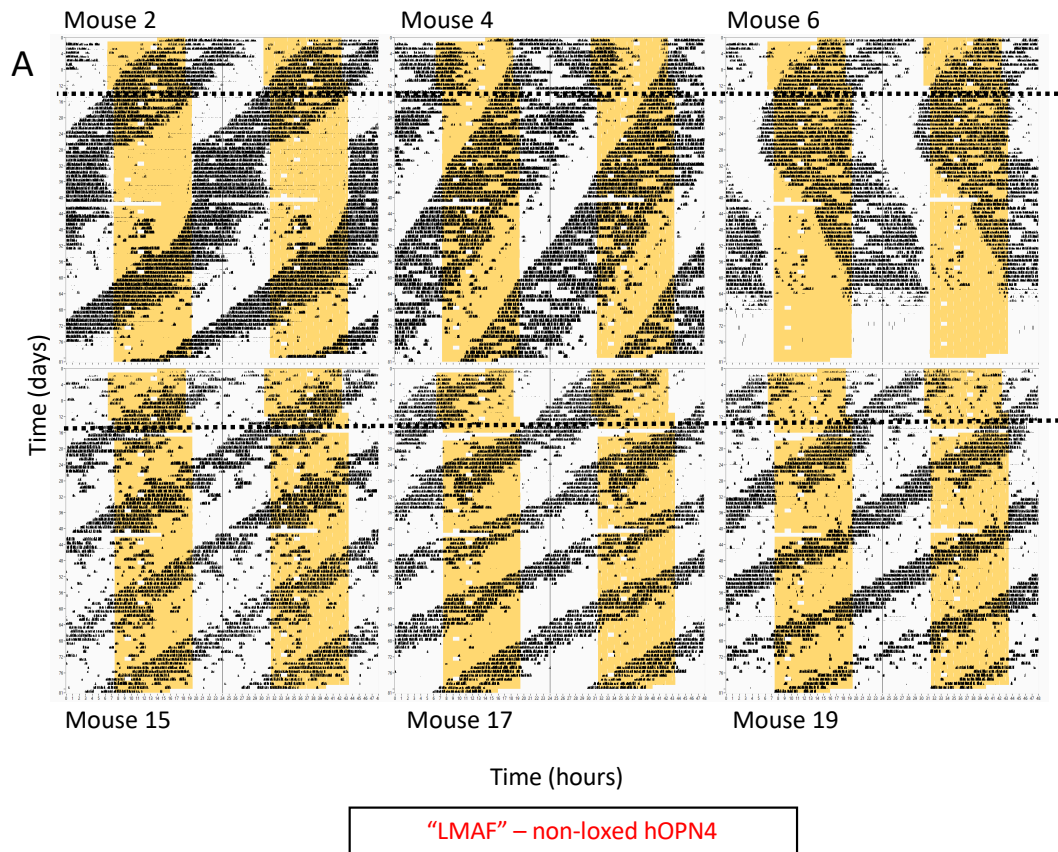


Figure 5-34 A. Actograms (double plotted) of mouse running wheel activity. "LMAF" non-floxed hOPN4 group. The dashed line indicates the timing of the injection. The mouse number refers to the order of the cage in which it is housed (these mice were interspersed with mice from the "LMAF" – floxed hOPN4 group. Note mouse number 2 appearing to entrain for a period of approximately 14 days.

B. The mean periods for each mouse in the pre-injection (days 2 to 14) and post injection (days 19 to 90) periods.

C. Mean IHC scoring values of hOPN4 coverage in each of the mice. A mean of 5 would indicate high staining in all four quadrants, 0 no staining in any quadrant (see Chapter 2)

Discussion

In this Chapter, AAVs containing high efficiency promoters driving either *floxed* or *non-floxed* hOPN4 are validated in the “LMAF” and “CGRK” models: both were able to express protein appropriately and drive spike firing in retinal ganglion cells. This allowed, for the first time, a direct comparison of electrophysiological retina responses between melanopsin targeted specifically to subsets of retinal cells. In addition, this was the first optogenetic restoration of light responses in a degenerate retina without native melanopsin responses. However, the reconstituted retinal light responses seen were unable to drive detectable light dependant behaviour in three tests.

AAV delivery

In planning these experiments, I hypothesised that the retinal processing gained by targeting ON-bipolar cells with AAV delivered human melanopsin would lead to improved restoration of electrophysiological and behavioural light responses compared to non-specific and RGC targeting.

In assessing the success of exogenous melanopsin expression following injections, both the quantity of the transfection and the specificity must be considered. Intravitreal delivery was used in this study given its reduced surgical complication rate and proven efficacy for non-specific delivery of melanopsin to RGCs in rodent and non-human primate models(160, 291). However, current clinical trials of gene therapy have relied on subretinal injections (also successful in delivering melanopsin to inner and outer retinal cells in a degenerate retina(159)), therefore, care must be taken when extrapolating results towards potential translation. A theoretical advantage of intravitreal delivery is the ability to transduce potentially the whole retina evenly, without being restricted by the confines of a subretinal bleb.

While good transduction was seen across large areas of the retinae in this study, variability in transduction was seen from area to area in most retina and quantified for each retina using a manual scoring system (Chapter 2).

Despite this variability, in areas of good transduction, the *non-floxed* (CBA.hOPN4) construct was able to access the whole depth of the retina and reach cells in the outer inner nuclear layer, just as it had been shown to do following subretinal injection(159, 175) while also reaching cells in the ganglion cell layer, which it was not demonstrated to do with subretinal injection. One explanation for this, is that areas of poorer transduction represent areas where viral access to the retina is impeded by variations in the ILM and local vitreous increasing sequestration of virus by glycosaminoglycans(149). Therefore, the addition of glycosidic enzymes to intravitreal injections could be considered in future to reduce observed variability in retinal coverage(133).

The specificity of viral delivery of melanopsin is particularly important in this study. Cre models were chosen to combine the good expression levels afforded by high efficiency non-specific promoters (e.g.CBA or EF1 α) with the cell population specific expression of specific promoters - such as L7 and Grk4 – too large to fit inside the AAV envelope (4kbp). There are inherent limitations in these models to be borne in mind: for example, the “*CGRK*” model expresses Cre in only a small subset of direction sensitive retinal ganglion cells rather than *all* RGCs (Chapter 3).

Similarly, the L7.Cre model is known to express Cre in a small subset of retinal ganglion cells (c.1,000 per retina(221)) in addition to ON-bipolar cells: like most promoters it is not absolutely specific for a single cell type(213, 221) and has off target expression (see Chapter 7). Reflecting previous descriptions(221), hOPN4 staining is

seen in a subset of retinal ganglion cells in this study too. It cannot therefore be excluded that a contribution of the light responses seen when floxed hOPN4 is delivered to this model originate in this RGC subset as well as ON-bipolar cells. A comparison to an alternative Cre line (such as the grm6.Cre line(256)) with *different* off-target expression would be informative in placing the responses seen here in context.

Grm6 based promoter constructs (without the use of the cre.lox system) have been used to deliver other optogenetic tools to ON-bipolar cells(99, 101, 105, 106) to good effect. However there have been indications that similarly, this promoter also has a degree off target retinal expression, especially the compressed construct used in many AAV studies (Grm6.SV40)(289).

On balance, these limitations must be weighed against the fact that targeted melanopsin expression was achieved in these lines, adequate to drive electrophysiological light responses (but not behavioural responses, see below). With much work to be done of optimising cell specific expression, the problem of off target expression is likely to remain as these techniques develop towards clinical use, therefore comparisons, even with a defined amount off target transfection are likely to remain useful in practically comparing the relative merits of individual promoters and delivery techniques.

Electrophysiology: Kinetics

Indeed, differences were observed in *ex vivo* electrophysiological responses when melanopsin was targeted to specific cell types: most notably, when targeted to bipolar cells in the “LMAF” model, offset kinetics (half-life and duration) of responses were significantly shorter, especially at higher light intensities. This, along with (albeit less

marked) slower rates of rise of response in this group could suggest differences in how melanopsin light responses are generated and quenched in these cells.

The mechanics of melanopsin deactivation after light responses are beginning to be elucidated in its native ipRGC environment. It has been proposed that activity dependant phosphorylation of a region of melanopsin's C-terminal tail(292-295) by an unknown kinase (295, 296) after light exposure allows the binding of arrestin- β 2(53, 297, 298) leading to deactivation of melanopsin's GPCR activity.

Arrestin- β 2 is found in many retinal cell types (including bipolar cells)(297) and Mure et al(298) found heterologous expression of melanopsin in (non ipRGC) retinal ganglion cells led to *expedited* offset kinetics compared to ipRGC s. They surmised that this implied mechanisms able to quench melanopsin were not unique to ipRGC s, but conversely, additional specific factors are to be found within ipRGC s that *slow* offset kinetics.

If these principal quenching mechanisms are likely to be present in other retinal cells – what may be different about ON-bipolar cells in order to lead to observed differences in offset kinetics in our study? Protein Kinase C – alpha ($PKC\alpha$) in the retina is mainly expressed in rod ON-bipolar cells(299) and has been implicated in the expedient termination of the native ON-bipolar light response by phosphorylating the mGluR6 G-protein coupled receptor in these cells(23, 37, 282, 283, 300). $PKC\alpha$ is a serine / threonine kinase, activated by increasing calcium and DAG levels(282) and therefore a potential candidate to efficiently phosphorylate the C terminal Ser and Thr residues of melanopsin implicated in arrestin binding, in an activity dependent manner(294, 301). $PKC\alpha$'s restricted expression in these cells could therefore

contribute to the expedited response kinetics seen when melanopsin is specifically targeted.

An alternative candidate represented by the atypical PKC ζ , which co-localises with PKC α , also in rod ON-bipolar cells(16), and has previously been implicated in melanopsin signalling in ipRGC s(302). However, PKC ζ does not possess DAG or Ca²⁺ binding domains and is therefore unlikely to *directly* mediate activity dependent phosphorylation of melanopsin in bipolar cells (50). Manipulation of these two enzymes in respect to melanopsin expressed in bipolar cells *in vitro* would be an interesting next step to investigate them as potential contributors to the functional responses seen *in vivo*.

Indeed, it is not known exactly which intracellular effector pathways melanopsin couples to when expressed in non ipRGC cells– such as bipolar cells. *In vitro*, melanopsin has been shown to be promiscuous – able to couple to G_{i/o} pathways (303, 304) (like those of the native bipolar cell signalling cascade(23)). *In vivo*, melanopsin coupling was classically described as to be with the G_{q/11} cascade in M1 cells(50, 305), but there is emerging evidence of coupling to cyclonucleotide mediated pathways in other ipRGC subtypes(57). As our models demonstrate a light induced *increase* in RGC spike firing with ON-bipolar cell targeted melanopsin – such an excitatory coupling within bipolar cell, leading to depolarisation in response to light - is perhaps more likely. Direct electrophysiological recordings from transduced bipolar cells would therefore be useful in further characterising this. If the G_{q/11} cascade is indeed coupled to in exogenous cells, differences in the cascade itself in these host cells could, in themselves, lead to changes in onset and offset kinetics as well as sensitivity.

These potential differences in coupling, as well as heterogeneity of response quenching in different cell populations may explain the observed differences in response kinetics and sensitivity and certainly warrant further investigation (with a view to potential manipulations to improve reconstituted responses).

Electrophysiology: sensitivity and amplitude of responses.

When expression of melanopsin is targeted to a sub-population of ganglion cells, such as *Grk4* RGCs, the lack of converging input from upstream cells could be predicted to result in a smaller amplitude of response to the same stimulus. To a degree, this is borne out with mean and maximum post firing rates in the *non-floxed* group higher than in the “*CGRK*” – *floxed* group (Figure 5-21) and little difference in the same metrics when the *non-floxed* group is compared to the bipolar restricted “*LMAF*” – *floxed* group. However, intrinsic differences in *Grk4* RGC physiology and indeed coupling of melanopsin in these cells could also contribute to this difference.

One of the major differences (and proposed advantages) between the restricted targeting of melanopsin to bipolar cells as opposed to a sub-population of retinal ganglion cells is the retention of an additional layer of retinal image processing. This preservation of afferent RGC synapses and gap junctions - downstream of targeted cells - could be a factor allowing the coding of a wider dynamic range(38, 306). Similarly, the fitting of a sigmoidal curve to “*CGRK*” – *floxed* normalised response sizes vs. intensity, implicated a lower EC_{50} than the other groups (and so higher sensitivity of responses with saturation at a lower intensity). The EC_{50} ($13.0 \pm 0.06 \log_{10}$ photons $cm^{-2} s^{-1}$) found in this group was also lower than values reported previously for non-specific delivery ($EC_{50} = 13.4 \log_{10}$ photons $cm^{-2} s^{-1}$ (159)) in a similar

experimental set up. Perhaps suggesting the presence of factors, leading to the saturation of responses within the RGC population targeted in the “*CGRK*” mice.

The “*CGRK*” – *floxed* EC_{50} however, was, like all groups, higher than values for native ipRGC melanopsin responses in a wildtype retina ($EC_{50}=12.7 \log_{10} \text{ photons cm}^{-2} \text{ s}^{-1}(182)$). This difference from native responses could again be accounted for by differences when melanopsin is expressed outside of its native cells where it may not have access to its preferred G protein or regulatory pathways which are found in ipRGCs.

Conversely, targeting of melanopsin to ON-bipolar cells in the “*LMAF*” – *floxed* group conferred no significant advantage ($EC_{50}=13.64\pm0.19$) over non-specific delivery ($EC_{50}=13.74\pm0.10$) in terms of IRC fit (or firing rate – Figure 5-25). This is especially interesting as after intravitreal delivery, many of transfected cells in the “*LMAF*” - *non-floxed* group are likely to be retinal ganglion cells(159, 160, 298) and may have been predicted to demonstrate response characteristics closer to that of the “*CGRK*” – *floxed* group. However, the numbers of electrodes with a dose response relationship fitting well to the sigmoidal relationship was particularly low in the “*LMAF*” - *flox* group (20 of 84 electrodes) even when the Hill slope value was not fixed at 1 - this could suggest that for many electrodes in this group, across different retinæ, either there was no dose-response relationship in post stimulus firing rate, or that the relationship was not sigmoidal in the range tested ($10^{10.1}$ to $10^{16.1} \text{ photons cm}^{-2} \text{ s}^{-1}$) - an “all-or-nothing” response for example. Again, this may suggest differences either in melanopsin coupling or the network connectivity of these cells in the context of the degenerate retina compared to those in the non-targeted group.

Behavioural Responses

With an EC₅₀ for retinal responses in all three groups within around an order of magnitude of a wildtype ipRGC response, why were results in behavioural assays so poor (no behavioural assay demonstrated a convincing restoration of light responses in any group)? While mice are often not considered to be good models of visual behaviour, they are able to perform visually guided tasks(189, 234). Therefore three assays were chosen that assessed 'vision' at different levels (reflex(307), sub-cortical(177) and cortical(308)). All three had been validated in similar projects in the literature aiming to restore light responses in mice of a similar genetic background(159, 233) as well as validated with wildtype control mice in this study. Therefore, it may have been expected to see some positive restoration of behavioural responses given good electrophysiological restoration at the level of the retina – however several factors may have contributed to this not happening in this context.

The first could be quantity: as the first demonstration of optogenetics in a retina without any residual light response, the number of cells transfected may simply have been too few to produce a signal of large enough amplitude to reach a threshold to drive a behavioural response by itself (without being added to residual ipRGC responses). This is likely to be especially true for pupillary responses where ipRGC responses are central to wild type pupil constriction(205, 206, 307, 309).

Also, the projections of specific cell populations targeted are central to reconstitution of behavioural responses. For example, the subset of RGC cells in which *Grk4* ("*CGRK*" line) is expressed has recently been characterised anatomically as a mono-stratified ON direction sensitive retinal ganglion cell(213, 222). Such cells are known to project to several areas of the brain (including the superior colliculus) but

mainly to the medial terminal nucleus(240) which is fundamental to the accessory optic system(310) and so optokinetic responses(34). Therefore, in this group, while failure to restore pupillary and novel object responses could be explained by anatomy, a robust restoration of optokinetic responses would be *expected* – but was not seen.

Similarly, as the main target cell in the “*LMAF*” – *floxed* group is the rod ON-bipolar cell which is known to connect via All amacrine cells to both cone ON/OFF and ipRGC streams (11, 51, 206, 311). Onward communication of restored light signals would be predicted to occur in both pupillary circuits, the accessory optical system and cortex based on anatomical connections, but again was not seen.

If anatomy were the only factor restricting where in the brain light signals are communicated to, it may have been expected that a *non-targeted* delivery would overcome these obstacles by restoring responses in a variety of cell types. However, this group, like those with targeted delivery, did not demonstrate a convincing behavioural response either.

Another possible important explanation for the poor behavioural response may lie in the models themselves: as mentioned above, all previous optogenetic restoration experiments have used models which have native melanopsin expression, whereas both “*LMAF*” and “*CGRK*” are melanopsin knock out. This lack of melanopsin during development could be relevant, given melanopsin’s role in development of the visual system.

In mice, the melanopsin system is active long before rods and cones during development (312) providing information on irradiance that allows behaviour modification, such as negative phototaxis(313) in this period. This melanopsin mediated light signal has been implicated in the development of the retina and its

projections – regulating the waves of activity present as retino-fugal projections develop (314), (315-317). Mice such as ours, will have no light signal in early development and only a transitory rod and cone mediated one in early maturity on account of their *Pde6b^{rd1/rd1}* degeneration. Potentially, this could have lead to a situation in these animals where retino-fugal projections do not develop in such a way to allow the visual behaviour responses required in our assays. Again, this would appear unlikely given previous work demonstrating central ipRGC projections in mice without melanopsin(48, 162, 318).

Interesting in this context however were results from pilot circadian entrainment experiments: while intravitreal delivery of a floxed melanopsin construct to “*LMAF*” mice did not lead to appreciable entrainment, however a single “*LMAF*” mouse did entrain for a period following injection of a non-floxed hOPN4 injection (although this was not sustained). This would further confirm the appropriate development of retino-SCN pathway in this mouse if it were entraining to a reconstituted light signal. Repeating this experiment (and incorporation of a further arm delivering floxed hOPN4 intravitreally to mice with ipRGC Cre expression (*Pde6b^{rd1/rd1} Opn4^{cre/-}*) and saline injected control groups will be particularly useful in confirming if functional retina-SCN transmission is able to be restored in these mice after AAV injection.

Further investigation of central projections of restored light responses in these models would be informative – for example with non-behavioural metrics of retina to brain transmission such as *in vivo* electrophysiology (visual evoked potential - VEP) or laser speckle recording(319). Or indeed investigation with anatomical methods such as

trans-synaptic retrograde labelling or brain c-fos staining to map projections in these models.

Two further points of note arise in the assessment of behavioural responses in these models. The novel object, visual context recognition task proved especially noisy – with even wild type mice not showing a significantly different recognition ratio from either chance or saline injected controls, which is curious given its recent validation in two studies(159, 178). However, as a behavioural assay, this is particularly sensitive to other environmental factors – such as noise or odour(178) (which may not be detectable to humans). Findings from two independent researchers using the same facility for other novel object recognition tasks(320) were informative as they too found control groups not to perform above chance, perhaps suggesting the need for greater operator training, or indeed an investigator or environmental ‘contaminant’ in the (new) facility where these tasks were carried out.

The second point presented by these behavioural tasks is the performance of a single mouse in the “*LMAF*” - *floxed* group in pupillometry (Figure 5-29) – who appeared to have a much larger constriction than others in the group. This could have been due to particularly good transduction in this animal (perhaps pushing him above a “threshold” for pupillary responses) or indeed an error in genotyping resulting in either a non-degenerate or native melanopsin phenotype (the later eliminated by re-genotyping a second tissue sample post-mortem). Regardless of the cause – exclusion or inclusion of the animal from analysis did not alter the resultant statistical conclusion given the low variance in the rest of the group.

Implications for further research and translation

With demonstration of restoration of responses at the retinal, if not behavioural, level an interesting next step would be to map the receptive fields of the responding ganglion cells and determine their character (ON or OFF type, direction selectivity, centre surround organisation and size). Previous work has suggested a preponderance of ON type responses when melanopsin is delivered non-specifically(160) via the vitreous, but that specific delivery to bipolar cells (albeit of a melanopsin chimera, transgenetically) may allow restoration of ON and OFF responses(105): perhaps due to retained intra-retinal processing. Investigation of alternative assays of mouse visual function will also be useful (such as responses to complex visual scenes(106, 321) or light avoidance assays(319)).

In addition, given the potentially improved offset kinetics of melanopsin responses when expressed specifically in bipolar cells, further investigation of the biochemistry in this situation would be informative. What is melanopsin coupling to in bipolar cells and what is quenching its response? The answers to these questions are likely to enable optimisation of functional responses in optogenetic restoration.

Conclusion

In this Chapter, targeted expression of human melanopsin to specific retinal cell types is demonstrated for the first time with successful restoration of light driven RGC spike firing in a retina devoid of all native light responses. A direct comparison of such responses to those attained when melanopsin is delivered using a non-specific, high efficiency promoter is also demonstrated for the first time. Notably, expedited offset response kinetics in bipolar cell targeted melanopsin are seen as is significantly increased sensitivity when melanopsin is targeted to a subset of RGCs. Despite

reconstitution of retinal electrophysiological light responses, behavioural responses to light were not demonstrated in any treatment group.

In summary, these data suggest that retinal ON-bipolar cells remain a viable target for melanopsin based optogenetic approaches to vision restoration, but further investigation of their responses and how these are transmitted to the brain to allow light dependant behaviour is required. In returning to our consideration of which delivery approach is preferable, it would appear that while none of the approaches explored fully overcome melanopsin's disadvantageous prolonged responses, specific targeting can be used to restore responses with differing kinetics and sensitivities. This could potentially be exploited in future to more faithfully restore specific aspects of visual responses, with melanopsin used in concert with other optogenetic tools with complimentary response characteristics.

In this Chapter, consideration has focused on comparison of different targets for our favoured optogenetic tool (melanopsin), however an increasing number of proteins with differing characteristics are being proposed and validated as such tools. Therefore, in the following Chapter, the same models will be utilised to allow comparison of the benefits of other potential tools.

Chapter 6 – Results: Comparing specific delivery of optogenetic tools in the degenerate retina

Introduction

In the preceding Chapters the first direct comparison of melanopsin targeted either generically or to a specific cell type as an optogenetic tool was described. However, an increasing number of proteins are being validated as potential optogenetic tools for restoration of vision in the inherited retinal degenerations and in the following Chapter, a comparison will be made between melanopsin and two alternative candidate tools.

Vertebrate Opsins

In addition to melanopsin, other human opsins have been delivered to the degenerate mouse retina in attempts to restore light responses – most notably perhaps is human rhodopsin, the specialised native opsin of rods(103, 106). While, like melanopsin, rhodopsin is a native, human retinal protein and so less likely to spark immunogenic responses than microbial opsins, it was initially believed to have some disadvantages as an optogenetic tool. Rhodopsin is highly specialised for phototransduction in its native cells coupling to the inhibitory, photoreceptor specific(322), $G_{i/o}$ G-protein transducin (323-325) and while it can couple to inhibitory cascades when exogenously expressed(326, 327) it does not appear to activate excitatory G-proteins(327, 328). In addition, rhodopsin, unlike melanopsin(62), does not form a stable association with all *trans*-retinal and therefore relies on exogenous regeneration of retinal chromophore either through the visual cycle or via Müller cells(329).

Despite these theoretical disadvantages, several investigators have found that in practice, *in vivo* expression of rhodopsin in surviving retinal cells in *Pde6b^{rd1/rd1}* can

indeed restore electrophysiological and behavioural responses to light(103, 106). Both groups noted increases in RGC spike firing rates in response to light, suggesting that rhodopsin is either able to couple to inhibitory second messengers in inhibitory cells (leading to disinhibition of RGC firing) or indeed couple directly to *excitatory* second messenger systems when exogenously expressed.

The work of Cehajic-Kapetanovic *et al*(106) is particularly interesting as they target rhodopsin using AAV using both a non-specific and ON-bipolar cell specific (grm6-SV40) promoter constructs and find excitatory responses in both groups – providing evidence for an excitatory coupling. Similarly, Gaub *et al*(103) target rhodopsin to bipolar cells using the 4xgrm6 promoter construct and find depolarisation of bipolar cells. Like melanopsin, the exact nature of coupling of rhodopsin when exogenously expressed is unclear, but unlike melanopsin, the native partners of rhodopsin are known not to be expressed outside its native cell. This would suggest that rhodopsin coupling with exogenous expression differs to that in rods.

Recently, Berry *et al* have extended the canon of potential vertebrate opsin optogenetic tools with the description of an intravitreal AAV being used to deliver medium wavelength (MW) cone opsin to mouse *Pde6b^{rd1/rd1}* retina(158), driven by the non-specific high efficiency promoter hSyn. While cone opsins may share rhodopsin's theoretical disadvantages, they report restoration of light responses using this method with a similar sensitivity to rhodopsin, but with faster kinetics and an ability to adapt to ambient illumination.

Alternative tools

Modifications of vertebrate opsins, notably a melanopsin-mGluR6 chimera are also able to restore light responses *in vivo* with more favourable kinetics and sensitivity than their wild type analogues (105) (see Chapters 5 and 7). However, non-vertebrate and engineered tools – often with very fast kinetics - have also been trialled in mouse models. This includes variants of acrylamide-azobenzene-quaternary-ammonium (AAQ) - a sensitizer of potassium channels that can induce light sensitivity in surviving retinal cells when delivered intravitreally(330, 331). Also, light gated glutamate receptors engineered to generate excitatory or inhibitory responses to light(104, 332-334) have also been described, as has their co-expression in the same retina, leading to improved visual discrimination(334). While boasting huge potential utility as scientific tools, the translational prospects of these approaches is likely to be limited by the need for (repeated) intraocular delivery of chemical substrate. Therefore, these tools were not prioritised for comparison in this study.

Microbial Opsins

The largest, and perhaps best studied, group of optogenetic tools available are the microbial opsins – which act directly through a cell membrane ion channel, rather than coupling to a G-protein cascade. Like engineered receptors, excitatory and inhibitory tools exist in this family: for example, haplorhodopsin hyperpolarises the cell in response to light. This allows it to restore ON responses when expressed in surviving photoreceptors of the degenerate retina (335) and OFF responses when expressed non-specifically in inner retinal cells (210). Indeed, when haplorhodopsin is targeted to the dendritic arbour of RGCs and channelrhodopsin (which depolarises the membrane) to the soma, opponent centre surround responses can be restored(336).

Channelrhodopsin was first described in the green Algae *Chlamydomonas reinhardtii*(151-153, 337, 338) and along with its variants represents perhaps the most investigated optogenetic tools for visual restoration in the preclinical arena. Despite early concerns regarding xenotransplantation, major immune reactions in rodent models have not yet been described at doses used(100, 339) and therefore such tools have entered non-human primate(340) and phase 1 clinical trials (clinicaltrials.gov reference NCT03326336, NCT02556736). Several variants of channelrhodopsin have been targeted to various cell populations within the retina, but few studies have directly compared either the channelrhodopsin used, or the cell types targeted(103, 341).

Several studies have used high efficiency, generic promoters to deliver channelrhodopsin to the degenerate retina. Bi *et al*.(342) were the first group to exploit nascent channelrhodopsins, delivering native channelrhodopsin 2 under a CAG promoter via an intravitreal AAV injection, demonstrating robust restoration of retinal and cortical electrophysiological responses to light in *Pde6b^{rd1/rd1}* mice. Similar findings have been reported in rat models (343, 344) with expression (and physiological responses) seen to persist in injected wildtype mice for the whole lifespan of the animals(345).

While these studies demonstrated the potential for AAV delivery of channelrhodopsins – some early descriptions of cell population targeted delivery relied on other methods, for example Tyagarajan *et al*.(346) attempted to restrict expression to retinal ganglion cells in a transgenic mouse by placing it under the control of the *thy1* promoter. While they found electrophysiological evidence of channelrhodopsin 2 mediated ON-responses in response to light, they showed no

improvement of behavioural performance over control mice. This may be in part due sensitivity – their single cell RGC responses being around 10^7 photon $\text{cm}^{-2} \text{s}^{-1}$ less sensitive than wildtype response. Or, as the authors argue, that *thy1* induces expression in both ON and OFF ganglion cells (as well as subsets of amacrine and bipolar cells(347, 348)) and therefore retinal responses may “cancel each other” when they reach higher centres.

Specific targeting of channelrhodopsin 2 to ON-bipolar cells using a *Grm6* promoter by Lagali *et al*(99) did restore behavioural responses to light; as well as centre-surround opponency in single cells recordings from RGC and cortical neurons. While this study, by demonstrating the maintenance of basic retinal processing, adds to the attractiveness of ON-bipolar cells as targets for optogenetics, like Thyagarajan *et al*(346), it used a technique of delivery (electroporation) that is not readily translatable.

Several subsequent studies however, have developed translatable methods of ON-bipolar specific delivery of channelrhodopsin using AAV delivery. Doroudchi *et al* (100) demonstrate long lasting (10 months) retinal channelrhodopsin 2 expression in bipolar cells of a *Pde6b*^{rd1/rd1} retinae after AAV delivery under the *grm6-sm40* promoter construct. This study also demonstrated the restoration of electrophysiological responses to full field illumination in the retina and at the behavioural level. Unlike Lagali *et al*, however, it did not include central electrophysiological recordings(99). Macé *et al* (102) do demonstrate the restoration of cortical ON and OFF responses with AAV delivered ChR2 under the same promoter construct. Whereas Cronin *et al*(101) use a modified *Grm6* based promoter construct (4xgrm6.SV40) and optimised AAV capsid to deliver channelrhodopsin 2, using full field

illumination and specific stimuli to show the restoration of RGC ON, OFF and ON-OFF responses as well as centre-surround opponency.

Taken together, these studies would suggest that AAV mediated, cell specific delivery of channelrhodopsin is possible in degenerate retinae; that this can restore ON, OFF and centre surround opponency type responses both at the level of the retinal ganglion cell(101) and in visual cortex(99, 102) and animals can use this information to perform light dependent behaviours(99, 100, 102).

Channelrhodopsin variants

Despite this compelling array of findings, several obstacles exist to the successful translation of channelrhodopsins as clinical optogenetic tools in the degenerate retina. Principal among these is that of sensitivity: native channelrhodopsin 2 requires intensities of light for activation (upwards of 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$ (107, 342)) at the edge of those permitted to avoid retinal toxicity in humans(349). Also, without second messenger coupling, channelrhodopsins are unable to amplify small responses to less intense stimuli. In early clinical trials (NCT03326336) these obstacles have been ameliorated to a degree by the use of “stimulator glasses” which amplify light signals from the visual scene.

This practical solution is not desirable and may even induce phototoxicity due to the high intensity of the stimulus and its spectral composition: the peak spectral sensitivity (λ_{max}) of native channelrhodopsin being at the high energy blue-violet end of the spectrum ($\lambda_{\text{max}} = 460\text{nm}$ (153)). Alternative approaches are therefore required and in this regard, multiple channelrhodopsins variants have been engineered with increasing sensitivity and red shifted peak sensitivities (λ_{max}), several of which have been trialled in mouse models of retinal degeneration. Searching and do novo

sequencing of novel opsins in the many hundreds of species photosensitive algae has provided one method of discovery of new native channelrhodopsins. For example, Crimson R, a red shifted ($\lambda_{\max} = \text{c.}600\text{nm}$) alternative microbial opsin was discovered in this way(350) and is in use in a clinical trial (NCT03326336).

An alternative approach has been to systematically alter native channelrhodopsin 2 by point mutations predicted to alter sensitivity and specificity. For example Kleinlogel *et al*(351) replaced the leucine residue at position 132 with cystine, creating a channel with greatly increased Ca^{2+} conductance and so a light response c.70 fold more sensitive than native channelrhodopsin 2. This tool was able to drive retinal ganglion cell firing and cortical visual evoked potentials when delivered specifically to RGCs of macaque and *Pde6b^{rd1/rd1}* mouse retinae ($\text{EC}_{50} = \text{c.}10^{15}$ photons $\text{cm}^{-2} \text{s}^{-1}$; $\lambda_{\max} = \text{c.}480\text{nm}$ (340)).

Ganjawala *et al* (208) have combined these approaches by inducing a point mutation in a novel microbial opsin CoChr(350); this modified tool demonstrates improved sensitivity ($\text{EC}_{50} = 10^{14}$ photons $\text{cm}^{-2} \text{s}^{-1}$; $\lambda_{\max} = \text{c.}470\text{nm}$). When delivered to the mouse retina by intravitreal AAV, under a CAG promoter, the construct restored light dependant increases in RGC firing. Additionally, their mice were able to perform behavioural assays requiring light at intensities comparable to those found in the environment. However, these findings are potentially confounded by their use of the triple-knock-out (“TKO”) mouse model(205). While lacking melanopsin (*Opn4^{-/-}*) and principal components of the rod and cone signalling pathways (*Gnat1^{-/-}*, *Cnga3^{-/-}*), this model is not degenerate, and indeed has been shown to retain light signalling though accessory transduction pathways, to the degree that it has a measurable electroretinogram (ERG) response(209).

Tomita *et al*(352) made use of a degenerate model (the RCS rat) when they assessed another novel construct: the channelrhodopsin ortholog VChR1 which had been discovered in *Volvox carteri*(353) and demonstrated to have similar channel properties to ChR2, but with a red shifted peak sensitivity ($\lambda_{\text{max}} = \text{c. } 530\text{nm}$). Noting poor trafficking to the membrane of VChR1, they developed a chimera of VChR1 and *Chlamydomonas* channelrhodopsin-1, termed mVChR1, which not only trafficked to the membrane well, but demonstrated a wide range of spectral sensitivity (c.460nm to 640nm) usefully within the visual range. This construct was delivered, under a generic, high efficiency promoter (CAG) via intravitreal injection of AAV to the degenerate animals with restoration of light sensitive RGC firing, VEPs and behavioural (optokinetic) responses. The same group went on to co-express both ChR2 and mVChR1 in the same degenerate retina with the aim of increasing sensitivity and improving spectral range – however both constructs were delivered under (different) non-specific promoters and so colour opponent responses are unlikely, although they do demonstrate increased sensitivity to light with co-expression.

The poor membrane trafficking of native VChR1 was also noted by Lin *et al*(354) who addressed the challenge by replacing its N-terminus with that from another channelrhodopsin variant (ChIEf)(355) known to have good membrane trafficking properties (Figure 6-1). They also replaced transmembrane domain F of VChR1 with that of ChR2 in order to increase expression levels(356) and carried out a point mutation (L171I) to reduce desensitization at higher wavelengths, as well as adding a fluorescent tag (YFP-citrine)(355) (Figure 6-1).

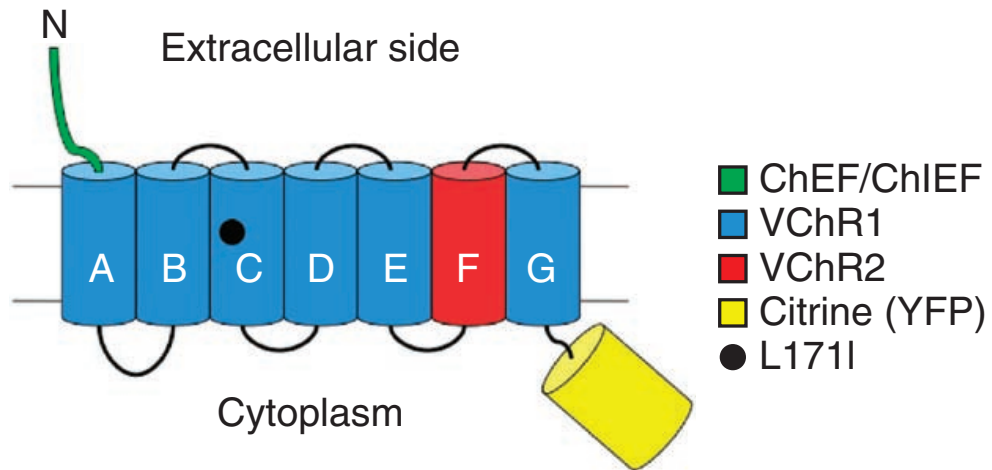


Figure 6-1 Schematic design of ReaChR, modified from Lin et al 2013(354). ReaChR consists of the N-terminus of the ChEF/ChIEFChR variant of channelrhodopsin, transmembrane domains A–E and G of the VChR1 variant, transmembrane domain F of the VChR2 variant as well as an L171I point mutation.

Sengupta(357) made use of this construct, styled *ReaChR*, in *Pde6b^{rd1/rd1}* mice, delivering it under the control of a high efficiency, non-specific promoter (hSyn) via intravitreal AAV, observing mostly RGC transfection. Restoration of electrophysiological responses was seen at the RGC ($\lambda_{\text{max}} = \text{c.}520\text{nm}$, responses in the range 10^{14} to 10^{18} photons $\text{cm}^{-2} \text{s}^{-1}$ with temporal fidelity up to 30Hz) and cortical level (with ON, but not OFF responses). While the intensity range is still at the upper safety limit for human use(349), as the stimuli are red shifted, the risk of toxicity is lower. In addition, behavioural responses to light were seen in the open field test, but only with light intensities above $\text{c.}10^{15.5}$ photons $\text{cm}^{-2} \text{s}^{-1}$. In addition, Sengupta *et al*(357) demonstrate the ability to express the ReaChR construct in macaque and human retina tissue with restoration of RGC spike firing in response to light.

ReaChR therefore has a collection of properties making it particularly attractive for clinical translation: a spectral sensitivity within the visual spectrum, activation at intensities likely to be within safe ranges, good temporal fidelity and effective membrane trafficking after AAV delivery to mouse, non-human primate and human retina. Therefore, I selected this variant of channelrhodopsin to compare to two other candidate optogenetic tools for clinical translation in retinal degeneration (melanopsin and rhodopsin).

Comparing optogenetic tools

With such a range of candidate optogenetic tools available for potential translation as visual restoration therapies in degenerate retinæ, there is a pressing need to directly compare these in the preclinical arena in order to select the most promising to bring forward to clinical testing. To date, only a small number of studies have directly compared different candidate tools and target cell populations in the same model

system. These include a direct comparison of Grm6 driven rhodopsin and channelrhodopsin2 (H134R mutant variant) AAV delivery to bipolar cells(103) and a comparison of grm6 driven and non-specific targeting of rhodopsin(106) in *Pde6b^{rd1/rd1}* retinæ. AAV delivery of rhodopsin under a generic promoter has also been compared to MW-cone opsin(158) in degenerate mouse models, but not to melanopsin. As described in Chapter 5, bipolar targeted (L7) delivery of melanopsin has been compared to AAV delivery under a generic promoter as well. Additionally, even with the large number of microbial opsins available, only three channelrhodopsin 2 and two *Chloromonas oogama* channelrhodopsin point mutation constructs have been directly compared(208, 341).

With this small number of directly comparative studies, many of the relative advantages and disadvantages of each of these candidate tools remain somewhat theoretical in relation to approaches to visual restoration in IRDs, presenting an important unmet need for direct comparison. In addition, none of these studies compare responses in a degenerate retina devoid of both rod cone and melanopsin responses.

Therefore, an appraisal of sensitivity and kinetics in such a context of three main candidates for clinical translation (melanopsin, rhodopsin and ReaChR), targeted to defined cell populations (ON-bipolar or a subset of retinal ganglion cells) will be essential to determine the most appropriate tool(s) for clinical translation. Such a comparison will form the basis of the following Chapter: it was hypothesised that ReaChR would give faster kinetics, but lower sensitivity compared to opsin-based tools due to its lack of second messenger coupling. It was also hypothesised that differences between ON-bipolar and RGC specific delivery would be more marked with opsin

based tools, compared to ReaChR given a potentially greater role of the host cell in response quenching and coupling due to second messenger coupling.

To investigate this, three AAV vectors containing floxed melanopsin, rhodopsin and ReaChR were produced and validated. These were then delivered by intravitreal injection to *Pde6b*^{rd1/rd1}; *OPN4*^{-/-} mice expressing the Cre enzyme under either an *L7* or *Grik4* promoter. Reconstituted electrophysiological retinal light responses were then compared in terms of size, kinetics and sensitivity.

Note on nomenclature

During this Chapter, for ease of labelling, mouse lines and treatment groups are abbreviated,

5. “LMAF” is used in place of *Tg(L7-Cre)*^{+/-} *Pde6b*^{rd1/rd} *Opn4*^{tm1yau/tm1yau}
6. “CGRK” is used in place of *Tg(grk4-Cre)*^{+/-} *Pde6b*^{rd1/rd} *Opn4*^{tm1yau/tm1yau}
7. “ReaChR” in place of *hSyn-ReaCgR* (floxed) virus – see Figure 6-1a
8. “Rhodopsin” in place of *EF1α-Rhodopsin* (floxed) virus – see Figure 6-1b
9. “hOPN4” in place of *EF1α-hOPN4* (floxed) virus – see Figure 6-1c

Experimental design and results

In order to test these hypotheses, two further AAV vectors were produced containing floxed human rhodopsin or ReaChR(354, 357), in addition to the floxed hOPN4 virus described in Chapter 5. Both were under the control of high efficiency promoters with LoxP motifs present to restrict expression to those cells expressing Cre recombinase (Figure 6-2). These viral preparations were validated *in vitro* before administration to “LMAF” or “CGRK” mice (see Chapter 3). Subsequent histology was used to confirm protein expression in the expected cell groups, *ex vivo* multiple electrode array recordings were then undertaken to assess function at the retinal level. Detailed methodology for all techniques is described in Chapter 2.

Production and *in vitro* validation of viral vectors

Two insert plasmids(165, 175, 354, 357) were used to construct two viral vectors (Figure 6-2). Pilot work in our group had previously established the rhodopsin construct to restrict high efficiency rhodopsin expression to bipolar cells in an “LMAC” mouse after injection of associated AAV(165) (see Chapter 3). The *ReaChR* construct was identical to that used in a previously published *in vivo* study(357). The *hOPN4* construct is described in Chapter 5.

Viral vectors were produced from these constructs with an AAV 2/2 quad-mutant capsid (Chapter 2) at the University of North Carolina (floxed ReaChR virus) or in house (floxed rhodopsin virus). Quantitative polymerase chain reaction (qPCR) was used to determine the viral titre of each: 1.5×10^{13} vg/ml (viral genomes per millilitre) for the ReaChR virus and 1.88×10^{10} vg/ml for the rhodopsin (hOPN4 1.72×10^{12} vg – Chapter 5).

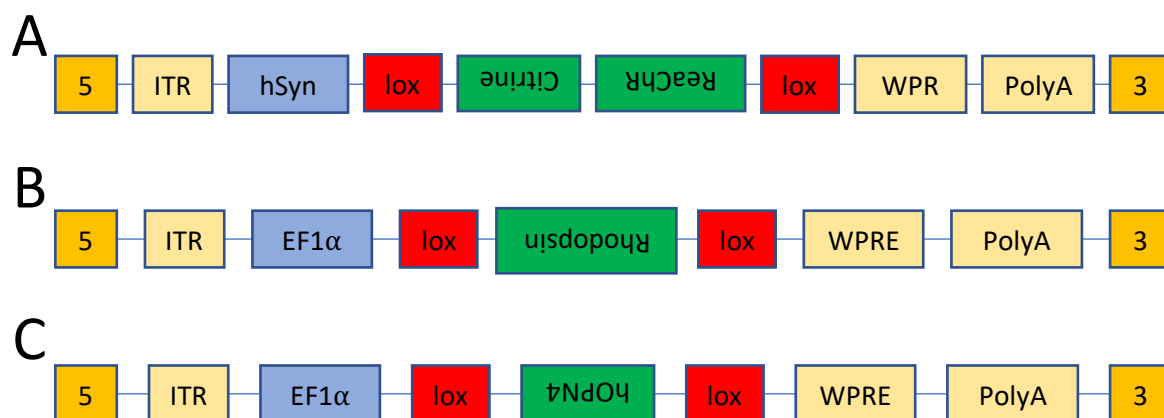


Figure 6-2 Linearized representation of insert plasmids used to make (A) floxed ReaChR (B) floxed Rhodopsin and (C) floxed hOPN4 (see Chapter 5) vectors. Inverted sequences are shown upside down. Elements required for effective AAV transfection are in yellow: ITR – inverted terminal repeat, WPRE – Woodchuck hepatitis virus Post-transcriptional Regulatory Element, PolyA – PolyAdenosine tail. Promoters are in blue. EF1 α - Elongation factor 1 α , hSyn – human Synapsin 1. LoxP sequences are in red. Payload (translated in target cells) is in green: Citrine – Citrine Yellow Fluorescent Protein.

On SDS-PAGE electrophoresis, the ReaChR virus displayed three bands corresponding in size to the viral proteins VP1, VP2 & VP3 (Figure 6-3a) without additional bands, demonstrating an absence of protein contaminants. The rhodopsin virus also demonstrated bands corresponding to VP1, VP2 and VP3, with additional, faint bands at higher molecular weights. Demonstrating the presence of a small quantity of additional higher molecular weight material, in addition to the expected viral proteins.

Given the presence of the expected viral proteins in the preparations, an assessment the vectors' ability to transfect cells *in vitro* was assessed prior to *in vivo* use. Dissociated retinal cell cultures were prepared from "LMAF" mice and allowed to culture on 1cm² laminin coated coverslips for two weeks before inoculation for 24hours with a volume of AAV corresponding to 5x10⁹vg per coverslip (see Chapter 2 for methods). Immunocytochemistry (ICC) of the cultures was performed three weeks later. This demonstrated staining in 16.25±1.25% (n=212) of DAPI stained cells counted in the *ReaChR* group and 10.5±1.38% (n=192) in the *rhodopsin* group (Figure 6-4). Demonstrating that both preparations contain intact AAV and all the elements necessary to enter retinal cells and produce protein. While successful expression in cultured retinal cells is certainly promising, it cannot be taken as a direct metric of efficacy: these cells are unlikely to be in their native state given the artificial nature of both dissociation and culture, however it did validate that progression to *in vivo* testing was indicated.

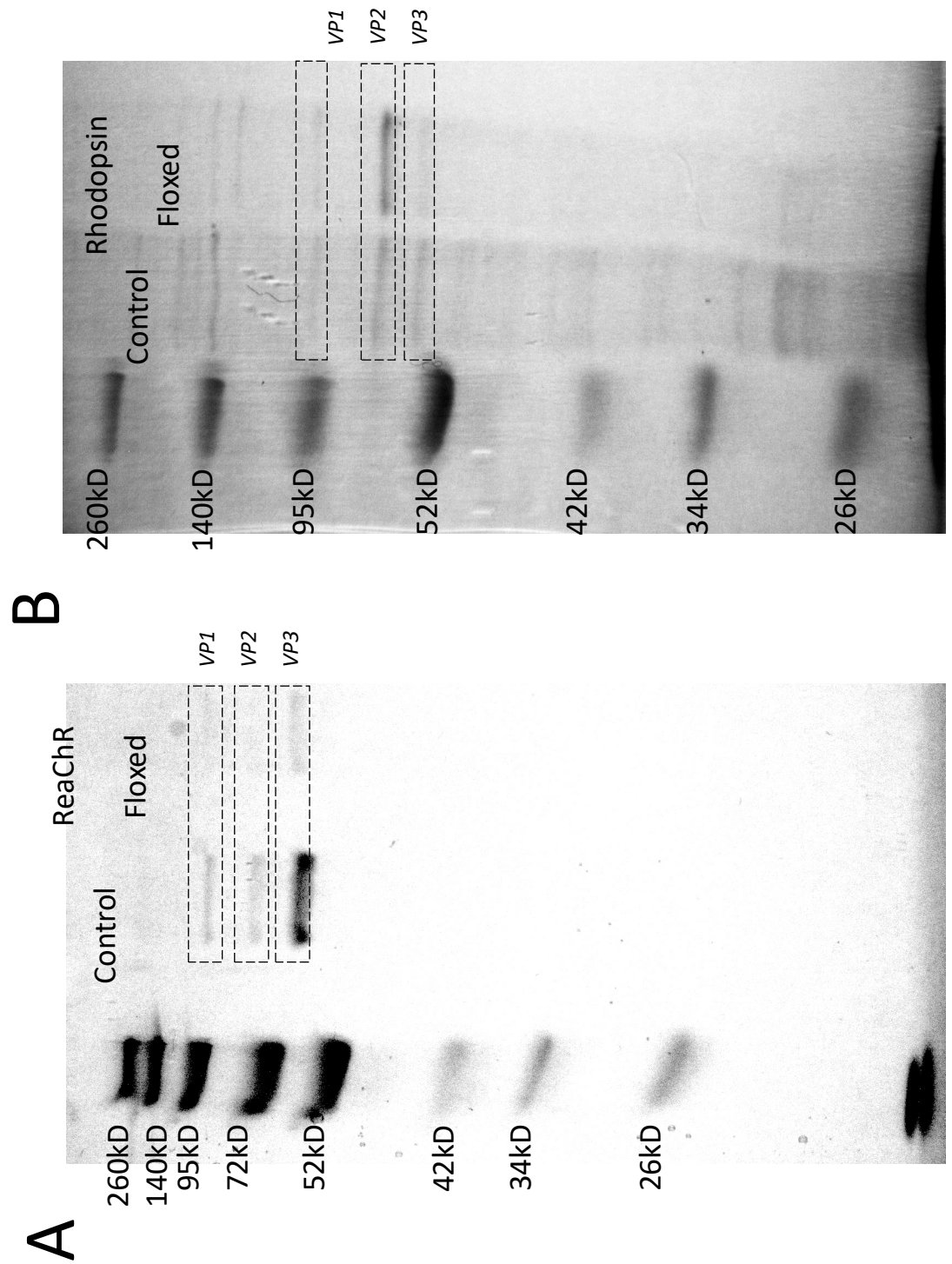


Figure 6-3 Denaturing SDS-PAGE page gel of samples of AAV containing either floxed ReaChr (A) or rhodopsin (B). Three discrete bands are visible at the expected sizes for viral proteins (62, 72 and 87kD) with no other prominent bands in A, indicating lack of contamination in A. In the rhodopsin preparation, there were a few (especially heavier molecular weight) bands, but the majority of these appear in the control. The control represents an AAV previously validated *in vivo*. VP1,2,3 – Viral protein 1,2,3.

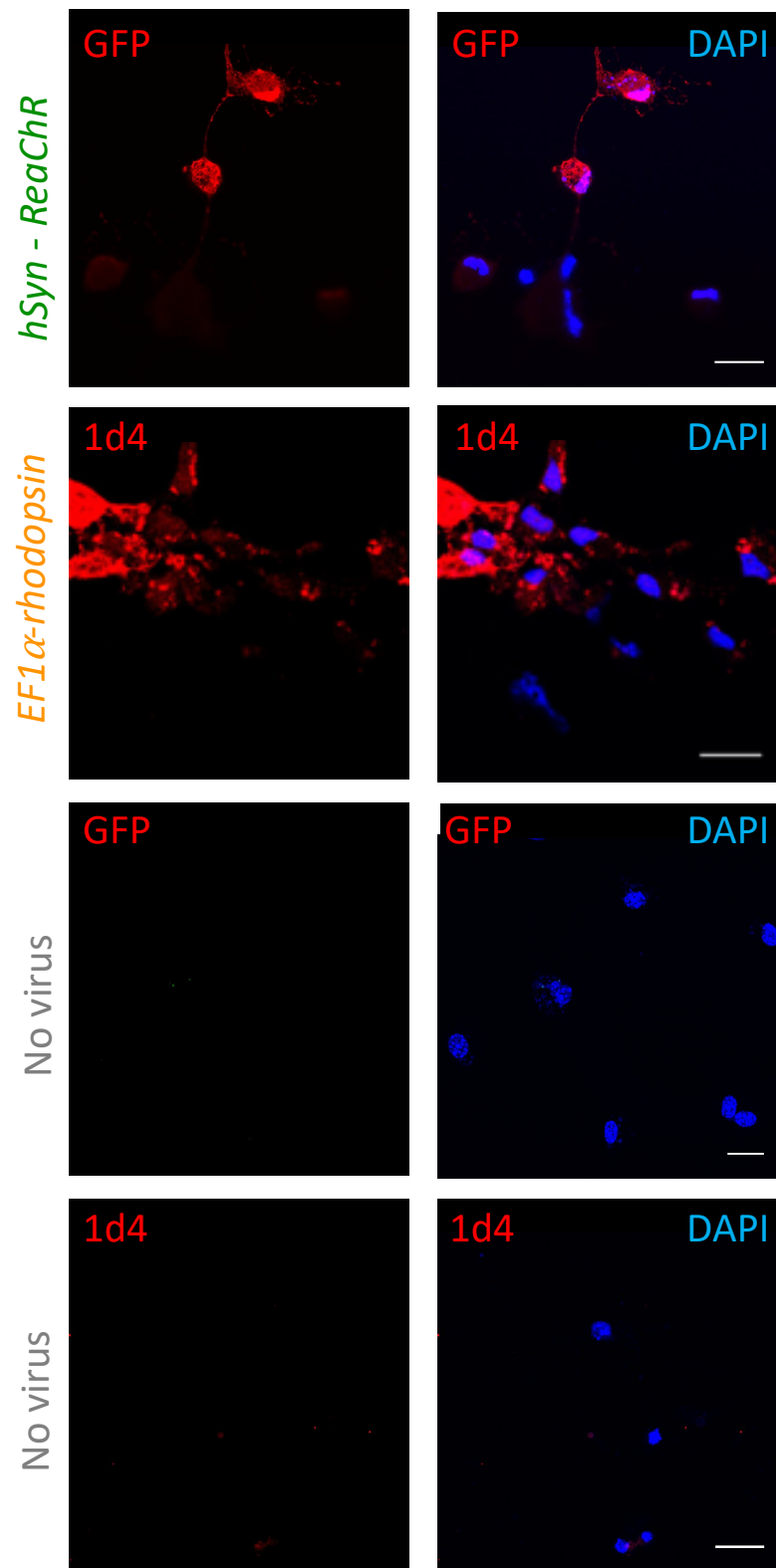


Figure 6-4 Immunocytochemistry of dissociated retinal cell culture incubated with an AAVs containing the ReaChR or rhodopsin constructs. Control panels, also represent dissociated retinal cell culture, but incubated with 0.9% saline in place of AAV. DAPI - nuclear stain; GFP – green fluorescent protein – an antibody that stains the YFP-Citrine tag of the ReaChR construct. Scale bar 20μm

In vivo validation of viral vectors

Given this evidence for efficacy *in vitro*, *in vivo* validation was required, not only to ensure quantitative efficacy of AAV induced protein production, but to qualitatively assess cell-specificity of the vectors. Do these viruses drive expression solely within the expected cells? One previous study has delivered a similar hSyn-ReaChR construct to the retina via intravitreal injection(357) and three studies have delivered rhodopsin via intravitreal injections either under generic or bipolar cell specific promoters(103, 106, 158), but none have restricted expression using the Cre.lox system or similar. With these previous reports of efficacy intravitreal delivery was again used (see Chapter 5 for further discussion).

Both the ReaChR and rhodopsin AAVs were administered intravitreally to seven-week-old “*LMAF*” or “*CGRK*” mice which were culled four weeks later to allow flat mount and cryo-section IHC for GFP or 1d4 (GFP antibodies stain the YFP-citrine incorporated into the ReaChR construct, 1d4 is an epitope of rhodopsin). GFP or 1d4 has differing staining patterns depending on the Cre line (“*LMAF*” or “*CGRK*”) which had been injected and is summarised in Figure 6-5 and further detailed in Figure 6-6 to Figure 6-14. For the floxed hOPN4 construct, a similar validation has been detailed in Chapter 5 and summary values for comparison are included in Figure 6-5.

Intravitreal delivery of floxed ReaChR to “*LMAF*” mice was seen to lead to staining in the inner nuclear layer (INL) ($17.5 \pm 3.9\%$ of cells ($n=386$)) as well as in the ganglion cell layer (GCL) ($7.1 \pm 1.6\%$ of cells ($n=198$)) and inner plexiform layer (Figure 6-6 to Figure 6-8). This distribution of protein expression reflects the expected pattern with a predominance in bipolar cells & a sub-population of retinal ganglion cells(213,

221) as previously described and seen for the melanopsin construct in Chapter 5. The co-staining of these cells for human melanopsin and L7 protein (Figure 6-8) was in keeping with these cells representing the population previously described by Ivanova *et al*(221).

Similarly, intravitreal delivery of the floxed ReaChR construct to “*CGRK*” mice demonstrated, as expected, staining restricted to a subpopulation of cells in the ganglion cell layer ($7.8 \pm 1.6\%$ of cells staining $n=218$) and dendrites in the inner plexiform layer, with no staining ($0.00 \pm 0.00\%$ of cells staining, $n=406$) in the inner nuclear layer (Figure 6-9 to Figure 6-11). Co-staining of these cells with Brn3a is in keeping with the expected restriction of expression by the Cre-lox system to a subpopulation retinal ganglion cells(213, 222) (Figure 6-11).

Staining following intravitreal delivery of the floxed rhodopsin construct to “*LMAF*” mice was most prominent in the ganglion cell layer (GCL) ($2.2 \pm 0.5\%$ of cells staining, $n=196$), inner plexiform layer and to a lesser degree, the inner nuclear layer (INL) ($4.1 \pm 1.4\%$ of cells staining ($n=374$)) (Figure 6-12 to Figure 6-14). This distribution of protein again reflects the expected predominance in bipolar cells & a subpopulation of retinal ganglion cells(213, 221). Staining in the INL was however was much less pronounced in this group than with either the hOPN4 or ReaChR construct in the same animals suggesting either poorer access of the virus to these cells, or a reduced ability to transfect them – likely secondary to the lower titre of the rhodopsin AAV.

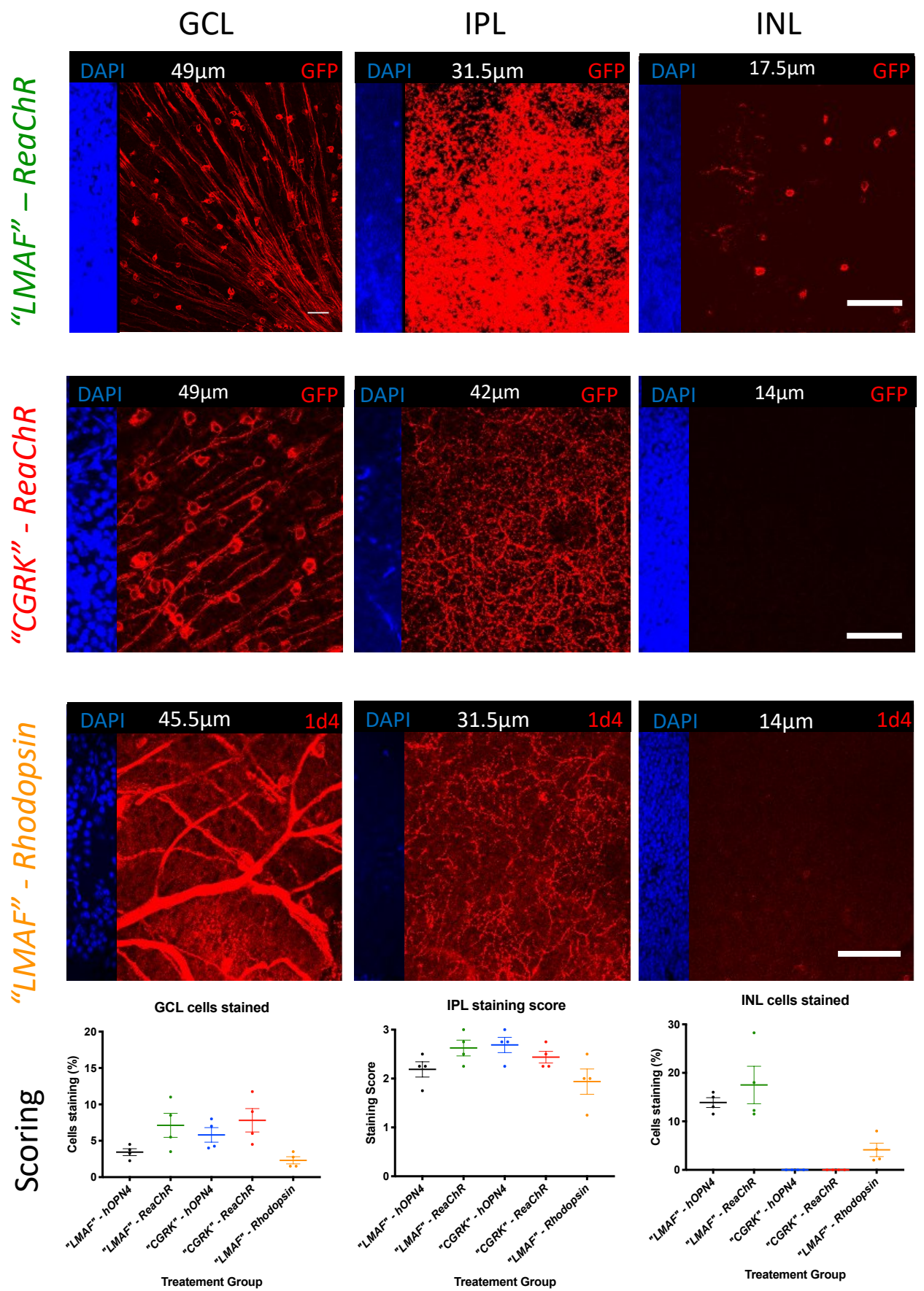


Figure 6-5 Example of immunohistochemical staining of retina from transfected retina flat mounts at layers three layers. (INL, IPL & GCL). The bottom graph panels represent the average number of stained cells in 100x100µm areas at that retinal layer at the four areas of best staining in each of four retina. In the inner plexiform layer a score was given, rather than a cell count (1= no stain, 2= moderate stain, 3= high staining).

The depths here refer to this images position in the Z stacks of Figure 6-7, Figure 6-10 & Figure 6-13 in which more detailed analysis of each group is shown.

INL – Inner nuclear layer, IPL – inner plexiform layer, RGL – retinal ganglion cell layer. Scale bars 50µm

“LMAF” – ReaChR

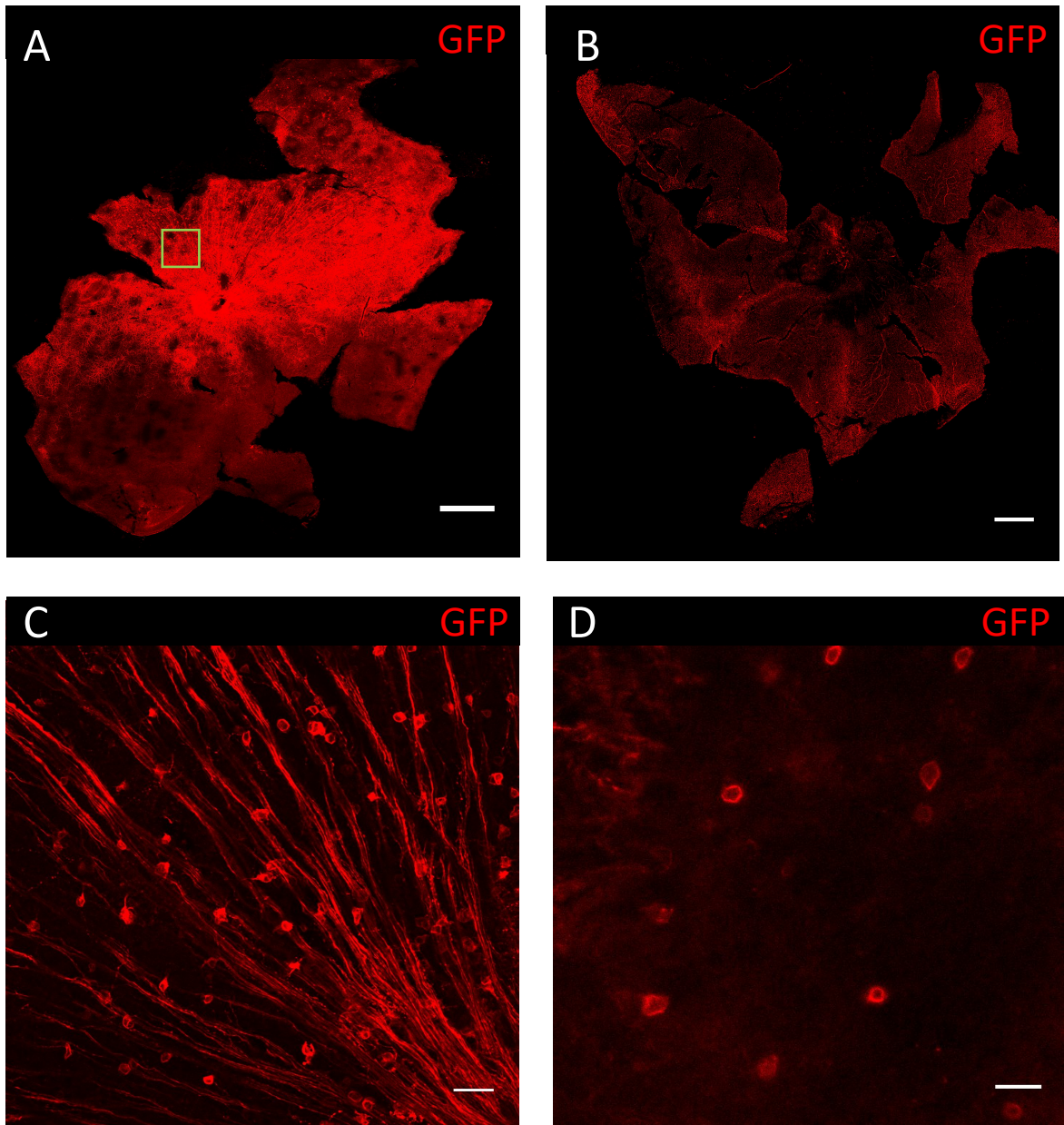


Figure 6-6 Confocal micrographs of a flat mount of a “LMAF” retina four weeks after injection with the hSyn-ReaChR construct.

- A. Low power tile of confocal images stitched to give view of whole retina – note areas of absence of staining in the lower retina. Green box gives approximation of location of the image in panel B. Scale bar 500µm
- B. A saline injected control stained for GFP and imaged as in panel A. Scale bar 500µm
- C. Single low power (x20) image from figure A at ganglion cell layer. Again, note staining of RGC bodies and axons) Scale bar 50µm
- D. High power (x40) image of the Inner nuclear layer from same retina. Scale bar 20µm

“LMAF” – *ReaChR*

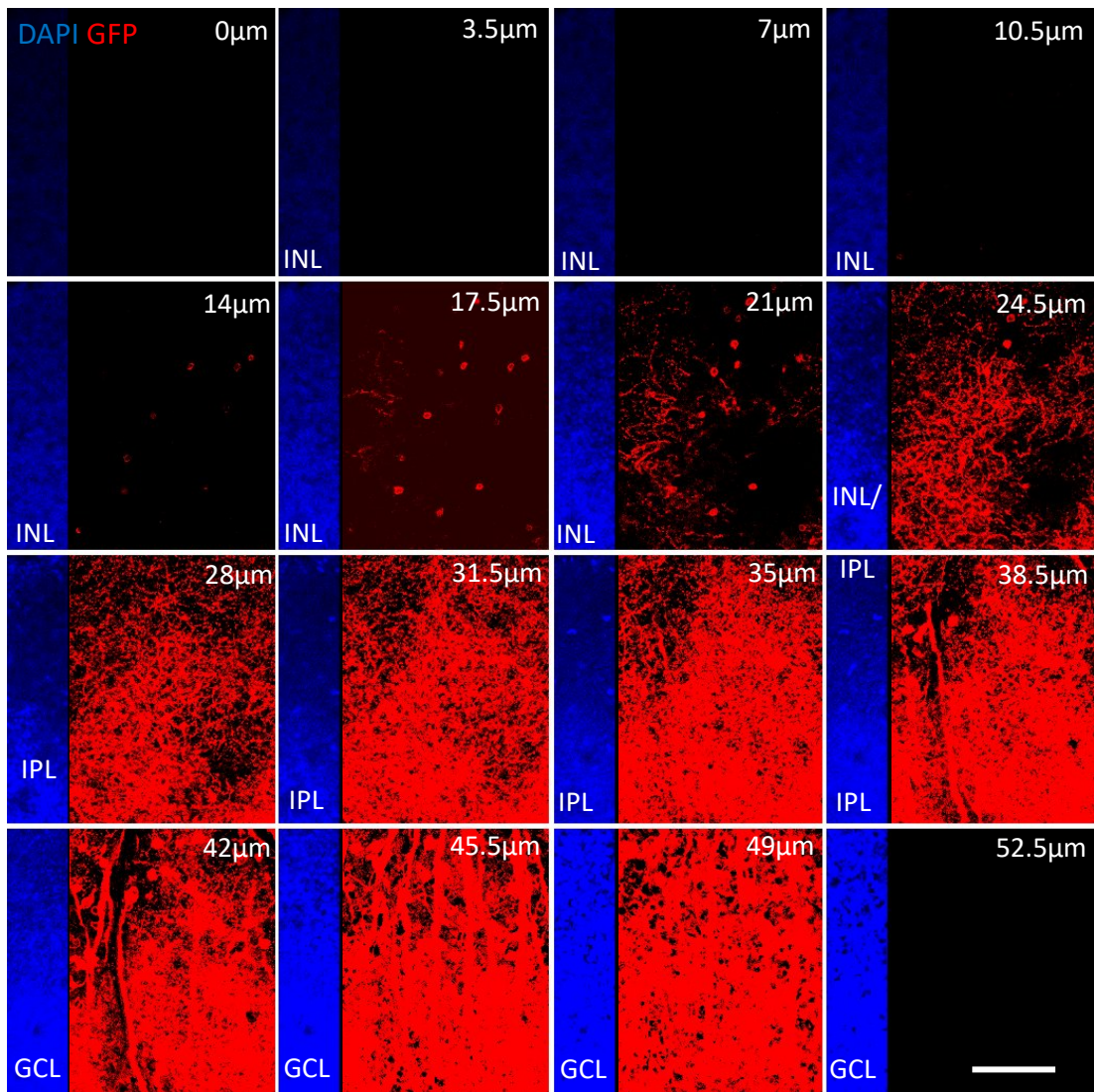


Figure 6-7 Montage of confocal micrographs of an “LMAF” retina four weeks after injection with the *hSyn-reaChR* construct. These represent slices of the same “Z stack” from the retina described in Figure Y. For clarity, the red channel (representing GFP (YFP-citrine) staining) is presented. Note stain of cells in the GCL and INL. The depths described in each panel refer to the distance in the Z plane from the top left panel (just below the retina). INL – inner nuclear layer. IPL – Inner plexiform layer. GCL – Retinal ganglion cell layer. Scale bar is 50μm

“LMAF” – ReaChR

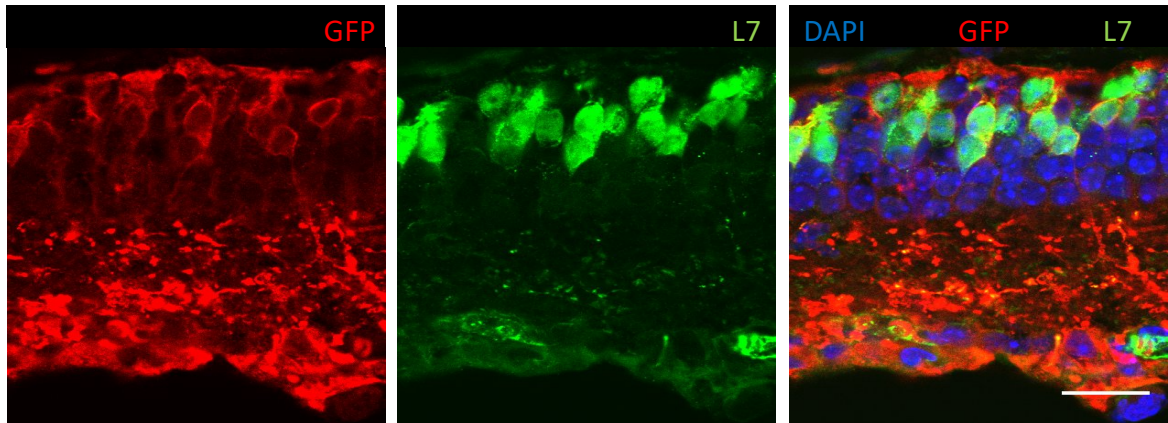


Figure 6-8 Cryosection from an “LMAF” retina four weeks after intravitreal injection with the hSyn-ReaChR construct. Note containing of cells in the INL for L7 and GFP indicating expression of the ReaChR construct in ON bipolar cells. Scale bar 50 μ m.

“CGRK” - ReaChR

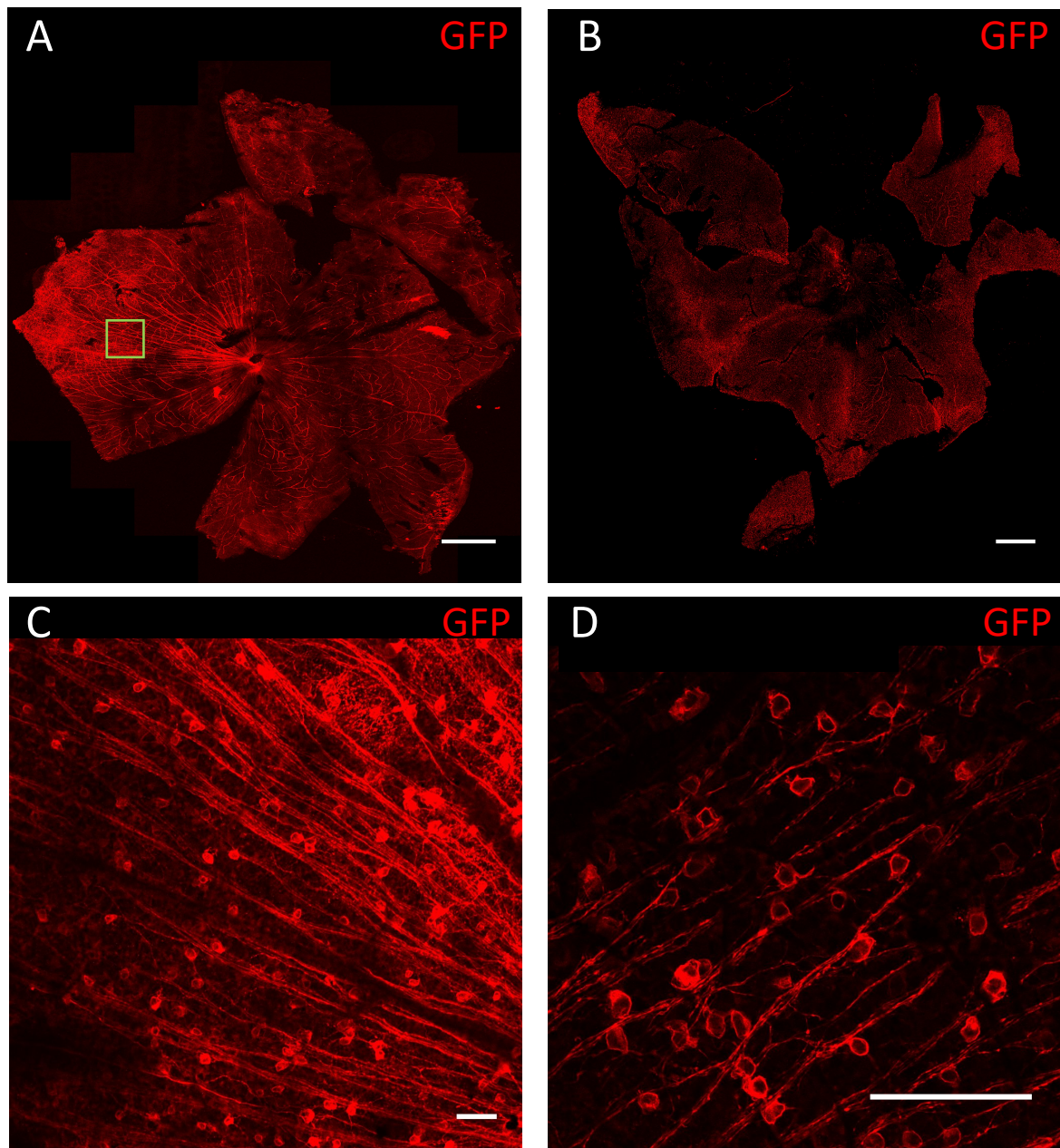


Figure 6-9 Confocal micrographs of a flat mount of a “CGRK” retina four weeks after injection with the hSyn-ReaChR construct.

- A. Low power tile of confocal images stitched to give view of whole retina – note areas of absence of staining in the lower retina. Green box gives approximation of location of the image in panel B. Scale bar 500µm
- B. A saline injected control stained for GFP and imaged as figure A. Scale bar 500µm
- C. Single low power (x20) image from figure A at ganglion cell layer. Again, note staining of RGC bodies and axons) Scale bar 50µm
- D. High power (x40) image of the Ganglion Cell layer from same retina. Note staining of cell bodies and axons of stained cells - indicating RGC identity. Scale bar 50µm

“CGRK” - ReaChR

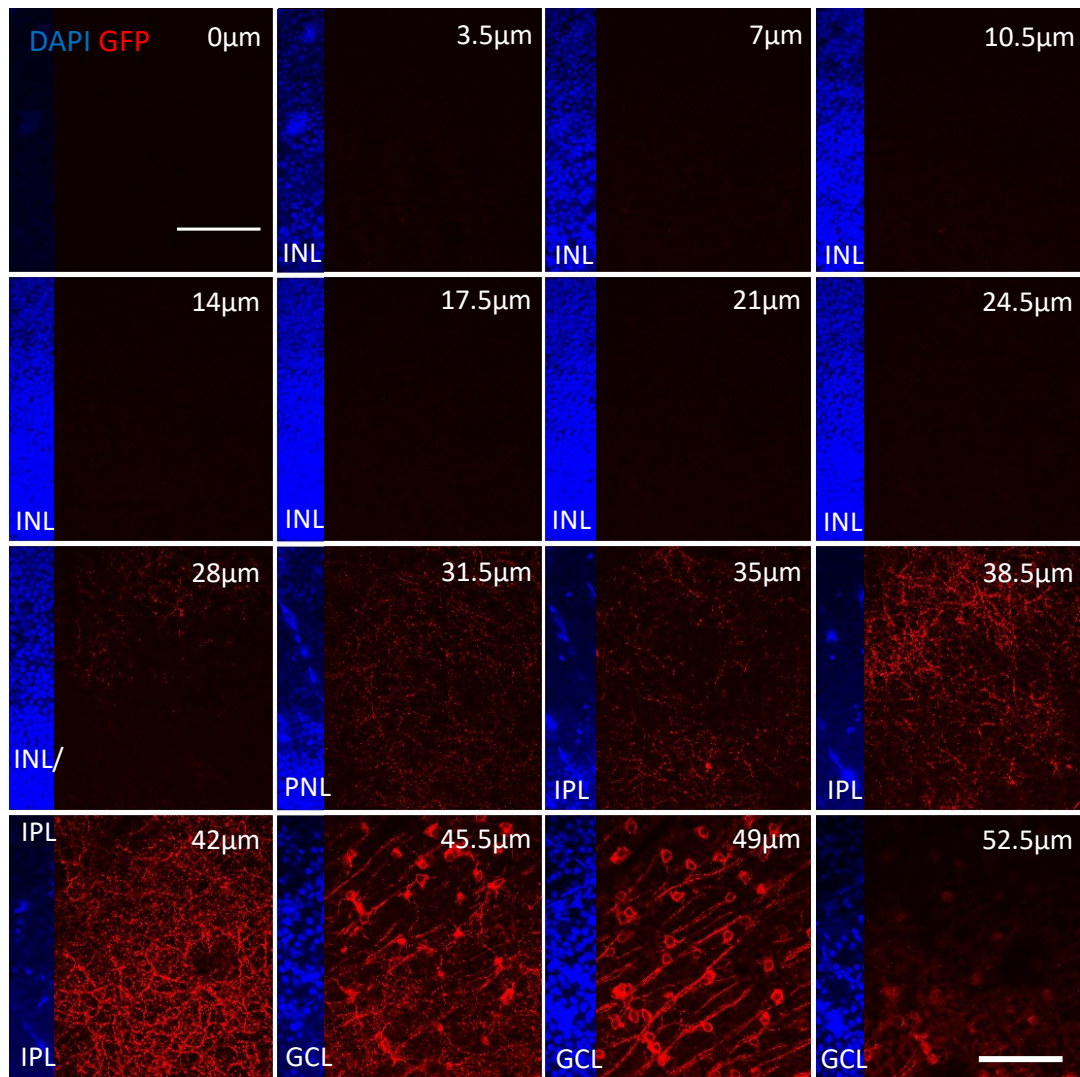


Figure 6-10 Montage of confocal micrographs of an “CGRK” retina four weeks after injection with the hSyn-reaChR construct. These represent slices of the same “Z stack” from the retina described in Figure Y. For clarity, the red channel (representing GFP (YFP-citrine) staining) is presented. Note in RGL staining of axons of transduced cells suggesting that they are retinal ganglion cells. The side panel in each pane represents the DAPI (nuclear) staining associated with that for orientation. The depths described in each panel refer to the distance in the Z plane from the top left panel (just below the retina). INL – inner nuclear layer. IPL – Inner plexiform layer. GCL – Retinal ganglion cell layer. Scale bar is 50μm

“CGRK” - ReaChR

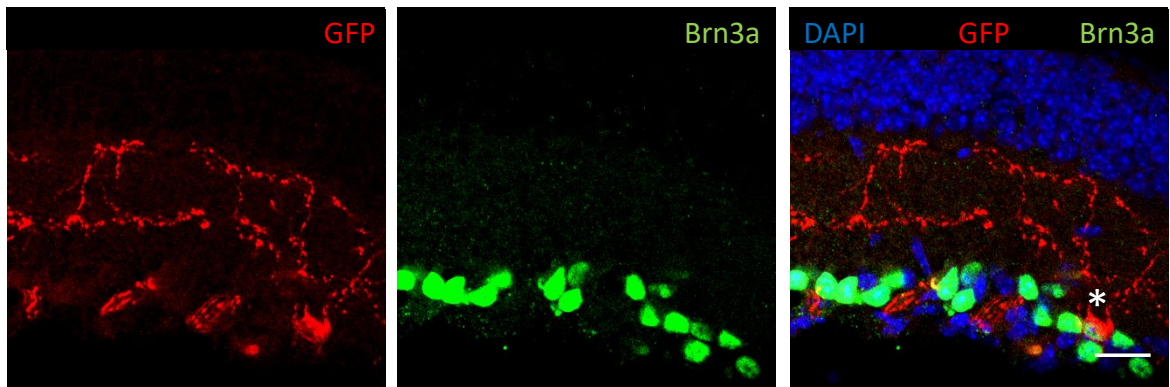


Figure 6-11 Cryosection from an “CGRK” retina four weeks after intravitreal injection with the hSyn ReaChR construct. Note staining of cell in GCL for Brn3a (RGC marker) and for GFP(ReaChR marker), indicating expression of the ReaChR construct in a RGC. (asterisk) Scale bar 20 μ m.

“LMAF” - Rhodopsin

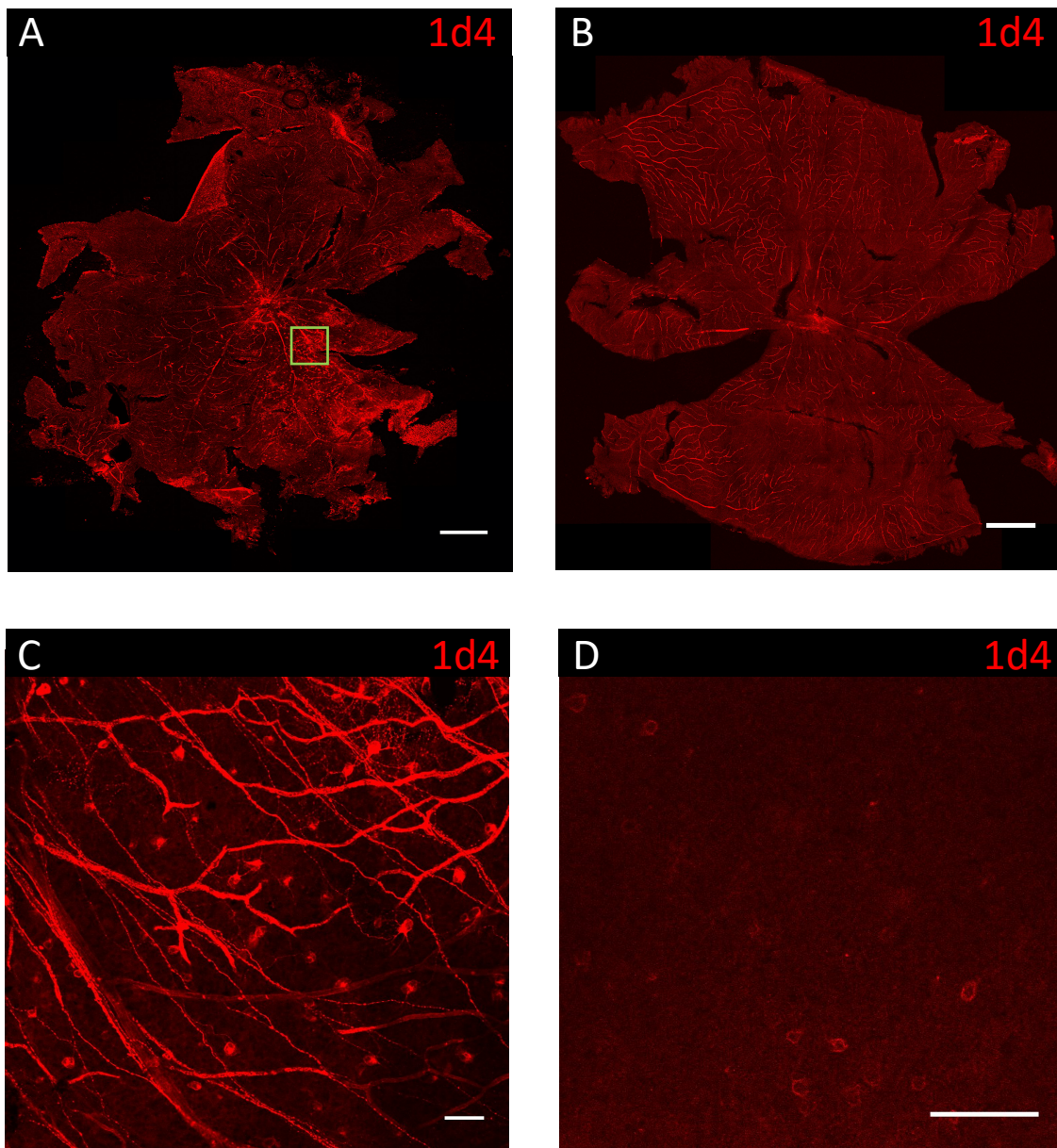


Figure 6-12 Confocal micrographs of a flat mount of an “LMAF” retina four weeks after injection with the “floxed” EF1 α -Rhodopsin construct.

- A. Low power tile of confocal images stitched to give view of whole retina – note poor staining other than inferiorly. NB. The 1d4 antibody is raised in a mouse, hence staining of vasculature in both treated and control retinæ. Green box gives approximation of location of the image in panel C. Scale bar 500 μ m
- B. A saline injected control stained for 1d4 and imaged as figure A. Scale bar 500 μ m
- C. Single low power (x20) image from figure A at ganglion cell layer. Again, note staining of RGC bodies and axons) Scale bar 50 μ m
- D. High power (x40) image of the inner nuclear layer from same retina. Overexposed compared to Figure 6-13 to highlight faint staining of cells in this layer. Scale bar 50 μ m

“LMAF” - Rhodopsin

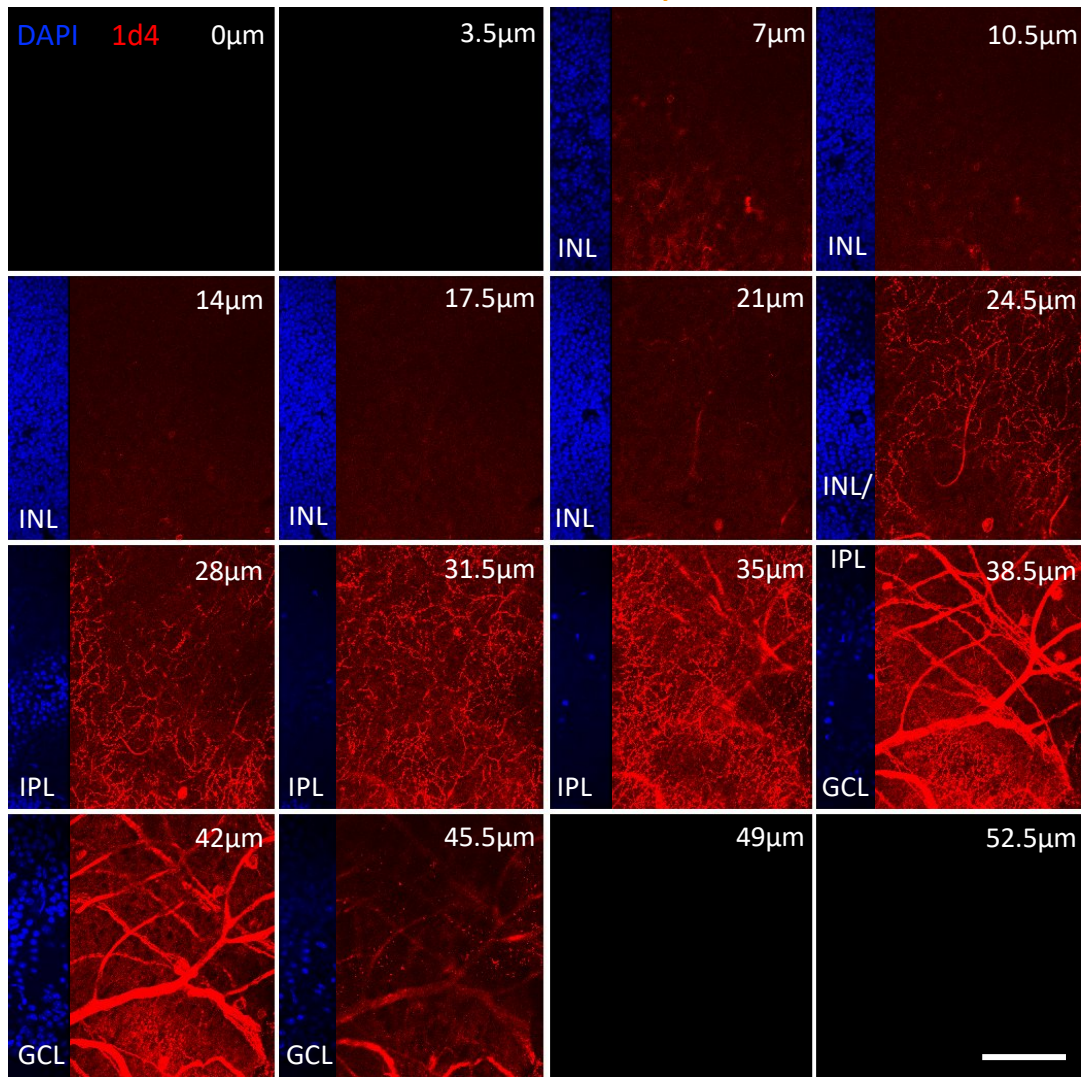


Figure 6-13 Montage of confocal micrographs of an “LMAF” retina four weeks after injection with the floxed EF1 α -Rhodopsin construct at x40 magnification. These represent slices of the same “Z stack.” The depths described in each panel refer to the distance in the Z plane from the top left panel (just below the retina). Side panels show DAPI nuclear staining to orientate to depth. INL – inner nuclear layer. IPL – Inner plexiform layer. GCL – Retinal ganglion cell layer. 1d4 antibody is raised in mouse, hence staining of vasculature. Scale bar is 50 μ m

“LMAF” - Rhodopsin

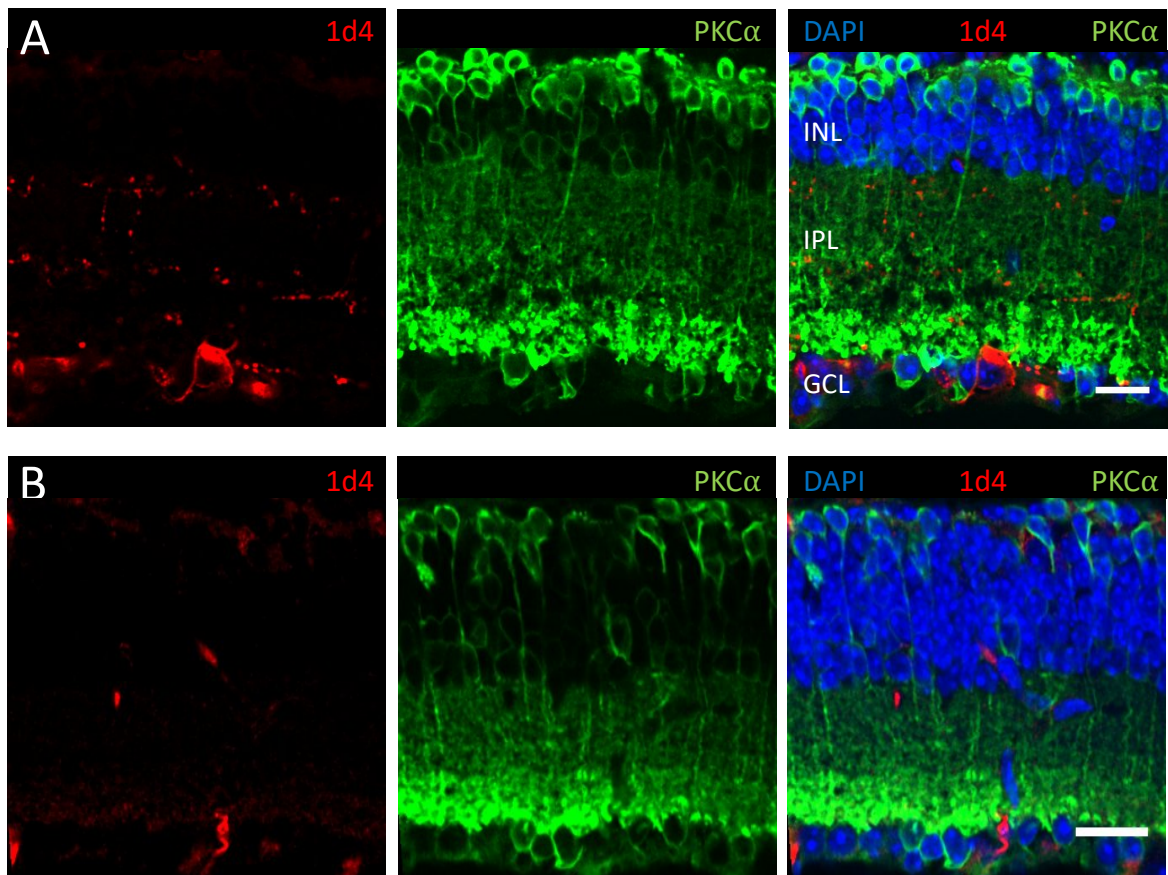


Figure 6-14 Cryo-sections from an “LMAF” retina four weeks after injection with the floxed EF1α-Rhodopsin construct.

- A. Cryo-section from an “LMAF” retina four weeks after injection with the floxed EF1α-Rhodopsin construct. Co-staining with 1d4 and PKCα, a marker for ON-bipolar cells. Note staining for 1d4 in ganglion cell layer with little in inner nuclear layer. Scale bar 20μm.
- B. Cryo-section from an “LMAF” retina four weeks after injection with the saline. Co-staining with 1d4 and PKCα, a marker for ON-bipolar cells. Scale bar 20μm

Retinal Coverage

A semi-quantitative index of retinal protein expression over whole retinae was obtained by manually scoring retina flat mounts, stained for GFP or 1d4, on a quadrant basis (see Chapter 2) (Figure 6-15). Both ReaChR groups show consistent retinal coverage in general, however staining with the rhodopsin construct was lower and more variable, suggesting patchy expression.

Retinal Electrophysiology

Reconstitution of electrophysiological responses to light

With evidence of protein expression in the expected cell populations of the degenerate retina, further investigation at a functional level was indicated. Therefore, eight “LMAF” and eight “CGRK” mice received bilateral intravitreal injections of the ReaChR virus, a further eight “LMAF” mice received the rhodopsin virus and a further eight of each a saline sham injection to act as controls. Mice were between six and eight weeks of age at injection and were culled nine weeks later for ex-vivo electrophysiology using multiple electrode arrays (MEAs).

As neither “LMAF” nor “CGRK” have any residual retinal photo-responses (being both *Pde6b*^{rd1/rd1} and *Opn4*^{-/-} - Chapter 3) the presence of any response to light would be indicative of successful transfection with, and function of, an optogenetic tool (Figure 6-16 & Figure 6-17).

Responsive electrodes

Recordings were successfully made of RGC firing from between five and eleven retinae in each group with light responses seen on between 5.0% and 24.1% of all possible electrodes (Figure 6-18 & Table 6-1).

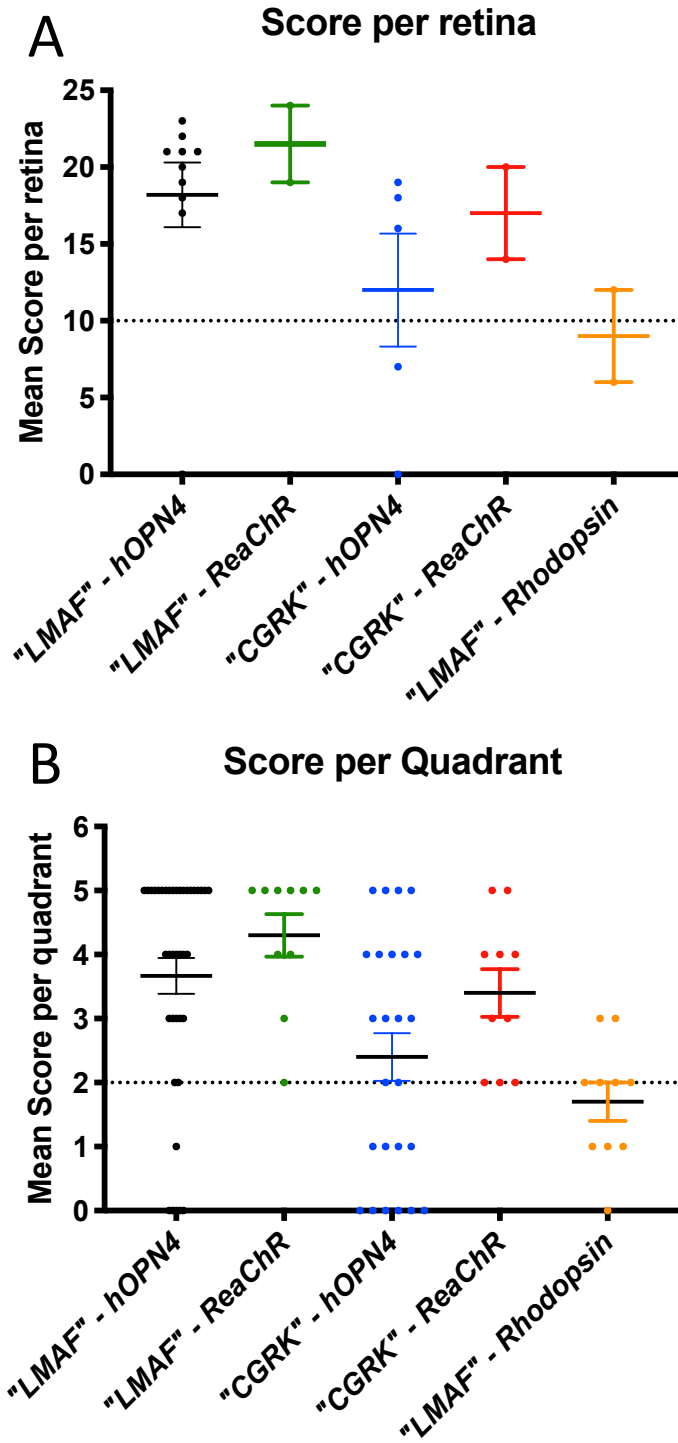


Figure 6-15 Semiquantitative indices of retinal flat mount staining of retina from either "LMAF" or "CGRK" mice treated with AAV containing either ReaChR or rhodopsin and IHC stained for GFP or 1D4 (see Chapter 2 for scoring methodology). Scores for hOPN4 virus from Chapter 5 are included on the graph for comparison.

A – Mean score per whole retina

B – Mean score per retinal quadrant

A light responsive electrode was defined as one with a mean rise of 10Hz in spike firing rate, maintained for 10 seconds (rolling 10 second bins) when compared to mean baseline firing rate in the 10 seconds preceding the stimulus. As discussed in Chapter 5, a small number (c.1%) of electrodes in the control groups were scored as reactive with this metric, but on manual review were seen to be spurious and so excluded.

There was a significant difference in the number of responsive electrodes between groups (One way ANOVA, $F_{(6,42)} = 6.270$; $p < 0.0001$), on post hoc testing this was seen only to be between treatment groups and their respective controls, not between treatment groups (Tukey's correction, "*LMAF*"- hOPN4 $p = 0.0113$, "*LMAF*"- ReaChR $p = 0.0093$, "*CGRK*" hOPN4 $p = 0.0069$, "*CGRK*" ReaChR $p = 0.0840$, "*CGRK*" hOPN4 $p = 0.0069$; "*LMAF*" – rhodopsin $p = 0.9975$ Figure 6-18).

Not every electrode was responsive to light in the treatment groups, which is to be expected due not only to variable AAV coverage, but also technical limitations in the preparation and apposition of the retinal tissue to the array. Without residual native responses however, only cells apposed to an electrode with optogenetically transduced light signals would generate an increase in spike firing rate in response to light. As areas of transfection are variable across the retina and the MEA only covers a very small area of the retina (c. 2mm²), the number of responsive electrodes on any individual trial will be stochastic.

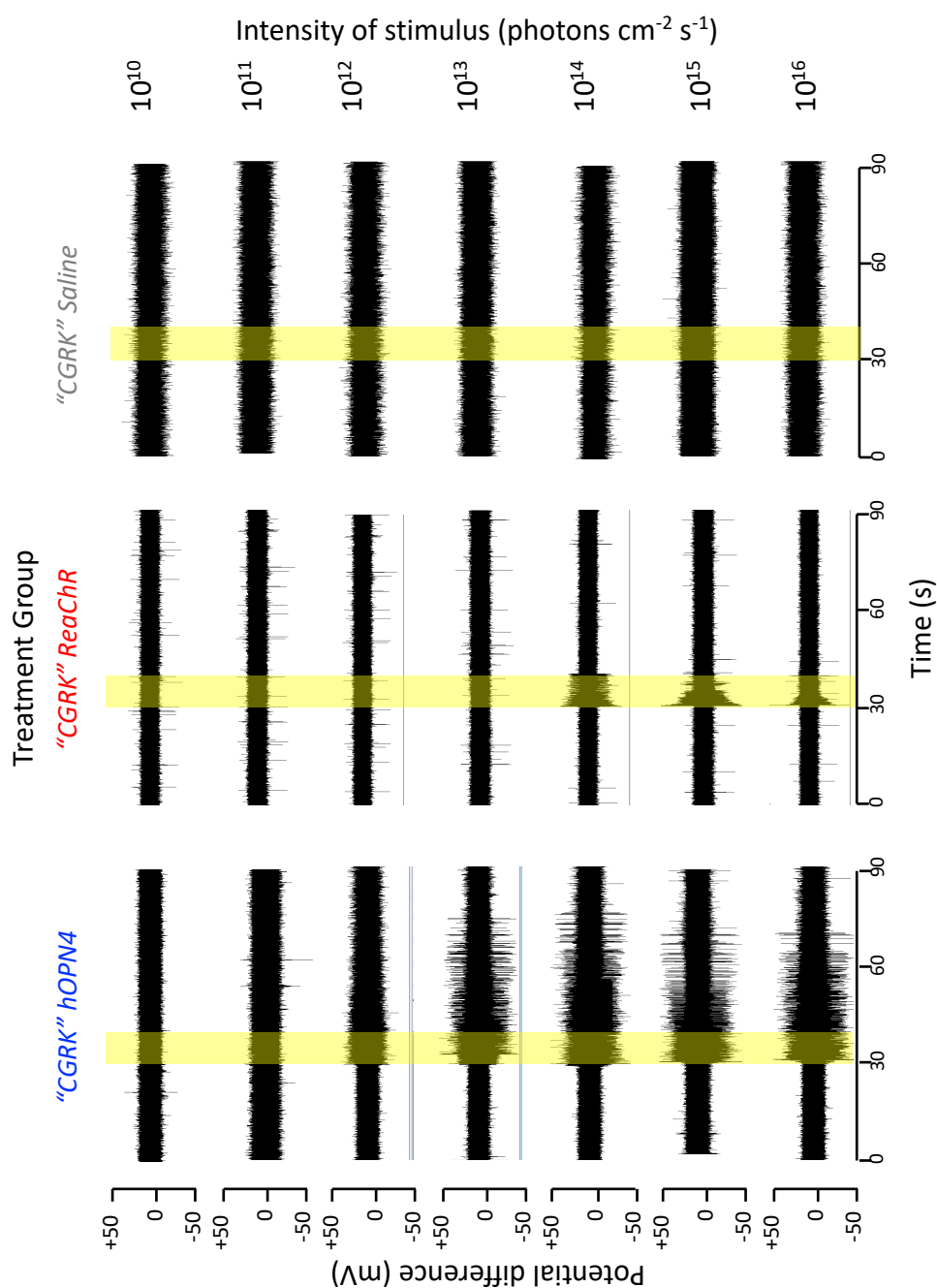


Figure 6-17 (this page) Example raw recording traces from a single electrode in each “CGRK” treatment group & controls stimulated with a ten second pulse of 480 or 510nm light (yellow bar) at seven light intensities (starting with 10^{10} photons $\text{cm}^{-2} \text{s}^{-1}$, with a 10 minute gap between each recording).

Figure 6-16 (previous page) Example raw recording traces from a single electrode in each “LMAF” treatment group & controls stimulated with a ten second pulse of 480 or 510nm light (yellow bar) at seven light intensities (starting with 10^{10} photons $\text{cm}^{-2} \text{s}^{-1}$, with a 10 minute gap between each recording).

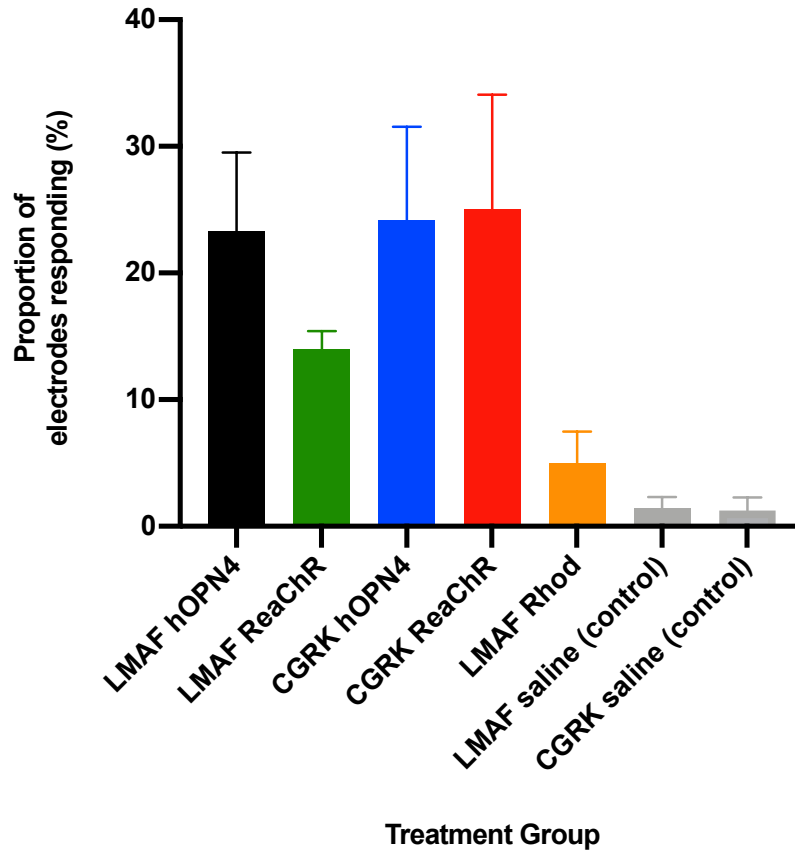


Figure 6-18 The proportion of electrodes in each group scored as responsive.

Treatments	LMAF hOPN4	LMAF ReaChR	CGRK hOPN4	CGRK ReaChR	LMAF Rhodopsin	LMAF Saline	CGRK Saline
Retinae (N=)	6	11	6	5	5	8	8
Total responsive electrodes (n=)	84	92	87	75	15 [†]	7*	6*
Total possible electrodes	360	660	360	300	300	480	480
Proportion	23.3%	14.0%	24.1%	25.0%	5%	1.4%	1.2%

Table 6-1 The proportion of electrodes in each group scored as responsive. Light responsiveness is defined as a rise in firing rate of 10Hz between 10 seconds before stimulus onset and ten seconds after onset.

*In both control groups, a small number of electrodes were scored as light responsive when the same criteria were applied. These traces were individually reviewed and found not to represent a light responses, but noise and were so excluded (see Chapter 5).

[†]The rhodopsin group, each of these was individually reviewed – see text for discussion.

Rhodopsin responses

Only a small number of electrodes in the "LMAF" - rhodopsin group were scored as demonstrating light responses by the above metric ($5.0 \pm 1.5\%$, over 5 retinae), in keeping with the lower titre of the rhodopsin AAV and lower levels of protein staining and patchy expression seen on IHC (see Figure 6-5). As can be seen in Figure 6-16, these responses appeared to comparatively transient. To further characterise these responses, detailed manual analysis was performed of these five retinae (but not directly compared to the other groups given the small number of responses seen).

To enable higher temporal resolution of these responses, data was re-examined using 100ms, rather than 1second binning and recordings from each retina manually reviewed. Responsive electrodes designated as those with an increase in spike firing of greater than 10Hz from the mean baseline rate (100ms bins) in the ten seconds preceding the stimulus and repeatably demonstrating light responses at least three intensities (10^{16} , 10^{15} and 10^{14} photons $\text{cm}^{-2} \text{ s}^{-1}$). This resulted in confirmation of 10 electrodes over 3 retinae demonstrating light responses (Figure 6-19a); the same manual review of the saline injected control retinae revealed no such responses in any electrode over both saline injected control groups. The finding of poor INL staining for rhodopsin (1d4) compared to GCL staining on immunohistochemistry would suggest a degree of caution in interpreting these findings too, the responses are likely to have a prominent contribution from transfected L7 ganglion cells(221), compared to ON-bipolar cells in this group. In addition, the fact that responses in rhodopsin treated retina result in increases in firing, could suggest coupling of rhodopsin to an excitatory second messenger system (rather than its native $G_{i/o}$) in these 'ON' cells.

Light responses in the rhodopsin treated retina were short (1.58 ± 0.13 s, $n=10$) and occurred in the absence of exogenous 9-*cis* retinal in the perfusion solution, in keeping with previous reports(103, 106) The addition of 9-*cis* retinal may potentiate responses (Figure 6-19c) with 3 additional electrodes becoming active (Figure 6-20b) when it was supplied. Maximum increases in spike firing rate showed an increase with brighter stimuli (Figure 6-20a) (two way ANOVA $F_{(6,182)}=22.20$, $p<0.0001$) and with increasing retinal concentration (Figure 6-20c) ($F_{(2,182)}=8.081$, $p=0.0004$) but no interaction between the two ($F_{(12,182)}=0.6823$, $p=0.7671$). Post Hoc testing (Dunnett's multiple comparisons test) however revealed significantly increased responses with $4\mu\text{mol/l}$ retinal at 10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$ and with $40\mu\text{mol/l}$ retinal at 10^{16} photons $\text{cm}^{-2} \text{s}^{-1}$. Together, this may suggest a potentiation of responses by addition of retinal in this study, however the low number of responses seen here is noted.

In summary, rhodopsin delivered by intravitreal AAV and restricted to L7 expression ON-bipolar cells is able to induce transient (ON-type) light responses and these are potentially able to be potentiated by exogenous 9-*cis* retinal. However, few such responses were recorded, most likely due to poor viral transfection and are therefore not included further in the comparison between treatment groups.

Comparison of electrophysiological light responses

In comparing the remaining treatment groups, raw recordings from an example electrode in each group are provided in Figure 6-16 & Figure 6-17, these raw recordings were processed in real time to give an instantaneous rate of spike firing, per electrode, that could be plotted against time. These are shown in Figure 6-21 & Figure 6-22 for all electrodes on a single retina as retinotopic arrays of graphs with a magnified example of a representative electrode's trace. Examples of hOPN4 treated

retina (Chapter 5) are provided alongside for comparison. A mean of spike firing traces (incorporating all active electrodes) in response to full field light pulses of increasing intensity is presented in Figure 6-23 with all groups superimposed in the lower panel to facilitate comparison. To aid this comparison, the mean spike firing trace response of each group is redrawn at a larger scale in Figure 6-24b - this is in response to a 10 second, 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$ light pulse (480nm for hOPN4, or 510nm for ReaChR ($\lambda_{\text{max}} = 510$ in retinal cells (357))).

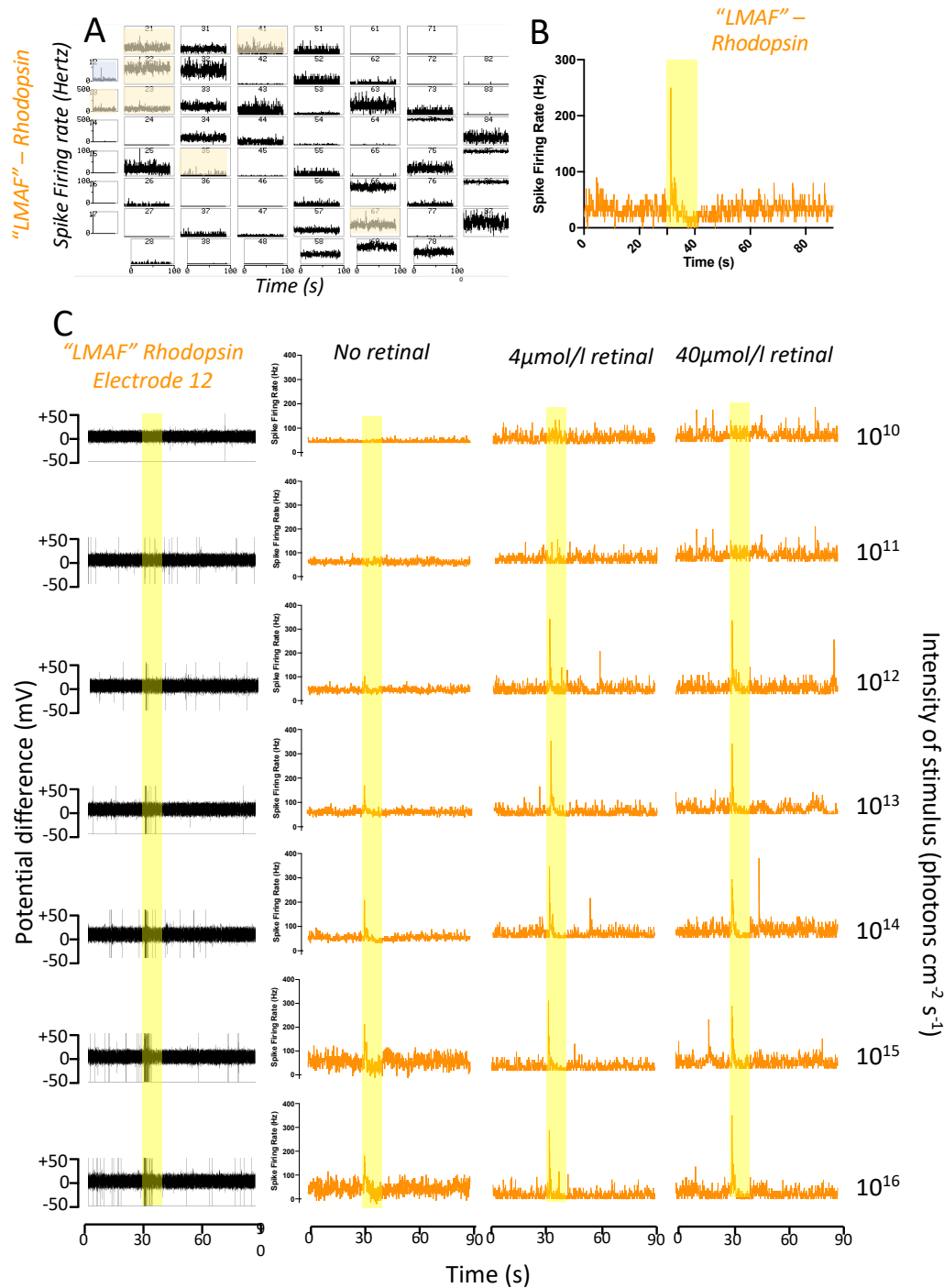


Figure 6-19

- A. An array of spike firing rate traces from a rhodopsin treated retina in response to a 10 second light stimulus (410nm, 10^{14} photons $\text{cm}^{-2} \text{s}^{-1}$) after 30 seconds of recording (100ms bins). Electrodes manually scored as responsive are shaded in yellow. Electrode No. 12 (highlighted in blue) has been redrawn in the right panel. NB. Electrode 25, this response was not repeatable and was therefore not scored as light responsive.
- B. The spike firing response of electrode 12 redrawn.
- C. The Raw data and spike firing traces (100ms bins) from this same electrode (12) to a 10 second light stimulus (410nm, after 30 seconds of recording each of seven intensities of light stimulus (10^{10} to 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$) either without, or with two concentrations of 9-cis retinal in the perfusion solution. (Stock solution of 40mmol/l 9-cis-retinal diluted in stock solution 4 or 40 $\mu\text{mol/l}$)

"LMAF" Rhodopsin – mean response of active electrodes

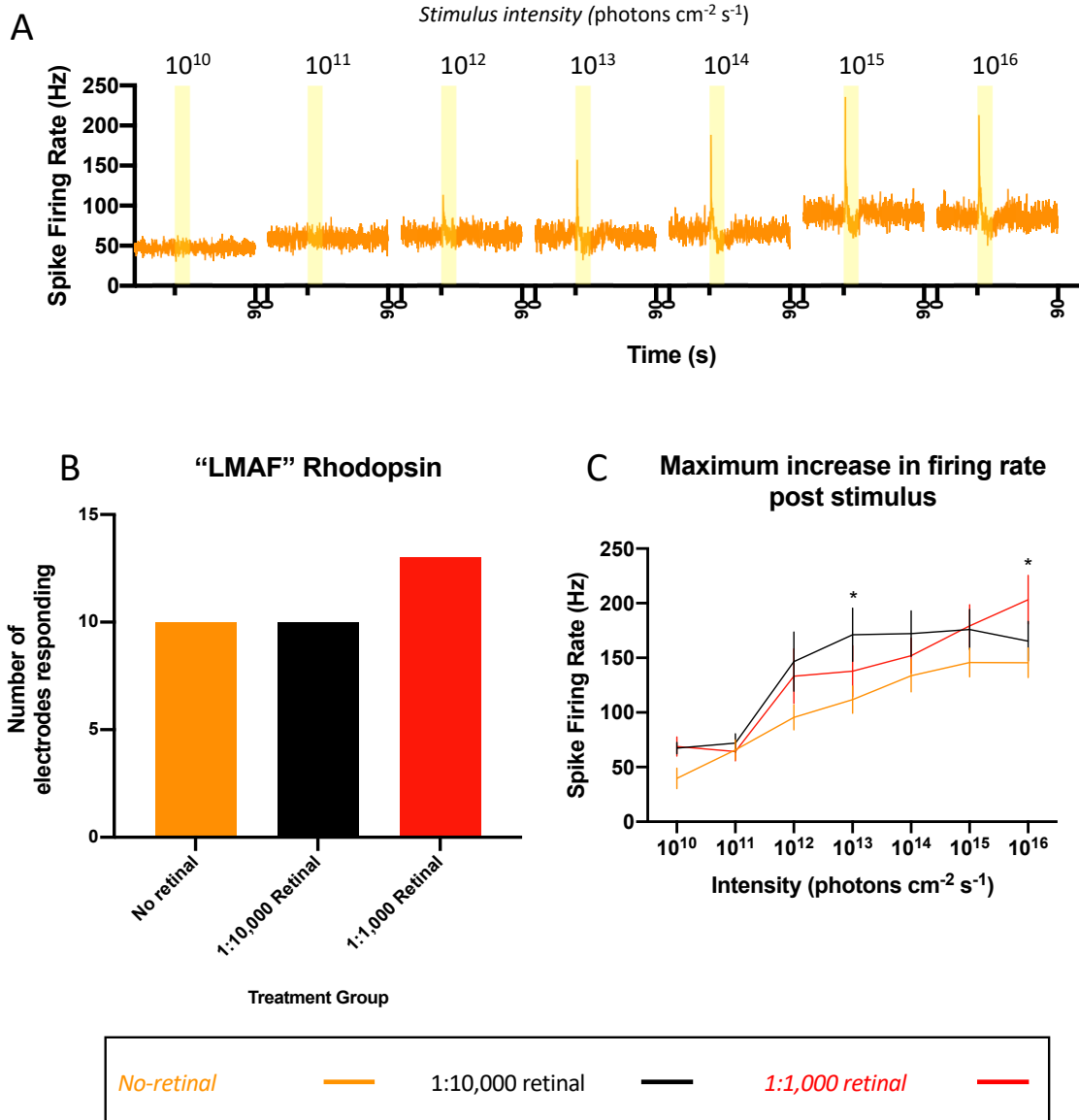


Figure 6-20

A. An averaged spike firing response obtained by taking the mean of responses from all responsive electrodes in each group in response to a 10 second light pulse (510nm) after 30 seconds of recording at increasing intensities. Timing of light pulse is indicated by yellow bar.

B. Traces from Rhodopsin treated retinæ were manually reviewed with ten being seen to display responses following a 510nm, 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$ light pulse (see text). 40mmol/l 9-cis Retinal was added to the perfusion solution at either 1:10,000 or 1:1,000 and trials repeated. This was seen to lead to an additional three electrodes demonstrating responses.

C. Stimuli at 7 intensities (510nm, 10^{10} to 10^{16} photons $\text{cm}^{-2} \text{s}^{-1}$) were performed with each concentration of 9-cis retinal in the solution. A two way ANOVA showed an increase with intensity ($F_{(6,182)}=22.20$, $p<0.0001$) and a variation with retinal concentration ($F_{(2,182)}=8.081$, $p=0.0004$) but no interaction ($F_{(12,182)}=0.6823$, $p=0.7671$). Post Hoc testing (Dunnnett's multiple comparisons test) however revealed only instances of significantly increased response 1:10,000 at 10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$ and 1:1,000 at 10^{16} photons $\text{cm}^{-2} \text{s}^{-1}$

In order to further investigate this impression, traces from light responsive electrodes were analysed in order to further characterise the size, kinetics and sensitivity of these responses.

Firing rates

In order to compare the size of responses between groups, firing rates before and in response to stimulus onset were obtained (see Chapter 2, Figure 6-25) and compared using two-way ANOVAs with post hoc testing between groups using Tukey's correction for multiple comparisons. For brevity, ANOVA results are first presented here in the text, with significant post hoc test results detailed in Figure 6-25, followed by a description in the text of the significance of these results under the next heading "Firing rates conclusions" (full ANOVA & post hoc tables are included in the appendix 5).

Mean baseline (pre-stimulus) firing, as may be expected, did not vary with intensity ($F_{(6,2371)}=0.3862$, $p=0.8883$) but did vary with group ($F_{(3,2371)}=133.5$, $p<0.0001$), with generally higher firing rates in ReaChR transfected groups (especially "CGRK" - *ReaChR*, there was no interaction between intensity and treatment group ($F_{(18,2371)}=0.4955$, $p=0.9613$).

Similarly, the maximum baseline (pre-stimulus) firing rate did not vary with intensity ($F_{(6,2336)}=0.1481$, $p=0.9895$) but did with group ($F_{(3,2336)}=90.78$, $p<0.0001$), again with generally higher rates in the ReaChR groups. There was no interaction between intensity and treatment group ($F_{(18,2336)}=0.6683$, $p=0.8452$).

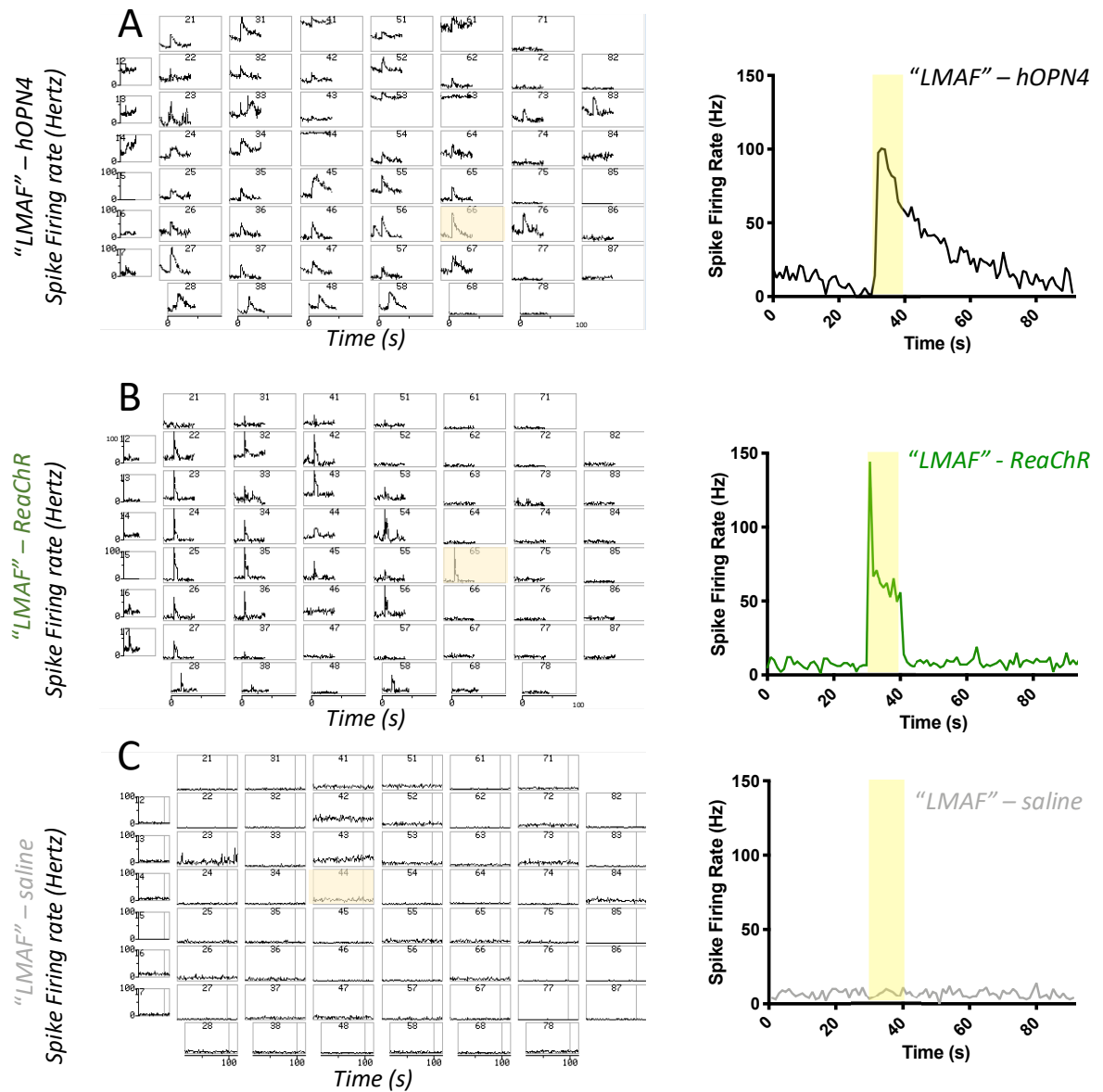


Figure 6-21 For each "LMAF" group, an array of spike firing rate traces from one retina is shown on the left.

On the right, one of the electrode traces (indicated by yellow shading on array) is redrawn at higher magnification. The yellow bar indicates the 10 second light stimulus (510nm , $10^{15}\text{ photons cm}^{-2}\text{ s}^{-1}$) after 30 seconds of recording.

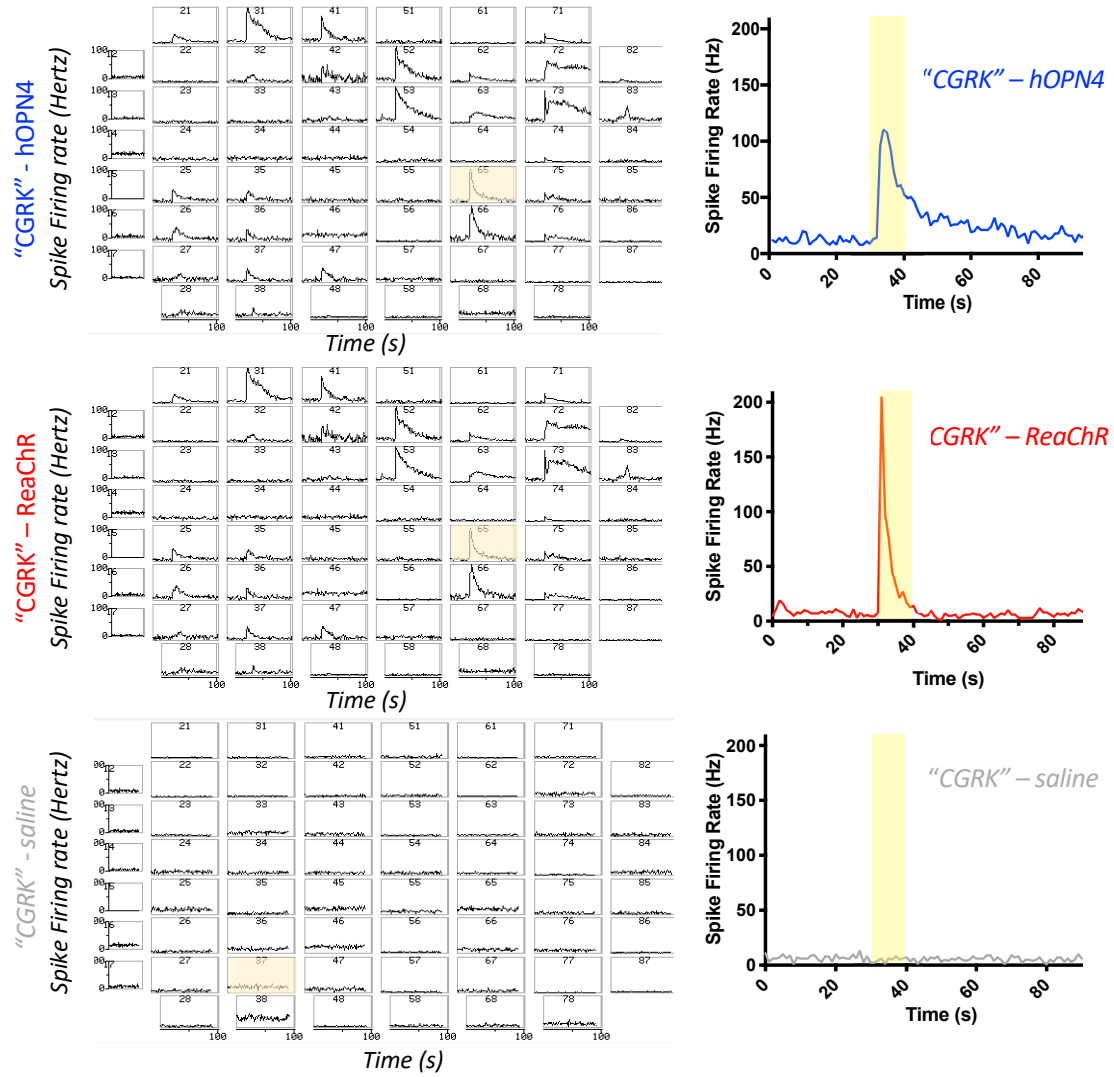


Figure 6-22 For each "CGRK" group, an array of spike firing rate traces from one retina is shown on the left. On the right, one of the electrode traces (indicated by yellow shading on array) is redrawn at higher magnification. The yellow bar indicates the 10 second light stimulus (510nm, 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$) after 30 seconds of recording.

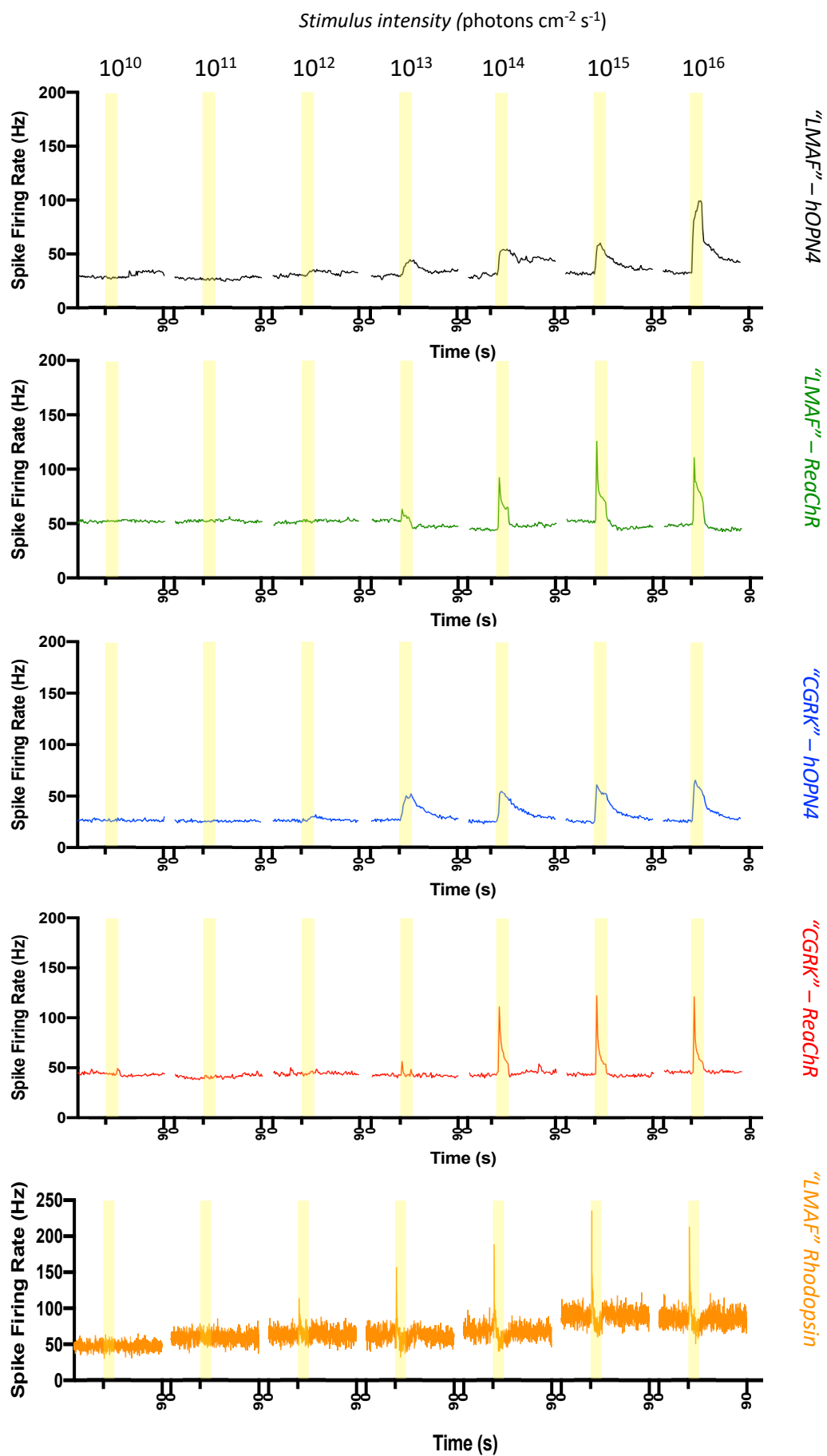


Figure 6-23 - see two pages on for figure legend

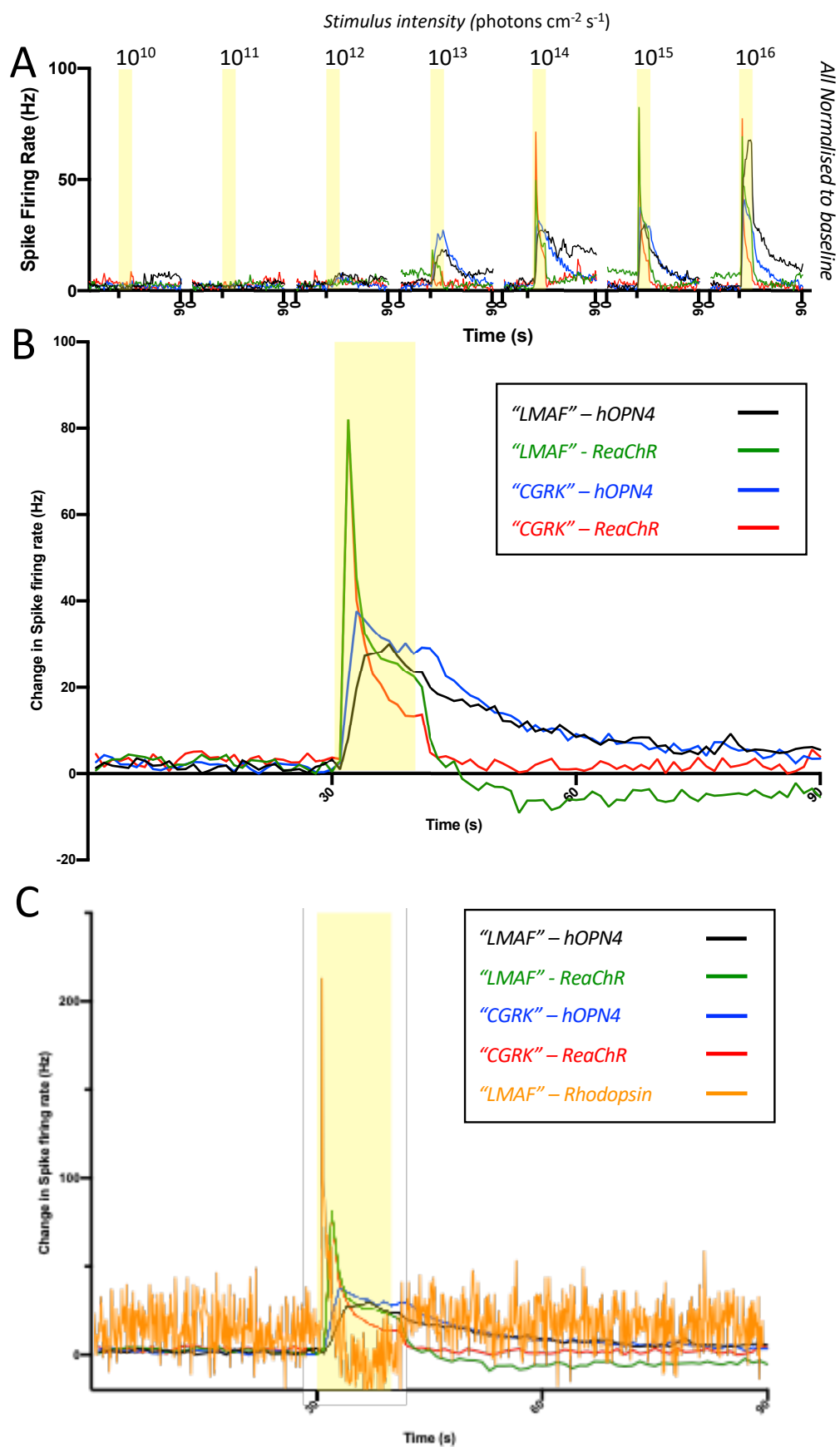


Figure 6-24 – see next page for figure legend

Figure 6-23 – An averaged spike firing response obtained by taking the mean of responses from all responsive electrodes in each group in response to a 10 second light pulse after 30 seconds of recording at increasing intensities. Timing of light pulse is indicated by yellow bar.

The same traces, each normalised to their lowest value and superimposed on a single graph to facilitate comparison are provided in Figure 6-24.

For control retina, please see Chapter 5

Figure 6-24 Mean of spike trace recordings averaged from all responsive electrodes in each group and normalised to lowest recorded baseline firing rate. Yellow bars represent light pulses.

- A. Responses at 7 intensities, superimposed.*
- B. Responses to a (480 / 510nm, 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$) light pulse (yellow box) for the four treatment groups*
- C. The same traces as in “B” redrawn at larger scale and corresponding rhodopsin response superimposed for comparison (note much larger and more transient response).*

Mean post stimulus firing rates also demonstrated significant difference: increasing with brighter stimuli ($F_{(6, 2371)}=87.01$, $p<0.0001$) and varying by treatment group ($F_{(3,2371)}=66.51$, $p<0.0001$), with an interaction between the two factors ($F_{(18,2371)}=3.153$, $p<0.0001$). The magnitude of spike firing changes is largest in ReaChR groups, especially the “LMAF” - ReaChR group.

A similar relationship is seen in maximum post stimulus firing rates: with responses increasing with brighter stimuli ($F_{(6,2336)}=152.9$, $p<0.0001$) and varying between treatment group ($F_{(3,2336)}=61.51$, $p<0.0001$) with an interaction between the two ($F_{(18,2336)}=8.481$, $p<0.0001$) again with larger increases in firing rate in ReaChR treated groups in response to brighter stimuli.

Firing rates: conclusions

This study demonstrates, as may be expected, a relationship of higher firing rates in response to brighter stimuli in all treatment groups. This is particularly marked in ReaChR treated groups, with larger increases in spike firing compared to hOPN4 treated groups exposed to the same stimulus; but differences between ReaChR groups was equivocal. Comparable absolute values for baseline firing have not been reported for ReaChR in degenerate retinæ(354, 357), however for other channelrhodopsins, values (c. 30Hz(102)) have been: below the values recorded here. In addition, our firing rates for melanopsin treated cells appear congruent with previous reports in RGCs (around 20Hz(159)). In addition, higher baseline firing rates are seen in ReaChR treated groups, especially in the “CGRK” model, compared to hOPN4 treated ones.

Response Kinetics

Indices of onset and offset response kinetics were obtained (see Chapter 2, Figure 6-26) and similarly compared using two-way ANOVAs with post hoc testing between groups using Tukey's correction for multiple comparisons.

Rates of spike firing before and after stimulus

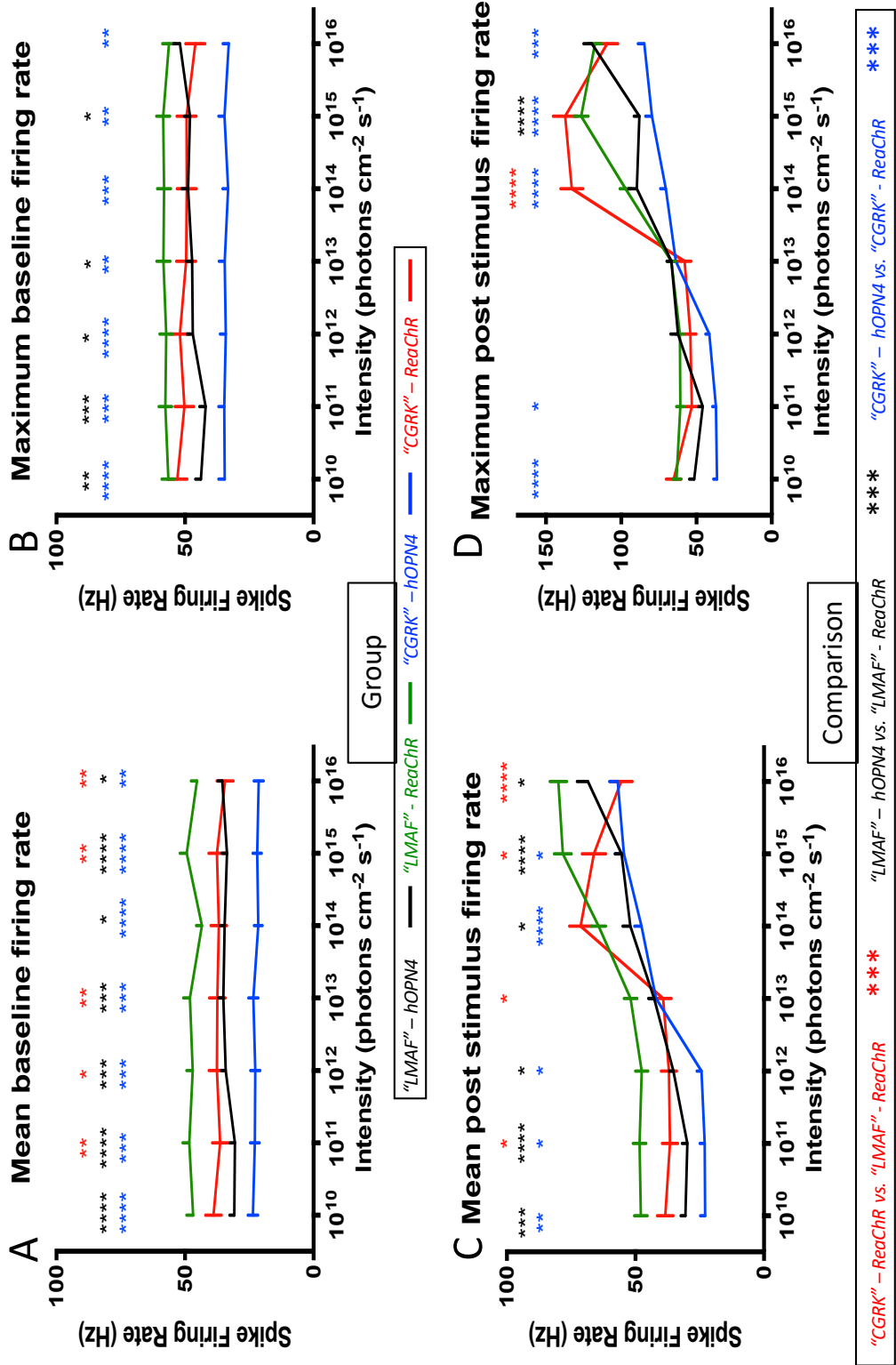


Figure 6-25 – please see next page for figure legend.

Figure 6-25 Base line and post stimulus firing rates (1 second bins) compared between groups.

For each metric, a two way ANOVA was performed with Tukey's correction for multiple comparisons. Asterisk in all panels refer to the p value of this post hoc test. Only significant differences are starred on the graph and included below. For ANOVA results, please see text and for full post hoc test results please see appendix.

"LMAF" – hOPN4 N=6 retinæ; n=84 electrodes

"LMAF" - ReaChr N=13 retinæ, n=108 electrodes

"CGRK" – hOPN4 N=7 retinæ; n=87 electrodes

"CGRK" – ReaChr N=5 retinæ; n=75 electrodes

Red asterisks refer to comparisons between ReaChr groups, black to comparisons between "LMAF" groups and blue to comparisons between "CGRK" groups.

A. The mean baseline firing rate in the 10 seconds preceding the stimulus.

Red asterisks: 10^{11} $p=0.0024$, 10^{12} $p=0.0295$, 10^{13} $p=0.0088$, 10^{15} $p=0.003$, 10^{16} $p=0.0079$

Black asterisks: 10^{10} $p<0.0001$, 10^{11} $p<0.0001$, 10^{12} $p=0.0006$, 10^{13} $p=0.0005$, 10^{14} $p=0.0361$, 10^{15} $p<0.0001$, 10^{16} $p=0.0145$

Blue asterisks: 10^{10} $p<0.0001$, 10^{11} $p=0.0006$, 10^{12} $p=0.0001$, 10^{13} $p=0.0004$, 10^{14} $p<0.0001$, 10^{15} $p<0.0001$, 10^{16} $p=0.0100$

B. The maximum baseline firing rate in the ten seconds preceding the stimulus.

Black asterisks: 10^{10} $p=0.0054$, 10^{11} $p=0.0003$, 10^{12} $p=0.0362$, 10^{13} $p=0.0194$, 10^{15} $p=0.0347$

Blue asterisks: 10^{10} $p<0.0001$, 10^{11} $p=0.0007$, 10^{12} $p<0.0001$, 10^{13} $p=0.0011$, 10^{14} $p=0.0004$, 10^{15} $p=0.0013$, 10^{16} $p=0.0074$

C. The mean firing rate in the 10 seconds following the stimulus onset

Red asterisks: 10^{11} $p=0.0272$, 10^{13} $p=0.0152$, 10^{15} $p=0.0224$, 10^{16} $p<0.0001$

Black asterisks: 10^{10} $p=0.0002$, 10^{11} $p<0.0001$, 10^{12} $p=0.0148$, 10^{14} $p=0.0145$, 10^{15} $p<0.0001$, 10^{16} $p=0.0263$

Blue asterisks: 10^{10} $p=0.0021$, 10^{11} $p<0.0001$, 10^{12} $p=0.0148$, 10^{14} $p<0.0001$, 10^{15} $p=0.0368$

D. The maximum firing rate in the 10 seconds following the stimulus onset

Red asterisks: 10^{14} $p<0.0001$

Black asterisks: 10^{15} $p<0.0001$

Blue asterisks: 10^{10} $p<0.0001$, 10^{11} $p=0.0496$, 10^{14} $p<0.0001$, 10^{15} $p<0.0001$, 10^{16} $p=0.0005$

Again, for brevity, ANOVA results are presented here in the text, with significant post hoc test results detailed in Figure 6-26, followed by a description in the text of the significance of these results under the next heading “Kinetics conclusions” (full ANOVA & post hoc tables are again included in the appendix).

The time between stimulus and response onset reduced with increasing intensity ($F_{(6,2424)}=45.42, p<0.0001$) but this did not differ between treatment groups, ($F_{(3,2424)}=1.090, p=0.3522$) and there was no interaction between intensity and treatment group ($F_{(18, 2424)}=0.7939, p=0.7097$).

Similarly, the time to maximum response rate reduced significantly with increasing stimulus intensity ($F_{(6,3352)}=116.8, p<0.0001$) this varied with treatment group ($F_{(3,3352)}=6.886, p=0.0001$) with the “LMAF” hOPN4 group taking significantly longer to reach maximum firing rate compared to “LMAF” – ReaChR. There was also an interaction between intensity and treatment group ($F_{(18,3352)}=4.250, p<0.0001$).

Metrics of the offset of responses also showed differences. Time to half maximum response ($t_{1/2}$) increased with increasing stimulus intensity ($F_{(6,3407)}=151.2, p<0.0001$), varied with treatment group ($F_{(3,3407)}=107.1, p<0.0001$) and there was an interaction between the two ($F_{(18,3407)}=14.81, p<0.0001$). Again suggesting, that brighter stimuli led to longer $t_{1/2}$, but that this was much more pronounced in melanopsin treated groups, particularly when targeted to retinal ganglion cells in the “CGRK” – hOPN4 group.

Response kinetics

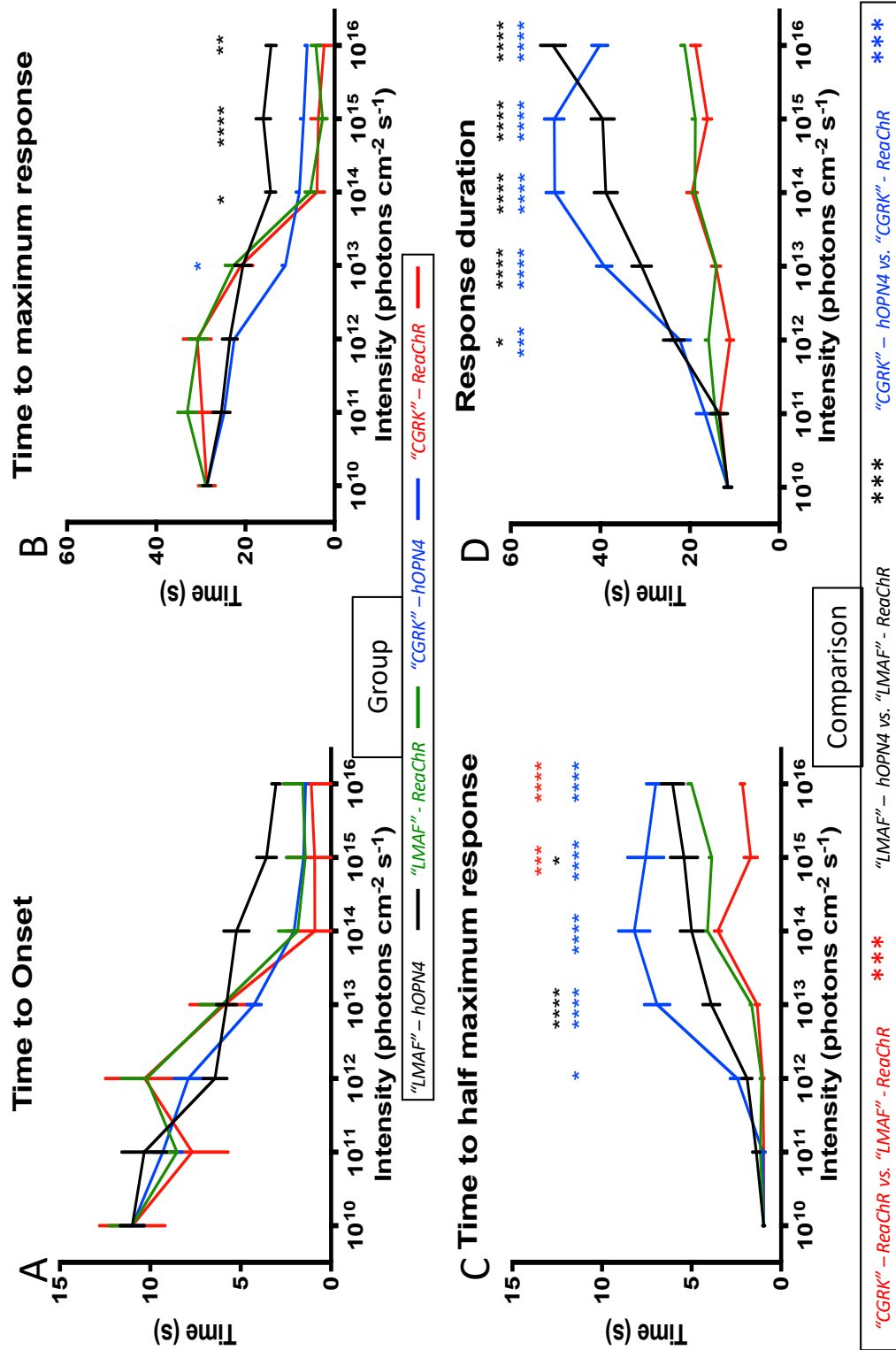


Figure 6-26 – please see next page for figure legend.

Figure 6-26 Response kinetics (1 second bins) compared between groups.

For each metric, a two-way ANOVA was performed with Tukey's correction for multiple comparisons. Asterisk in all panels refer to the p value of this post hoc test. Only significant differences are starred on the graph and included below. For ANOVA results, please see text and for full ANOVA table and post hoc test results please see appendix.

"LMAF" – hOPN4 N=6 retinæ; n=84 electrodes

"LMAF" - ReaChr N=13 retinæ, n=108 electrodes

"CGRK" – hOPN4 N=7 retinæ; n=87 electrodes

"CGRK" – ReaChr N=5 retinæ; n=75 electrodes

Red asterisks refer to comparisons between ReaChr groups, black to comparisons between "LMAF" groups and blue to comparisons between "CGRK" groups.

A. The time between stimulus onset and response onset

B. The time between stimulus onset and maximum firing rate (1 second bins)

Black asterisks: 10^{14} $p=0.0215$, 10^{15} $p<0.0001$, 10^{16} $p=0.0059$

Blue asterisk: 10^{13} $p=0.0105$

C. Time time for the response to fall from its maximum value to half that value (1 second bins)

Red asterisks: 10^{15} $p=0.0002$, 10^{16} $p<0.0001$

Black asterisks: 10^{13} $p<0.0001$, 10^{15} $p<0.0106$

Blue asterisks: 10^{12} $p=0.0347$, 10^{13} $p<0.0001$, 10^{14} $p<0.0001$, 10^{15} $p<0.0001$, 10^{16} $p<0.0001$

D. The duration of responses

Black asterisks: 10^{12} $p=0.0138$, 10^{13} $p<0.0001$, 10^{14} $p<0.0001$, 10^{15} $p<0.0001$, 10^{16} $p<0.0001$

Blue asterisks: 10^{12} $p=0.0002$, 10^{13} $p<0.0001$, 10^{14} $p<0.0001$, 10^{15} $p<0.0001$, 10^{16} $p<0.0001$

Similarly, the overall duration of response increased with increasing stimulus intensity ($F_{(6,2534)}=158.0$, $p<0.0001$) and varied with group ($F_{(3,2534)}=220.8$, $p<0.0001$) – with hOPN4 groups showing significantly longer responses at all intensities above 10^{12} photons $\text{cm}^{-2} \text{s}^{-1}$. There was also an interaction between intensity and treatment group ($F_{(18,2534)}=28.17$, $p<0.0001$).

Kinetics: conclusions

This study demonstrates, as expected, that brighter stimuli lead to faster onset of responses with slower returns to baseline. Between groups in this study, there were few differences in onset kinetics – notably hOPN4 in “CGRK” mice having a comparable time to maximal response to ReaChR in the same group (while hOPN4 in “LMAF” mice was slower than ReaChR).

Differences in offset kinetics were more marked, with significantly shorter responses in both ReaChR groups (but no significant difference between them). Times to half maximal responses were in general shorter in the ReaChR groups, however at higher intensities “CGRK” – ReaChR responses were shorter still than their “LMAF” – ReaChR counterparts.

Irradiance Response Curves

Stimuli of varying intensity also allowed a relationship between intensity and response size to be modelled (Chapter 2). A sigmoidal curve was fitted to the response of each responsive electrode. Fits were made either with a constant Hill slope value of 1, or with the Hill slope value allowed to vary to achieve the best fit (Figure 6-27)

With the latter, the mean Hill-slope values giving the best fit were significantly different from 1 in all groups (all $p < 0.0001$), except the “CGRK” – *ReaChR* ($p = 0.4903$, Wilcoxon signed rank test). Therefore, as described in Chapter 5, for consistency, a variable Hill slope approach to curve fitting was used in further comparisons (with fixed slope fits provided in Figure 6-28 for information).

The number of responsive electrodes with a dose response relationship fitting well to the sigmoidal relationship was 20 of the 84 total responsive electrodes (24%) in the “LMAF” – *floxed hOPN4* group, 68 of 108 (63%) in the “LMAF” – *floxed ReaChR*, 59 of 87 (68%) in the “CGRK” – *floxed hOPN4* and 34 of 75 (45%) in the “CGRK” – *floxed ReaChR*. A mean of the EC_{50} (excitatory concentration for 50% response) of best fit of all the electrodes in each treatment group were taken and used to produce a mean irradiance response curve (IRC) for each group (Figure 6-29 & Figure 6-30). Mean EC_{50} by group was respectively: “LMAF” – *floxed hOPN4* 13.64 ± 0.21 , “LMAF” – *floxed ReaChR* 13.03 ± 0.06 , “CGRK” – *floxed hOPN4* 13.03 ± 0.06 and “CGRK” – *floxed ReaChR*. $13.86 \pm 0.06 \log_{10} \text{ photons cm}^{-2} \text{ s}^{-1}$.

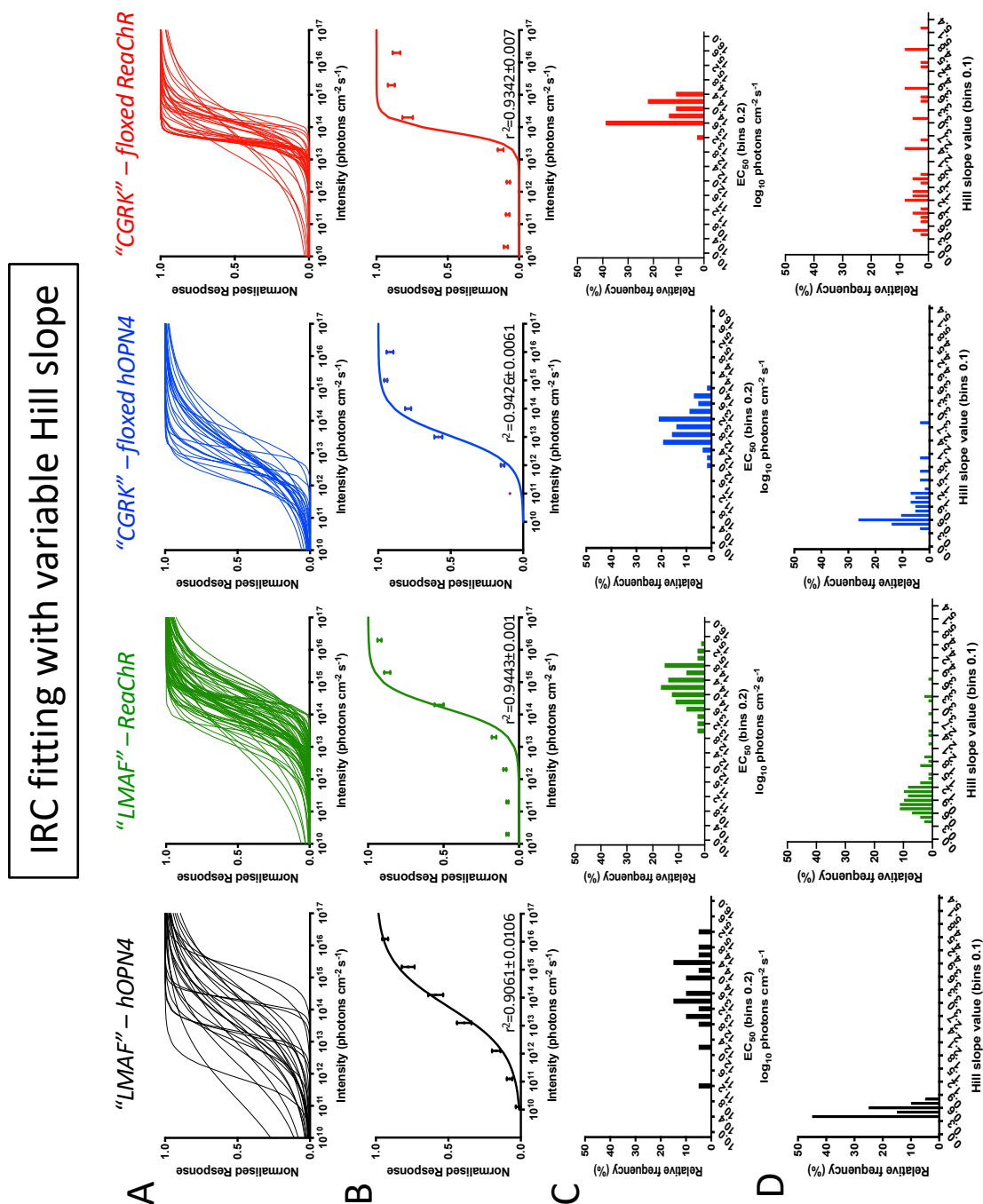


Figure 6-27 Curve fitting of Irradiance response curves (IRCs). In this figure, the Hill slope is allowed to vary to achieve the best fit.

A – All electrodes in each group, fitted using a variable Hill slope. Only electrodes with a good fit ($r^2 > 0.8$) are plotted.

B – The mean EC_{50} from all individual electrodes was used to plot a mean IRC curve; the mean $\pm$ SEM of r^2 values for the individual electrodes is shown. Also plotted on the graph (as round points) are the mean $\pm$ SEM of the normalised responses at each intensity tested.

"LMAF" – hOPN4; $EC_{50}=13.64 \pm 0.21$; Hill slope 0.5143 ± 0.03 (mean $\pm$ SEM); $n=20$ of 84 (electrodes) - (24%)

"LMAF" – ReaChR; $EC_{50}=14.21 \pm 0.06$; Hill slope = 1.203 ± 0.08 , $n=71$ of 92 (77%)

"CGRK" – floxed hOPN4; $EC_{50}=13.03 \pm 0.06$; Hill slope 0.9248 ± 0.07 $n=57$ of 87 (66%)

"CGRK" – ReaChR; $EC_{50}=13.86 \pm 0.06$; Hill slope 2.335 ± 0.24 $n=36$ of 75 (48%)

C – Histograms demonstrating the distribution of individual electrodes EC_{50} values in each group

D - Histograms demonstrating the distribution of individual electrode Hill slope values in each group

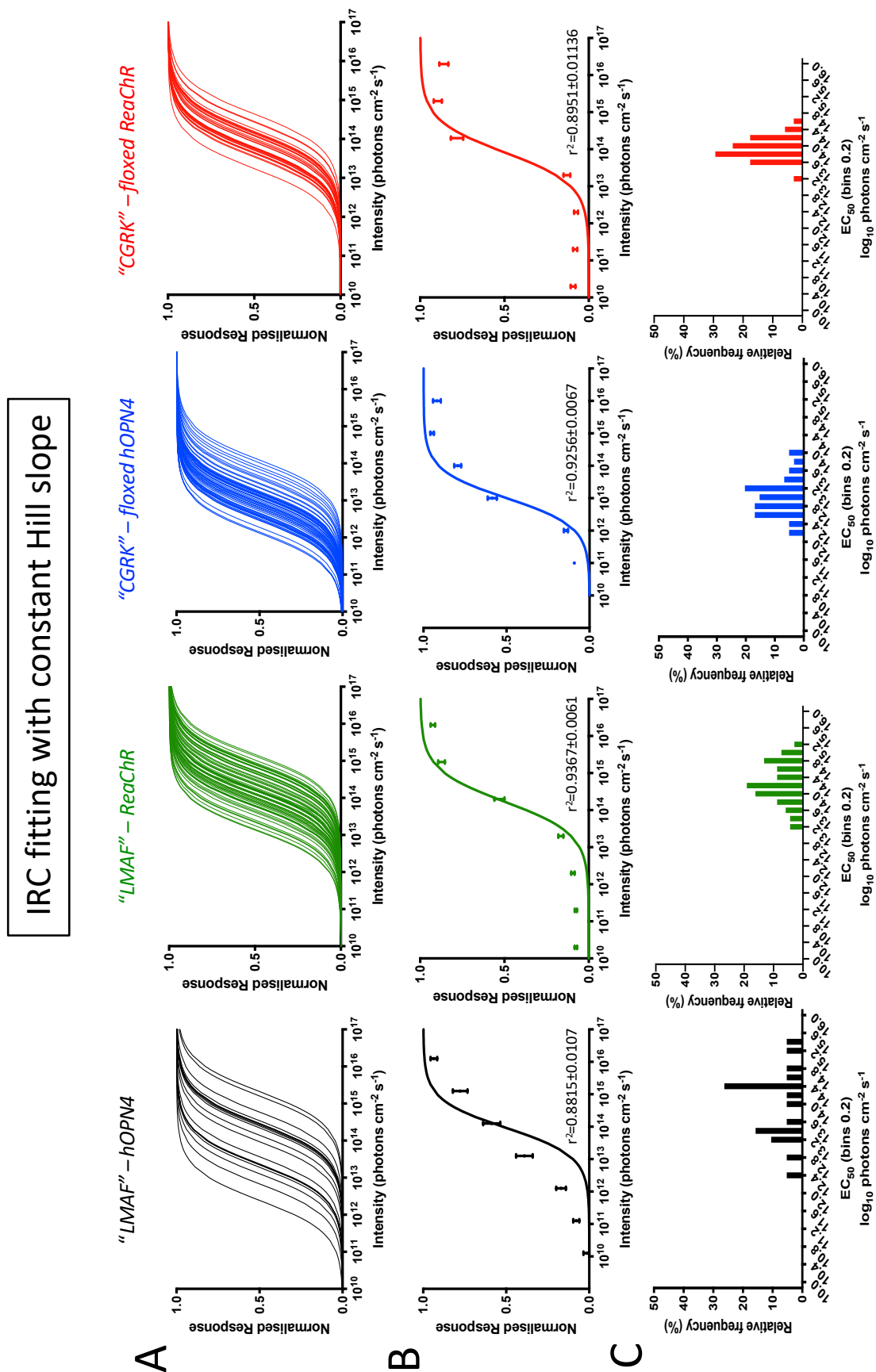


Figure 6-28 Please see next page for figure legend

Figure 6-28 Curve fitting of Irradiance response curves (IRCs)

In this figure, the Hill slope is fixed at 1.

A – All electrodes in each group, fitted using a Hill slope =1. Only electrodes with a good fit ($r^2 > 0.8$) are plotted.

B – The mean EC_{50} from all individual electrodes was used to plot a mean IRC curve; the mean $\pm$ SEM of r^2 values for the individual electrodes is shown. Also plotted on the graph (as round points) are the mean $\pm$ SEM of the normalised responses at each intensity tested.

“LMAF” – hOPN4; $EC_{50} = 13.96 \pm 0.18$ (mean $\pm$ SEM); $n = 19$ of 84 (electrodes) - (23%)

“LMAF” – ReaChR; $EC_{50} = 14.23 \pm 0.06$; $n = 68$ of 92 (74%)* **

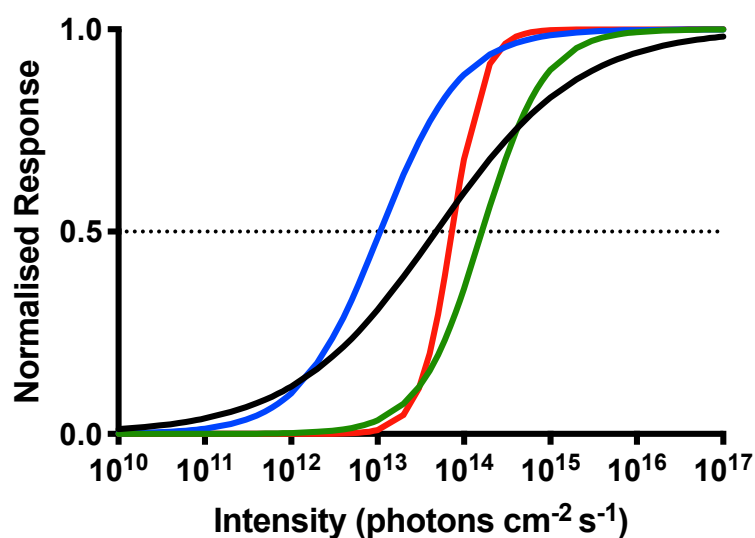
“CGRK” – floxed hOPN4; $EC_{50} = 13.00 \pm 0.06$ $n = 59$ of 87 (68%)

“CGRK” – ReaChR; $EC_{50} = 13.91 \pm 0.05$; $n = 34$ of 75 (45%)

C – Histograms demonstrating the distribution of individual electrodes EC_{50} values in each group

A

Mean Irradiance Response Curves Variable Hill Slope



"LMAF" - hOPN4 — "LMAF" - ReaChR — "CGRK" - hOPN4 — "CGRK" - ReaChR —

B

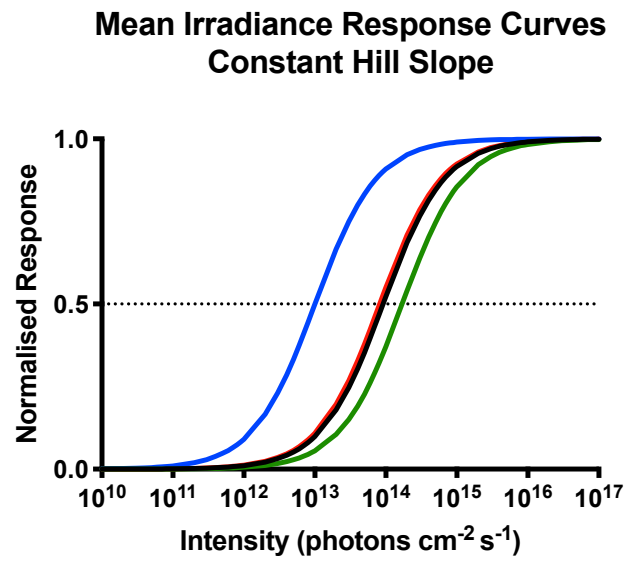
EC ₅₀			
"LMAF" - hOPN4	"LMAF" - ReaChR	"CGRK" - hOPN4	"CGRK" - ReaChR
13.63±0.21	14.21±0.06	13.03±0.06	13.86±0.06
Dunn's multiple comparison test, <i>p</i> =			
	"LMAF" - ReaChR	"CGRK" - hOPN4	"CGRK" - ReaChR
"LMAF" - hOPN4	0.021	0.0013	>0.9999
"LMAF" - ReaChR	x	<0.0001	0.0924
"CGRK" - hOPN4	x	x	<0.0001

C

Hill Slope			
"LMAF" - hOPN4	"LMAF" - ReaChR	"CGRK" - hOPN4	"CGRK" - ReaChR
0.5143±0.03	1.203±0.08	0.9248±0.07	2.335±0.24
Dunn's multiple comparison test, <i>p</i> =			
	"LMAF" - ReaChR	"CGRK" - hOPN4	"CGRK" - ReaChR
"LMAF" - hOPN4	<0.0002	0.0028	<0.0001
"LMAF" - ReaChR	x	0.0634	0.0043
"CGRK" - hOPN4	x	x	<0.0001

Figure 6-29 Irradiance response curve fitting

- Irradiance response curve fits for each group, plotted on same axis, Hill slope allowed to vary.
- A Kruskal-Wallis test with Dunn's multiple comparison test was used to test for difference between EC₅₀ of each of the four groups. *p* values for the post hoc test are shown here (see text for full details).
- A Kruskal-Wallis test with Dunn's multiple comparison test was used to test for difference between Hill slope of each of the four groups. *p* values for the post hoc test are shown here.



— "LMAF" - hOPN4
 — "LMAF" - ReaChR
 — "CGRK" - hOPN4
 — "CGRK" - ReaChR
 —

A. Figure 6-30 Irradiance response curve fits for each group, plotted on same axis, from mean EC_{50} ; Hill slope fixed as = 1.

These mean EC₅₀ values could also be used to compare sensitivity between groups, a Kruskal-Wallis test demonstrated a significant difference between groups (KW statistic = 93.46, $p < 0.0001$). Post hoc testing between groups using Dunn's multiple comparison test was performed (Figure 6-27) showing that while the mean EC₅₀ of ReaChR was not significantly different whether used in the "LMAF" model or the "CGRK" ($p = 0.0924$) and EC₅₀ hOPN4 in "LMAF" retinæ was not significantly different to ReaChR in "CGRK" ($p > 0.9999$), all other comparisons between groups were significantly different.

Similarly, the mean Hill slope of each group gives an indication of the mean dynamic range of responses and can be compared between groups, a Kruskal-Wallis test demonstrating a significant difference between groups (KW statistic = 61.93, $p < 0.0001$). Dunn's multiple comparison test was performed (Figure 6-27) showing that while the mean Hill slope values were not significantly different between ReaChR in the "LMAF" group and ReaChR in the "CGRK," all other comparisons of Hill slope values were significantly different (Figure 6-27).

[Irradiance response curves: dynamic range](#)

As discussed in Chapter 5, the responses of a significant number of electrodes, especially in the "LMAF" – hOPN4 group (64 of 84 electrodes -76%) did not fit a sigmoidal relationship well – suggesting a non-sigmoidal (or indeed lack of) dose-response relationship in the range tested for many electrodes (these could also represent "all or nothing" responses or a response at lower, but not higher intensities for example). As discussed, curve fits were therefore performed by allowing the Hill slope value to vary from 1, and while this did not lead to more electrodes to fit (20, rather than 19 in the "LMAF" – hOPN4 group) it did lead to significantly better r^2

values for curve fits (especially in CGRK groups (Figure 6-27). The Hill slope value is the gradient of the linear portion of the sigmoidal curve and therefore gives an index of the width of the dynamic range of the electrode (or group, when averaged). The values of these varied for the four treatment groups (Figure 6-29) with melanopsin groups (with second messenger coupling to facilitate extension of dynamic range via intracellular factors) and “LMAF” groups (with the advantage of an additional layer of retinal processing to allow extension of range by convergent processing) having significantly shallower gradients: suggesting wider dynamic ranges. The narrower dynamic range of microbial compared to vertebrate opsins has been previously noted (103), and these data would suggest that although ReaChR does have a wider range than native channelrhodopsin2(354, 357), it is not comparable to that of melanopsin in the same host cell.

Irradiance response curves: conclusions

In summary, based on the EC₅₀ values produced by these fits, hOPN4 in the “CGRK” model produces significantly more sensitive responses than all other groups. Additionally, there is no difference in sensitivity with ReaChR, regardless of which cell population it is targeted to, however the sensitivity of hOPN4 in the “LMAF” model overlaps with ReaChR targeted to the “CGRK.” Mean Hill slopes are steeper where ReaChR is used and in “CGRK” animals, indicating narrower dynamic ranges in these groups.

Behavioural assays

Given the poor responses obtained using these models (discussed in Chapter 5), behavioural assessment of vision was not carried out for these ReaChR groups. The levels of sensitivity obtained from electrophysiological recordings when ReaChR is

targeted to both “*LMAF*” (EC_{50} $14.21 \pm 0.06 \log_{10}$ photons $\text{cm}^{-2} \text{s}^{-1}$) and “*CGRK*” (EC_{50} $13.86 \pm 0.06 \log_{10}$ photons $\text{cm}^{-2} \text{s}^{-1}$), are several orders of magnitude above the highest levels of light used in both the described optokinetic (1.15×10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$) and novel object visual context recognition assays (1.89×10^{11} photons $\text{cm}^{-2} \text{s}^{-1}$). Indeed Sengupta *et al*(357), required illumination levels above 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$ to induce any behavioural response in a light dark box test for ReaChR with Ganjawala *et al*(208) requiring similar levels in order to record optokinetic responses (in a TKO mouse) - above even the maximum intensity able to be delivered by the pupillometer described in Chapter 2 (c. 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$).

As discussed in Chapter 5, it would be informative to use other assays to determine if the demonstrated retinal light responses reached higher centres in these models. These could include for example, alternative behavioural light response assays, post light exposure brain c-fos staining, visual evoked potentials, or direct LGN or cortical electrophysiological recording in response to light stimuli.

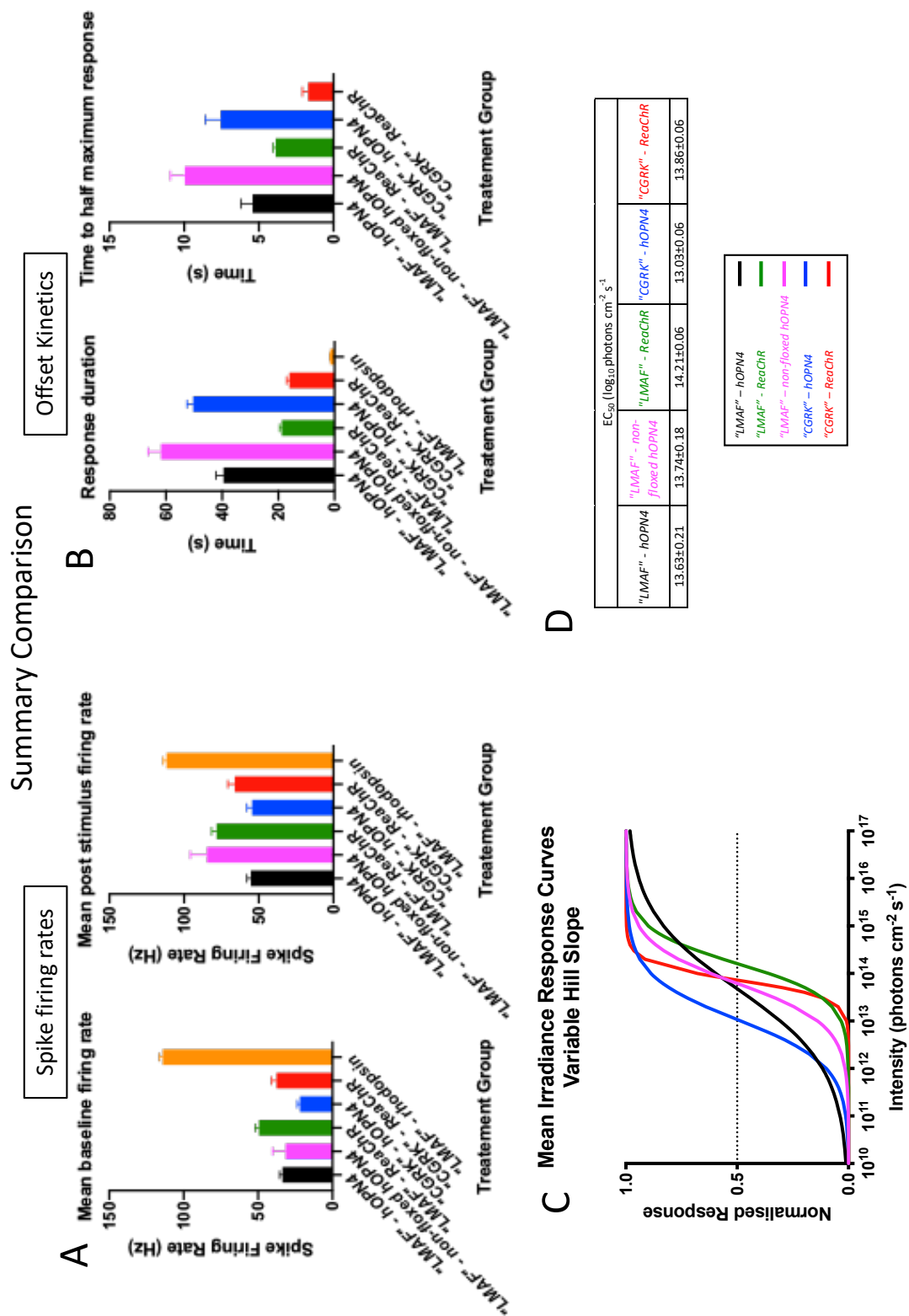


Figure 6-31 A summary of data from all five treatment group, values for non-floxed (CBA-hOPN4) delivery of melanopsin to "LMAF" mice from Chapter 5 is plotted alongside for comparison.

- Spike firing rates in response to a 10^{15} photons $cm^{-2} s^{-1}$ light pulse – redrawn from Figure 6-25 A & C
- Offset kinetics values in response to a 10^{15} photons $cm^{-2} s^{-1}$ light pulse – redrawn from Figure 6-26 C & D
- Sigmoidal curve fits of irradiance response relationships, redrawn from Figure 6-29
- Mean EC_{50} values for each group, as in Figure 6-29

Discussion

This Chapter has explored targeted expression of ReaChR, human rhodopsin and human melanopsin in two specific retinal cell types and demonstrated successful restoration of light driven RGC spike firing. This was achieved in retinae devoid of all native light responses, allowing responses driven by exogenous protein to be compared in isolation. A direct comparison was undertaken of the size, kinetics and sensitivity of electrophysiological responses to light between the ReaChR and hOPN4 groups. The aim being to answer the question: which of these optogenetic tools is likely to be the best for cell specific optogenetic visual restoration in the inherited retinal degenerations.

In summary, (Figure 6-31) this comparison demonstrated significantly higher baseline firing and larger responses in ReaChR treated retinae which were also significantly shorter in duration and half-life than hOPN4, however hOPN4 had superior sensitivity and a wider dynamic range. As there were relatively few responsive electrodes in the rhodopsin group, descriptions of response characteristics were presented without direct statistical comparisons.

An idealised tool

The ideal response characteristics of an optogenetic tool for use in survivor cell stimulation in the degenerate retina would closely emulate those of native visual responses. Specifically, it would give large amplitude responses with a fast onset and offset in response to light stimuli; it would also be sensitive and maintain responses over a wide dynamic range of intensities. Importantly, native retinal ganglion cell responses compromise both transient and sustained type responses.

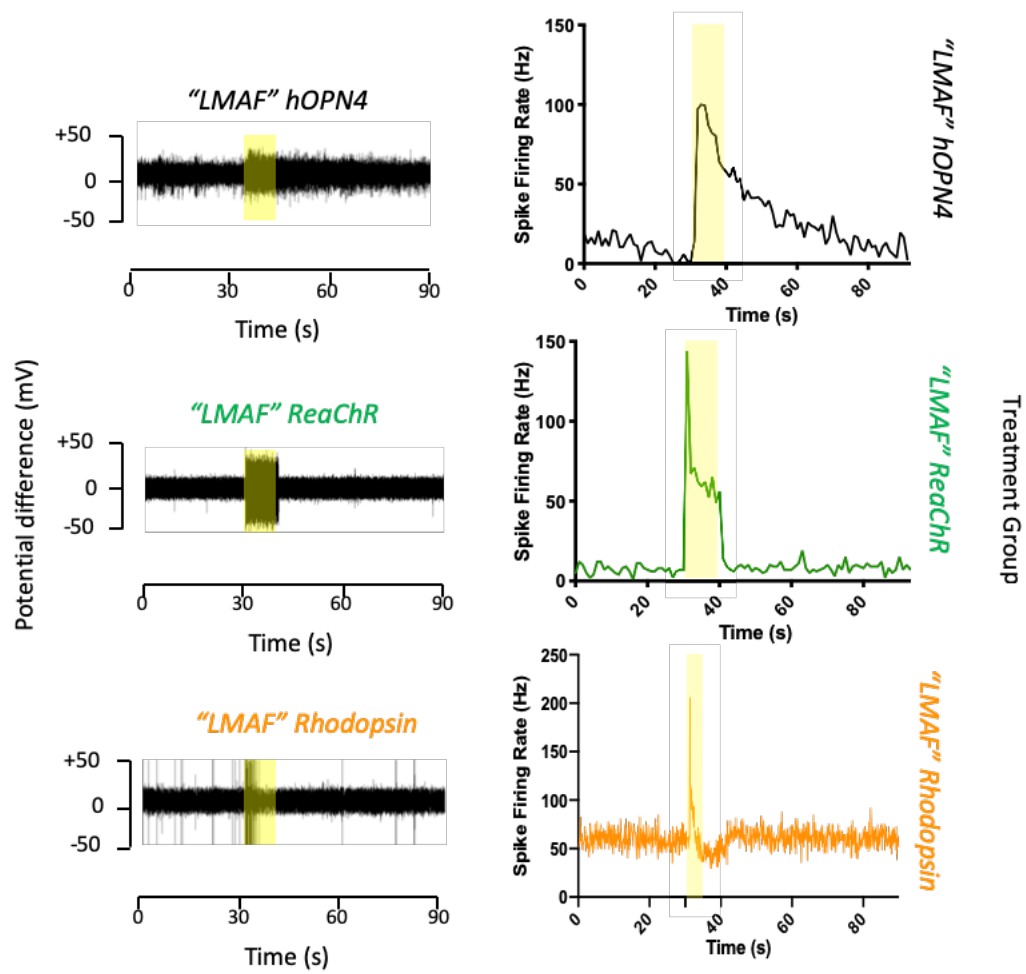


Figure 6-32 Raw response & spike firing traces demonstrating differing forms of RGC firing in response to a 10 second 10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$ light pulse. NB the extension of the hOPN4 response beyond the end of the stimulus, the maintained response of ReaChR during the stimulus and the transient "ON" nature of Rhodopsin response.

As can be seen in (Figure 6-31) no one tool would appear to unequivocally meet all of these criteria, but interestingly there is marked diversity in the forms of response that are seen when different optogenetic tools are targeted to specific cell populations (Figure 6-32).

Comparison of tools

Forms of response and kinetics

Fast kinetics will be necessary for optogenetic vision restoration in order to effectively encode important aspects of natural visual scenes such as motion. As can be seen in Figure 6-32, differences in the kinetics of responses when each of our three candidate tools are targeted to the same cell type in the “*LMAF*” mouse model lead to markedly different responses: a transient “ON” type response with rhodopsin lasting for around the first second of the light response, a sustained response throughout the length of the light pulse with ReaChR, which quickly extinguishes at the end of the stimuli and a prolonged response with hOPN4, lasting beyond the end of the stimulus.

If our metric for selection would be on speed of response onset and decay alone, that of rhodopsin may be seen as preferable, however the functional properties of both the hOPN4 and ReaChR responses could allow quite different information to be encoded – for example, the persistence of the stimuli, rather than solely its onset. In this regard, it should be emphasised that native RGC response do indeed comprise discrete populations of both sustained and transient light responses.

Sensitivity & dynamic range

Sensitivity of visual responses is important in normal visual processing and IRD sufferers are most disabled in low light environments, therefore restoring light

responses to low level scotopic stimuli is particularly important. A large dynamic range is also important to allow patients to render normal real-world stimuli.

In this study, rhodopsin used in the “*LMAF*” model led to sensitive responses (responses at 10^{-12} photons $\text{cm}^{-2} \text{s}^{-1}$), in keeping with previous reports (103, 106, 158) and similar to values reported for melanopsin(159) and other tools such as MW cone opsin(158). Therefore, it would be important to repeat this experiment with a new higher titre viral preparation in order to attempt to achieve a larger number of responsive electrodes and more reliably describe the irradiance response relationship in this group (both with and without exogenous 9-*cis* retinal). The low rhodopsin AAV titre is likely to have been a contributing factor to the small number of responsive electrodes found, especially given the low levels of IHC staining seen in this group.

Certainly, based on the small number of electrodes in the group, rhodopsin would appear still to have a useful level of sensitivity compared to other tools in this study. As expected, a direct comparison of hOPN4 and ReaChR confirmed hOPN4’s superior sensitivity in both models – presumably due to its ability to amplify responses through second messenger coupling. However, the finding that hOPN4 sensitivity was enhanced (rather than reduced) in the “*CGRK*” compared to “*LMAF*” models was interesting: the converging input available with bipolar cell, rather than RGC targeting could have been hypothesised to lead to enhanced sensitivity. One explanation for this could be more efficient coupling of melanopsin or the presence of other response enhancing factors in the RGCs of the “*CGRK*” model. The lack of a significant difference when ReaChR was targeted to either the “*LMAF*” or “*CGRK*” (without any second messenger coupling) would be consistent with this explanation.

In this regard, the lack of significant difference in EC_{50} between the most sensitive ReaChR group ("*CGRK*") and least sensitive hOPN4 group ("*LMAF*") is also interesting: suggesting that sensitivities for engineered microbial tools are not as far from vertebrate opsins as their native counterparts - the closest direct comparison previously reported showed a difference in EC_{50} of around two orders of magnitude(103).

To counter this, ReaChR response amplitudes were seen to plateau soon after threshold: potentially representing the "all or nothing" nature of ReaChR channel opening. This would agree with the narrower dynamic range suggested by the steeper Hill slope (figure 1-29C) value needed to achieve a good fit for ReaChR IRCs in both models.

Therefore, at present, melanopsin would still appear to represent the most sensitive tool available, with a superior dynamic range to ReaChR (despite ReaChR's much improved characteristics compared to native melanopsin(357)). However, preliminary data presented in this study would indicate that if problems of delivery can be overcome, rhodopsin too may potentially also have levels of sensitivity within the visual range, similar to that of melanopsin.

Magnitude – firing rates

Optogenetic tools, deployed for visual restoration in IRDs, will need to produce changes in RGC firing large enough to be detected above noise and be transmitted onto retinorecipient centres in the brain. It was hypothesised that with the advantage of second messenger coupling, melanopsin would be able to induce larger changes in RGC spike firing rates. However, in both the "*LMAF*" and "*CGRK*" models, ReaChR,

induced larger increases in spike firing from baseline, a potential reason for this becomes apparent when mean baseline firing rates are also considered.

In all ReaChR treated groups baseline firing rates were significantly higher than hOPN4 treated groups. *In vitro* studies have suggested a small amount of “leak current” is present with exogenously expressed channelrhodopsins, allowing ingress of cations (notably Ca^{2+}) with repeated activation(351, 358). This could presumably lead to a slight, relative depolarisation of the membrane potential, so accounting for both an increased (spontaneous) baseline and post response spike firing rate compared to hOPN4 groups. The potential long-term toxicity of this leak current to retinal neurons has not been investigated and certainly should be further studied, prior to clinical translation. However, in the experiments described here, it does not appear to impair cells’ ability to respond to light stimuli with spike trains.

By far the largest changes in spike firing in response to light stimuli observed were in the rhodopsin group, however, as described above, these were transient. Therefore, we see again that no one tool is absolutely preferable in terms of response magnitude: melanopsin had the smallest amplitude responses, rhodopsin has very large responses, but they are transient; ReaChR too has large responses, but there is a danger these, associated with a higher baseline firing rate, may be toxic.

Strengths and weaknesses of each tool

From the above comparison of three candidate tools in terms of sensitivity, dynamic range, response size and speed we can see that our three tools each have differing strengths and weaknesses when considering them as potential tools for clinical restoration of vision in the context of the IRDs.

While ReaChR may meet much of our criteria for an ideal tool - giving large, sustained responses with quick on and offset kinetics it may potentially induce a degree of excitotoxicity which requires urgent further investigation before it is used clinically.

Melanopsin certainly has the advantage when it comes to sensitivity and range, however the prolonged nature of its response and slower kinetics (even with cell specific targeting) could represent disadvantages in attempts to restore aspects of vision with high temporal fidelity.

In this study, difficulty in attaining good viral delivery of rhodopsin to the appropriate cells is likely to have hindered our ability to fully contrast it with the other two candidates. However, for the small number of electrodes for which we were able to describe responses, rhodopsin would potentially appear to have a great advantage in terms of kinetics and size of response with sensitivities comparable to melanopsin (if it can be successfully delivered to the correct cells in sufficient quantity).

Conclusion: an idealised tool

With this comparison of optogenetic light responses, it is apparent that no one of the tools is presented is likely to be the best for cell specific optogenetic visual restoration in inherited retinal degenerations, but that each presents a particular array of advantages and disadvantages. An idealised tool would need to combine aspects of each tool: for example the sensitivity and range of melanopsin, the kinetics of ReaChR and amplitude of rhodopsin responses could provide a tool approaching our ideal. Indeed engineering of recombinant tools(105, 334) to incorporate these features may well be a fruitful approach in the long term.

In the medium term, the variety of responses provided with these demonstrated tools could conceivably be used, in combination with cell specific targeting, to improve restored visual experience. Sustained, prolonged and transient ON-type responses such as those demonstrated in Figure 6-32 are not dissimilar to those found in normal neural retinal cells encoding aspects of the visual scene. So targeting specific tools to surviving cells with complimentary native response properties (for example rhodopsin to fast ON-RGCs(38)) could conceivably restore light responses closer to those found in normal visual processing. Indeed, in the longer term, specific targeting of multiple different optogenetic tools in such a way in the same retina may provide a fruitful approach to restore more naturalistic visual perception to IRD patients.

Chapter 7 – Delivery of Melanopsin to the degenerate retina using compressed L7 promoters

Introduction

As described in Chapters 4 to 6, targeted, cell population specific approaches to visual restoration in the IRDs is possible: ON-bipolar cells survive in a relatively normal physiological state with no apparent down regulation of essential second messenger systems which may allow the use of opsin based optogenetic tools. In addition, the use of the powerful Cre-lox system has allowed the combination of high efficiency promoters (such as CBA, h-Syn or EF1 α) with cell population specific expression. This has permitted investigation of the practical advantages of specifically targeting defined cell types – especially ON-bipolar cells – in the preclinical arena. However, cre-lox models are not appropriate for translational work: a non-transgenic approach to bipolar-cell specific delivery is urgently required if these techniques are to be used in clinical approaches to optogenetic visual restoration.

AAV delivery & bipolar cell specific promoters: challenges

An ideal, eminently translatable, solution to this problem could originate from the use of cell specific promoters in association with clinically proven AAV capsids(128). There are general impediments to this – not least the small payload of the AAV (at around 4kb(359)) – many cell specific promoters in their native form can be much too large to fit inside this envelope, let alone in addition to a gene of interest, such as an optogenetic tool. In addition, alterations to the AAV capsid can markedly alter its propensity to enter different cell populations (138, 360).

In relation to retinal bipolar cells, and specifically ON-bipolar cells, cell specific promoters have been developed: the most ubiquitous in the literature being a

composite of a 200bp enhancer from *Grm6* (the gene coding for the ON-bipolar specific metabotropic glutamate receptor mGluR6) and a basal SV40 (simian virus - 40) promotor(361). Indeed this construct has been used to deliver optogenetic tools (ChR(99-102), rhodopsin(103, 106) and a composite melanopsin(105)) to bipolar cells using AAV. Early problems with quantity of expression were overcome to some degree by enhancement of the AAV capsids with which the promotor construct was paired. However in careful direct comparison of the performance of *grm6*-sv40 paired with such capsids, Van Wyk et al(289) found the promotor not to be entirely specific for bipolar cells and significant differences in expression between wild type and *Pde6b^{rd1/rd1}* retinæ. They ascribe the latter to possible changes in the level of *Grm6* expression in ON-bipolar cells in the degenerate retina, which interestingly was *not* found when specifically investigated in this study (Chapter four). It would appear that while this “bipolar-cell specific” promotor construct has been in use for some time, as its efficiency has been optimised, its specificity has been called into question.

Therefore, if optogenetics is to be translated in an efficacious manner, there remains a pressing unmet need for a promotor specific for retinal ON-bipolar cells, yet small enough to fit inside an AAV. While one recent approach has been to modify the (relatively short) *grm6* basal promoter(362), others have focused on another (relatively large) promotor altogether, which while not specific solely for retinal bipolar cells, is specific for a subsection of ON-bipolar cells within the retina: the L7 promotor.

The L7-6 compressed promotor

The L7, or *Pcp2* gene codes four splice variants(166, 363-366) (Figure 7-1) variously expressed in a subset of retinal ON-bipolar cells (rod and cone types 2,5,6,7)(213, 221)

where its action as a regulator of $G\alpha_1$ and G_i function is essential to normal bipolar cell light responses(365). L7 is also expressed in cerebellar Purkinje cells – where, while its exact function is unclear, it has been used as a cell specific promotor for many years.

Oberdick et al (229, 230) initially described and cloned the L7 gene before producing a transgenic mouse with a 3kbp L7 promotor construct driving beta-galactadase (LacZ)(229). This construct was modified by Smeyne et al to improve specificity, and styled their modified construct “L7-1.(367)” This construct formed the basis of their L7-1.diphtheria toxin mouse model(367), subsequently widely used in cerebellar research and forming the basis of the L7.Cre lines described in Chapter 3(163, 368).

While L7-1’s large size (2.9kbp) and low efficiency were not overly problematic in its applications in transgenic Cre lines, they did preclude its use, unmodified, in AAV based vectors. Additionally, the concurrent development of *Grm6* based promotor constructs(361) negated the pressure to develop shortened L7 based constructs for AAV use – at least in the retina.

In cerebellar research however, this pressure remained with the Hirai group particularly modifying L7-1 to both decrease size and increase efficiency and specificity(166). Nitta et al removed either sections of the 5’ end (L7-2), 3’ end (L7-3) or both (L7-4, 1kbp) without detriment to efficiency in cell culture.

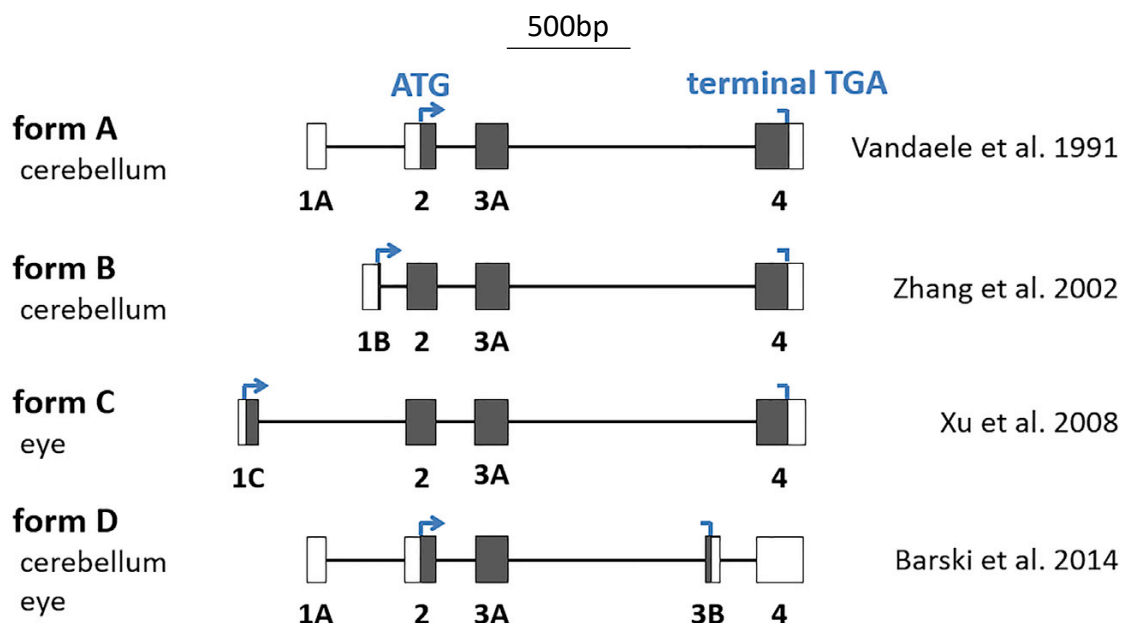


Figure 7-1 The four splice variants of L7 with their predominant tissue of expression. Boxes indicate exons, shaded areas coding regions, blue arrows and lines start (ATG) and stop (TGA) codons. References to first description of that variant are included on the right. Modified from(166).

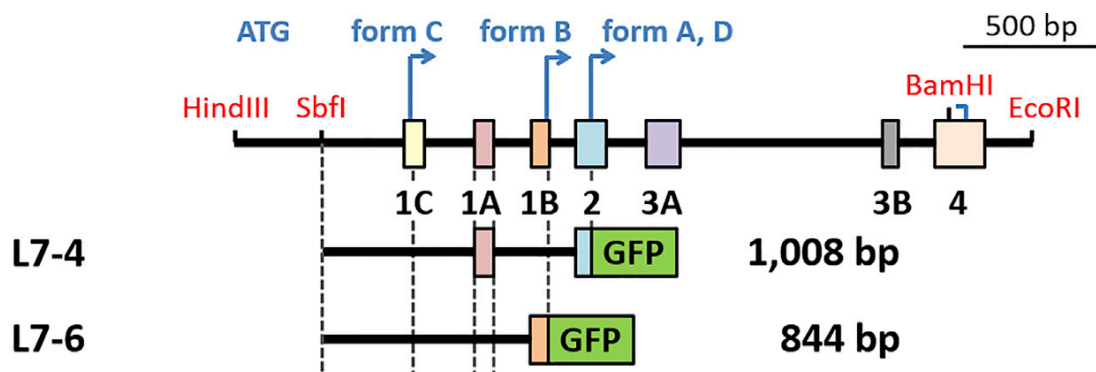


Figure 7-2 L7-4 is a modification of the L7-1 promoter construct: it lacks 1.6kb from the 3' end and 300bp from the 5' end compared to L7-1. The L7-6 modification has a further 3' deletion with the gene of interest appended to the start codon in exon 1b. Modified from(166).

In vivo however this was found to drive off target expression in cerebellar granule cells, leading to further modifications to remove splice sites preceding the GOI (GFP in Figure 7-2) (termed L7-5 to L7-9). Of these L7-6 was seen in mice to reduce off target expression whilst maintaining the efficiency of L7-4 and was so developed for AAV use in the cerebellum and tested on non-human primate brain tissue with similar results (166). Therefore, given the success of this particular construct in brain, I aimed to assess its efficacy in murine retina as a potentially novel cell specific, AAV compatible promoter for delivering retinal optogenetics.

L7 mini-promoters

Over and above work on the L7 promotor, there is a general demand in both basic and clinical science for such cell specific, short promoters for use in brain and eye tissues, however the process described above(166, 361, 369) of sequential promotor deletion and assessment is laborious. Therefore, an alternative approach has been developed by the Pleiades promoter project(370) to systematically identify cell population specific regulatory elements and integrate these into small, high efficiency cell specific promoters of less than 4kbp using high throughput methods.

In short, this approach(370) involves three stages: potentially useful genes expressed in the cell populations of interest are first identified from previous studies and prioritised for a second stage of bioinformatic analysis to predict regulatory regions for specific expression. Finally, these predicted sequences are integrated into a Minipromoter construct driving an indicator (such as GFP) and tested, ultimately in vivo, for efficiency and specificity of expression.

In 2014, a collection of such mini-reporters was reported including 17 with expression in the eye and one derived from the L7 promotor: Pleiades-155

(Ple155)(246). This 1652bp construct was derived from the two alternative promoters of variants A and B of L7(Figure 7-1) with the later inverted and added 5' to the former to avoid confusion with its alternative start codon(246, 371).

The construct showed robust expression in retinal bipolar cells and interestingly *not* in cerebellar Purkinje cells, but non-specifically in multiple cell types throughout the cortex and thalamus(167, 246). The ple155 construct has subsequently been functionally validated in vivo – with the rescue of a functional ERG b-wave in the *Nyx^{nob/nob}* mouse model when the *Nyx* gene under Ple155 control was delivered to retinal bipolar cells via intravitreal AAV(371).

Notably, while the *Nyx^{nob/nob}* mouse is blind due to bipolar cell dysfunction (analogous to human congenital stationary night blindness), it does not have a degenerate retina, nor has the Ple155 been tested in the context of a degenerate retina. Therefore, given its demonstrated efficacy in vivo, an assessment of the efficacy of Ple155 in delivering optogenetic tools to the bipolar cells of the degenerate retina was also proposed.

Experimental design & Results

Published evidence therefore suggests that there are at least two compressed L7 promoter constructs that are able to deliver proteins via AAV to relevant specific cell populations – potentially pivotal in translating targeted optogenetic strategies for visual restoration. However, neither have been assessed in the context of a degenerate retina nor used to deliver opsins. Therefore, I aimed to clone each of these promoters into AAV insert plasmids containing hOPN4 and then produce AAV using these. I then validated these viruses in vitro before injecting intravitreally into *L7^{+/+}Pde6b^{rd1/rd1}Opn4^{-/-}* (“LMAF”) mice to assess specificity of protein expression in the retina by IHC and the functionality of the delivered protein by MEA electrophysiology. Detailed methodology is described in Chapter 2.

Preparation of plasmids

Plasmids containing L7-6 (Figure 7-3) and Ple155 (Figure 7-4) promoter constructs were kindly received from the Hirai(166) and Simpson groups respectively(246). A backbone plasmid containing hOPN4 driven by a ubiquitous promotor (EF1 α) and other elements essential to the production of efficacious AAV vector, previously validated in murine retina(165) (Figure 7-3, Figure 7-4) was selected.

The initial strategy was to use traditional restriction cloning to remove the respective promotor from all three plasmids and then ligate the L7-6 or Ple155 construct in place of EF1 α in the backbone to form L7-6.hOPN4 (Figure 7-3) or Ple155.hOPN4 (Figure 7-4) insert plasmids for AAV production.

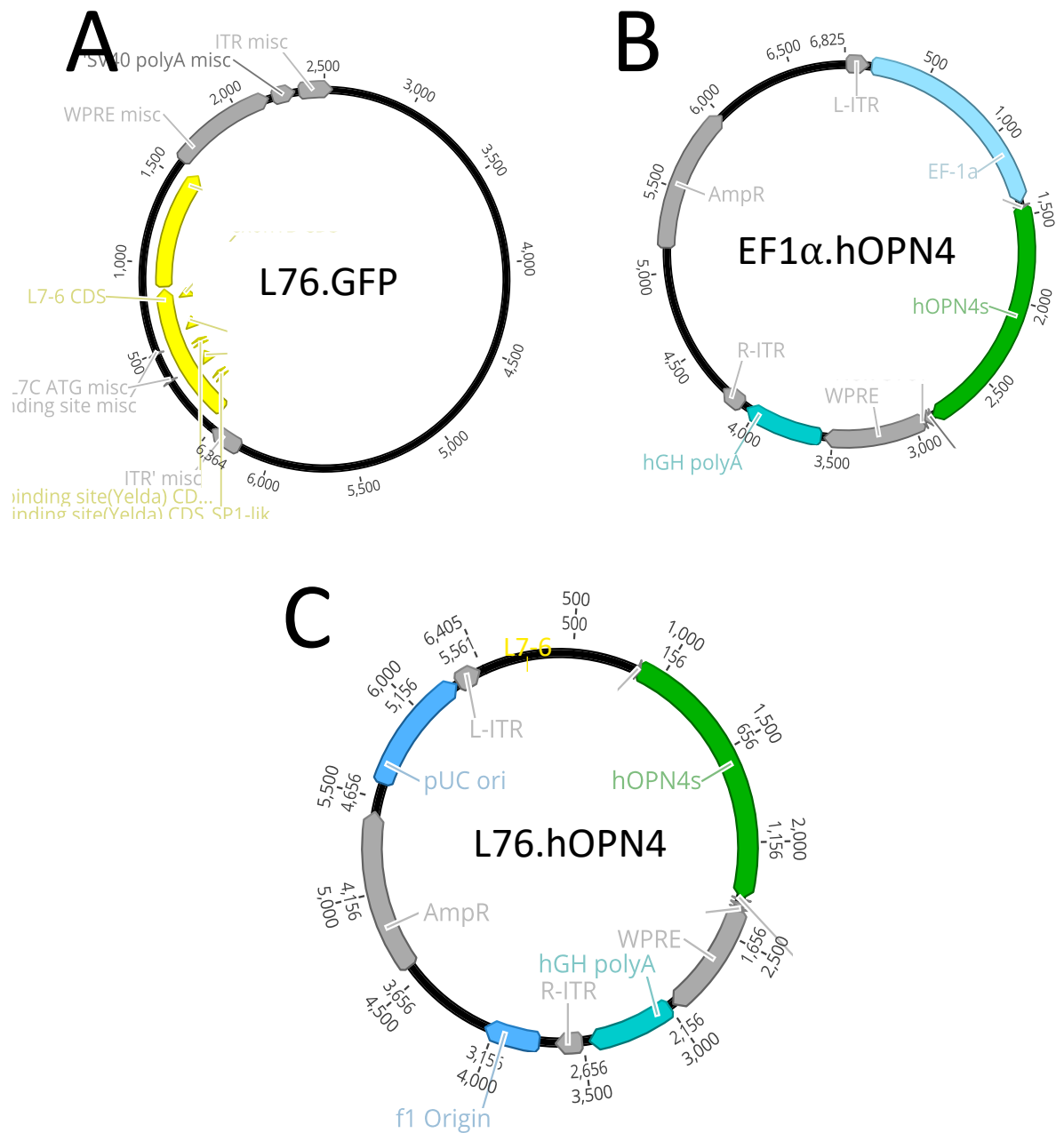


Figure 7-3

- The L7-6 promotor construct was received on a plasmid where it was driving GFP (not shown) and also incorporating a WPRE and ITRs.
- The EF1 α .hOPN4 was used as an AAV back bone, incorporating hOPN4 driven by EF1 α and incorporating ITRs, WPRE and polyA sequence
- The L7-6 promotor was excised from plasmid A, while the EF1 α was similarly removed from B. L7-6 was then ligated into the backbone of B (without EF1 α) to give a product plasmid.

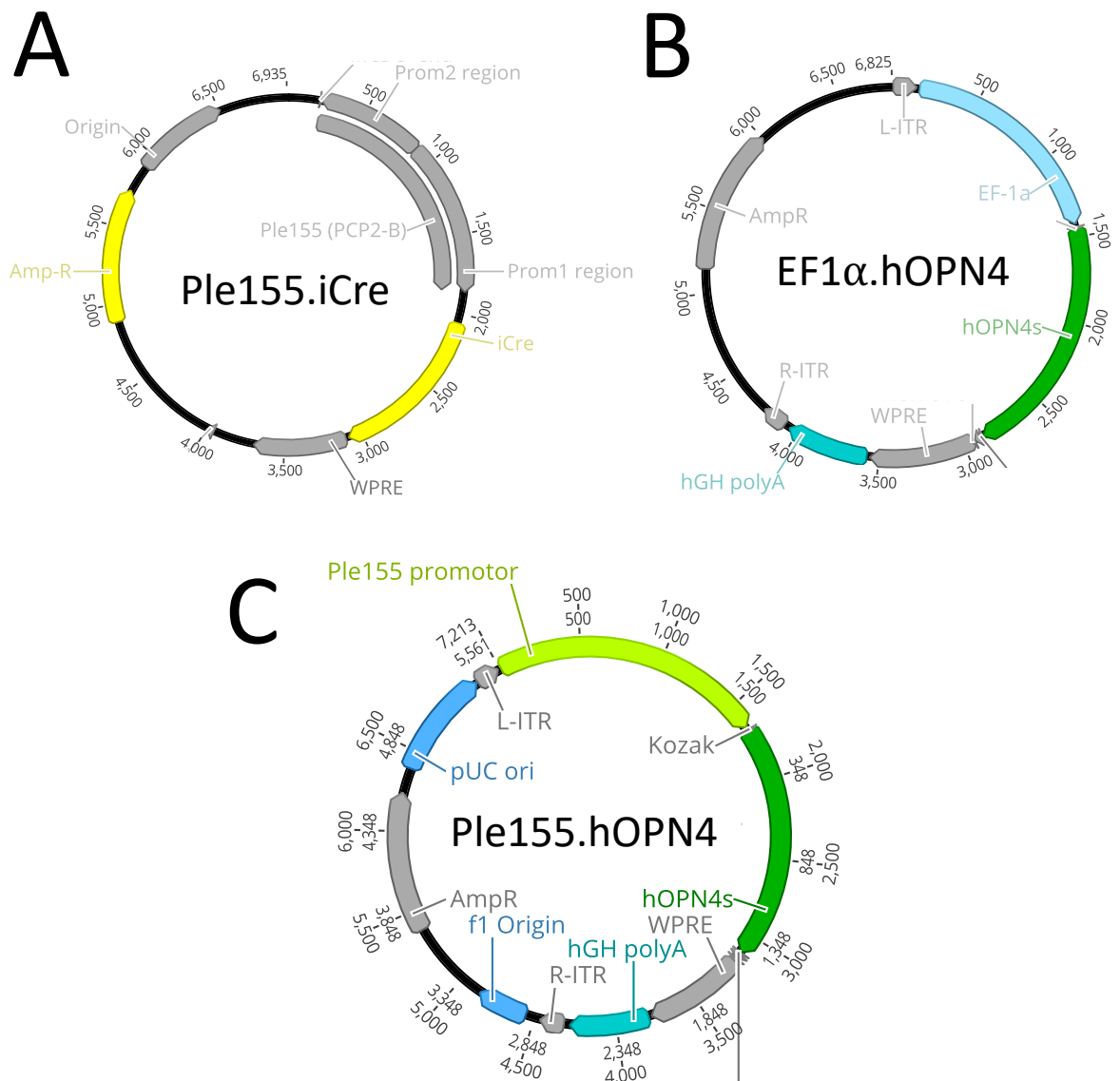


Figure 7-4

- The Ple155 promoter construct was received on a plasmid where it was driving iCre
- The EF1α.hOPN4 was used as an AAV back bone, incorporating hOPN4 driven by EF1α and incorporating ITRs, WPRE and polyA sequence
- The Ple155 promoter was excised from plasmid A, while EF1α was similarly removed from B. Ple155 was then ligated into the backbone of B (without EF1α) to give a product plasmid

This proved unsuccessful, as did a “blunt-end-ligation” approach likely due to the incompatibility of restriction enzymes required in this strategy, resulting in a complex multi-step cloning protocol. Eventually an (external) synthetic PCR based approach was used to good effect with the sequence of the resulting plasmids as expected (see appendix)

Preparation of viral vectors

With validation of two insert plasmids containing hOPN4, each driven by one of the compressed promoters, AAV production could be undertaken following the same approaches as in Chapters 5 & 6, using the same helper plasmid and quad-mutant capsid.

In house preparations utilising both plasmids were initially undertaken giving a titre of 7.33×10^{12} vg/ml for L7-6.hOPN4 with three clear bands (Figure 7-5a) of the appropriate size on SDS-PAGE gel electrophoresis and so was taken onto cellular (and in-vivo) testing. For the Ple155.hOPN4 construct, AAV was synthesised by the commercial vector core at the University of North Carolina and found to have a titre of 3.15×10^{12} vg/ml and three clear bands of the appropriate size on SDS-PAGE electrophoresis (Figure 7-5). This preparation was therefore taken forward for further validation along with the L7-6.hOPN4.

As described in Chapters 5&6, dissociated retinal cell cultures were prepared from “LMAF” mice and allowed to culture on 1cm laminin coated coverslips for two weeks before inoculation for 24hours with AAV with subsequent ICC three weeks later showing expression of hOPN4 with both preparations (L7-6hOPN4 19.17 ± 2.88 , n= 197 cells; Ple155.hOPN4 10.17 ± 0.98 of n=204 cells, Figure 7-6). As in Chapters 5 & 6, this result demonstrates the presence of viable viral particles, able to produce hOPN4

protein in retinal cells, but as cultured cells are unlikely to be in their native state given the artificial nature of both dissociation and culture, progression to in vivo testing was indicated.

In vivo validation of compressed L7 promoters in degenerate retina

In vivo validation was required, not only to ensure quantitative efficacy of AAV

induced protein production, but to qualitatively assess the specificity of the vectors:

do these constructs drive expression within the ON-bipolar cells of a degenerate retina

as expected? Both L7-6.hOPN4 and Ple155.hOPN4 AAV were administered

intravitreally to five 7 week old “LMAF” mice each who were culled four weeks later to

allow flat mount IHC for hOPN4. As demonstrated in Figure 7-7 & Figure 7-8 hOPN4

was expressed in the inner nuclear layer in in the L7-6.hOPN4 group ($10.6 \pm 3.7\%$ cells,

n=397), with staining cells seen to co-localise with L7 staining. hOPN4 staining in the

Ple.155.hOPN4 group – interestingly the one previously validated in non-degenerate

retinae – was not seen on any of the five retinae assessed. Similar images from a

Ple155.hOPN4 retina are also shown for comparison in Figure 7-9 & Figure 7-10.

Additionally, four slides of retinal cryosections (n=10 per slide) from each treated

retina were stained for hOPN4, over two batches, however none demonstrated any

positive staining.

As previously, a semi-quantitative index of IHC staining over whole retinae was obtained by manually scoring retina flat mounts, stained for hOPN4, on a sectoral basis. As can be seen (Figure 7-11) both the mean score per retina and for individual quadrants were variable in L76.hOPN4, again describing the patchiness of transfection that was seen in Chapters 5 and 6.

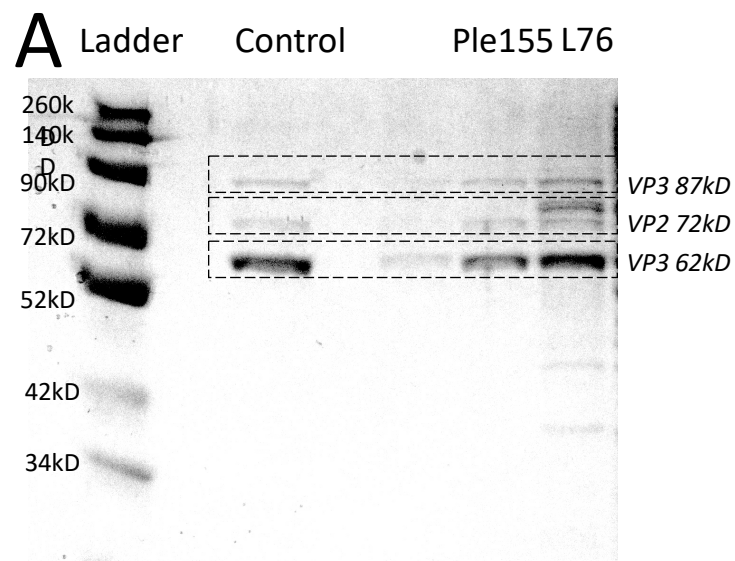


Figure 7-5

- A. Denaturing SDS page gel of samples of AAV containing hOPN4 driven by various promotors (subsequently used for *in vivo* validation) shows the presence of three viral proteins at the expected sizes (62, 72 and 87kD) and relatively few other bands, indicating lack of contamination.

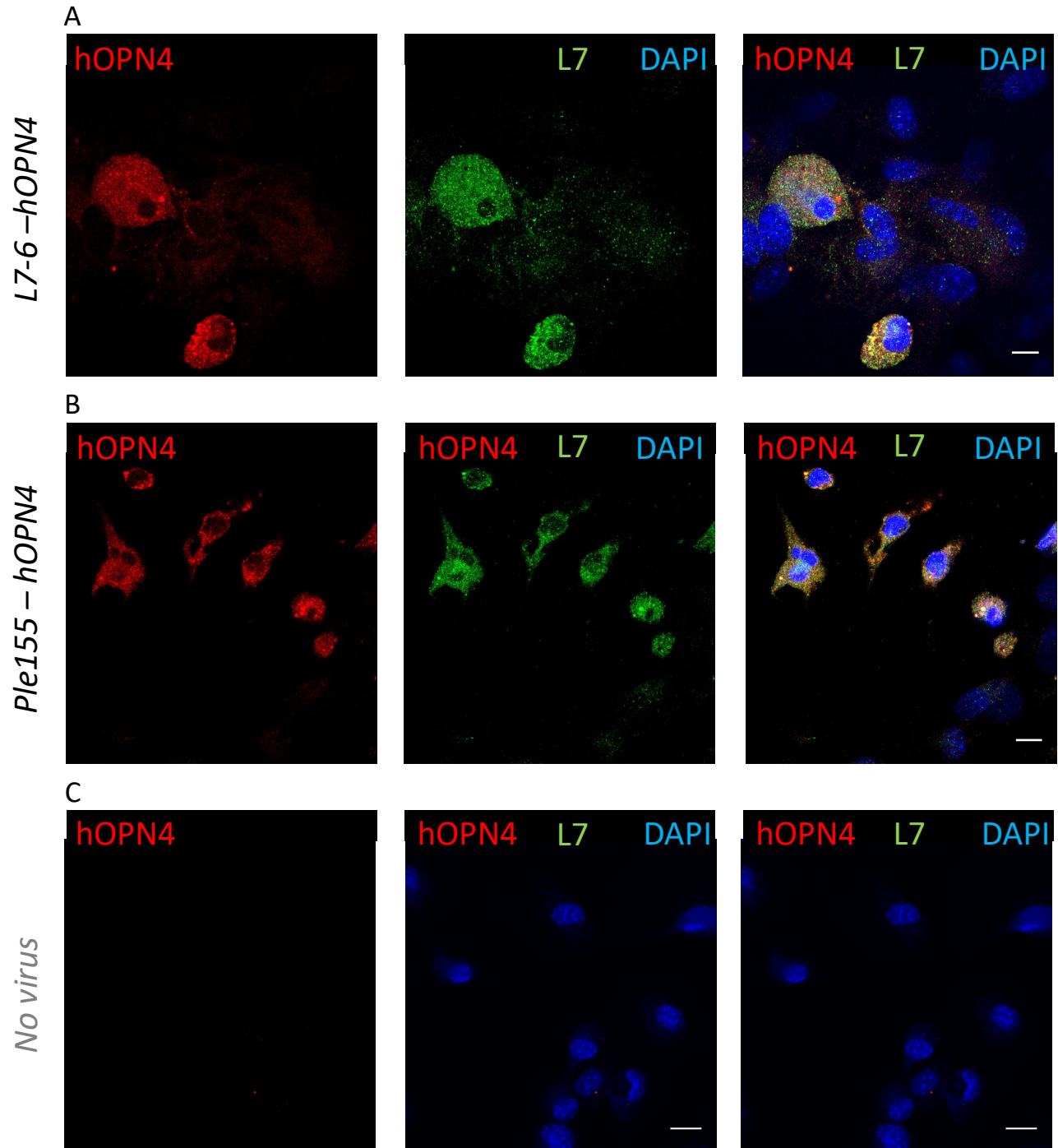


Figure 7-6

- Immunocytochemistry of dissociated retinal cell culture incubated with an AAV containing hOPN4 driven by the L7-6 promotor construct. DAPI = nuclear stain
- Immunocytochemistry of dissociated retinal cell culture incubated with an AAV containing hOPN4 driven by the Ple155 promotor construct. DAPI = nuclear stain
- Immunocytochemistry of dissociated retinal cell culture incubated without AAV alongside samples A&B. DAPI = nuclear stain

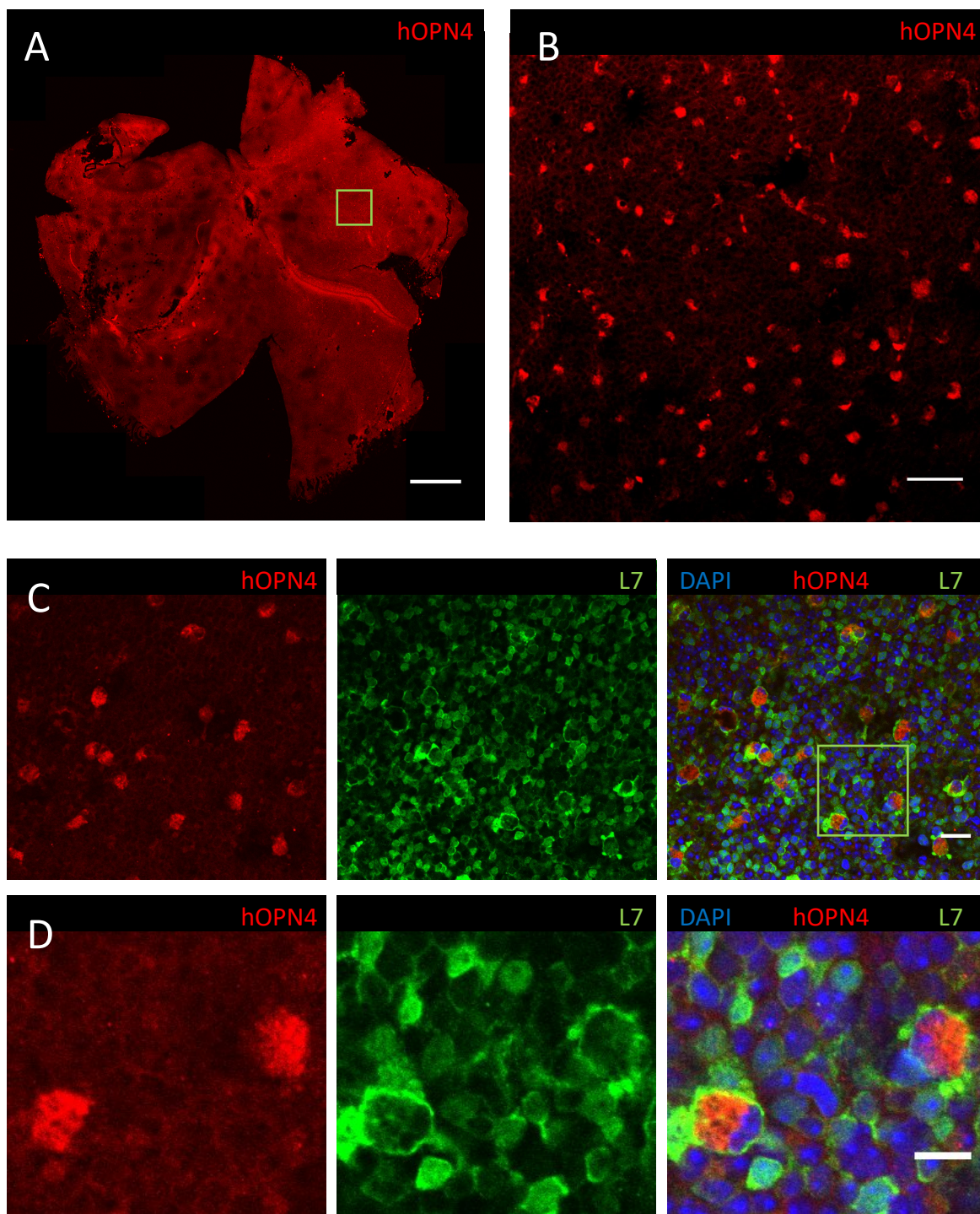


Figure 7-7 Confocal micrographs of a flat mount of an "LMAF" retina four weeks after injection with the L7-6 hOPN4 construct.

- A. Low power tile of confocal images stitched to give view of whole retina – note areas of lower staining inferiorly and on the left of the image. Green box gives approximation of location of the image in panel B. Scale bar 500µm
- B. Single low power (x20) image from figure A taken at level of the inner nuclear layer. Scale bar 50µm
- C. High power (x40) image of the inner nuclear layer from same retina. Note colocalization of hOPN4 and L7 staining. Green box gives approximation of location of the image in panel D. Scale bar 10µm
- D. Close up of high power (x40) image of the inner nuclear layer. image from figure A. Scale bar 10µm

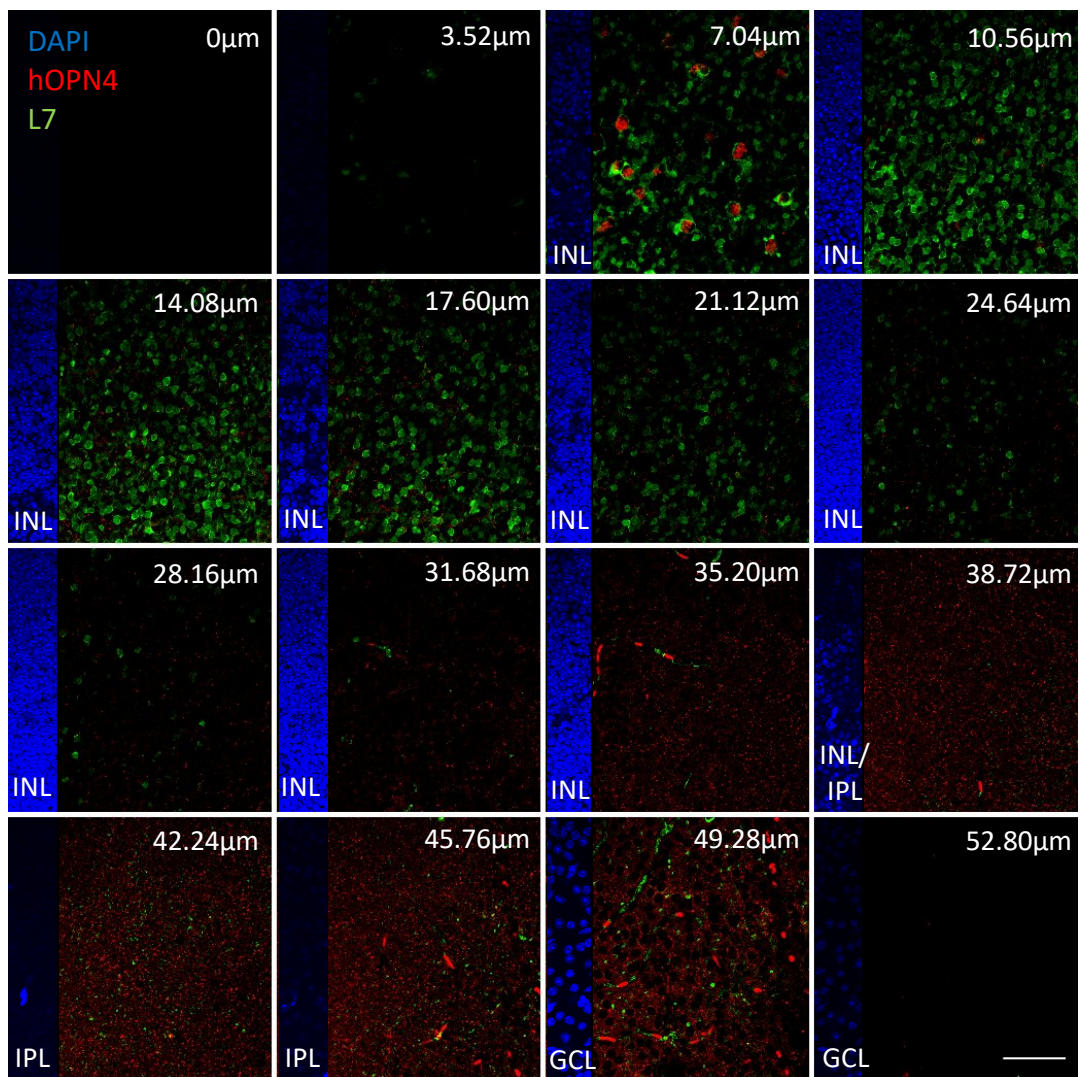


Figure 7-8 Montage of confocal micrographs of an "LMAF" retina four weeks after injection with the L7-6 hOPN4 construct. These represent slices of the same "Z stack" from the same retina described in Figure Y. For clarity, the red and green channel (representing hOPN4 and L7 staining) are presented as a composite. The side panel in each pane represents the DAPI (nuclear) staining associated with that pane as an indication of location within the layers of the retina. The depths described in each panel refer to the distance in the Z plane from the top left panel (just below the retina). INL – inner nuclear layer. IPL – Inner plexiform layer. GCL – Retinal ganglion cell layer. Scale bar is 50μm

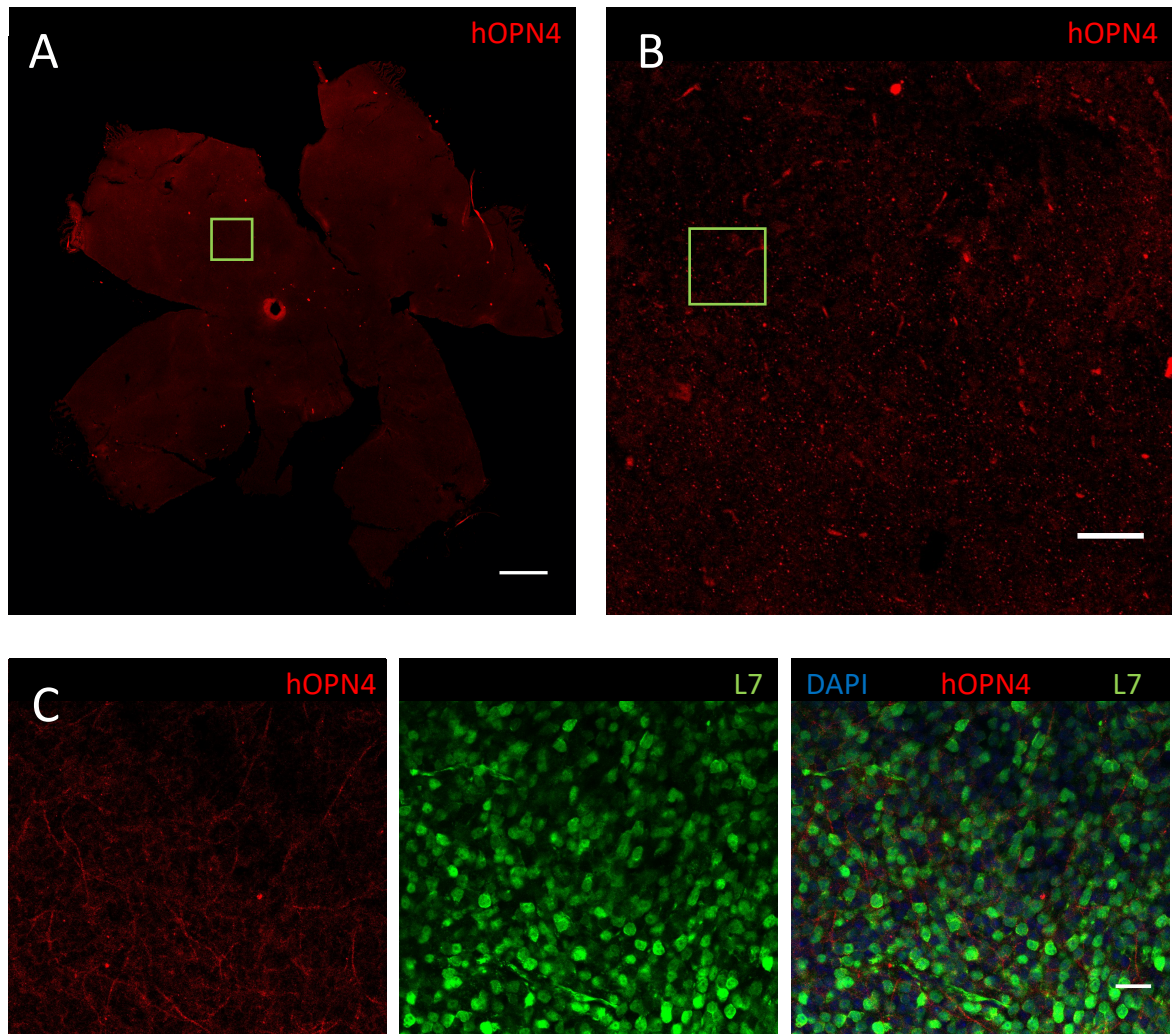


Figure 7-9 Confocal micrographs of a flat mount of an "LMAF" retina four weeks after injection with the Ple155 hOPN4 construct. Note poor staining for hOPN4.

- A. Low power tile of confocal images stitched to give view of whole retina – note areas of absence of staining over whole retina. Green box gives approximation of location of the image in panel B. Scale bar 500µm
- B. Single low power (x20) image from figure A. Scale bar 50µm
- C. High power (x40) image of the inner nuclear layer from same retina. Note colocalization of hOPN4 and L7 staining. Scale bar 10µm

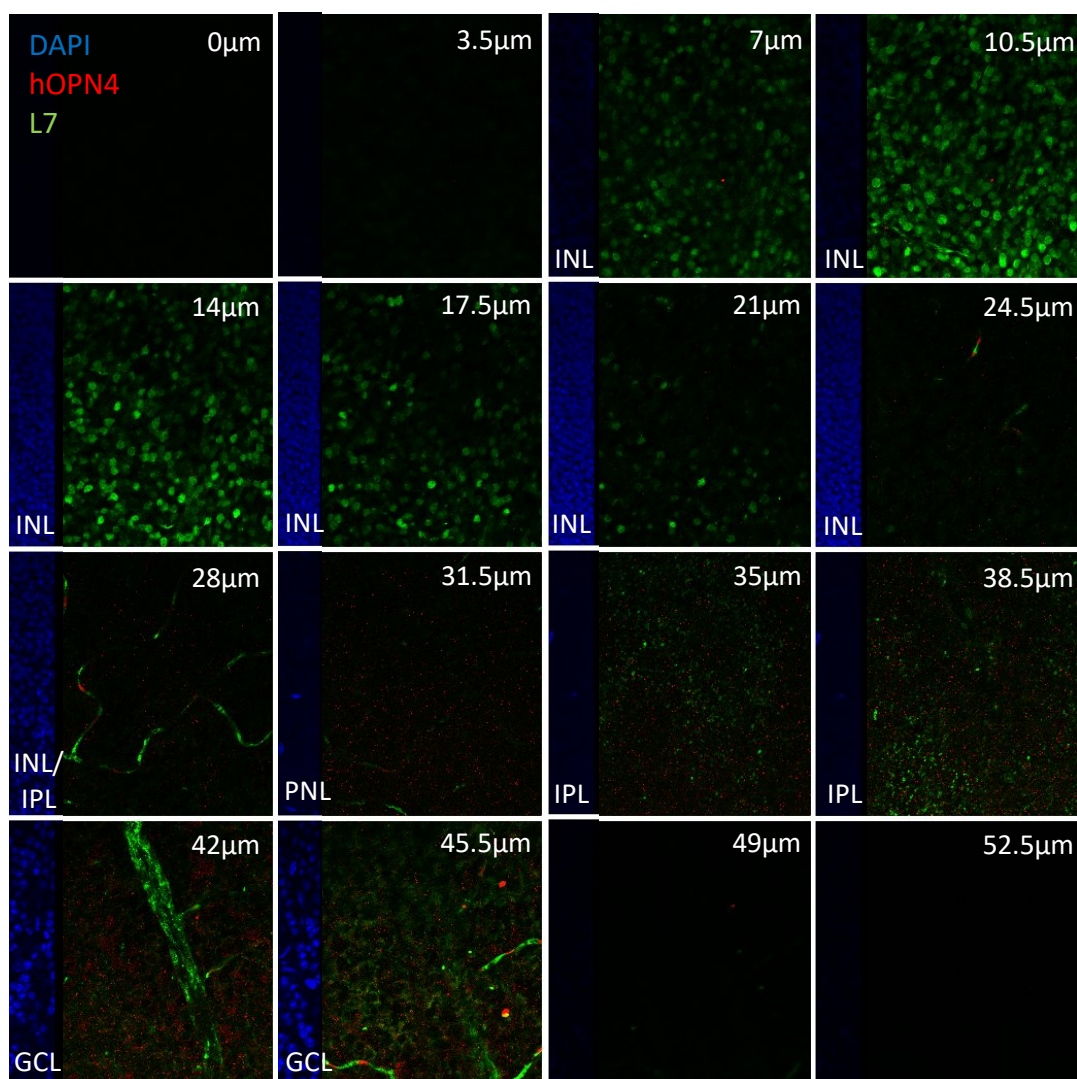


Figure 7-10 Montage of confocal micrographs of an “LMAF” retina four weeks after injection with the Ple155 hOPN4 construct. These represent slices of the same “Z stack” from the same retina described in Figure Y. For clarity, the red and green channel (representing hOPN4 and L7 staining) are presented as a composite. The side panel in each pane represents the DAPI (nuclear) staining associated with that pane as an indication of location within the layers of the retina. The depths described in each panel refer to the distance in the Z plane from the top left panel (just below the retina). INL – inner nuclear layer. IPL – Inner plexiform layer. RGL – Retinal ganglion cell layer. Scale bar is 50μm

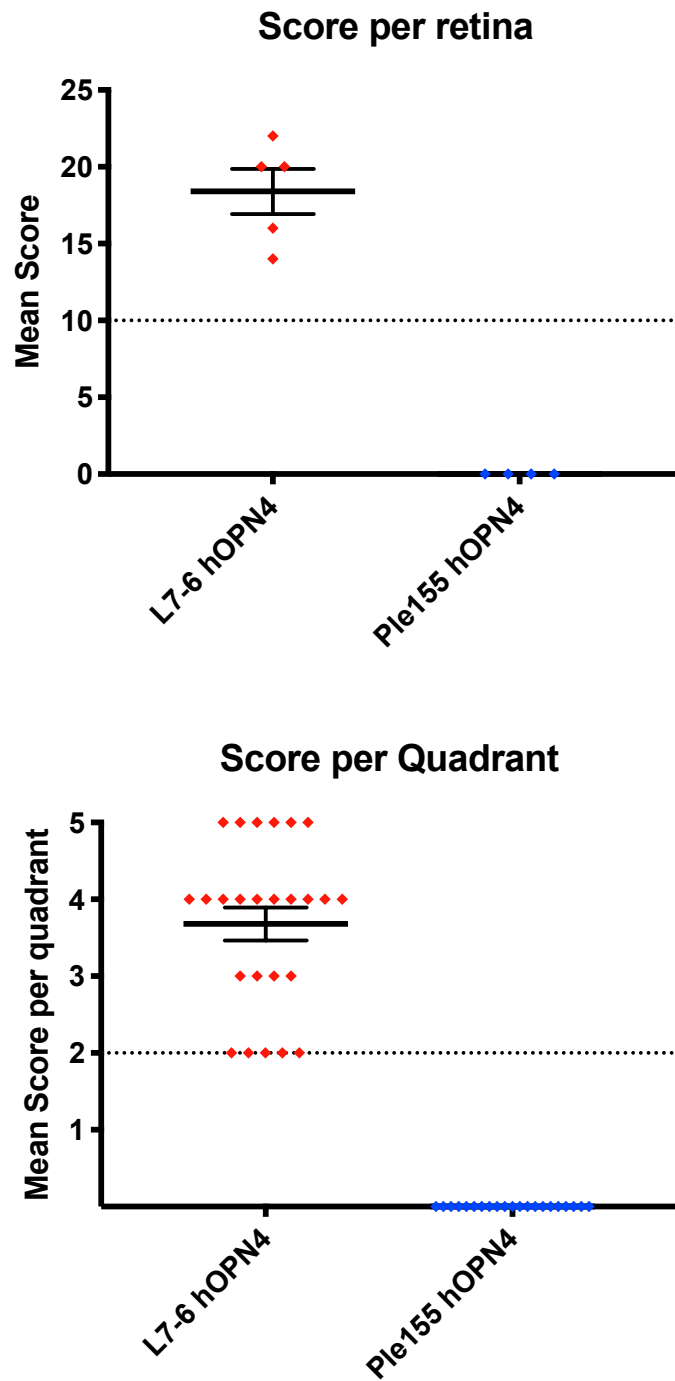


Figure 7-11 Semiquantitative indices of retinal flat mount staining of retina treated with either L76 or Ple155 driven hOPN4 AAV and IHC stained for hOPN4 (see Chapter 2 for description of technique).

Functional validation of compressed promoter targeted Melanopsin

With demonstration of hOPN4 expression in L7 expressing ON-bipolar cells of the degenerate retina using the L7-6 construct, further investigation at a functional level was undertaken. Therefore, two “*LMAF*” mice (genotyped to be wildtype at the Tg(Cre) locus received intravitreal L7-6.hOPN4 at the age of seven weeks before culling eight weeks later for multiple electrode array electrophysiology.

Of the four retinae injected with L7-6.hOPN4 good preparations and spike recordings were achieved in three, two of these had multiple electrodes (n= 28 over two retinae) responsive to a 10 second light pulse (480nm, 10^{15} photons $\text{cm}^{-2}\text{s}^{-1}$). Responsive electrodes were, as in Chapters 5 &6 defined as those showing an increase in firing rate of 10Hz above the mean baseline firing rate. An example raw recording and arrays of spike firing graphs from both retinae with responsive electrodes are shown in Figure 7-12.

Given the small number of retinae and electrodes recorded from in this pilot experiment, expansive statistical comparison of responses were not made, but light response metrics exogenous hOPN4 delivered by either L7-6 or the Cre.lox system are presented side by side for contrast – showing that there may indeed be differences in the observed response characteristics, but that this warrants further investigation by repeating electrophysiology in sufficient numbers of L7-6.hOPN4 retinae.

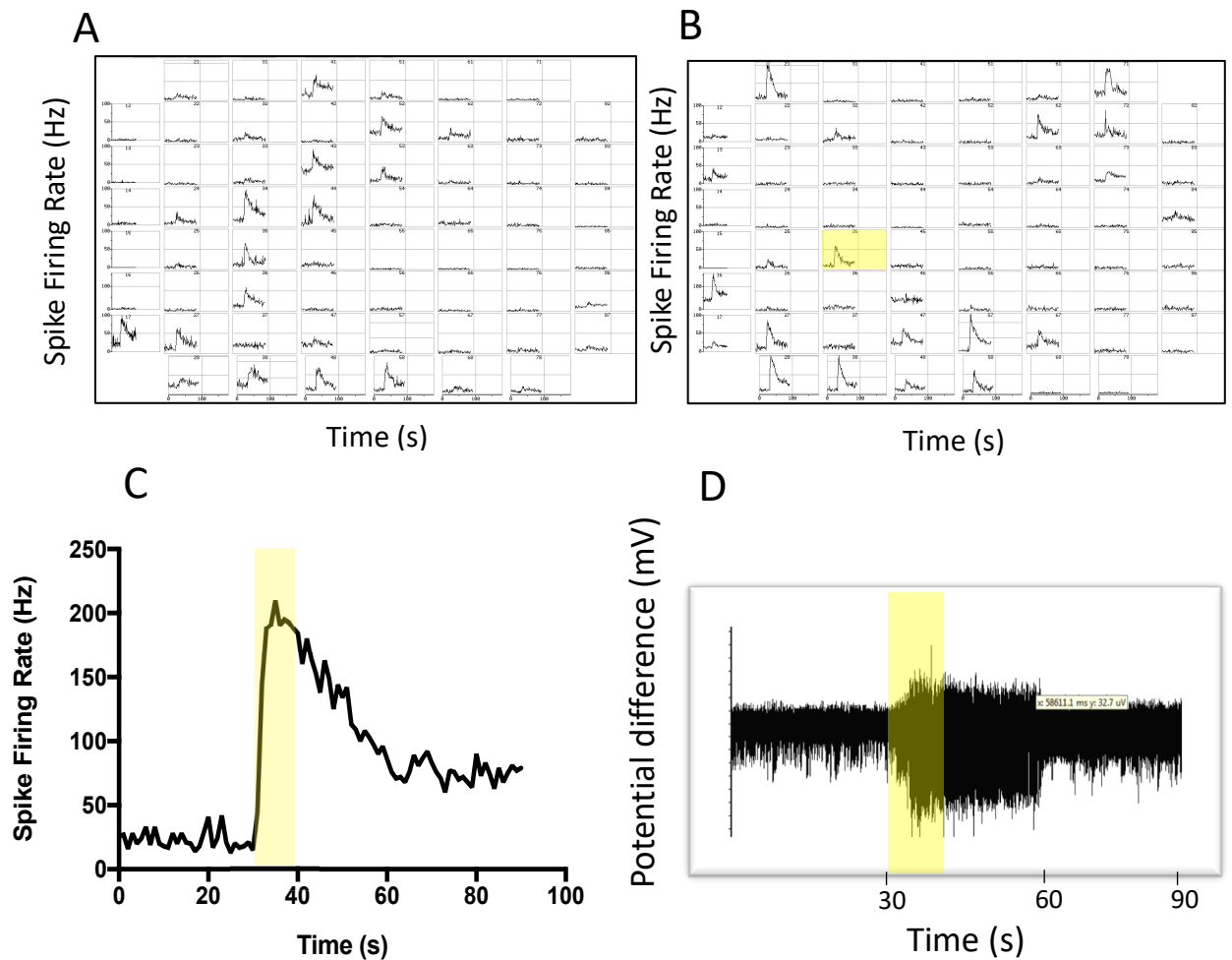


Figure 7-12

A & B – Arrays of spike firing traces from each retina in the L7-6 group from which responses were recorded in response to a 10 second pulse of 480nm light, $10^{15} \text{ photons cm}^{-2} \text{ s}^{-1}$ at 30 seconds after beginning of recording.

C - The spike firing rate vs time for electrode 28 (highlighted) of B to the same stimulus (indicated by yellow shaded bar)

D – The raw recording from the same electrode

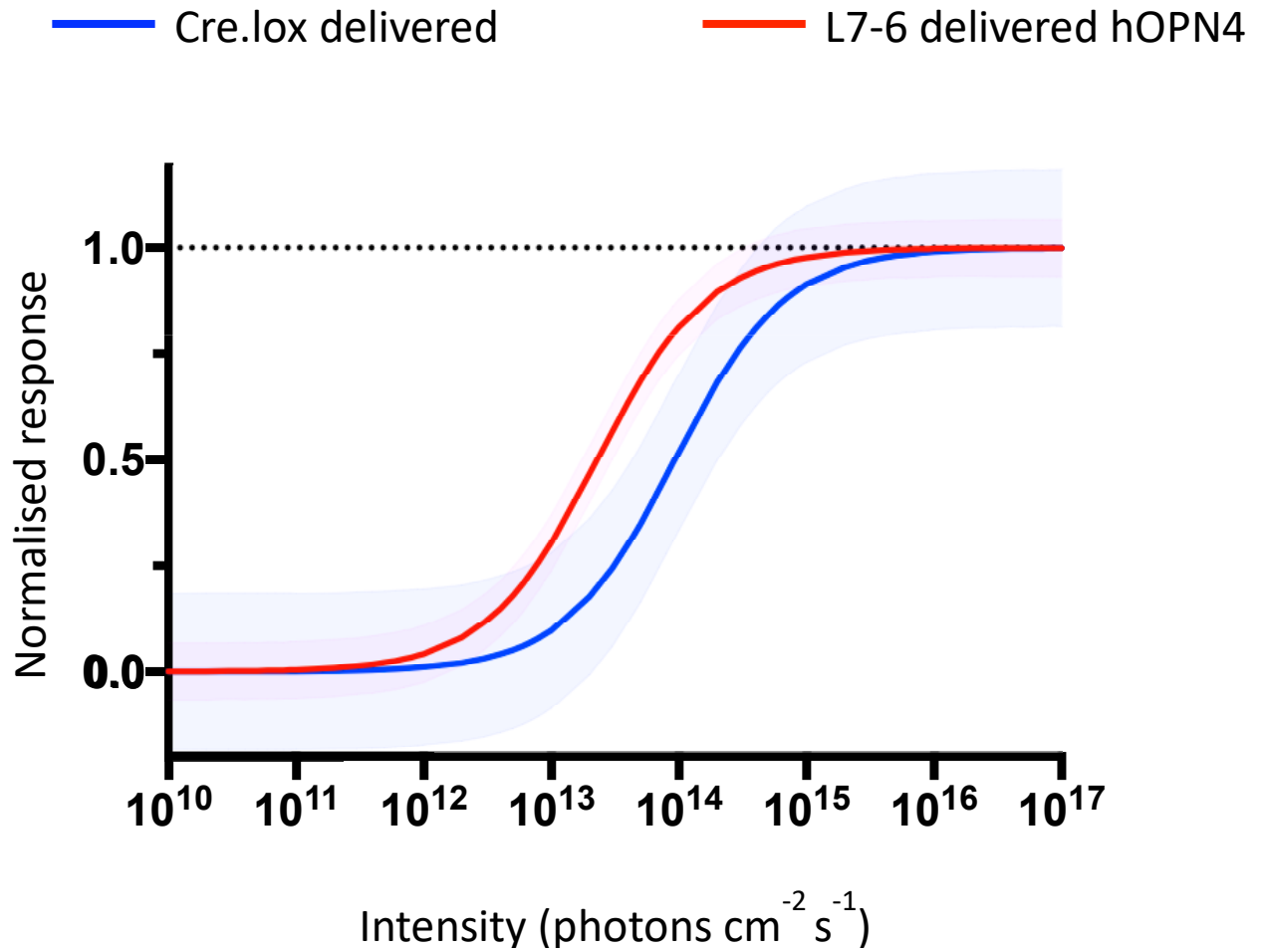


Figure 7-13 Mean irradiance response curves of light responsive electrodes from L7^{+/+}Pde6b^{rd1/rd1} OPN4^{-/-} ("LMAF") retina where hOPN4 has been delivered by AAV and restricted to ON-bipolar cells either using the L7.Cre or L7-6 compressed promotor system. In both cases the hill-slope value was fixed at 1; for L7-6 delivered hOPN4 $EC_{50}=13.36\pm0.066$ (shaded area - SEM) $N=2$ $n=28$, for Cre.lox delivered hOPN4 $EC_{50}=13.96\pm0.1846$ (shaded area - SEM) $N=5$ $n=19$. Mann Whitney test $p=0.0020$

— Cre.lox delivered

— L76 delivered hOPN4

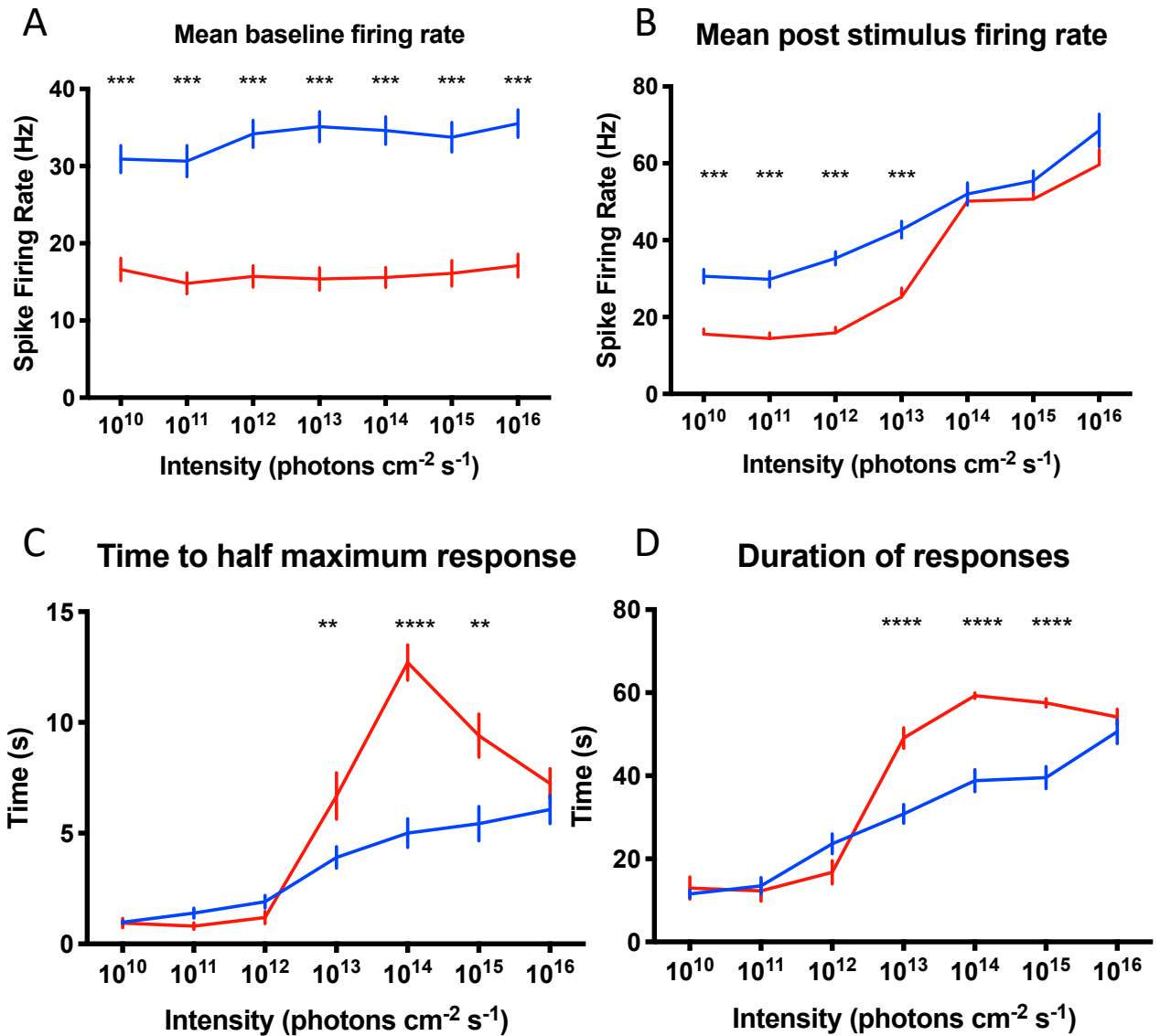


Figure 7-14 Spike firing characteristic and response decay kinetics for L7-6 delivered hOPN4 compared to Cre.lox delivery. N=2; n=37. For all values, differences tested with 2way ANOVA (see appendix for results table) with Sidak's test for multiple comparisons. A. $p = t$ -test comparing mean values at each intensity tested. $p = 0.0001$ at all intensities. B. $p = 0.0064, 0.0050, 0.0001$, respectively. C. $p = 0.0050, <0.0001, <0.0001$. D. $p < 0.0001$ for all intensities.

Discussion

In this Chapter, I describe the cloning and production of AAV vectors containing hOPN4 driven by one of two compressed L7 promotor constructs. These were then validated in vitro and in vivo showing for the first time that the L7-6 construct was able to drive production of protein in L7 ON-bipolar cells in a degenerate retina. The L7-6.hOPN4 construct additionally was able to drive sufficient hOPN4 expression to drive functional light responses in a degenerate retina, additionally devoid of melanopsin.

Differences in restored hOPN4 responses depending of method of delivery

Even from the small number of retina recorded from in this exploratory study (n=2), there are suggestions of differences in the characteristics the functional responses seen with L7-6 delivered hOPN4 in comparison to Cre.lox delivered hOPN4. Which is particularly interesting given the well circumscribed expression seen in the expected, L7 expressing cells on immunohistochemical staining.

There may be several reasons for this – firstly, the small number of retinae recorded from in this exploratory study (n=2), the findings could be particular to these retinae. However, the absolute number of responsive electrodes was relatively large at n=28 and the effect (especially on offset kinetics) was particularly pronounced, so repeating this work in further mice will be useful.

However, there are potentially other reasons for these findings that need to be addressed: given the complexity of the L7 promotor (with multiple isoforms) it is possible that the L7-6 modification may alter the cell specificity, even restricting to a subpopulation of L7 cell types within the retina and therefore altering electrophysiological responses. This possibility is heightened with the observation of

multiple L7 staining cells in treated retina that do not co-stain for hOPN4 (Figure 7-7) – although this could also simply be explained these cells simply not being transfected by an AAV. Certainly an in-depth investigation of which cell types are targeted by the L7-6 construct is warranted with co-staining with alternative bipolar cell markers - as has been reported for the L7-1.Cre mouse (213). In addition, co-staining with other retinal cell markers (especially for ganglion cells) would be informative given the presence of off target expression in a population of direction sensitive RGCs in the L7-1.Cre line(221) as well as a comparison to grm6.sv40 driven delivery and of the L7-6.hOPN4 construct's action in the wildtype (*Pde6^{+/+}*) retina.

Even if the L7-6 construct does restrict expression to the expected cell types, if it were to alter the expression of native L7 within bipolar cells in a way that L7-1 did not, this could potentially alter response offset kinetics. The L7 protein is intimately connected to the quenching of G protein responses in the retinal bipolar cells, with L7 knockouts demonstrating elongated ERG b-wave responses (365). Thus, the elongation of responses we see in L7-6 delivered melanopsin pilot could suggest alterations to the function of native L7 (perhaps an alteration the balance of isoforms that is produced) within these cells – although gross IHC staining for L7 protein remained robust in all treated retina. Nevertheless, this incongruity warrants further, functional, investigation.

Ple155 mini-promotor in the degenerate retina

The lack of protein expression or restoration of electrophysiological light responses when the Ple155 minipromoter was used *in vivo* was perhaps surprising given both the ability of the construct to drive protein expression *in vitro* and the promoter's

previous validation to deliver a functional gene to ON-bipolar cells in a diseased (but not degenerate) retina (371).

The main difference in this study compared to that previously described by Scalabrino *et al*(371) is the presence of retinal degeneration in our model. While investigations into bipolar cell gene expression (Chapter 4) suggest no overt changes in expression of genes associated with the main bipolar cell signalling cascade or the L7 gene itself, the action of the L7 protein to this cascade is only poorly characterised(365) in wildtype retina, let alone the degenerate. Therefore, the Ple155 promotor construct could potentially act rather differently in the context of the degenerate retina. During preparation of this thesis, I became aware of a new L7 mini-promotor “Ple265” which has been recently described as particularly specific for retinal bipolar cells (with no brain expression at all) when delivered by AAV(372) – this would be particularly interesting to compare to Ple155 in the context of a degenerate retina.

Conclusion

These unexpected failure of the Ple155.hOPN4 AAV to produce protein and the possibility that electrophysiological responses restored by L7-6.hOPN4 may differ from cre.lox delivered hOPN4 may perhaps pose more questions than are answered. However, these findings do represent the first demonstration of compressed L7 promoters in a degenerate retina (and for L7-6, in any retina); showing hOPN4 expression driven in the INL in cells co-staining for L7: ON-bipolar cells. This led to the first reported optogenetic restoration of electrophysiological responses to light in a degenerate retina using this promotor construct.

This is especially exciting as the L7 promotor, gene and protein are becoming increasingly understood both in the retina and cerebellum. In tandem, organised efforts to systematically create and evaluate cell specific promotors(370) are bearing fruit – with an exponential number of such constructs being reported. In addition, the high homology of mouse L7 to its human analogue and success of compressed L7 promotors in ex vivo non-human-primate tissue(166) highlight this is a particularly promising avenue of translational investigation. A compressed L7 promotor driven construct may well produce a cell specific optogenetic approach to visual restoration in inherited retinal degenerations that can be used in clinic.

Chapter 8 – General Conclusion

Overview

The overarching aim of this research study was to compare optogenetic strategies to restore light responses in a model of retinal degeneration with particular reference to cell specific targeting. The suitability of the theoretically most attractive cellular targets (ON-bipolar cells) was investigated in the context of retinal degeneration; the delivery of human melanopsin, human rhodopsin and ReaChR to the retina by different methods was assessed.

Mouse models of retinal degeneration - *Pde6b*^{rd1/rd1} - were bred to be additionally devoid of their native melanopsin and to express the enzyme Cre in either ON-bipolar or a population of retinal ganglion cells. These were validated anatomically and functionally and provided a system in which optogenetic tools could be targeted specifically and efficiently to cells of interest in a model retina without native light responses. All previous studies of optogenetic restoration have used mouse models that retain native melanopsin dependant light responses.

These mouse models were initially used to label ON-bipolar cells to allow their isolation by FACS. This formed the basis of the first comparison of gene expression profiles specifically within these cells in the context of retinal degeneration. Perhaps unexpectedly, given the marked morphological changes in bipolar cells during retinal degeneration, a comparatively small number of genes were found to be differentially expressed. Promisingly, this list did not include genes relating to increased oxidative stress, second messenger systems nor the cells' native light signalling cascade – areas where dramatic changes may have pointed towards unsuitability as targets for optogenetic therapy. However, several genes potentially associated with cell shape

regulation, neurotransmitter release and viral entry to the cell were however seen to be altered, four of which were further characterised using immunohistochemistry. The changes seen in these genes were generally reflected the known morphological and neurotransmission alterations of bipolar cells seen in degenerating retinae(75, 76, 85, 87, 238). These findings would appear not to provide evidence that might preclude bipolar cells as optogenetic targets and there appears to be no *a priori* reason to exclude them as potential therapeutic targets.

The optogenetic tool melanopsin was then targeted specifically to these cells, for the first time, using floxed AAV constructs in order to allow a direct comparison with non-specific delivery and retinal ganglion cell targeted expression. At the level of retinal electrophysiology, this demonstrated good reconstitution of light responses (compared to sham saline injected control retinae where no responses were seen). Response kinetics when melanopsin was targeted to ON-bipolar cells were expedited and sensitivity increased when it was targeted to RGCs. Despite this, robust reconstitution of behavioural light dependant responses were not seen in any group. As discussed in Chapter 5, this could have several explanations, including that isolated reconstituted retinal responses are not large enough to drive behavioural responses, that targeted cells do not project to the correct centres in the brain necessary to drive the assay, or that the absence of melanopsin in development, in the context of retinal degeneration, leads to deficiencies in projections or processing necessary to drive functional vision as measured with our assays. The current studies presented here do, however, demonstrate the value of the degenerate and melanopsin-less retina as a powerful model system to directly explore the properties of specific optogenetic tools at the retinal network level.

It was planned to compare the electrophysiological responses attained when melanopsin was targeted specifically to bipolar and RGCs to a similar targeting of two other candidate tools: human rhodopsin and the recombinant channelrhodopsin ReaChR(357). The rhodopsin AAV produced comparatively poor expression of protein in a degenerate retina but did lead to the restoration of electrophysiological light responses in a small number of recordings: the most likely explanation for this being the low viral preparation titre(103, 106, 158). However, these data confirm the potential of human-rhodopsin as an optogenetic tool.

Robust responses were however seen with ReaChR delivery which, as expected, had much faster response kinetics compared to melanopsin, and sensitivities much closer to those of vertebrate opsin-based tools than for native channelrhodopsins. These findings in favour of ReaChR as a tool were tempered by evidence of significantly elevated baseline firing rates. This will require further investigation before clinical translation, in order to determine if they represent a relative depolarisation of the cell membrane due to a deleterious “leak” of Ca^{2+} through the channel into the cell, potentially leading to excitotoxicity and cell death(97, 358).

In comparing responses of these three candidate optogenetic tools, perhaps the most notable contrast was the form of the responses that were achieved when targeted to the same cells: rhodopsin producing a transient ON type response, ReaChR a sustained type response (following the onset and offset of the stimulus closely) and hOPN4 producing a prolonged response (extending beyond the end of the stimulus). This diversity of response forms is particularly interesting for the future development of cell specific optogenetic approaches. If tools with specific responses can be targeted

to surviving cells with complimentary native response types (for example Rhodopsin to fast ON type RGC), there may be potential to restore light responses much closer to those found in normal visual processing. This could potentially contribute to restoring a percept closer to normal visual experience for animals and ultimately IRD patients.

The Cre-lox system used in the described models, used to restrict expression to the cell populations of interest, is not however clinically translatable to patients. To address this, two “compressed” versions of the bipolar cell specific promotor (L7-6 and Ple155), previously optimised for AAV use in the cerebellum, were cloned into the human melanopsin backbone plasmid and used to produce AAVs containing human melanopsin driven by either promotor. These were injected intravitreally into retinal degenerate mice with the L7-6 promotor found to successfully (for the first time) drive the expression of protein in the retina – specifically in L7 expressing cells.

[Implications, future directions and suggestions for further work](#)

In summary, this study demonstrates that no one optogenetic tool is unequivocally superior for optogenetic approaches to visual restoration in the IRDs, but suggests that the tools’ differing properties could, in the longer term be exploited to restore different aspects of visual processing. While further preclinical work is warranted and required to fully maximise deployment of optogenetics as a clinical therapeutic (especially in terms of safety in the case of ReaChR) all three tools would appear to have much potential. In addition, this work has confirmed the viability of AAV cre.lox cell specific targeting of optogenetics tools in the preclinical setting and demonstrated that the melanopsin tools can have differing response properties when specifically targeted to different cell populations.

Melanopsin coupling

This is especially interesting in the case of melanopsin where expedited kinetics with ON-bipolar cells specific expression were found, which could be accounted for by differences in coupling or quenching mechanisms for melanopsin within these cells. If there are indeed such differences between cell populations, then these could conceivably be maximised to improve melanopsin's response kinetics. The current study did not extend to direct recording from individual retinal cells which may prove useful in further characterising the kinetics of the exogenous melanopsin response in ON-bipolar cells. First in determining that the expedited kinetics occur at this level (rather than being a network effect) and then, with inhibition of candidate second messenger and quenching pathways, in determining which of these melanopsin is interacting with in this context.

ReaChR responses

While the superior kinetics of ReaChR mediated responses was perhaps to be expected, its level of sensitivity was particularly interesting. This recombinant channelrhodopsin would appear, in this context, to demonstrate comparatively favourable levels of sensitivity, superior kinetics and a previously demonstrated spectral sensitivity well within the visual spectrum ($\lambda_{\text{max}} = 510\text{nm}(357)$). However, while comparatively raised baseline firing rates could suggest a potential risk of excitotoxicity, this study did not specifically track the loss of transduced bipolar cells through over the course of degeneration. This could be further investigated in the first instance by histological tracking of cell death at time points following AAV delivery, or by monitoring levels of YFP-Citrine fluorescence *in vivo* following AAV injection (for

example using scanning laser ophthalmoscopy) or indeed by monitoring the thickness of the inner nuclear and plexiform layers using optical coherence tomography (OCT).

Rhodopsin responses

The small number of responsive electrodes in the rhodopsin treated group limited the power of comparison that could be made. However, the marked transience, sensitivity and large size of responses noted suggest that rhodopsin could well combine some of the positive attributes of the other tools. Therefore, a repetition of this experiment (with an improved AAV titre) will be important in the first instance in order to fully elucidate irradiance response relationship and kinetics of targeted melanopsin delivery.

Retinal processing

This study has demonstrated the efficacy of Cre-lox models as a powerful method for cell specific expression of AAV delivered optogenetic tools and shown that such tools can have differing response kinetics and sensitivities depending on which cell populations they are targeted to. With this, a useful next step would be to further characterise these electrophysiological responses by mapping receptive fields: this study has used full field illumination stimuli, so investigating the character of responses in more detail with stimuli specifically designed to stimulate centre surround opponent, edge detection and direction selective responses is indicated. This will be important in investigating cell specific delivery as an improvement to current survivor cell stimulation techniques: targeting of ON-bipolar cells is attractive, in theory, as it allows retention of the retinal processing necessary to achieve these complex responses - so specifically interrogating metrics of this processing will be essential.

Viral delivery

The differences seen in this study, while indicating the utility of further study, add weight to the general importance of targeting optogenetic tools to specific cell types. However, if this is to be realised, translatable cell specific delivery systems will need to be further improved. While the work presented in Chapter 7, demonstrating a novel mini-promoter, able to drive melanopsin expression in the degenerate retina is promising, there remain several practical challenges to overcome in terms of vector development, especially for bipolar cell specific targeting. For example, the most effective surgical route of delivery of AAV (intravitreal, subretinal) is an ongoing discussion with pros and cons on each side, as are the relative merits of AAV capsid serotype and promoter choice. The finding in this study of changes in expression, during retinal degeneration, of enzymes such as Sulphatase 2 may be particularly relevant to the success of viral delivery. Sulphatase 2 is involved in the regulation of heparin sulphate Proteoglycans (HSPG) on the cell surface -involved in binding of AAV during cell entry - therefore further investigation of the functional effects of these enzymes on AAV efficacy in the context of retinal degeneration could be particularly informative. For example, by investigating the transfection efficacy of current AAV combinations in *Slf2* knock out mice or otherwise interfering with enzyme action.

Models without melanopsin

In addition to their retinal degeneration phenotype, the mice used in this study were melanopsin knockouts. While this novel approach to assessing visual restoration interventions allows easier isolation of exogenously induced light responses – especially on MEA, it may have contributed to the poor behavioural responses seen in treated mice. Indeed, previous studies in degenerate, but *Opn4*^{+/+} mice, using the

same optogenetic tools have demonstrated robust reconstitution of behavioural responses(103, 106, 159, 357). It would therefore be useful to expand the range of “central assays” used on these treated mice to determine if, in the context of good retinal electrophysiology responses, such light dependant information reaches higher retinorecipient centres. For example, *in vivo* visual evoked potentials, post light pulse c-Fos staining of the brain or direct LGN or cortical recordings could be informative in determining if the poor responses seen represent an intrinsic property of the model.

If indeed light responses returned to degenerate mice by optogenetics are too small to drive higher responses, this may suggest that the robust reconstitution seen in previous reports represents an augmentation of residual responses, rather than purely the induced signal. From a translational point of view, this is a moot point: in IRD patients, the melanopsin ipRGC system will be intact (not to mention a number of rods and cones depending on their individual phenotype), therefore regardless if optogenetics potentiates residual responses, or induces entirely new ones, if the patient is able to utilise the light information, its origin is less important.

Conversely, making this differentiation in long term will be hugely important – the development of new optogenetic tools is progressing with great pace: many of the disadvantages evident with our current options conceivably could soon be overcome. Understanding the intrinsic functional properties of individual optogenetic tools, in the cellular context in which they will be used, will facilitate the selection of the most appropriate tools. This will be paramount as we move not towards a prototype optogenetic therapy in humans, but to an optimised therapy, restoring the most useful form of vision to these patients as possible.

Conclusion

Retinal ON-bipolar cells are valid targets for optogenetic approaches to restoring vision in the context of retinal degeneration. While work remains to select the optimum tool and vector with which to specifically target these cells, the approach holds increasingly great promise to improve the quality of vision that may be restored to IRD patients.

Chapter 9 – Appendices

Appendix 1 - Sequencing primers

Sequencing primers used

Helper plasmid

HELP_SEQ_F1	GCGCATTTCAGCAGGCCG
HELP_SEQ_F2	GACGCTCAAGTCAGAG
HELP_SEQ_F3	CTGACGCTCAGTGGAAC
HELP_SEQ_F4	CTCCGATCGTTGTC
HELP_SEQ_F5	GACAGCTTATCATCG
HELP_SEQ_F5a	TTCGCTACTTGGAGCCACT
HELP_SEQ_F6a	CTTGGTTATGCCGGTACTGC
HELP_SEQ_F7	TGACCGAGGAGCTGT
HELP_SEQ_F8	TACAATGGCCGGACT
HELP_SEQ_F9	ATGACACTACGACCA
HELP_SEQ_F10	CAGTTACTGTGCTGA
HELP_SEQ_F11	TCTTGGGCAATGTATG
HELP_SEQ_F12	TAGGAACCATAGCCTTG
HELP_SEQ_F13	GTA CTGCTATCGGTG
HELP_SEQ_F14	GCAAGGAGGTGCTGCTG
HELP_SEQ_F15	CAAGCGAGGAGCTCAC
HELP_SEQ_F16	GATCGCAGAGACCTG
HELP_SEQ_F17	GTCTGACACCTCTTCG
HELP_SEQ_F18	AGACCGTCACCGCTCA
HELP_SEQ_F19	ATCTCCGGGTCAGCA
HELP_SEQ_F20	TGGGCTCGCATGTGCCGT
HELP_SEQ_F21	CGGATGAGGCGGCGTATC
HELP_SEQ_F22	CAGAGGTGACCTACAAGC
HELP_SEQ_F23	CGACTTCAAGATATC
HELP_SEQ_F24	GCTACGGCGCAGGAGTG
HELP_SEQ_F25	CGGCATCACCTGGATGTC

Capsid (AAV2.2) Quad Mutant

Cap_1	GGGTATTTAAGCCCGAGTGA
Cap_2	GCTCAGTGGAACGAAAACCTC
Cap_3	GATTTAGAGCTTGACGGGGA
Cap_4	ACTTTCCCTTCAACGACTGT
Cap_5	CCAATCAGGAGCTTCGAACGACAATCACTTCTTTGGCTACAG
Cap_6	CGACCAGTACCTGTATTTCTTAAGCAGAACAAACACTCCAAG
Cap_7	CCTCGCCCCATTGGTACCAGATTCTGACTCGTAATC

EF1 α -DIO-Melanopsin

EF1a_hOPN4_6,785_F	TTTACGGTTCCTGGCCTTTT
EF1a_hOPN4_501_F	CTGGCCTCTTTACGGGTTAT
EF1a_hOPN4_1,322_F	TCTCCTTGGAATTTGCCCTT
EF1a_hOPN4_6,709_F	TCTGACTTGAGCGTCGATTT

EF1a_hOPN4_3,078_R	AAGCCATACGGGAAGCAATA
EF1a_hOPN4_2,438_R	CTCATGTAGGGTGTGAGGAC
EF1a_hOPN4_1,807_R	TGAAGGACATGAGGAAGTCG
EF1a_hOPN4_1,207_R	AGAACTAATCGAGGTGCCTG
EF1a_hOPN4_520_R	ATAACCCGTAAAGAGGCCAG

CBA-Melanopsin

ITR F	GGAACCCCTAGTGATGGAGTT
ITR R	CGGCCTCAGTGAGCGA
Dsred F 1	ACCTTCAGCTTCACGGTGTT
DSred R 1	GGGCAACGTGCTGGTTATTG
Dsred F 3	ACAATAACCAGCACGTTGCC
DSred R 3	GGAGCCGAAATCTGGGAGG
Dsred F 7	TGAAGCGCATGAACTCCTTG
DSred R 7	CGTGCTGGTTATTGTGCTGTC
OPN4 H F	GGGGTTGCTGACATCCTGCTCCTGGG
OPN4 H R	GGGAGCCCAGGAGAGCACGAAGAGGAGG
M CITRINE F	CTTCATGTGGTCGGGGTAGC
M CITRINE R	GACGTAAACGGCCACAAGTT

EF1 α -Rhodopsin

EF1a_FI_Rho_6,583_F	TATGGAAAAACGCCAGCAAC
EF1a_FI_Rho_501_F	CTGGCCTCTTTACGGGTTAT
EF1a_FI_Rho_1,221_F	TACGTCGTCTTTAGGTTGGG
EF1a_FI_Rho_1,838_F	ATCAGGAAAGCGATGACCAT
EF1a_FI_Rho_1,451_R	ATTCCGGAGTCGACTCTAGA
EF1a_FI_Rho_3,508_R	TCCAGAGGTTGATTATCGATAAGC
EF1a_FI_Rho_571_R	CAAGAATCACGTACTGCAGC
EF1a_FI_Rho_2,465_R	TCAACTTCCTCACGCTCTAC

hSyn-DIO-ReaChR-citrine

SBC_EGFP C F	CATGGTCCTGCTGGAGTTCGTG
SBC_30nt_M13R	AGCGGATAACAATTTACACAGGA
BCEGFPNrev	CGTCGCCGTCCAGCTCGACCAG
flox_ReaChR_F1	TGCAGAATGGTAGCTGGATTGT
flox_ReaChR_R1	GCTGTACAAGTAGTAGGGCG
flox_ReaChR_F2	TGCAGAATGGTAGCTGGATTG
flox_ReaChR_R2	GGCGCGCCATAACTTCGTA

L7-6 plasmid

L7_6_GFP_6,319_F	ATGATTAACCCGCCATGCTA
L7_6_GFP_691_F	AGGACAGTGGTAGAATGCTG
L7_6_GFP_1586_R	CGCTTTACTTGTACAGCTCG

L7_6_GFP_599_R	ATCTCTTGGGGGACAGTACT
----------------	----------------------

Ple155 plasmid

PLE155 F 1	TCAATCATGGGCTCTGAGAC
PLE155 F 2	GGCTAGGGTGATAGAGGAGA
PLE155 R 1	TCTCCTCTATCACCCCTAGCC
PLE155 R2	GCCTTTTAAACGTCCAGGAC

Appendix 2 - Commercial laboratory cloning reports (Genescript Ltd)

Certificate of Analysis

Project ID: U2599DI210-2

Construct Information:

Gene Name: L76 promotor_EF1a-hOPN4

Clone ID: X95347

Cloning Vector: EF1a-hOPN4

Gene Length: 856 bp

Cloning Strategy: MluI / KpnI

QC Items	Specifications	Results	
Sequencing Alignment	Sequencing results are consistent with the targeted insert sequence.	Pass	Consistent
Vector Sequence	The flanking sequences of the cloning site are correct.	Pass	Correct Shown in the SQD file
Restriction Digests	The size of inserted fragment is correct and free of unexpected bands suggesting contamination.	Pass	Correct Shown in attachment 1
DNA Quality	Miniprep: 10 µg OD260/280=1.8~2.0 Free of contamination	Pass	≥ 10 µg OD260/280=1.86 Pure
Quality grade	Research Grade	Pass	Research Grade
Appearance	Clear and free of foreign particles.	Pass	Clear Free of foreign particles
Additional Test		N/A	

NOTE

Shipping at	Plasmid Storing at	Bacstab Storing at	Glycerol Stock Storing at
Room Temperature	-20°C	4°C	-20°C/-80°C

Morgan

Certified by:

Date: 10/31/2018

Thank you for your patronage to our Gene services! To maintain this working relationship, we shall be grateful if you can add our webpage URL into your lab website. As a token of appreciation, you will be rewarded by 1,000 EZcoupon™ points. For more information, please contact us by e-mail at web@genscript.com.

For research use only

860 Centennial Ave., Piscataway, NJ 08854, USA

Toll-Free: 1-877-436-7274

Tel: 1-732-885-9188

Fax: 1-732-210-0262

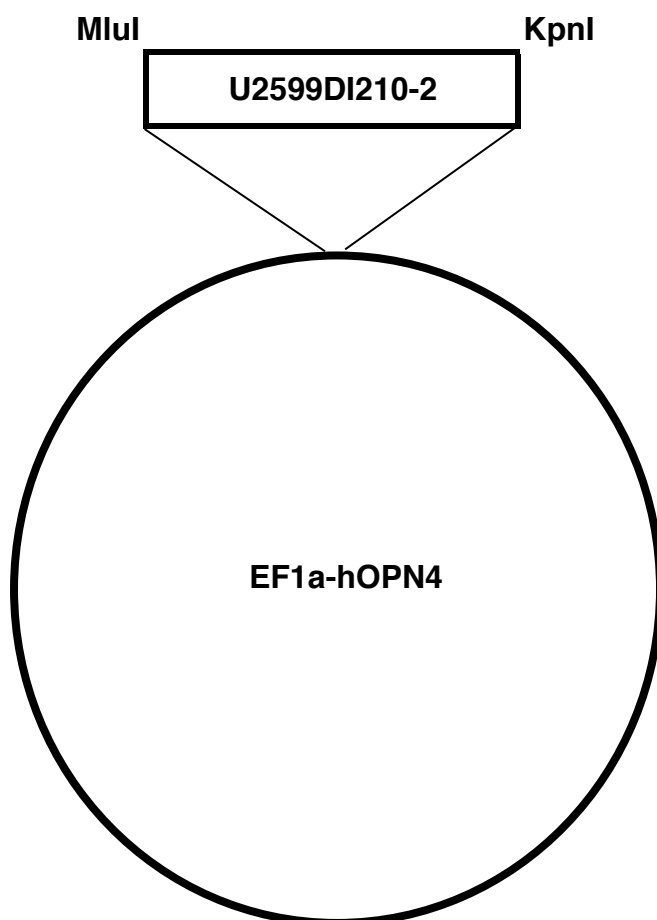
Email: order@genscript.com

Web: www.genscript.com

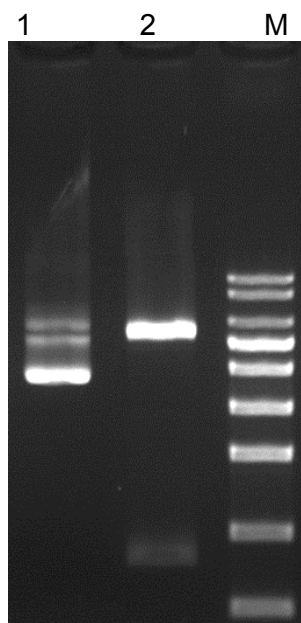
Attachment 1

Plasmid Construct Map

The gene was cloned in EF1a-hOPN4 by MluI / KpnI.

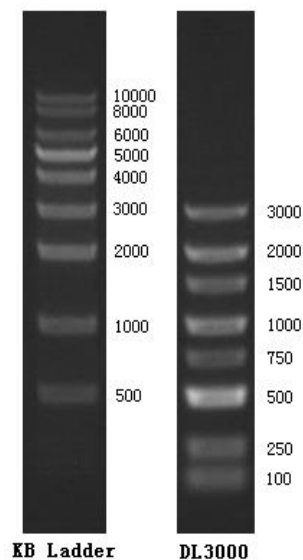


Enzyme Digestion



Lane M: KB Ladder
Lane 1: U2599DI210-2 plasmid
Lane 2: U2599DI210-2 plasmid digested
by MluI and KpnI

Digestion Conditions:
About 300ng plasmid digested
Digestion in water-bath, 37°C for 40 minutes
1% Agarose Gel



For research use only

860 Centennial Ave., Piscataway, NJ 08854, USA

Toll-Free: 1-877-436-7274

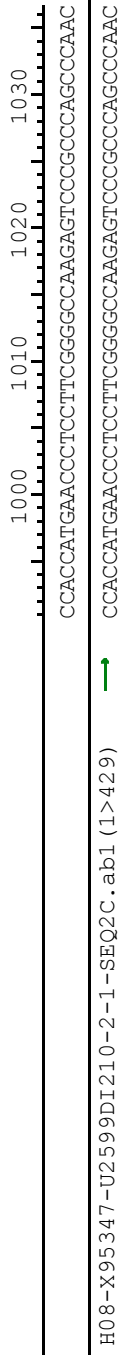
Tel: 1-732-885-9188

Fax: 1-732-210-0262

Email: order@genscript.com

Web: www.genscript.com

[illegible]



Certificate of Analysis

Project ID: U2599DI210-4

Construct Information:

Gene Name: Ple155 promotor_EF1a-hOPN4

Clone ID: X95348

Cloning Vector: EF1a-hOPN4

Gene Length: 1664 bp

Cloning Strategy: MluI / KpnI

QC Items	Specifications	Results	
Sequencing Alignment	Sequencing results are consistent with the targeted insert sequence.	Pass	Consistent
Vector Sequence	The flanking sequences of the cloning site are correct.	Pass	Correct Shown in the SQD file
Restriction Digests	The size of inserted fragment is correct and free of unexpected bands suggesting contamination.	Pass	Correct Shown in attachment 1
DNA Quality	Miniprep: 10 µg OD260/280=1.8~2.0 Free of contamination	Pass	≥ 10 µg OD260/280=1.83 Pure
Quality grade	Research Grade	Pass	Research Grade
Appearance	Clear and free of foreign particles.	Pass	Clear Free of foreign particles
Additional Test		N/A	

NOTE

Shipping at	Plasmid Storing at	Bacstab Storing at	Glycerol Stock Storing at
Room Temperature	-20°C	4°C	-20°C/-80°C

Morgan

Certified by:

Date: 11/04/2018

Thank you for your patronage to our Gene services! To maintain this working relationship, we shall be grateful if you can add our webpage URL into your lab website. As a token of appreciation, you will be rewarded by 1,000 EZcoupon™ points. For more information, please contact us by e-mail at web@genscript.com.

For research use only

860 Centennial Ave., Piscataway, NJ 08854, USA

Toll-Free: 1-877-436-7274

Tel: 1-732-885-9188

Fax: 1-732-210-0262

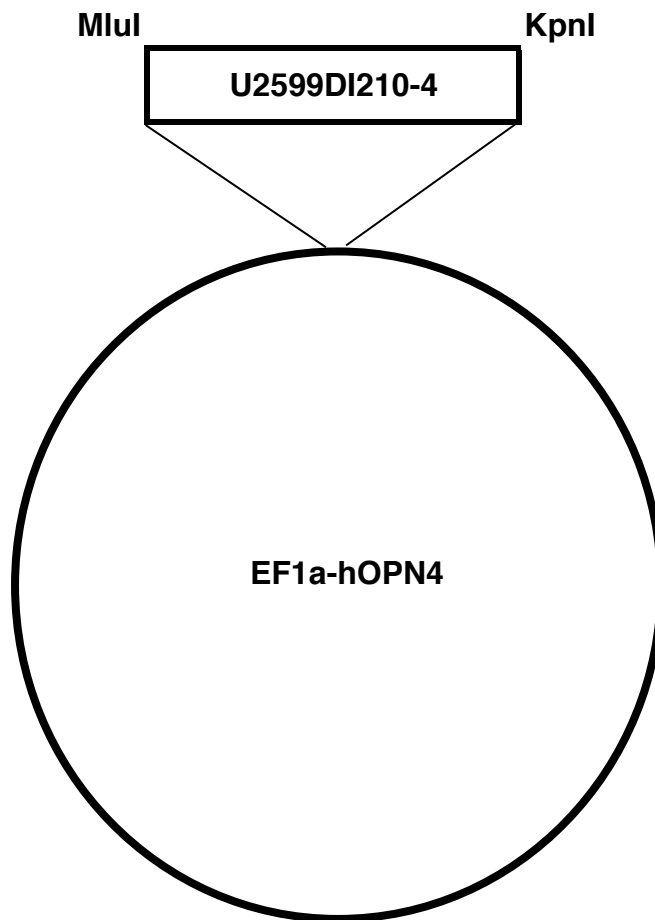
Email: order@genscript.com

Web: www.genscript.com

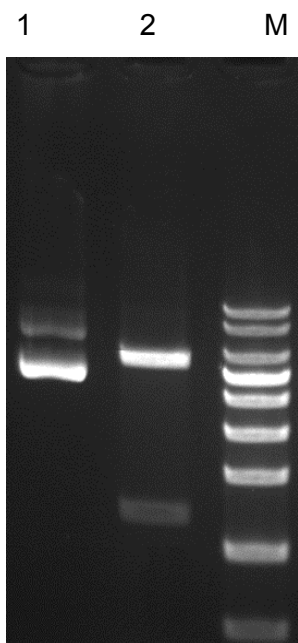
Attachment 1

Plasmid Construct Map

The gene was cloned in EF1a-hOPN4 by MluI / KpnI.

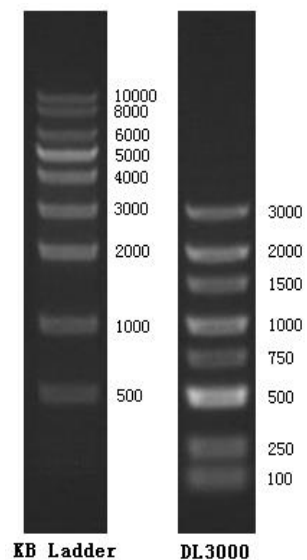


Enzyme Digestion



Lane M: KB Ladder
Lane 1: U2599DI210-4 plasmid
Lane 2: U2599DI210-4 plasmid digested by KpnI

Digestion Conditions:
About 300ng plasmid digested
Digestion in water-bath, 37°C for 40 minutes
1% Agarose Gel



For research use only

860 Centennial Ave., Piscataway, NJ 08854, USA

Toll-Free: 1-877-436-7274

Tel: 1-732-885-9188

Fax: 1-732-210-0262

Email: order@genscript.com

Web: www.genscript.com

1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
GATCCTCTAGAGCCACCATGAACCCCTCCTTCGGGGCCAAAGAGTCCCGGCCAGCCCAACCCAAAGAGCCCAGCTGCATGGCCACCCAGCACCCAGCTG

E12-X95348-U2599DI210-4-V-SEQRC.ab1 (1>806) ↵ GATCCTCTAGAGCCACCATGAACCCCTCCTTCGGGGCCAAAGAGTCCCGGCCAGCCCAACCCAAAGAGCCCAGCTGCATGGCCACCCAGCACCCAGCTG

1810
GTGGGA

E12-X95348-U2599DI210-4-V-SEQRC.ab1 (1>806) ↵ GTGGGA

Appendix 3 - Bipolar Cell Gene array report

1 Material and Methods

1.1 Array processing

RNA was assessed for concentration and quality using a SpectroStar (BMG Labtech, Aylesbury, UK) and a Bioanalyser (Agilent Technologies, Cheadle, UK). Microarray experiments were performed at Cambridge Genomic Services, University of Cambridge, using the MouseWG-6 v2 Expression BeadChip (Illumina, Chesterford, UK) according to the manufacturer's instructions. Briefly, total RNA was amplified using the Ovation Pico WTA v2 kit (NuGEN Technologies, Leek, Netherlands) and subsequently labelled using the BiotinIL kit (NuGEN Technologies) following the manufacturer's instructions. The concentration, purity and integrity of the resulting cDNA was measured using the Nanodrop ND-1000 (Thermo Scientific, Paisely, UK) and by Bioanalyser. Finally cDNA was hybridised to the MouseWG-6 v2 BeadChip overnight followed by washing, staining and scanning using the Bead Array Reader (Illumina).

1.2 Bioinformatics

After scanning the data are load in Illumina proprietary software, GenomeStudio. No background correction or normalisation is applied at this stage. A "final report" is generated, generating a text file containing the sample probe profile and the control probe information. The report is then processed in R using the lumi package [1] and the limma package [2]. The data are loaded and divided into subsets according to the groups being compared; only the samples involved in a given comparison are used. Subsets were then filtered to remove any non-expressed probes using the detection p-value from Illumina. Across all samples probes for which the intensity values were not significantly different ($p > 0.01$) from the negative controls were removed from the analysis. Following filtering the data was transformed using the Variance Stabilization Transformation (VST [1]) from lumi and then normalised to remove technical variation between arrays using quantile normalisation. Comparisons were performed using the limma package with results corrected for multiple testing using False Discovery Rate (FDR) testing. Finally the quality of the data was assed and the correlation of the samples in the groups compared. In the interest of reproducibility the packages version and R session information [3] are provided in the reference.

2 Technical QC report

This section focuses on the technical aspect of the array, using the Illumina controls integrated to the array to check if the data generated is of good quality

2.1 Hybridisation control

The hybridisation controls are spiked in the hybridisation buffer at 3 different concentrations, low, medium and high. If the hybridisation steps as performed optimally the controls should display an increasing signal intensity from low to high.

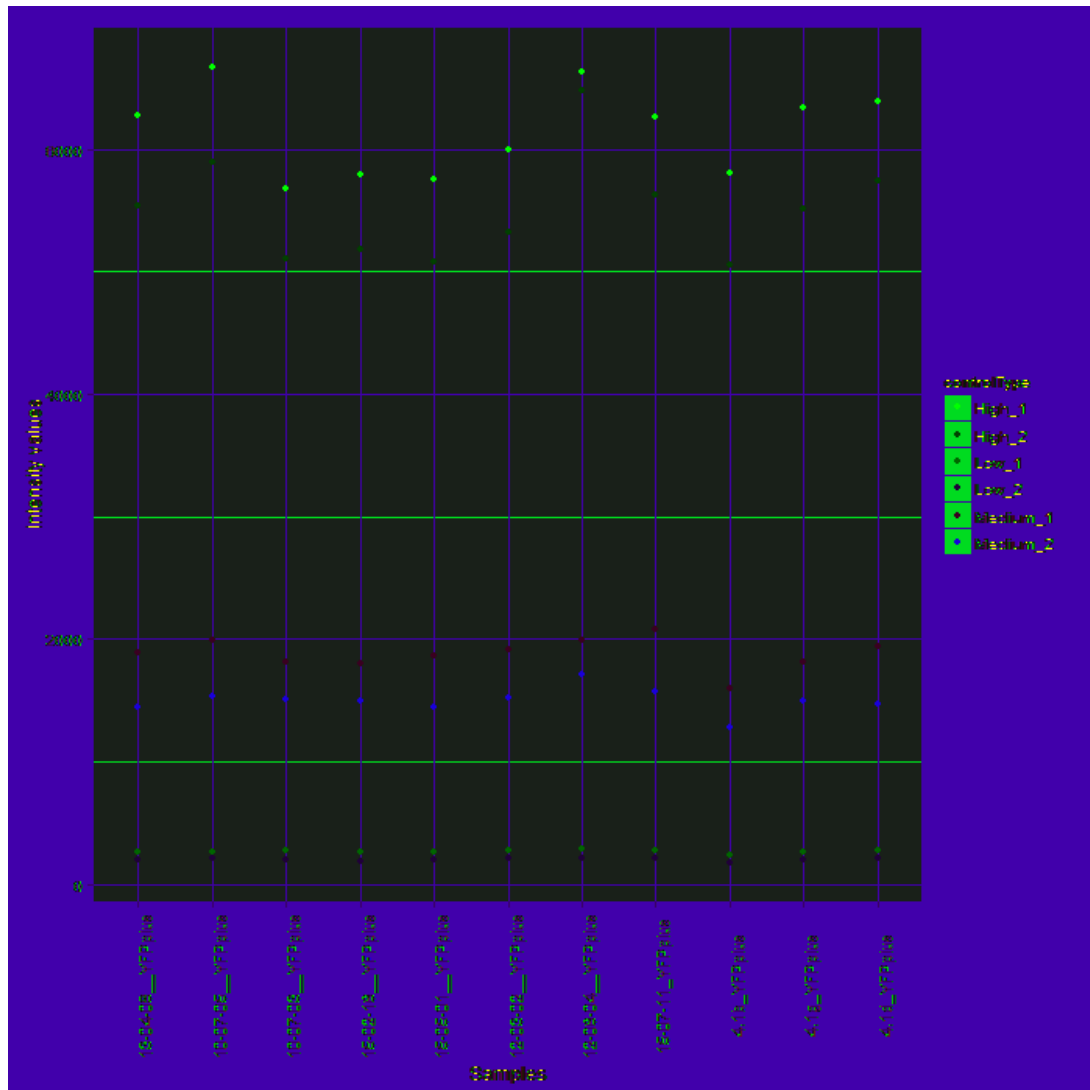


Figure 1: Hybridisation controls

2.2 Stringency controls

Stringency controls are design to assess the likelihood of cross hybridisation during the assay. Two types of probes are present on the array:

- Mismatch probes, with 2 mismatches
- Perfect match probes with no mismatches

Ideally the perfect match probes should have a signal at least three times higher than the mismatch ones.

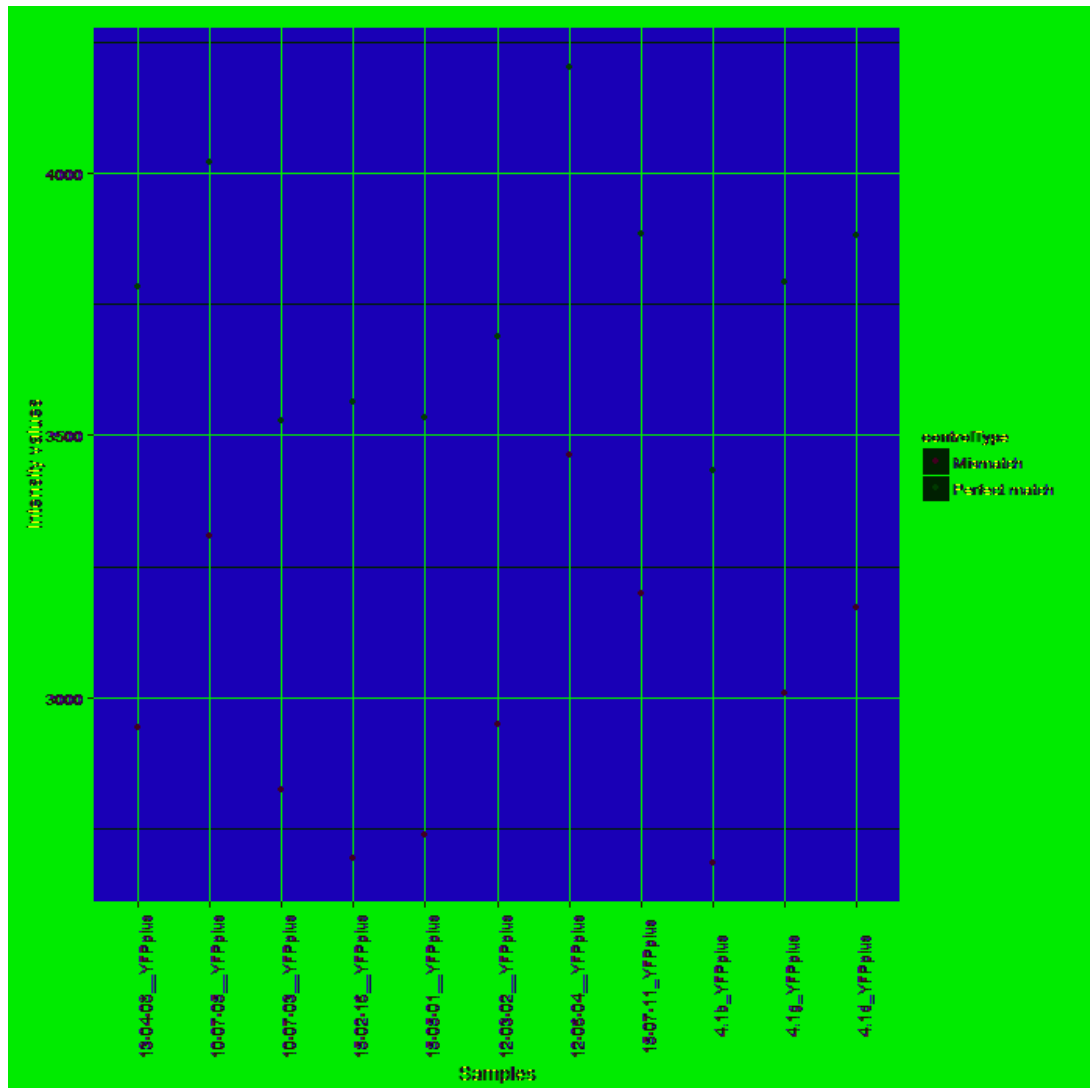


Figure 2: Stringency controls

2.3 Labelling controls

This controls are design to look at the efficiency of labelling, if the biotin has been well integrated it should give a high signal compare to background.

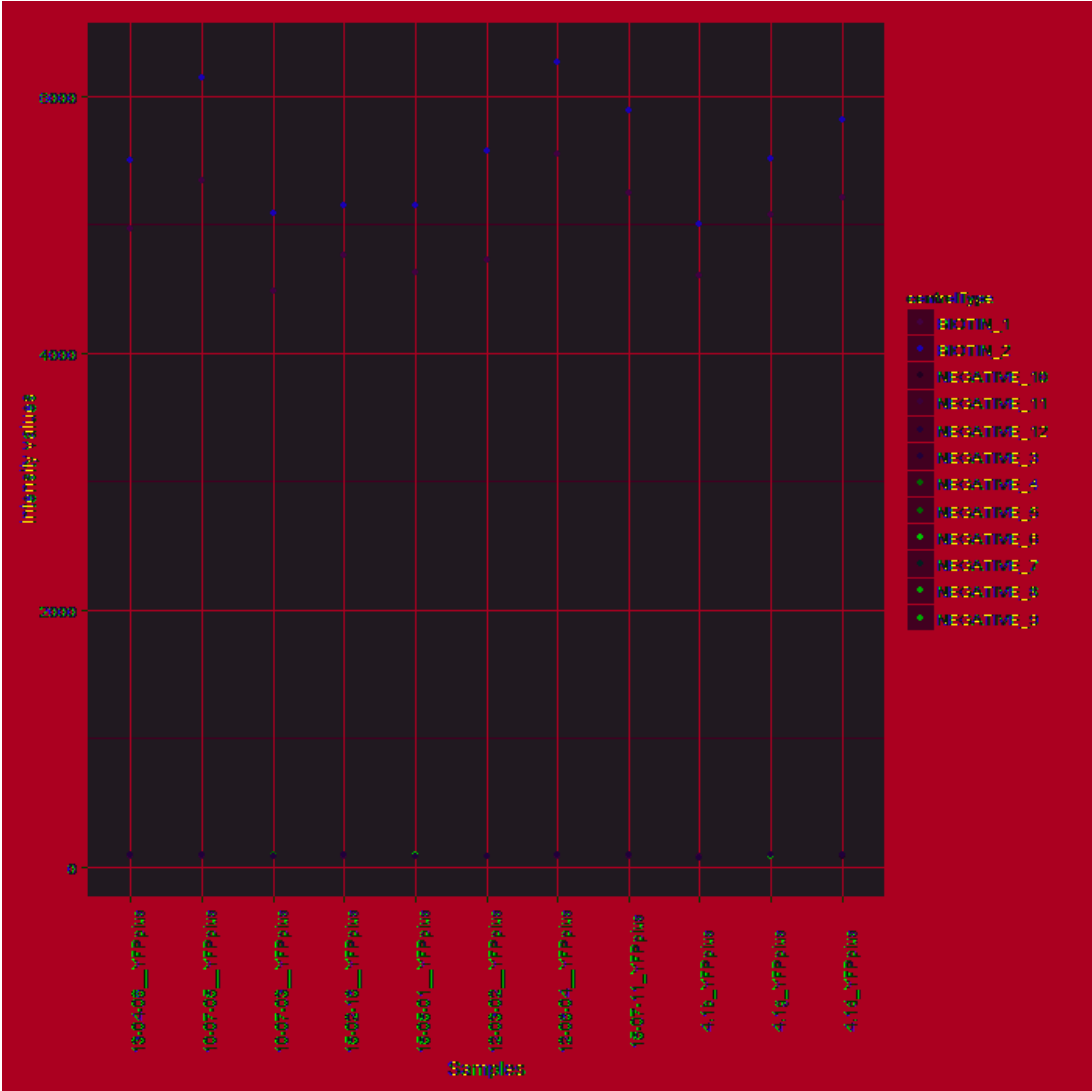


Figure 3: Labelling controls

2.4 Negative controls

Negative controls are empty beads on the array, they are a measure of the level of background on the array(s). Ideally the background should not vary too much between sections of the arrays. Increase in background signal could indicate issues with the array processing.

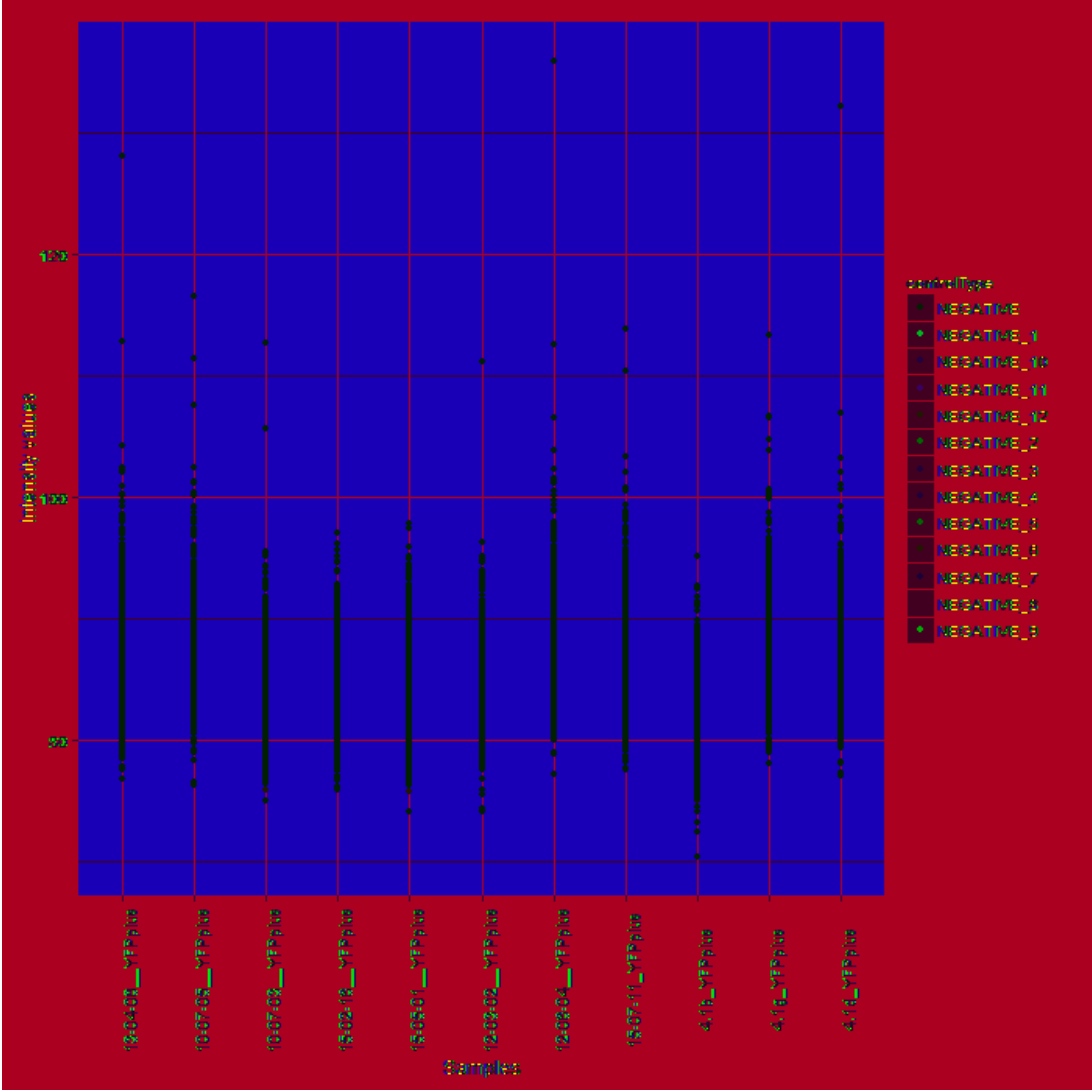


Figure 4: Labelling controls

3 Biological samples QC

Biological samples QC assess the relationship between samples within groups and between groups.

3.1 Hierarchical clustering

Hierarchical clustering plots the distance between samples as a tree, the longer the branches and the more nodes between the samples, the more distant they are. It is expected that sample should cluster according to their biological group.

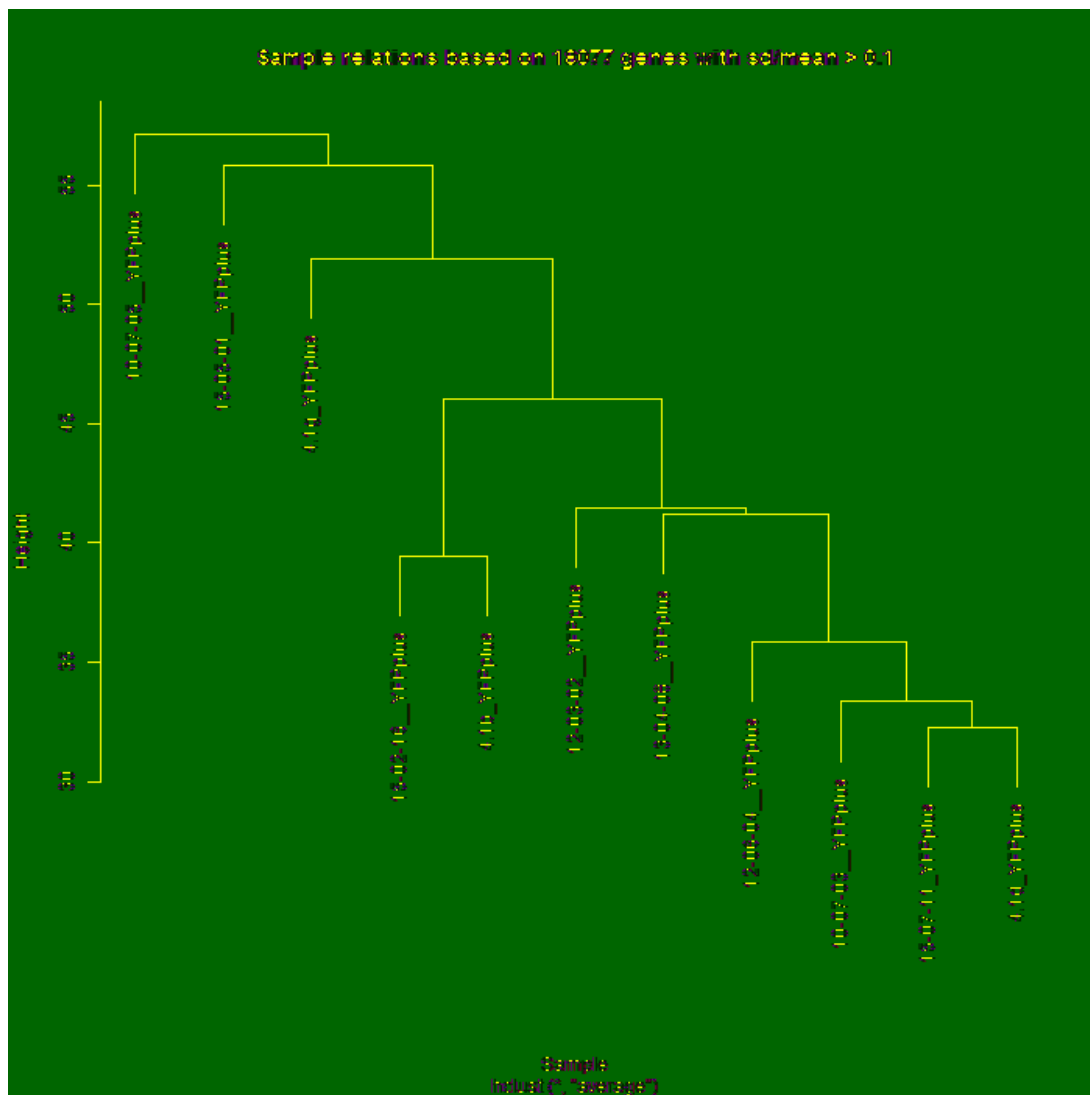


Figure 5: Hierarchical Clustering

3.2 MultiDimensional Scaling (MDS) clustering

MDS clustering clusters the samples in two dimensions (or more) instead of one, decomposing the variance into several components. Below are plotted the samples in relation to the two largest sources of potential variations. MDS can identify sources of variation potentially missed by relying on hierarchical clustering alone.

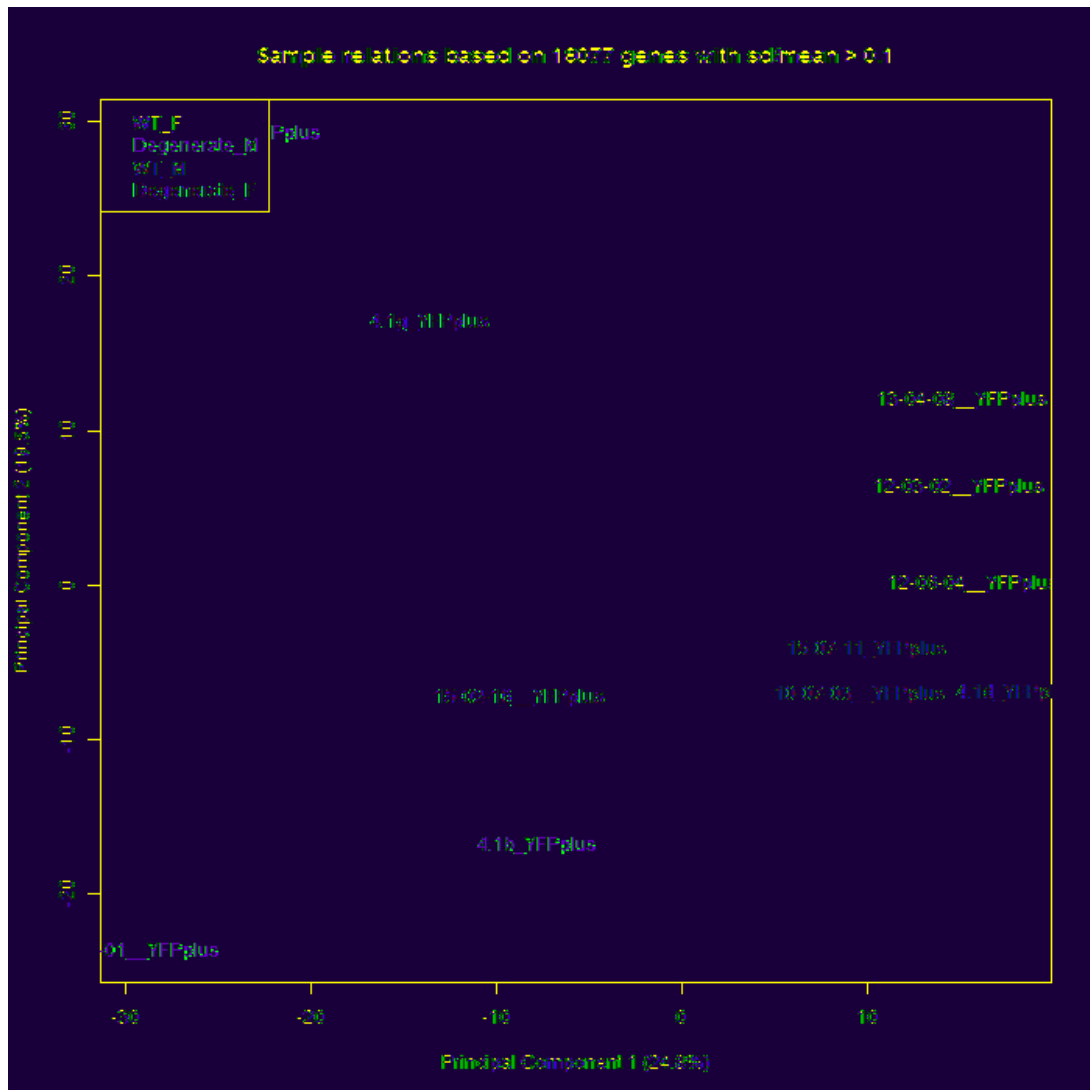


Figure 6: MDS clustering

3.3 Boxplots

Boxplots show the distribution of the intensity as a box with whiskers, the bottom of the box delimits the 25th percentile, the middle of the box the median, the top of the box the 75th percentile. Samples should have similar distribution of intensity. The intensity scale is log base 2.

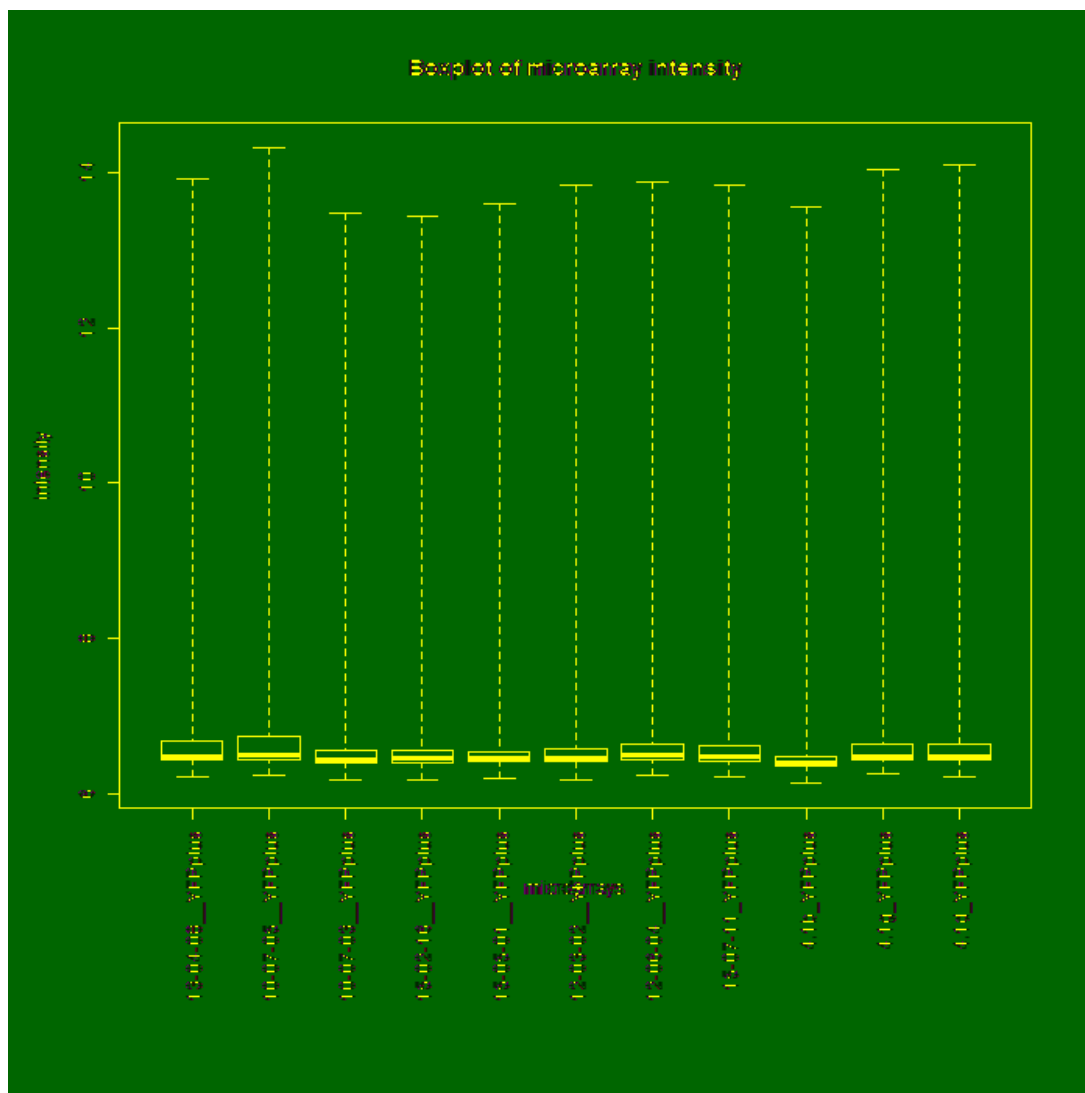


Figure 7: Boxplot

3.4 Density

The density plot represents the distribution of the intensity by plotting the intensities on the x axis against the frequency on the y axis, it is a less schematic representation of the distribution of the intensities.

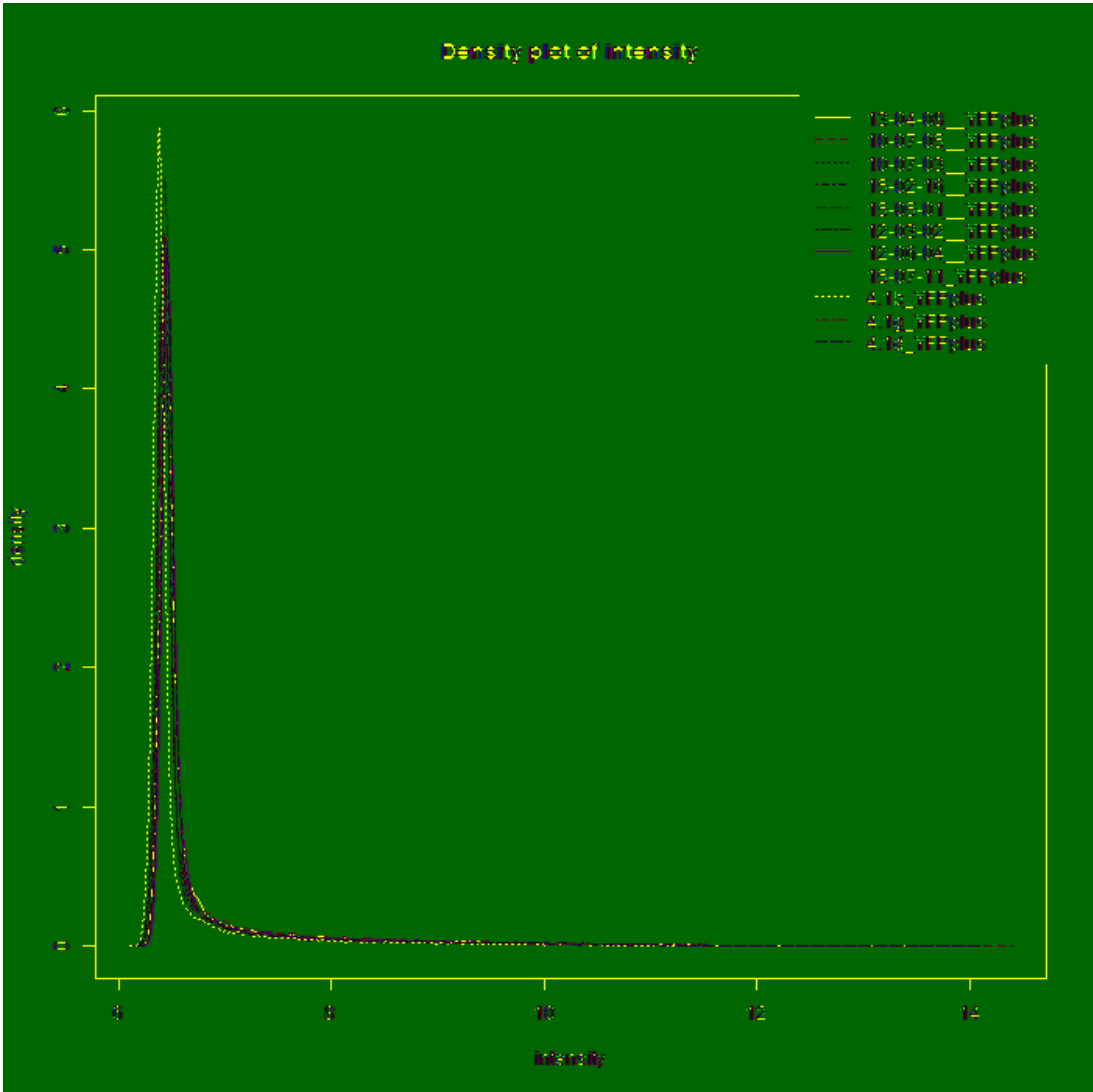


Figure 8: Boxplot

4.3 Scatter plots between groups

Scatter plots between two groups, these type of plots allow a closer inspection of the correlation within and between groups. These graphs are also important when assessing potential outliers, their profiles should be markedly different from the other samples within their group.

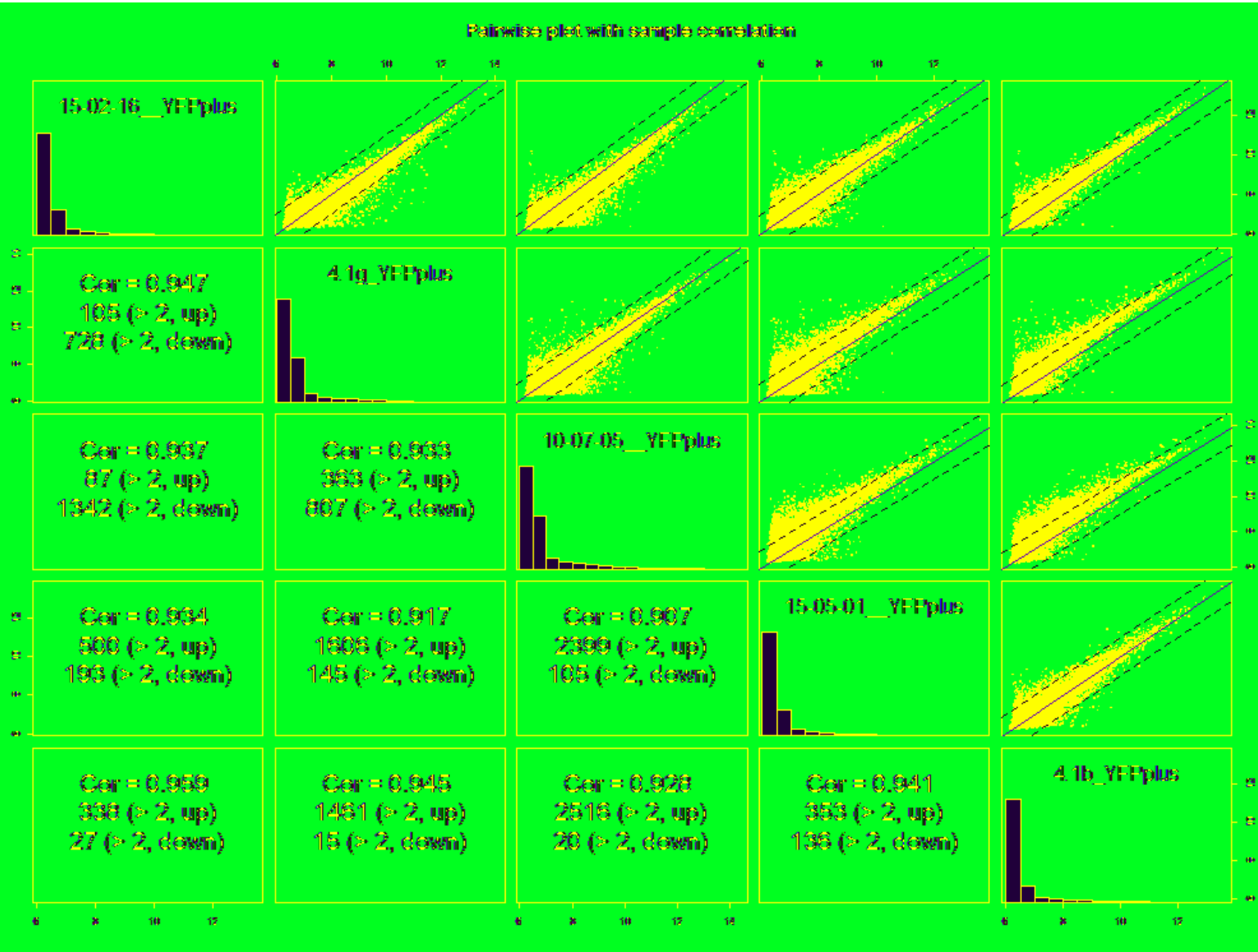


Figure 11: Scatter plots between two groups
[Click here to access the other graphs](#)

5 Normalisation

5.1 Normalisation Method

The normalisation method use by default is Quantile normalisation. Briefly, a reference distribution is constructed using the sample data. Then the intensity value of each sample and the reference distribution are ranked from highest to lowest. The sample values are fitted to the reference distribution, hence all the samples now share the same distribution of intensity. Sharing the same distribution of intensity allows for more powerful comparisons as the samples only differ by the change in gene expression and not change due to the intensity measurement, the assumption of the sample originating from the same population is validated. It is important to remove any source of noise (eg. outlier samples) before normalisation as otherwise their values will be used to construct the reference distribution. This is also why when performing a comparison between two groups, the rest of the data is removed.

5.2 Normalisation QC

All the QC graphs that were generated for the raw data are also regenerated for the normalisation data in order to check if there are any issue caused by the procedure. The expected results is that all the samples share the same distribution of intensities. [Click here to access the normalised graphs](#)

6 Comparisons

6.1 How to interpret your results

This section contains information about the comparisons performed. The datasets to compare are processed on a per comparison basis to make sure only the relevant samples are used for the analysis, therefore avoiding adding noise to the datasets when processing them which could be detrimental to the analysis. The steps used in the analysis are:

- Subset the data to include only the sample to be compared (usually from 2 groups)
- Perform pre-processing on the subset of data (filter, transformation and normalisation)
- Comparisons between the groups is performed using limma [2]

This is done for all the possible comparisons.

A summary table is generated (see below) to give an overview of the results. For each comparison a table with the top 10 genes is displayed along with a volcano plot and a heatmap. The volcano plot is useful to get an overview of the level of change and the level of significance, the x axis shows the level of change, the fold change (log2 scale), while the y axis represent the significance, $-\log_{10}(\text{pvalue})$. The heatmap displays the levels of expression for both groups as a color map for the top 50 genes. A direct link to the table is provided for each comparison.

To work on the results there are 2 important columns to use, logFC and adj.pvalue. The adj.pvalue contains the p-value corrected for multiple testing using False Discovery Rate. The p.value column should **not** be used as it is not corrected for multiple testing, resulting in a large number of false positives. The adjusted p-value should be used as a primary filtering column. If there are still too many results after filtering using "classic" levels (0.05 or 0.01) consider lowering it further or using the logFC column as a secondary filter to look at the higher rates of change in your data. The logFC represents the log in base 2 of the fold change. A value of 1 is equivalent to a change of 2 folds, 2 is 4 folds, 3 is 8, etc... Using the value as a power of 2 gives you the real fold change.

If there are still a large number of probes to study, it is recommended to take a functional approach to analyse the results. The aim of a functional analysis is to group genes according to function/pathways in order to reduce the complexity of the set. For this Gene Ontology tools or pathway finder can be used.

A note on the p-value: in some case the adjusted p-value will have the same value for several probes, which might look like an error has occurred during the correction. This can happen as a results of how the correction is performed. The steps are as follow:

- Sort the p-value from low (rank 1) to high (rank N, with N the number of comparisons performed)
- Multiply the p-value by N
- Divide the p-value by its rank
- Check that the rank is kept

The last point is the reason why the adjusted p-value can share the same value, if the ranks are not kept and a higher ranked probe ends up with a lower adjusted p-value than a lower rank probe, the lower value of the higher ranked probe will be used for the lower ranked p-value.

6.3.6 Degenerate vs WT

PROBE_ID	SYMBOL	logFC	adj.P.Val	B
ILMN_2765547	Gnat1	-2.65	0.00	8.85
ILMN_2595543	Pde6b	-1.08	0.00	7.84
ILMN_2603504	Car2	-0.94	0.00	7.66
ILMN_2859960	Adcy1	-0.95	0.00	7.47
ILMN_2944168	C80008	-1.17	0.00	7.28
ILMN_2512149	LOC100047936	-1.29	0.00	6.90
ILMN_2730436	Sag	-1.56	0.00	6.44
ILMN_3065373	4930572J05Rik	-1.02	0.00	6.22
ILMN_1257965	Ldha	-1.33	0.00	5.93
ILMN_2596522	Mt1	-2.06	0.00	5.80

[Click here to open the comparison table](#)

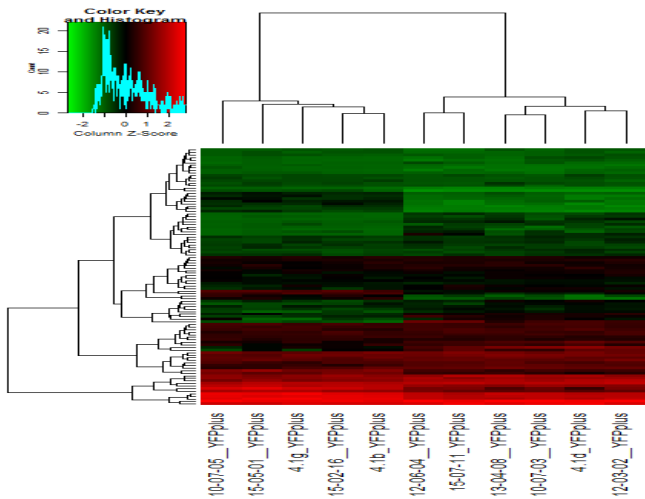


Figure 22: Heatmap

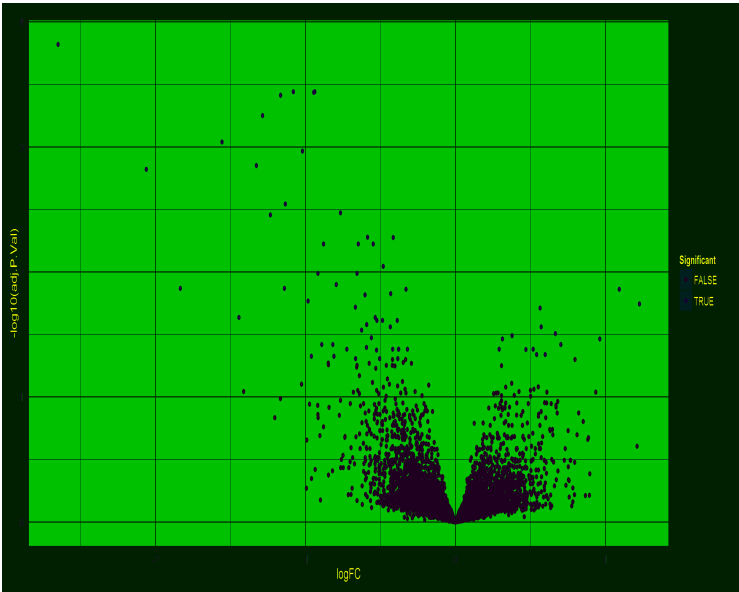


Figure 23: Volcano Plot

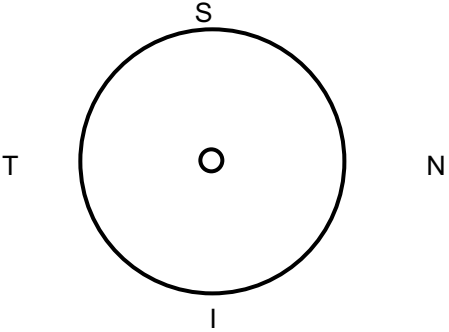
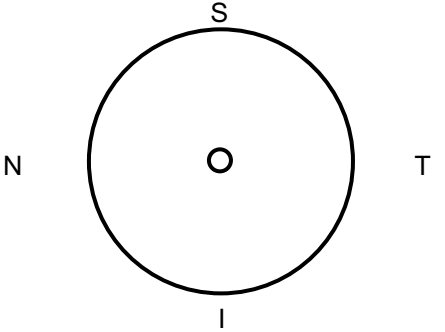
References

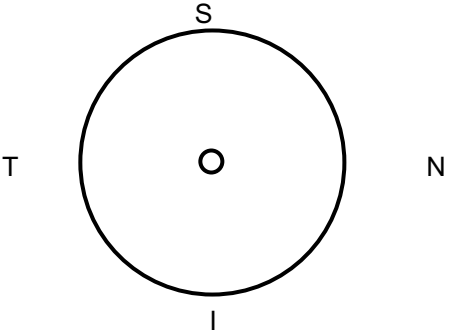
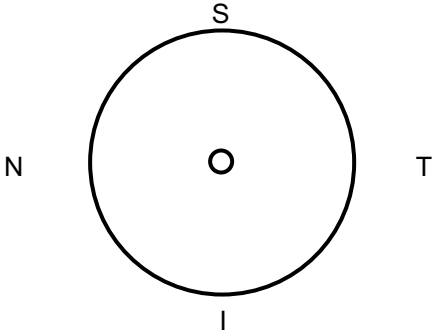
- [1] Du, P., Kibbe, W.A. and Lin, S.M., (2008) 'lumi: a pipeline for processing Illumina microarray', *Bioinformatics* 24(13):1547-1548
- Lin, S.M., Du, P., Kibbe, W.A., (2008) 'Model-based Variance-stabilizing Transformation for Illumina Microarray Data', *Nucleic Acids Res.* 36, e11
- Package version: 2.22.0
- [2] Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research* 43(7), e47.
- A BibTeX entry for LaTeX users is
- @Article, author = Matthew E Ritchie and Belinda Phipson and Di Wu and Yifang Hu and Charity W Law and Wei Shi and Gordon K Smyth, title = limma powers differential expression analyses for RNA-sequencing and microarray studies, journal = Nucleic Acids Research, year = 2015, volume = 43, number = 7, pages = e47,
- Package version: 3.26.3
- [3] R version 3.2.2 (2015-08-14)
- Platform: x86_64-w64-mingw32/x64 (64-bit)
- Running under: Windows 7 x64 (build 7601) Service Pack 1
- locale:
- [1] LC_COLLATE=English_United Kingdom.1252
- [2] LC_CTYPE=English_United Kingdom.1252
- [3] LC_MONETARY=English_United Kingdom.1252
- [4] LC_NUMERIC=C
- [5] LC_TIME=English_United Kingdom.1252
- attached base packages:
- [1] parallel stats graphics grDevices utils datasets methods
- [8] base
- other attached packages:
- [1] xtable_1.8-0 lumi_2.22.0 Biobase_2.30.0
- [4] BiocGenerics_0.16.1 limma_3.26.3 knitr_1.11
- loaded via a namespace (and not attached):
- [1] nor1mix_1.2-1 splines_3.2.2
- [3] foreach_1.4.3 ellipse_0.3-8
- [5] bumpHunter_1.10.0 affy_1.48.0
- [7] stats4_3.2.2 doRNG_1.6
- [9] Rsamtools_1.22.0 methylumi_2.16.0
- [11] minfi_1.16.0 RSQLite_1.0.0
- [13] lattice_0.20-33 quadprog_1.5-5
- [15] digest_0.6.8 GenomicRanges_1.22.1
- [17] RColorBrewer_1.1-2 XVector_0.10.0
- [19] colorspace_1.2-6 Matrix_1.2-3
- [21] preprocessCore_1.32.0 plyr_1.8.3
- [23] GEOquery_2.36.0 siggenes_1.44.0
- [25] XML_3.98-1.3 mixOmics_5.2.0
- [27] biomaRt_2.26.1 genefilter_1.52.0
- [29] zlibbioc_1.16.0 corpcor_1.6.8
- [31] scales_0.3.0 affyio_1.40.0
- [33] BiocParallel_1.4.0 nleqslv_2.9.1
- [35] annotate_1.48.0 mgcv_1.8-9
- [37] beanplot_1.2 pkgmaker_0.22
- [39] IRanges_2.4.4 ggplot2_1.0.1

Appendix 4 - Surgical note proforma

Ocular Injection Record Sheet

Page _____ of _____

Date			Time		
Location					
Identifier	No.:	Sex:	Notch:	Cage:	
Anaesthesia	Body weight (g):				
	Injected induction mix (ml):				
Reversal	Injected reversal mix (ml):				
Right Eye	Intravitreal / Subretinal No Injection		Left Eye	Intravitreal / Subretinal No Injection	
Injection done by			Injection done by		
Material Injected			Material Injected		
					
Comments			Comments		

Date			Time		
Location					
Identifier	No.:	Sex:	Notch:	Cage:	
Anaesthesia	Body weight (g):				
	Injected induction mix (ml):				
Reversal	Injected reversal mix (ml):				
Right Eye	Intravitreal / Subretinal No Injection		Left Eye	Intravitreal / Subretinal No Injection	
Injection done by			Injection done by		
Material Injected			Material Injected		
					
Comments			Comments		
Initials _____					

Appendix 5 – Two-way ANOVA and post hoc test result tables for MEA responses

Two Way ANOVA Comparing Treatment Group Kinetics, Post Hoc Tests

Time to maximum response

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
1010					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-0.1772	-3.978 to 3.623	No	ns	0.9994
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-0.00000304	-4.654 to 4.654	No	ns	>0.9999
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	0	-4.437 to 4.437	No	ns	>0.9999
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.1772	-3.874 to 4.228	No	ns	0.9995
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	0.1772	-3.623 to 3.978	No	ns	0.9994
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	0.00000304	-4.654 to 4.654	No	ns	>0.9999
1011					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-7.65	-15.58 to 0.2798	No	ns	0.0633
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-4.278	-12.56 to 4.003	No	ns	0.5452
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	0.6585	-7.316 to 8.632	No	ns	0.9966
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	3.372	-4.799 to 11.54	No	ns	0.7135
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	8.309	0.4493 to 16.17	Yes	*	0.0335
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	4.937	-3.277 to 13.15	No	ns	0.4108
1012					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-7.14	-15.07 to 0.7894	No	ns	0.0949
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-7.313	-15.59 to 0.9681	No	ns	0.1053
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	0.9828	-6.991 to 8.957	No	ns	0.989
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-0.1729	-8.344 to 7.998	No	ns	>0.9999
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	8.123	0.2640 to 15.98	Yes	*	0.0396
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	8.296	0.08231 to 16.51	Yes	*	0.0467
1013					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-2.103	-10.03 to 5.826	No	ns	0.9041
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-0.4405	-8.722 to 7.841	No	ns	0.9991
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	9.468	1.494 to 17.44	Yes	*	0.0123
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	1.663	-6.508 to 9.834	No	ns	0.9535
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	11.57	3.712 to 19.43	Yes	***	0.0009
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	9.908	1.694 to 18.12	Yes	*	0.0105
1014					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	8.88	0.9282 to 16.83	Yes	*	0.0215
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	10.37	2.089 to 18.65	Yes	**	0.0071
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	6.426	-1.548 to 14.40	No	ns	0.1627
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	1.491	-6.702 to 9.683	No	ns	0.9661
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-2.454	-10.33 to 5.427	No	ns	0.8543
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-3.944	-12.16 to 4.269	No	ns	0.605
1015					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	13.29	5.362 to 21.22	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	12.35	4.065 to 20.63	Yes	***	0.0007
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	9.011	1.038 to 16.99	Yes	*	0.0194
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-0.9455	-9.116 to 7.225	No	ns	0.9909
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-4.281	-12.14 to 3.579	No	ns	0.4994
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-3.335	-11.55 to 4.879	No	ns	0.7237
1016					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	10.1	2.166 to 18.03	Yes	**	0.0059
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	11.9	3.559 to 20.24	Yes	**	0.0014
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	8.102	0.1282 to 16.08	Yes	*	0.0448
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	1.805	-6.426 to 10.04	No	ns	0.9428
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-1.993	-9.852 to 5.866	No	ns	0.9149
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-3.798	-12.07 to 4.476	No	ns	0.6396

Average Rate Post Stimulation

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
1010					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-17.29	-27.86 to -6.717	Yes	***	0.0002
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-7.823	-19.09 to 3.442	No	ns	0.2806
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	7.673	-3.174 to 18.52	No	ns	0.2648
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	9.463	-1.440 to 20.37	No	ns	0.1151
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	24.96	14.49 to 35.43	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	15.5	4.322 to 26.67	Yes	**	0.0021
1011					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-18.57	-29.14 to -8.000	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-6.734	-18.00 to 4.532	No	ns	0.4156
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	6.752	-4.096 to 17.60	No	ns	0.3787
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	11.84	0.9317 to 22.74	Yes	*	0.0272
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	25.32	14.85 to 35.79	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	13.49	2.312 to 24.66	Yes	*	0.0105
1012					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-12.31	-22.88 to -1.740	Yes	*	0.0148
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-1.726	-12.99 to 9.539	No	ns	0.9793
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	10.98	0.1310 to 21.83	Yes	*	0.046
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	10.58	-0.3208 to 21.49	No	ns	0.0609
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.29	12.82 to 33.76	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	12.7	1.531 to 23.88	Yes	*	0.0184
1013					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-9.185	-19.75 to 1.384	No	ns	0.1144
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	3.47	-7.796 to 14.74	No	ns	0.8582
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	0.7277	-10.12 to 11.58	No	ns	0.9982
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	12.66	1.752 to 23.56	Yes	*	0.0152
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	9.913	-0.5582 to 20.38	No	ns	0.0711
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-2.742	-13.92 to 8.431	No	ns	0.9221
1014					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-12.34	-22.91 to -1.768	Yes	*	0.0145
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-19.34	-30.61 to -8.077	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	4.324	-6.524 to 15.17	No	ns	0.7349
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-7.006	-17.91 to 3.898	No	ns	0.3498
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	16.66	6.190 to 27.13	Yes	***	0.0003
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.67	12.49 to 34.84	Yes	****	<0.0001
1015					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-22.88	-33.45 to -12.31	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-10.77	-22.03 to 0.4998	No	ns	0.0671
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	0.901	-9.946 to 11.75	No	ns	0.9966
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	12.12	1.213 to 23.02	Yes	*	0.0224
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.78	13.31 to 34.26	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	11.67	0.4933 to 22.84	Yes	*	0.0368
1016					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-11.52	-22.09 to -0.9487	Yes	*	0.0263
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	12.95	1.600 to 24.29	Yes	*	0.0178
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	11.59	0.7395 to 22.43	Yes	*	0.0309
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	24.46	13.48 to 35.45	Yes	****	<0.0001
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.1	12.63 to 33.58	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-1.36	-12.62 to 9.896	No	ns	0.9896

Average Firing for 10 seoncds before stimulus

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
1010					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-15.96	-24.41 to -7.501	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-8.024	-17.04 to 0.9895	No	ns	0.1009
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	7.283	-1.396 to 15.96	No	ns	0.1356
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	7.933	-0.7907 to 16.66	No	ns	0.0899
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.24	14.86 to 31.62	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	15.31	6.367 to 24.25	Yes	****	<0.0001
1011					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-17.69	-26.15 to -9.236	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-5.712	-14.73 to 3.301	No	ns	0.3623
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	7.763	-0.9155 to 16.44	No	ns	0.0984
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	11.98	3.256 to 20.70	Yes	**	0.0024
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	25.46	17.08 to 33.83	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	13.48	4.536 to 22.42	Yes	***	0.0006
1012					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-12.84	-21.30 to -4.387	Yes	***	0.0006
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-3.471	-12.48 to 5.542	No	ns	0.7552
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	11.44	2.757 to 20.11	Yes	**	0.004
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	9.372	0.6482 to 18.10	Yes	*	0.0295
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	24.28	15.90 to 32.66	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	14.91	5.967 to 23.85	Yes	***	0.0001
1013					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-13.02	-21.47 to -4.560	Yes	***	0.0005
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-2.305	-11.32 to 6.708	No	ns	0.9129
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	11.65	2.970 to 20.33	Yes	**	0.0032
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	10.71	1.987 to 19.44	Yes	**	0.0088
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	24.67	16.29 to 33.04	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	13.95	5.014 to 22.89	Yes	***	0.0004
1014					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-8.852	-17.31 to -0.3954	Yes	*	0.0361
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-2.289	-11.30 to 6.724	No	ns	0.9145
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	13.04	4.363 to 21.72	Yes	***	0.0007
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	6.562	-2.162 to 15.29	No	ns	0.2141
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	21.89	13.52 to 30.27	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	15.33	6.391 to 24.27	Yes	****	<0.0001
1015					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-15.68	-24.13 to -7.222	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-3.908	-12.92 to 5.106	No	ns	0.6805
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	11.77	3.087 to 20.45	Yes	**	0.0028
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	11.77	3.046 to 20.49	Yes	**	0.003
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	27.44	19.07 to 35.82	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	15.67	6.734 to 24.61	Yes	****	<0.0001
1016					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-9.867	-18.32 to -1.411	Yes	*	0.0145
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	1.034	-8.045 to 10.11	No	ns	0.9913
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	14.18	5.501 to 22.86	Yes	***	0.0002
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	10.9	2.110 to 19.69	Yes	**	0.0079
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	24.05	15.67 to 32.43	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	13.15	4.141 to 22.15	Yes	**	0.001

Maximum Baseline Firing Rate

Tukey's multiple comparisons test

Predicted (LS) mean diff. 95.00% CI of diff. Significant? Summary Adjusted P Value

1010					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-12.69 -22.57 to -2.801	Yes	**		0.0054
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-9.196 -19.60 to 1.211	No	ns		0.1049
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	9.202 -0.8185 to 19.22	No	ns		0.0851
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	3.49 -6.701 to 13.68	No	ns		0.815
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	21.89 12.09 to 31.68	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	18.4 8.076 to 28.72	Yes	****		<0.0001
1011					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-15.67 -25.56 to -5.784	Yes	***		0.0003
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-8.259 -18.67 to 2.148	No	ns		0.1735
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	7.243 -2.777 to 17.26	No	ns		0.2465
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	7.411 -2.780 to 17.60	No	ns		0.2415
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	22.91 13.12 to 32.71	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	15.5 5.180 to 25.82	Yes	***		0.0007
1012					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-10.34 -20.23 to -0.4568	Yes	*		0.0362
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-4.967 -15.37 to 5.440	No	ns		0.6096
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	12.88 2.855 to 22.90	Yes	**		0.0054
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	5.376 -4.816 to 15.57	No	ns		0.5272
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.22 13.42 to 33.01	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	17.84 7.521 to 28.16	Yes	****		<0.0001
1013					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-11.17 -21.06 to -1.284	Yes	*		0.0194
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-2.302 -12.71 to 8.104	No	ns		0.9414
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	12.71 2.691 to 22.73	Yes	**		0.0062
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	8.867 -1.324 to 19.06	No	ns		0.1137
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.88 14.08 to 33.68	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	15.01 4.692 to 25.34	Yes	**		0.0011
1014					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-9.315 -19.20 to 0.5711	No	ns		0.0732
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-0.5057 -10.91 to 9.901	No	ns		0.9993
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	15.57 5.550 to 25.59	Yes	***		0.0004
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	8.809 -1.382 to 19.00	No	ns		0.1175
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	24.89 15.09 to 34.68	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	16.08 5.755 to 26.40	Yes	***		0.0004
1015					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-10.4 -20.29 to -0.5169	Yes	*		0.0347
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-1.348 -11.75 to 9.059	No	ns		0.9873
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	13.44 3.420 to 23.46	Yes	**		0.0032
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	9.055 -1.136 to 19.25	No	ns		0.102
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.84 14.05 to 33.64	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	14.79 4.467 to 25.11	Yes	**		0.0013
1016					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-4.234 -14.12 to 5.652	No	ns		0.6889
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	6.032 -4.450 to 16.51	No	ns		0.4501
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	19.01 8.993 to 29.03	Yes	****		<0.0001
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	10.27 -0.001682 to 20.53	No	ns		0.0501
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.25 13.45 to 33.04	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	12.98 2.584 to 23.38	Yes	**		0.0074

Maximum Firing Rate After Stimulus Onset

Tukey's multiple comparisons test

	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
1010					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-11.7	-27.06 to 3.661	No	ns	0.2043
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-13.48	-29.65 to 2.688	No	ns	0.1398
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	15.23	-0.3400 to 30.80	No	ns	0.0579
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-1.782	-17.62 to 14.05	No	ns	0.9916
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	26.93	11.71 to 42.15	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	28.71	12.67 to 44.75	Yes	****	<0.0001
1011					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-14.93	-30.29 to 0.4303	No	ns	0.0603
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-7.323	-23.49 to 8.848	No	ns	0.6495
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	8.733	-6.838 to 24.30	No	ns	0.4733
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	7.608	-8.228 to 23.44	No	ns	0.6045
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	23.66	8.442 to 38.89	Yes	***	0.0004
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	16.06	0.01712 to 32.10	Yes	*	0.0496
1012					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	1.249	-14.11 to 16.61	No	ns	0.9968
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	8.249	-7.922 to 24.42	No	ns	0.5557
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	20.75	5.178 to 36.32	Yes	**	0.0035
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	7	-8.836 to 22.84	No	ns	0.667
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	19.5	4.277 to 34.72	Yes	**	0.0055
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	12.5	-3.540 to 28.54	No	ns	0.1869
1013					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-0.07712	-15.44 to 15.28	No	ns	>0.9999
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	8.711	-7.460 to 24.88	No	ns	0.5089
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	2.968	-12.60 to 18.54	No	ns	0.9613
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	8.789	-7.047 to 24.62	No	ns	0.4827
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	3.045	-12.18 to 18.27	No	ns	0.9557
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-5.743	-21.78 to 10.30	No	ns	0.7938
1014					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-7.453	-22.81 to 7.908	No	ns	0.5967
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-43.13	-59.31 to -26.96	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	19.33	3.755 to 34.90	Yes	**	0.0078
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-35.68	-51.52 to -19.84	Yes	****	<0.0001
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	26.78	11.56 to 42.00	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	62.46	46.42 to 78.50	Yes	****	<0.0001
1015					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-38.73	-54.09 to -23.36	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-49.35	-65.52 to -33.18	Yes	****	<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	8.392	-7.179 to 23.96	No	ns	0.5085
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-10.62	-26.46 to 5.213	No	ns	0.311
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	47.12	31.90 to 62.34	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	57.74	41.70 to 73.78	Yes	****	<0.0001
1016					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	2.144	-13.22 to 17.51	No	ns	0.9842
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	10.23	-6.057 to 26.52	No	ns	0.3704
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	34.86	19.29 to 50.43	Yes	****	<0.0001
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	8.086	-7.869 to 24.04	No	ns	0.5611
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	32.72	17.50 to 47.94	Yes	****	<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	24.63	8.475 to 40.79	Yes	***	0.0005

Time of Response Onset

Tukey's multiple comparisons test

Predicted (LS) mean diff. 95.00% CI of diff. Significant? Summary Adjusted P Value

1010					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-0.0249	-3.026 to 2.976	No	ns	>0.9999
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	0	-3.442 to 3.442	No	ns	>0.9999
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	0	-3.282 to 3.282	No	ns	>0.9999
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.0249	-3.151 to 3.200	No	ns	>0.9999
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	0.0249	-2.976 to 3.026	No	ns	>0.9999
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	0	-3.442 to 3.442	No	ns	>0.9999
1011					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	1.814	-3.974 to 7.603	No	ns	0.8517
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	2.656	-3.323 to 8.634	No	ns	0.6636
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	1.028	-5.007 to 7.063	No	ns	0.9719
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.841	-4.756 to 6.438	No	ns	0.9804
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-0.7867	-6.443 to 4.870	No	ns	0.9843
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-1.628	-7.478 to 4.223	No	ns	0.8911
1012					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-3.788	-9.117 to 1.540	No	ns	0.2605
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-3.89	-9.693 to 1.914	No	ns	0.3118
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-1.478	-6.526 to 3.570	No	ns	0.8755
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-0.1015	-5.964 to 5.761	No	ns	>0.9999
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	2.31	-2.806 to 7.426	No	ns	0.6517
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	2.412	-3.198 to 8.021	No	ns	0.6863
1013					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-0.2315	-5.057 to 4.594	No	ns	0.9993
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-0.1129	-5.415 to 5.189	No	ns	>0.9999
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	1.53	-3.158 to 6.219	No	ns	0.8358
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.1186	-5.124 to 5.362	No	ns	>0.9999
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	1.762	-2.860 to 6.383	No	ns	0.761
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	1.643	-3.474 to 6.760	No	ns	0.8424
1014					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	3.39	-1.059 to 7.839	No	ns	0.2041
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	4.336	-0.3871 to 9.058	No	ns	0.0852
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	3.183	-1.362 to 7.728	No	ns	0.2734
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.9457	-3.645 to 5.536	No	ns	0.9519
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-0.2071	-4.615 to 4.201	No	ns	0.9994
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-1.153	-5.836 to 3.531	No	ns	0.9215
1015					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	2.149	-2.314 to 6.613	No	ns	0.6028
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	2.665	-2.054 to 7.385	No	ns	0.4671
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	2.056	-2.516 to 6.628	No	ns	0.6547
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.5162	-4.057 to 5.090	No	ns	0.9915
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-0.09324	-4.514 to 4.328	No	ns	>0.9999
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-0.6094	-5.289 to 4.070	No	ns	0.9871
1016					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	1.478	-2.964 to 5.919	No	ns	0.8278
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	1.951	-2.802 to 6.705	No	ns	0.7167
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	1.647	-2.899 to 6.194	No	ns	0.788
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.4737	-4.113 to 5.061	No	ns	0.9935
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	0.1695	-4.203 to 4.542	No	ns	0.9996
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-0.3042	-4.993 to 4.384	No	ns	0.9983

Time to Half Response

Tukey's multiple comparisons test

Predicted (LS) mean diff. 95.00% CI of diff. Significant? Summary Adjusted P Value

1010					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	0.00000013 -0.6291 to 0.6291	No	ns		>0.9999
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	0.00000013 -0.7718 to 0.7718	No	ns		>0.9999
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-1.776E-15 -0.7359 to 0.7359	No	ns		>0.9999
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-8.882E-16 -0.6707 to 0.6707	No	ns		>0.9999
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-0.00000013 -0.6291 to 0.6291	No	ns		>0.9999
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-0.00000013 -0.7718 to 0.7718	No	ns		>0.9999
1011					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	0.2326 -1.060 to 1.525	No	ns		0.9672
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	0.4109 -0.9663 to 1.788	No	ns		0.8694
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	0.3631 -0.9633 to 1.690	No	ns		0.8957
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.1783 -1.151 to 1.508	No	ns		0.9859
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	0.1305 -1.146 to 1.407	No	ns		0.9937
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-0.04782 -1.410 to 1.314	No	ns		0.9997
1012					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	0.812 -0.4765 to 2.100	No	ns		0.3675
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	0.8781 -0.4953 to 2.251	No	ns		0.3543
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-0.555 -1.877 to 0.7674	No	ns		0.7026
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.06612 -1.263 to 1.395	No	ns		0.9993
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-1.367 -2.643 to -0.09049	Yes	*		0.0303
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-1.433 -2.795 to -0.07096	Yes	*		0.0347
1013					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	2.245 0.9565 to 3.533	Yes	****		<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	2.558 1.185 to 3.931	Yes	****		<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-3.026 -4.349 to -1.704	Yes	****		<0.0001
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.3131 -1.016 to 1.642	No	ns		0.9304
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-5.271 -6.548 to -3.995	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-5.584 -6.947 to -4.222	Yes	****		<0.0001
1014					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	0.8866 -0.4019 to 2.175	No	ns		0.2887
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	1.453 0.07998 to 2.827	Yes	*		0.0332
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-3.207 -4.529 to -1.885	Yes	****		<0.0001
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.5667 -0.7625 to 1.896	No	ns		0.6921
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-4.093 -5.370 to -2.817	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-4.66 -6.022 to -3.298	Yes	****		<0.0001
1015					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	1.552 0.2638 to 2.841	Yes	*		0.0106
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	3.722 2.349 to 5.095	Yes	****		<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-2.135 -3.457 to -0.8123	Yes	***		0.0002
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	2.17 0.8404 to 3.499	Yes	***		0.0002
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-3.687 -4.963 to -2.410	Yes	****		<0.0001
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-5.857 -7.219 to -4.494	Yes	****		<0.0001
1016					
"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	1.051 -0.2376 to 2.339	No	ns		0.1545
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	3.907 2.524 to 5.290	Yes	****		<0.0001
"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-0.9286 -2.251 to 0.3938	No	ns		0.2711
"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	2.856 1.517 to 4.196	Yes	****		<0.0001
"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-1.979 -3.256 to -0.7029	Yes	***		0.0004
"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-4.836 -6.208 to -3.463	Yes	****		<0.0001

Response Duration

Tukey's multiple comparisons test

Predicted (LS) mean diff. 95.00% CI of diff. Significant? Summary Adjusted P Value

1010	"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-0.00000259 -3.481 to 3.481	No	ns	>0.9999
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	-0.00000259 -4.271 to 4.271	No	ns	>0.9999
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-1.066E-14 -4.072 to 4.072	No	ns	>0.9999
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	7.105E-15 -3.711 to 3.711	No	ns	>0.9999
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	0.00000259 -3.481 to 3.481	No	ns	>0.9999
	"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	0.00000259 -4.271 to 4.271	No	ns	>0.9999
1011	"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	-0.8333 -8.015 to 6.348	No	ns	0.9908
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	0.1538 -7.264 to 7.571	No	ns	>0.9999
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-3.16 -10.65 to 4.327	No	ns	0.6987
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	0.9872 -5.956 to 7.930	No	ns	0.9833
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-2.327 -9.344 to 4.691	No	ns	0.8293
	"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-3.314 -10.57 to 3.945	No	ns	0.6436
1012	"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	7.752 1.141 to 14.36	Yes	*	0.0138
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	12.57 5.367 to 19.77	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	1.342 -4.921 to 7.604	No	ns	0.9464
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	4.815 -2.458 to 12.09	No	ns	0.3229
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-6.41 -12.76 to -0.06266	Yes	*	0.0467
	"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-11.23 -18.18 to -4.266	Yes	***	0.0002
1013	"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	16.73 10.75 to 22.72	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	16.63 10.05 to 23.20	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-8.323 -14.14 to -2.506	Yes	**	0.0014
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-0.1053 -6.610 to 6.399	No	ns	>0.9999
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-25.06 -30.79 to -19.32	Yes	****	<0.0001
	"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-24.95 -31.30 to -18.60	Yes	****	<0.0001
1014	"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	19.88 14.36 to 25.40	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	19.22 13.37 to 25.08	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-11.41 -17.05 to -5.774	Yes	****	<0.0001
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	-0.6584 -6.354 to 5.037	No	ns	0.9909
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-31.3 -36.76 to -25.83	Yes	****	<0.0001
	"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-30.64 -36.45 to -24.83	Yes	****	<0.0001
1015	"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	20.73 15.19 to 26.27	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	23.4 17.55 to 29.26	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	-10.8 -16.48 to -5.132	Yes	****	<0.0001
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	2.675 -2.999 to 8.349	No	ns	0.6192
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-31.53 -37.02 to -26.05	Yes	****	<0.0001
	"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-34.21 -40.01 to -28.40	Yes	****	<0.0001
1016	"LMAF" - floxed hOPN4 vs. "LMAF" - floxed ReaChR	29.43 23.92 to 34.94	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed ReaChR	31.87 25.97 to 37.76	Yes	****	<0.0001
	"LMAF" - floxed hOPN4 vs. "CGRK" - floxed hOPN4	10.49 4.845 to 16.13	Yes	****	<0.0001
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed ReaChR	2.438 -3.253 to 8.129	No	ns	0.6888
	"LMAF" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-18.94 -24.37 to -13.52	Yes	****	<0.0001
	"CGRK" - floxed ReaChR vs. "CGRK" - floxed hOPN4	-21.38 -27.20 to -15.56	Yes	****	<0.0001

References

- 1 Gray, H. and Lewis, W. (1918) *Anatomy of the Human Body*. Lea & Febiger, Philadelphia.
- 2 Forrester, J.V., Dick, A.D., McMenamin, P.G., Roberts, F. and Pearlman, E. (2015) *The eye E-book: basic sciences in practice*. Elsevier Health Sciences, London
- 3 Fain, G.L. and Fain, M.J. (2003) *Sensory transduction*. Sinauer Associates, Sunderland.
- 4 Lamb, T.D. and Pugh, E.N., Jr. (2006) Phototransduction, dark adaptation, and rhodopsin regeneration the proctor lecture. *Invest Ophthalmol Vis Sci*, **47**, 5137-5152.
- 5 Tang, P.H., Kono, M., Koutalos, Y., Ablonczy, Z. and Crouch, R.K. (2013) New insights into retinoid metabolism and cycling within the retina. *Prog Retin Eye Res*, **32**, 48-63.
- 6 Masland, R.H. (2012) The neuronal organization of the retina. *Neuron*, **76**, 266-280.
- 7 Fain, G.L. (2003) *Sensory transduction*. Sinauer Associates, Sunderland.
- 8 Staurengi, G., Sadda, S., Chakravarthy, U., Spaide, R.F. and International Nomenclature for Optical Coherence Tomography, P. (2014) Proposed lexicon for anatomic landmarks in normal posterior segment spectral-domain optical coherence tomography: the IN*OCT consensus. *Ophthalmology*, **121**, 1572-1578.
- 9 Klapper, S.D., Swiersy, A., Bamberg, E. and Busskamp, V. (2016) Biophysical Properties of Optogenetic Tools and Their Application for Vision Restoration Approaches. *Front Syst Neurosci*, **10**, 74.
- 10 Shang, Y.M., Wang, G.S., Sliney, D., Yang, C.H. and Lee, L.L. (2014) White light-emitting diodes (LEDs) at domestic lighting levels and retinal injury in a rat model. *Environ Health Perspect*, **122**, 269-276.
- 11 Euler, T., Haverkamp, S., Schubert, T. and Baden, T. (2014) Retinal bipolar cells: elementary building blocks of vision. *Nature Reviews Neuroscience*, **15**, 507-519.
- 12 Protti, D.A., Flores-Herr, N. and Von Gersdorff, H. (2000) Light evokes Ca²⁺ spikes in the axon terminal of a retinal bipolar cell. *Neuron*, **25**, 215-227.
- 13 Wassle, H., Puller, C., Muller, F. and Haverkamp, S. (2009) Cone contacts, mosaics, and territories of bipolar cells in the mouse retina. *J Neurosci*, **29**, 106-117.
- 14 Helmstaedter, M., Briggman, K.L., Turaga, S.C., Jain, V., Seung, H.S. and Denk, W. (2013) Connectomic reconstruction of the inner plexiform layer in the mouse retina. *Nature*, **500**, 168-174.
- 15 DeVries, S.H. (2000) Bipolar cells use kainate and AMPA receptors to filter visual information into separate channels. *Neuron*, **28**, 847-856.
- 16 Koistinaho, J. and Sagar, S.M. (1994) Localization of protein kinase C subspecies in the rabbit retina. *Neuroscience letters*, **177**, 15-18.
- 17 Nakajima, Y., Iwakabe, H., Akazawa, C., Nawa, H., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1993) Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate. *Journal of Biological Chemistry*, **268**, 11868-11873.
- 18 Nelson, R. and Connaughton, V., 2012. Bipolar cell pathways in the vertebrate retina. In *Webvision: The Organization of the Retina and Visual System* [<https://webvision.med.utah.edu>]. University of Utah Health Sciences Center.

- 19 Xu, Y., Orlandi, C., Cao, Y., Yang, S., Choi, C.I., Pagadala, V., Birnbaumer, L., Martemyanov, K.A. and Vardi, N. (2016) The TRPM1 channel in ON-bipolar cells is gated by both the alpha and the betagamma subunits of the G-protein Go. *Sci Rep*, **6**, 20940.
- 20 Regus-Leidig, H. and Brandstatter, J.H. (2012) Structure and function of a complex sensory synapse. *Acta Physiol*, **204**, 479-486.
- 21 Sasaki, T. and Kaneko, A. (1996) L-Glutamate-induced responses in OFF-type bipolar cells of the cat retina. *Vision research*, **36**, 787-795.
- 22 Lindstrom, S.H., Ryan, D.G., Shi, J. and DeVries, S.H. (2014) Kainate receptor subunit diversity underlying response diversity in retinal off bipolar cells. *J Physiol*, **592**, 1457-1477.
- 23 Martemyanov, K.A. and Sampath, A.P. (2017) The Transduction Cascade in Retinal ON-Bipolar Cells: Signal Processing and Disease. *Annual Review of Vision Science*, **3**, 25-51.
- 24 Cajal, S.R. y (1893) La rétine des vertébrés. *La cellule*, **9**, 119-257.
- 25 Volgyi, B., Deans, M.R., Paul, D.L. and Bloomfield, S.A. (2004) Convergence and segregation of the multiple rod pathways in mammalian retina. *J Neurosci*, **24**, 11182-11192.
- 26 Mariani, A.P. (1990) Amacrine cells of the rhesus monkey retina. *Journal of comparative neurology*, **301**, 382-400.
- 27 Euler, T. and Masland, R.H. (2000) Light-evoked responses of bipolar cells in a mammalian retina. *Journal of Neurophysiology*, **83**, 1817-1829.
- 28 Masland, R.H. (2012) The tasks of amacrine cells. *Visual Neuroscience*, **29**, 3-9.
- 29 Boije, H., Shirazi Fard, S., Edqvist, P.H. and Hallbook, F. (2016) Horizontal Cells, the Odd Ones Out in the Retina, Give Insights into Development and Disease. *Front Neuroanat*, **10**, 77.
- 30 Kolb, H. (2007), In *Webvision: The Organization of the Retina and Visual System* [<https://webvision.med.utah.edu>]. University of Utah Health Sciences Center.
- 31 Pelassa, I. and Lagnado, L. (2011) Synaptic ribbons: machines for priming vesicle release? *Curr Biol*, **21**, R819-821.
- 32 Thoreson, W.B., Rabl, K., Townes-Anderson, E. and Heidelberger, R. (2004) A highly Ca²⁺-sensitive pool of vesicles contributes to linearity at the rod photoreceptor ribbon synapse. *Neuron*, **42**, 595-605.
- 33 Rao-Mirotznik, R., Harkins, A., Buchsbaum, G. and Sterling, P. (1995) Mammalian rod terminal: architecture of a binary synapse. *Neuron*, **14**, 561-569.
- 34 Sanes, J.R. and Masland, R.H. (2015) The types of retinal ganglion cells: current status and implications for neuronal classification. *Annu Rev Neurosci*, **38**, 221-246.
- 35 Morgans, C.W., Zhang, J., Jeffrey, B.G., Nelson, S.M., Burke, N.S., Duvoisin, R.M. and Brown, R.L. (2009) TRPM1 is required for the depolarizing light response in retinal ON-bipolar cells. *Proceedings of the National Academy of Sciences*, **106**, 19174-19178.
- 36 Koike, C., Obara, T., Uriu, Y., Numata, T., Sanuki, R., Miyata, K., Koyasu, T., Ueno, S., Funabiki, K., Tani, A. *et al.* (2010) TRPM1 is a component of the retinal ON bipolar cell transduction channel in the mGluR6 cascade. *Proc Natl Acad Sci U S A*, **107**, 332-337.
- 37 Nawy, S. (2014) Modulation of TRPM1 and the mGluR6 Cascade in ON Bipolar Cells. *Spr Ser Vis Res*, **3**, 99-119.

- 38 Baden, T., Berens, P., Franke, K., Roman Roson, M., Bethge, M. and Euler, T. (2016) The functional diversity of retinal ganglion cells in the mouse. *Nature*, **529**, 345-350.
- 39 Wiesel, T.N. (1960) Receptive fields of ganglion cells in the cat's retina. *The Journal of Physiology*, **153**, 583-594.
- 40 Wiesel, T.N. and Hubel, D.H. (1966) Spatial and chromatic interactions in the lateral geniculate body of the rhesus monkey. *Journal of neurophysiology*, **29**, 1115-1156.
- 41 Provencio, I., Jiang, G., De Grip, W.J., Hayes, W.P. and Rollag, M.D. (1998) Melanopsin: An opsin in melanophores, brain, and eye. *Proc Natl Acad Sci U S A*, **95**, 340-345.
- 42 Provencio, I., Rodriguez, I.R., Jiang, G., Hayes, W.P., Moreira, E.F. and Rollag, M.D. (2000) A novel human opsin in the inner retina. *J Neurosci*, **20**, 600-605.
- 43 Foster, R.G., Provencio, I., Hudson, D., Fiske, S., De Grip, W. and Menaker, M. (1991) Circadian photoreception in the retinally degenerate mouse (rd/rd). *Journal of Comparative Physiology A*, **169**, 39-50.
- 44 Lall, G.S., Revell, V.L., Momiji, H., Al Enezi, J., Altimus, C.M., Guler, A.D., Aguilar, C., Cameron, M.A., Allender, S., Hankins, M.W. *et al.* (2010) Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance. *Neuron*, **66**, 417-428.
- 45 Barnard, A.R., Hattar, S., Hankins, M.W. and Lucas, R.J. (2006) Melanopsin regulates visual processing in the mouse retina. *Curr Biol*, **16**, 389-395.
- 46 Hankins, M.W. and Hughes, S. (2014) Vision: melanopsin as a novel irradiance detector at the heart of vision. *Current Biology*, **24**, R1055-R1057.
- 47 Dacey, D.M., Liao, H.-W., Peterson, B.B., Robinson, F.R., Smith, V.C., Pokorny, J., Yau, K.-W. and Gamlin, P.D. (2005) Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. *Nature*, **433**, 749.
- 48 Ecker, J.L., Dumitrescu, O.N., Wong, K.Y., Alam, N.M., Chen, S.K., LeGates, T., Renna, J.M., Prusky, G.T., Berson, D.M. and Hattar, S. (2010) Melanopsin-expressing retinal ganglion-cell photoreceptors: cellular diversity and role in pattern vision. *Neuron*, **67**, 49-60.
- 49 Zhu, Y., Tu, D.C., Denner, D., Shane, T., Fitzgerald, C.M. and Van Gelder, R.N. (2007) Melanopsin-dependent persistence and photopotential of murine pupillary light responses. *Investigative ophthalmology & visual science*, **48**, 1268-1275.
- 50 Hughes, S., Hankins, M.W., Foster, R.G. and Peirson, S.N. (2012) Melanopsin phototransduction: slowly emerging from the dark. *Prog Brain Res*, **199**, 19-40.
- 51 Hankins, M.W., Peirson, S.N. and Foster, R.G. (2008) Melanopsin: an exciting photopigment. *Trends Neurosci*, **31**, 27-36.
- 52 Hughes, S., Jagannath, A., Rodgers, J., Hankins, M.W., Peirson, S.N. and Foster, R.G. (2016) Signalling by melanopsin (OPN4) expressing photosensitive retinal ganglion cells. *Eye (Lond)*, **30**, 247-254.
- 53 Panda, S., Nayak, S.K., Campo, B., Walker, J.R., Hogenesch, J.B. and Jegla, T. (2005) Illumination of the melanopsin signaling pathway. *Science*, **307**, 600-604.
- 54 Mure, L.S., Cornut, P.L., Rieux, C., Drouyer, E., Denis, P., Gronfier, C. and Cooper, H.M. (2009) Melanopsin bistability: a fly's eye technology in the human retina. *PLoS One*, **4**, e5991.

- 55 Hughes, S., Potheary, C.A., Jagannath, A., Foster, R.G., Hankins, M.W. and Peirson, S.N. (2012) Profound defects in pupillary responses to light in TRPM-channel null mice: a role for TRPM channels in non-image-forming photoreception. *Eur J Neurosci*, **35**, 34-43.
- 56 Do, M.T.H. (2018) Mixed Palettes of Melanopsin Phototransduction. *Cell*, **175**, 637-639.
- 57 Jiang, Z., Yue, W.W.S., Chen, L., Sheng, Y. and Yau, K.-W. (2018) Cyclic-Nucleotide- and HCN-Channel-Mediated Phototransduction in Intrinsically Photosensitive Retinal Ganglion Cells. *Cell*.
- 58 Melyan, Z., Tarttelin, E.E., Bellingham, J., Lucas, R.J. and Hankins, M.W. (2005) Addition of human melanopsin renders mammalian cells photoresponsive. *Nature*, **433**, 741-745.
- 59 Qiu, X., Kumbalasiri, T., Carlson, S.M., Wong, K.Y., Krishna, V., Provencio, I. and Berson, D.M. (2005) Induction of photosensitivity by heterologous expression of melanopsin. *Nature*, **433**, 745-749.
- 60 Yasin, B., Kohn, E., Peters, M., Zaguri, R., Weiss, S., Schopf, K., Katz, B., Huber, A. and Minke, B. (2017) Ectopic Expression of Mouse Melanopsin in Drosophila Photoreceptors Reveals Fast Response Kinetics and Persistent Dark Excitation. *J Biol Chem*, **292**, 3624-3636.
- 61 Do, M.T., Kang, S.H., Xue, T., Zhong, H., Liao, H.W., Bergles, D.E. and Yau, K.W. (2009) Photon capture and signalling by melanopsin retinal ganglion cells. *Nature*, **457**, 281-287.
- 62 Emanuel, A.J. and Do, M.T. (2015) Melanopsin tristability for sustained and broadband phototransduction. *Neuron*, **85**, 1043-1055.
- 63 Lamb, T.D. and Pugh, E.N., Jr. (2004) Dark adaptation and the retinoid cycle of vision. *Prog Retin Eye Res*, **23**, 307-380.
- 64 Stockman, A., Sharpe, L.T., Merbs, S. and Nathans, J. (2000), In *Methods in enzymology*. Elsevier, Vol. 316, pp. 626-650.
- 65 Koberlein, J., Beifus, K., Schaffert, C. and Finger, R.P. (2013) The economic burden of visual impairment and blindness: a systematic review. *BMJ Open*, **3**, e003471.
- 66 Serge Resnikoff, D., Etya'ale, D. and Kocur, I. (2002) Global data on visual impairment in the year 2002. *Bulletin of the World Health Organization*.
- 67 Liew, G., Michaelides, M. and Bunce, C. (2014) A comparison of the causes of blindness certifications in England and Wales in working age adults (16-64 years), 1999-2000 with 2009-2010. *BMJ Open*, **4**, e004015.
- 68 Hartong, D.T., Berson, E.L. and Dryja, T.P. (2006) Retinitis pigmentosa. *Lancet*, **368**, 1795-1809.
- 69 Gilhooley, M.J. and Acheson, J. (2017) Artificial vision: principles and prospects. *Curr Opin Neurol*, **30**, 55-60.
- 70 Daiger, S.P., Sullivan, L.S. and Bowne, S.J. (2013) Genes and mutations causing retinitis pigmentosa. *Clin Genet*, **84**, 132-141.
- 71 Ait-Ali, N., Fridlich, R., Millet-Puel, G., Clerin, E., Delalande, F., Jaillard, C., Blond, F., Perrocheau, L., Reichman, S., Byrne, L.C. *et al.* (2015) Rod-derived cone viability factor promotes cone survival by stimulating aerobic glycolysis. *Cell*, **161**, 817-832.

- 72 Leveillard, T., Mohand-Said, S., Lorentz, O., Hicks, D., Fintz, A.C., Clerin, E., Simonutti, M., Forster, V., Cavusoglu, N., Chalmel, F. *et al.* (2004) Identification and characterization of rod-derived cone viability factor. *Nat Genet*, **36**, 755-759.
- 73 Hamel, C.P. (2007) Cone rod dystrophies. *Orphanet J Rare Dis*, **2**, 7.
- 74 Yue, L., Weiland, J.D., Roska, B. and Humayun, M.S. (2016) Retinal stimulation strategies to restore vision: Fundamentals and systems. *Prog Retin Eye Res*, **53**, 21-47.
- 75 Jones, B.W., Kondo, M., Terasaki, H., Lin, Y., McCall, M. and Marc, R.E. (2012) Retinal remodeling. *Jpn J Ophthalmol*, **56**, 289-306.
- 76 Jones, B.W., Pfeiffer, R.L., Ferrell, W.D., Watt, C.B., Marmor, M. and Marc, R.E. (2016) Retinal remodeling in human retinitis pigmentosa. *Exp Eye Res*, **150**, 149-165.
- 77 Keeler, C. (1966) Retinal degeneration in the mouse is rodless retina. *Journal of Heredity*, **57**, 47-50.
- 78 Keeler, C.E. (1924) The inheritance of a retinal abnormality in white mice. *Proceedings of the National Academy of Sciences of the United States of America*, **10**, 329.
- 79 Marc, R.E. and Jones, B.W. (2003) Retinal remodeling in inherited photoreceptor degenerations. *Mol Neurobiol*, **28**, 139-147.
- 80 Marc, R.E., Jones, B.W., Watt, C.B. and Strettoi, E. (2003) Neural remodeling in retinal degeneration. *Prog Retin Eye Res*, **22**, 607-655.
- 81 Strettoi, E. and Pignatelli, V. (2000) Modifications of retinal neurons in a mouse model of retinitis pigmentosa. *Proc Natl Acad Sci U S A*, **97**, 11020-11025.
- 82 Strettoi, E., Porciatti, V., Falsini, B., Pignatelli, V. and Rossi, C. (2002) Morphological and functional abnormalities in the inner retina of the rd/rd mouse. *J Neurosci*, **22**, 5492-5504.
- 83 Strettoi, E., Pignatelli, V., Rossi, C., Porciatti, V. and Falsini, B. (2003) Remodeling of second-order neurons in the retina of rd/rd mutant mice. *Vision Res*, **43**, 867-877.
- 84 Cuenca, N., Pinilla, I., Sauve, Y. and Lund, R. (2005) Early changes in synaptic connectivity following progressive photoreceptor degeneration in RCS rats. *Eur J Neurosci*, **22**, 1057-1072.
- 85 Kalloniatis, M., Nivison-Smith, L., Chua, J., Acosta, M.L. and Fletcher, E.L. (2016) Using the rd1 mouse to understand functional and anatomical retinal remodelling and treatment implications in retinitis pigmentosa: A review. *Exp Eye Res*, **150**, 106-121.
- 86 Cuenca, N., Fernandez-Sanchez, L., Campello, L., Maneu, V., De la Villa, P., Lax, P. and Pinilla, I. (2014) Cellular responses following retinal injuries and therapeutic approaches for neurodegenerative diseases. *Prog Retin Eye Res*, **43**, 17-75.
- 87 Chua, J., Fletcher, E.L. and Kalloniatis, M. (2009) Functional remodeling of glutamate receptors by inner retinal neurons occurs from an early stage of retinal degeneration. *J Comp Neurol*, **514**, 473-491.
- 88 Greferath, U., Anderson, E.E., Jobling, A.I., Vessey, K.A., Martinez, G., de longh, R.U., Kalloniatis, M. and Fletcher, E.L. (2015) Inner retinal change in a novel rd1-FTL mouse model of retinal degeneration. *Front Cell Neurosci*, **9**, 293.
- 89 Marc, R.E., Jones, B.W., Anderson, J.R., Kinard, K., Marshak, D.W., Wilson, J.H., Wensel, T. and Lucas, R.J. (2007) Neural reprogramming in retinal degeneration. *Invest Ophthalmol Vis Sci*, **48**, 3364-3371.
- 90 Dunn, F.A. (2015) Photoreceptor ablation initiates the immediate loss of glutamate receptors in postsynaptic bipolar cells in retina. *J Neurosci*, **35**, 2423-2431.

- 91 Michalakakis, S., Schaferhoff, K., Spiwoks-Becker, I., Zabouri, N., Koch, S., Koch, F., Bonin, M., Biel, M. and Haverkamp, S. (2013) Characterization of neurite outgrowth and ectopic synaptogenesis in response to photoreceptor dysfunction. *Cell Mol Life Sci*, **70**, 1831-1847.
- 92 Lin, B., Masland, R.H. and Strettoi, E. (2009) Remodeling of cone photoreceptor cells after rod degeneration in rd mice. *Experimental Eye Research*, **88**, 589-599.
- 93 Peng, Y.-W., Hao, Y., Petters, R.M. and Wong, F. (2000) Ectopic synaptogenesis in the mammalian retina caused by rod photoreceptor-specific mutations. *Nature neuroscience*, **3**, 1121.
- 94 Strettoi, E., Mears, A.J. and Swaroop, A. (2004) Recruitment of the rod pathway by cones in the absence of rods. *J Neurosci*, **24**, 7576-7582.
- 95 Wang, T., Pahlberg, J., Cafaro, J., Frederiksen, R., Cooper, A.J., Sampath, A.P., Field, G.D. and Chen, J. (2019) Activation of rod input in a model of retinal degeneration reverses retinal remodeling and induces formation of functional synapses and recovery of visual signaling in the adult retina. *J Neurosci*.
- 96 Gibson, R., Fletcher, E.L., Vingrys, A.J., Zhu, Y., Vessey, K.A. and Kalloniatis, M. (2013) Functional and neurochemical development in the normal and degenerating mouse retina. *J Comp Neurol*, **521**, 1251-1267.
- 97 Arundine, M. and Tymianski, M. (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium*, **34**, 325-337.
- 98 Puthusser, T., Gayet-Primo, J., Pandey, S., Duvoisin, R.M. and Taylor, W.R. (2009) Differential loss and preservation of glutamate receptor function in bipolar cells in the rd10 mouse model of retinitis pigmentosa. *Eur J Neurosci*, **29**, 1533-1542.
- 99 Lagali, P.S., Balya, D., Awatramani, G.B., Munch, T.A., Kim, D.S., Busskamp, V., Cepko, C.L. and Roska, B. (2008) Light-activated channels targeted to ON bipolar cells restore visual function in retinal degeneration. *Nat Neurosci*, **11**, 667-675.
- 100 Doroudchi, M.M., Greenberg, K.P., Liu, J., Silka, K.A., Boyden, E.S., Lockridge, J.A., Arman, A.C., Janani, R., Boye, S.E., Boye, S.L. *et al.* (2011) Virally delivered channelrhodopsin-2 safely and effectively restores visual function in multiple mouse models of blindness. *Mol Ther*, **19**, 1220-1229.
- 101 Cronin, T., Vandenbergh, L.H., Hantz, P., Jüttner, J., Reimann, A., Kacso, A.E., Huckfeldt, R.M., Busskamp, V., Kohler, H., Lagali, P.S. *et al.* (2014) Efficient transduction and optogenetic stimulation of retinal bipolar cells by a synthetic adeno-associated virus capsid and promoter. *EMBO Mol Med*, **6**, 1175-1190.
- 102 Macé, E., Caplette, R., Marre, O., Sengupta, A., Chaffiol, A., Barbe, P., Desrosiers, M., Bamberg, E., Sahel, J.-A. and Picaud, S. (2015) Targeting channelrhodopsin-2 to ON-bipolar cells with vitreally administered AAV Restores ON and OFF visual responses in blind mice. *Molecular Therapy*, **23**, 7-16.
- 103 Gaub, B.M., Berry, M.H., Holt, A.E., Isacoff, E.Y. and Flannery, J.G. (2015) Optogenetic Vision Restoration Using Rhodopsin for Enhanced Sensitivity. *Mol Ther*, **23**, 1562-1571.
- 104 Gaub, B.M., Berry, M.H., Holt, A.E., Reiner, A., Kienzler, M.A., Dolgova, N., Nikonov, S., Aguirre, G.D., Beltran, W.A., Flannery, J.G. *et al.* (2014) Restoration of visual function by expression of a light-gated mammalian ion channel in retinal ganglion cells or ON-bipolar cells. *Proc Natl Acad Sci U S A*, **111**, E5574-5583.

- 105 van Wyk, M., Pielecka-Fortuna, J., Lowel, S. and Kleinlogel, S. (2015) Restoring the ON Switch in Blind Retinas: Opto-mGluR6, a Next-Generation, Cell-Tailored Optogenetic Tool. *PLoS Biol*, **13**, e1002143.
- 106 Cehajic-Kapetanovic, J., Eleftheriou, C., Allen, A.E., Milosavljevic, N., Pienaar, A., Bedford, R., Davis, K.E., Bishop, P.N. and Lucas, R.J. (2015) Restoration of Vision with Ectopic Expression of Human Rod Opsin. *Curr Biol*, **25**, 2111-2122.
- 107 Baker, C.K. and Flannery, J.G. (2018) Innovative Optogenetic Strategies for Vision Restoration. *Front Cell Neurosci*, **12**, 316.
- 108 Ashtari, M., Cyckowski, L.L., Monroe, J.F., Marshall, K.A., Chung, D.C., Auricchio, A., Simonelli, F., Leroy, B.P., Maguire, A.M., Shindler, K.S. *et al.* (2011) The human visual cortex responds to gene therapy-mediated recovery of retinal function. *J Clin Invest*, **121**, 2160-2168.
- 109 Cunningham, S.I., Shi, Y., Weiland, J.D., Falabella, P., Olmos de Koo, L.C., Zacks, D.N. and Tjan, B.S. (2015) Feasibility of Structural and Functional MRI Acquisition with Unpowered Implants in Argus II Retinal Prosthesis Patients: A Case Study. *Transl Vis Sci Technol*, **4**, 6.
- 110 Dagnelie, G., Christopher, P., Arditi, A., da Cruz, L., Duncan, J.L., Ho, A.C., Olmos de Koo, L.C., Sahel, J.A., Stanga, P.E., Thumann, G. *et al.* (2017) Performance of real-world functional vision tasks by blind subjects improves after implantation with the Argus(R) II retinal prosthesis system. *Clin Exp Ophthalmol*, **45**, 152-159.
- 111 Luo, Y.H. and da Cruz, L. (2016) The Argus((R)) II Retinal Prosthesis System. *Prog Retin Eye Res*, **50**, 89-107.
- 112 Lewis, P.M., Ayton, L.N., Guymer, R.H., Lowery, A.J., Blamey, P.J., Allen, P.J., Luu, C.D. and Rosenfeld, J.V. (2016) Advances in implantable bionic devices for blindness: a review. *ANZ J Surg*, **86**, 654-659.
- 113 Besch, D., Sachs, H., Szurman, P., Gulicher, D., Wilke, R., Reinert, S., Zrenner, E., Bartz-Schmidt, K.U. and Gekeler, F. (2008) Extraocular surgery for implantation of an active subretinal visual prosthesis with external connections: feasibility and outcome in seven patients. *Br J Ophthalmol*, **92**, 1361-1368.
- 114 Zrenner, E., Bartz-Schmidt, K.U., Benav, H., Besch, D., Bruckmann, A., Gabel, V.P., Gekeler, F., Greppmaier, U., Harscher, A., Kibbel, S. *et al.* (2011) Subretinal electronic chips allow blind patients to read letters and combine them to words. *Proc Biol Sci*, **278**, 1489-1497.
- 115 da Cruz, L., Dorn, J.D., Humayun, M.S., Dagnelie, G., Handa, J., Barale, P.O., Sahel, J.A., Stanga, P.E., Hafezi, F., Safran, A.B. *et al.* (2016) Five-Year Safety and Performance Results from the Argus II Retinal Prosthesis System Clinical Trial. *Ophthalmology*, **123**, 2248-2254.
- 116 Ghodasra, D.H., Chen, A., Arevalo, J.F., Birch, D.G., Branham, K., Coley, B., Dagnelie, G., de Juan, E., Devenyi, R.G., Dorn, J.D. *et al.* (2016) Worldwide Argus II implantation: recommendations to optimize patient outcomes. *BMC Ophthalmol*, **16**, 52.
- 117 Ramsden, C.M., Powner, M.B., Carr, A.J., Smart, M.J., da Cruz, L. and Coffey, P.J. (2013) Stem cells in retinal regeneration: past, present and future. *Development*, **140**, 2576-2585.
- 118 Zarbin, M. (2016) Cell-Based Therapy for Degenerative Retinal Disease. *Trends Mol Med*, **22**, 115-134.

- 119 Jayakody, S.A., Gonzalez-Cordero, A., Ali, R.R. and Pearson, R.A. (2015) Cellular strategies for retinal repair by photoreceptor replacement. *Prog Retin Eye Res*, **46**, 31-66.
- 120 Barnea-Cramer, A.O., Wang, W., Lu, S.J., Singh, M.S., Luo, C., Huo, H., McClements, M.E., Barnard, A.R., MacLaren, R.E. and Lanza, R. (2016) Function of human pluripotent stem cell-derived photoreceptor progenitors in blind mice. *Sci Rep*, **6**, 29784.
- 121 Singh, M.S., Balmer, J., Barnard, A.R., Aslam, S.A., Moralli, D., Green, C.M., Barnea-Cramer, A., Duncan, I. and MacLaren, R.E. (2016) Transplanted photoreceptor precursors transfer proteins to host photoreceptors by a mechanism of cytoplasmic fusion. *Nat Commun*, **7**, 13537.
- 122 MacLaren, R.E., Pearson, R.A., MacNeil, A., Douglas, R.H., Salt, T.E., Akimoto, M., Swaroop, A., Sowden, J.C. and Ali, R.R. (2006) Retinal repair by transplantation of photoreceptor precursors. *Nature*, **444**, 203-207.
- 123 Schwartz, S.D., Regillo, C.D., Lam, B.L., Elliott, D., Rosenfeld, P.J., Gregori, N.Z., Hubschman, J.-P., Davis, J.L., Heilwell, G., Sporn, M. *et al.* (2015) Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *The Lancet*, **385**, 509-516.
- 124 Nazari, H., Zhang, L., Zhu, D., Chader, G.J., Falabella, P., Stefanini, F., Rowland, T., Clegg, D.O., Kashani, A.H., Hinton, D.R. *et al.* (2015) Stem cell based therapies for age-related macular degeneration: The promises and the challenges. *Prog Retin Eye Res*, **48**, 1-39.
- 125 Klassen, H. (2016) Stem cells in clinical trials for treatment of retinal degeneration. *Expert Opin Biol Ther*, **16**, 7-14.
- 126 MacLaren, R.E., Bennett, J. and Schwartz, S.D. (2016) Gene Therapy and Stem Cell Transplantation in Retinal Disease: The New Frontier. *Ophthalmology*, **123**, S98-S106.
- 127 Athanasiou, D., Aguila, M., Bellingham, J., Li, W., McCulley, C., Reeves, P.J. and Cheetham, M.E. (2018) The molecular and cellular basis of rhodopsin retinitis pigmentosa reveals potential strategies for therapy. *Prog Retin Eye Res*, **62**, 1-23.
- 128 Russell, S., Bennett, J., Wellman, J.A., Chung, D.C., Yu, Z.-F., Tillman, A., Wittes, J., Pappas, J., Elci, O. and McCague, S. (2017) Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *The Lancet*, **390**, 849-860.
- 129 Jacobson, S.G., Cideciyan, A.V., Roman, A.J., Sumaroka, A., Schwartz, S.B., Heon, E. and Hauswirth, W.W. (2015) Improvement and decline in vision with gene therapy in childhood blindness. *N Engl J Med*, **372**, 1920-1926.
- 130 MacLaren, R.E., Groppe, M., Barnard, A.R., Cottrill, C.L., Tolmachova, T., Seymour, L., Clark, K.R., Durr, M.J., Cremers, F.P., Black, G.C. *et al.* (2014) Retinal gene therapy in patients with choroideremia: initial findings from a phase 1/2 clinical trial. *Lancet*, **383**, 1129-1137.
- 131 Cukras, C., Wiley, H.E., Jeffrey, B.G., Sen, H.N., Turriff, A., Zeng, Y., Vijayasarathy, C., Marangoni, D., Ziccardi, L., Kjellstrom, S. *et al.* (2018) Retinal AAV8-RS1 Gene Therapy for X-Linked Retinoschisis: Initial Findings from a Phase I/IIa Trial by Intravitreal Delivery. *Mol Ther*, **26**, 2282-2294.

- 132 Ghazi, N.G., Abboud, E.B., Nowilaty, S.R., Alkuraya, H., Alhommedi, A., Cai, H., Hou, R., Deng, W.T., Boye, S.L., Almaghami, A. *et al.* (2016) Treatment of retinitis pigmentosa due to MERTK mutations by ocular subretinal injection of adeno-associated virus gene vector: results of a phase I trial. *Hum Genet*, **135**, 327-343.
- 133 Cehajic-Kapetanovic, J., Allen, A., Bishop, P. and Lucas, R. (2013) Glycosidic Enzymes Enhance Retinal Transduction Following Intravitreal Delivery of AAV2. *Investigative Ophthalmology & Visual Science*, **54**, 2736-2736.
- 134 de Silva, S.R., McClements, M.E., Hankins, M.W. and MacLaren, R.E. (2015) Adeno-Associated Viral Gene Therapy for Retinal Disorders. *Gene Delivery and Therapy for Neurological Disorders*, 203-228.
- 135 McClements, M.E., Charbel Issa, P., Blouin, V. and MacLaren, R.E. (2016) A fragmented adeno-associated viral dual vector strategy for treatment of diseases caused by mutations in large genes leads to expression of hybrid transcripts. *J Genet Syndr Gene Ther*, **7**.
- 136 Surace, E.M. and Auricchio, A. (2008) Versatility of AAV vectors for retinal gene transfer. *Vision Res*, **48**, 353-359.
- 137 Auricchio, A. (2003) Pseudotyped AAV vectors for constitutive and regulated gene expression in the eye. *Vision Research*, **43**, 913-918.
- 138 De Silva, S.R., Charbel Issa, P., Singh, M.S., Lipinski, D.M., Barnea-Cramer, A.O., Walker, N.J., Barnard, A.R., Hankins, M.W. and MacLaren, R.E. (2016) Single residue AAV capsid mutation improves transduction of photoreceptors in the Abca4^{-/-} mouse and bipolar cells in the rd1 mouse and human retina ex vivo. *Gene Ther*, **23**, 767-774.
- 139 Planul, A.D., Deniz. (2018) Vectors and Gene Delivery to the Retina. *Annual Review of Vision Science*, **3**, 121-140.
- 140 Dalkara, D., Byrne, L.C., Klimczak, R.R., Visel, M., Yin, L., Merigan, W.H., Flannery, J.G. and Schaffer, D.V. (2013) In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous. *Science translational medicine*, **5**, 189ra176-189ra176.
- 141 Petrs-Silva, H., Dinculescu, A., Li, Q., Deng, W.T., Pang, J.J., Min, S.H., Chiodo, V., Neeley, A.W., Govindasamy, L., Bennett, A. *et al.* (2011) Novel properties of tyrosine-mutant AAV2 vectors in the mouse retina. *Mol Ther*, **19**, 293-301.
- 142 Kotterman, M.A., Yin, L., Strazzeri, J.M., Flannery, J.G., Merigan, W.H. and Schaffer, D.V. (2015) Antibody neutralization poses a barrier to intravitreal adeno-associated viral vector gene delivery to non-human primates. *Gene Ther*, **22**, 116-126.
- 143 Boye, S.L., Bennett, A., Scalabrino, M.L., McCullough, K.T., Van Vliet, K., Choudhury, S., Ruan, Q., Peterson, J., Agbandje-McKenna, M. and Boye, S.E. (2016) Impact of Heparan Sulfate Binding on Transduction of Retina by Recombinant Adeno-Associated Virus Vectors. *J Virol*, **90**, 4215-4231.
- 144 Kern, A., Schmidt, K., Leder, C., Muller, O.J., Wobus, C.E., Bettinger, K., Von der Lieth, C.W., King, J.A. and Kleinschmidt, J.A. (2003) Identification of a heparin-binding motif on adeno-associated virus type 2 capsids. *J Virol*, **77**, 11072-11081.
- 145 Summerford, C. and Samulski, R.J. (1998) Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol*, **72**, 1438-1445.
- 146 Vives, R.R., Lortat-Jacob, H. and Fender, P. (2006) Heparan sulphate proteoglycans and viral vectors : ally or foe? *Curr Gene Ther*, **6**, 35-44.

- 147 Xie, Q., Spear, J.M., Noble, A.J., Sousa, D.R., Meyer, N.L., Davulcu, O., Zhang, F., Linhardt, R.J., Stagg, S.M. and Chapman, M.S. (2017) The 2.8 Å Electron Microscopy Structure of Adeno-Associated Virus-DJ Bound by a Heparinoid Pentasaccharide. *Mol Ther Methods Clin Dev*, **5**, 1-12.
- 148 Pillay, S., Meyer, N.L., Puschnik, A.S., Davulcu, O., Diep, J., Ishikawa, Y., Jae, L.T., Wosen, J.E., Nagamine, C.M., Chapman, M.S. *et al.* (2016) An essential receptor for adeno-associated virus infection. *Nature*, **530**, 108-112.
- 149 Dalkara, D., Kolstad, K.D., Caporale, N., Visel, M., Klimczak, R.R., Schaffer, D.V. and Flannery, J.G. (2009) Inner limiting membrane barriers to AAV-mediated retinal transduction from the vitreous. *Mol Ther*, **17**, 2096-2102.
- 150 Rein, M.L. and Deussing, J.M. (2012) The optogenetic (r)evolution. *Mol Genet Genomics*, **287**, 95-109.
- 151 Boyden, E.S., Zhang, F., Bamberg, E., Nagel, G. and Deisseroth, K. (2005) Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci*, **8**, 1263-1268.
- 152 Nagel, G., Ollig, D., Fuhrmann, M., Kateriya, S., Musti, A.M., Bamberg, E. and Hegemann, P. (2002) Channelrhodopsin-1: a light-gated proton channel in green algae. *Science*, **296**, 2395-2398.
- 153 Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P. and Bamberg, E. (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proceedings of the National Academy of Sciences*, **100**, 13940-13945.
- 154 Deisseroth, K., Feng, G., Majewska, A.K., Miesenbock, G., Ting, A. and Schnitzer, M.J. (2006) Next-generation optical technologies for illuminating genetically targeted brain circuits. *J Neurosci*, **26**, 10380-10386.
- 155 Stroh, A., Tsai, H.C., Wang, L.P., Zhang, F., Kressel, J., Aravanis, A., Santhanam, N., Deisseroth, K., Konnerth, A. and Schneider, M.B. (2011) Tracking stem cell differentiation in the setting of automated optogenetic stimulation. *Stem Cells*, **29**, 78-88.
- 156 Bruegmann, T., Malan, D., Hesse, M., Beiert, T., Fuegemann, C.J., Fleischmann, B.K. and Sasse, P. (2010) Optogenetic control of heart muscle in vitro and in vivo. *Nat Methods*, **7**, 897-900.
- 157 Llewellyn, M.E., Thompson, K.R., Deisseroth, K. and Delp, S.L. (2010) Orderly recruitment of motor units under optical control in vivo. *Nature medicine*, **16**, 1161.
- 158 Berry, M.H., Holt, A., Salari, A., Veit, J., Visel, M., Levitz, J., Aghi, K., Gaub, B.M., Sivyer, B., Flannery, J.G. *et al.* (2019) Restoration of high-sensitivity and adapting vision with a cone opsin. *Nature Communications*, **10**.
- 159 De Silva, S.R., Barnard, A.R., Hughes, S., Tam, S.K.E., Martin, C., Singh, M.S., Barnea-Cramer, A.O., McClements, M.E., During, M.J., Peirson, S.N. *et al.* (2017) Long-term restoration of visual function in end-stage retinal degeneration using subretinal human melanopsin gene therapy. *Proc Natl Acad Sci U S A*, **114**, 11211-11216.
- 160 Lin, B., Koizumi, A., Tanaka, N., Panda, S. and Masland, R.H. (2008) Restoration of visual function in retinal degeneration mice by ectopic expression of melanopsin. *Proc Natl Acad Sci U S A*, **105**, 16009-16014.
- 161 Pittler, S.J. and Baehr, W. (1991) Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase beta-subunit gene of the rd mouse. *Proc Natl Acad Sci U S A*, **88**, 8322-8326.

- 162 Hattar, S., Liao, H.W., Takao, M., Berson, D.M. and Yau, K.W. (2002) Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science*, **295**, 1065-1070.
- 163 Silvia Marino, P.K., Carly Leung, Hetty A. G. M. van der Korput, Jan Trapman, Isabelle Camenisch, Anton Berns and Sebastian Brandner. (2002) PTEN is essential for cell migration but not for fate determination and tumourigenesis in the cerebellum. *Development*, **129**, 3513-3522.
- 164 Nakazawa, K., Quirk, M.C., Chitwood, R.A., Watanabe, M., Yeckel, M.F., Sun, L.D., Kato, A., Carr, C.A., Johnston, D. and Wilson, M.A. (2002) Requirement for hippocampal CA3 NMDA receptors in associative memory recall. *Science*, **297**, 211-218.
- 165 Hickey, D.G. (2016), Thesis submitted for the degree of *D. Phil*, University of Oxford, Oxford.
- 166 Nitta, K., Matsuzaki, Y., Konno, A. and Hirai, H. (2017) Minimal Purkinje Cell-Specific PCP2/L7 Promoter Virally Available for Rodents and Non-human Primates. *Mol Ther Methods Clin Dev*, **6**, 159-170.
- 167 de Leeuw, C.N., Korecki, A.J., Berry, G.E., Hickmott, J.W., Lam, S.L., Lengyel, T.C., Bonaguro, R.J., Borretta, L.J., Chopra, V., Chou, A.Y. *et al.* (2016) rAAV-compatible MiniPromoters for restricted expression in the brain and eye. *Mol Brain*, **9**, 52.
- 168 Rowley, S. and Clare, S. (2011) ANTT: a standard approach to aseptic technique. *Nursing times*, **107**, 12-14.
- 169 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.
- 170 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S. and Schmid, B. (2012) Fiji: an open-source platform for biological-image analysis. *Nature methods*, **9**, 676.
- 171 Bolte, S. and Cordelieres, F. (2006) A guided tour into subcellular colocalization analysis in light microscopy. *Journal of microscopy*, **224**, 213-232.
- 172 Li, Q., Lau, A., Morris, T.J., Guo, L., Fordyce, C.B. and Stanley, E.F. (2004) A syntaxin 1, Gao, and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. *Journal of Neuroscience*, **24**, 4070-4081.
- 173 Costes, S.V., Daelemans, D., Cho, E.H., Dobbin, Z., Pavlakis, G. and Lockett, S. (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophysical journal*, **86**, 3993-4003.
- 174 Douglas, R.H. (2018) The pupillary light responses of animals; a review of their distribution, dynamics, mechanisms and functions. *Prog Retin Eye Res*.
- 175 de Silva, S (2015), Thesis submitted for the degree of *D. Phil*, University of Oxford
- 176 Watson, T.S. (2014), Thesis submitted for the degree of *D. Phil*, University of Oxford
- 177 Douglas, R.M., Alam, N.M., Silver, B.D., McGill, T.J., Tschetter, W.W. and Prusky, G.T. (2005) Independent visual threshold measurements in the two eyes of freely moving rats and mice using a virtual-reality optokinetic system. *Vis Neurosci*, **22**, 677-684.
- 178 Tam, S.K., Hasan, S., Choi, H.M., Brown, L.A., Jagannath, A., Hughes, S., Hankins, M.W., Foster, R.G., Vyazovskiy, V.V. and Bannerman, D.M. (2017) Constant

Light Desynchronizes Olfactory versus Object and Visuospatial Recognition Memory Performance. *Journal of Neuroscience*, **37**, 3555-3567.

179 Tam, S.K., Hasan, S., Hughes, S., Hankins, M.W., Foster, R.G., Bannerman, D.M. and Peirson, S.N. (2016) Modulation of recognition memory performance by light requires both melanopsin and classical photoreceptors. *Proc Biol Sci*, **283**.

180 Lucas, R.J., Peirson, S.N., Berson, D.M., Brown, T.M., Cooper, H.M., Czeisler, C.A., Figueiro, M.G., Gamlin, P.D., Lockley, S.W., O'Hagan, J.B. and Price, L.L., 2014. Measuring and using light in the melanopsin age. *Trends in neurosciences*, *37*(1), pp.1-9. ; <https://www.cell.com/cms/10.1016/j.tins.2013.10.004/attachment/baab3719-3769-4c31-886b-4fe56da7c66d/mmc1.pdf>

181 Hughes, S. (2017), Personal communication with Steven Hughes, Nuffield Laboratory of Ophthalmology, University of Oxford.

182 Rodgers, J., Hughes, S., Potheary, C.A., Brown, L.A., Hickey, D.G., Peirson, S.N. and Hankins, M.W. (2018) Defining the impact of melanopsin missense polymorphisms using in vivo functional rescue. *Hum Mol Genet*.

183 Kemmer, G. and Keller, S. (2010) Nonlinear least-squares data fitting in Excel spreadsheets. *Nat Protoc*, **5**, 267-281.

184 Peirson, S.N., Thompson, S., Hankins, M.W. and Foster, R.G. (2005), In *Methods in enzymology*. Elsevier, Vol. 393, pp. 697-726.

185 Govardovskii, V.I., Fyhrquist, N., Reuter, T.O.M., Kuzmin, D.G. and Donner, K. (2000) In search of the visual pigment template. *Visual Neuroscience*, **17**, 509-528.

186 Slijkerman, R.W., Song, F., Astuti, G.D., Huynen, M.A., van Wijk, E., Stieger, K. and Collin, R.W. (2015) The pros and cons of vertebrate animal models for functional and therapeutic research on inherited retinal dystrophies. *Prog Retin Eye Res*, **48**, 137-159.

187 Prusky, G.T. and Douglas, R.M. (2004) Characterization of mouse cortical spatial vision. *Vision Res*, **44**, 3411-3418.

188 Prusky, G.T. and Douglas, R.M. (2003) Developmental plasticity of mouse visual acuity. *European Journal of Neuroscience*, **17**, 167-173.

189 Prusky, G.T., West, P.W. and Douglas, R.M. (2000) Behavioral assessment of visual acuity in mice and rats. *Vision research*, **40**, 2201-2209.

190 Huberman, A.D. and Niell, C.M. (2011) What can mice tell us about how vision works? *Trends Neurosci*, **34**, 464-473.

191 Chang, B., Hurd, R., Wang, J. and Nishina, P. (2013) Survey of common eye diseases in laboratory mouse strains. *Investigative ophthalmology & visual science*, **54**, 4974-4981.

192 Samardzija, M., Neuhauss, S.C.F., Joly, S., Kurz-Levin, M. and Grimm, C. (2010), In *Animal Models for Retinal Diseases*. pp. 51-79.

193 Chang, B. (2012), In *Retinal Degeneration*. Springer, pp. 27-39.

194 Veleri, S., Lazar, C.H., Chang, B., Sieving, P.A., Banin, E. and Swaroop, A. (2015) Biology and therapy of inherited retinal degenerative disease: insights from mouse models. *Dis Model Mech*, **8**, 109-129.

195 Chang, B., Hawes, N., Hurd, R., Davisson, M., Nusinowitz, S. and Heckenlively, J. (2002) Retinal degeneration mutants in the mouse. *Vision research*, **42**, 517-525.

196 The Jackson Laboratory (2019); <https://www.jaxlabs.com>

- 197 Bowes, C., Li, T., Danciger, M., Baxter, L.C., Applebury, M.L. and Farber, D.B. (1990) Retinal degeneration in the rd mouse is caused by a defect in the β subunit of rod cGMP-phosphodiesterase. *Nature*, **347**, 677.
- 198 Fox, D.A., Poblenz, A.T. and He, L. (1999) Calcium overload triggers rod photoreceptor apoptotic cell death in chemical-induced and inherited retinal degenerations. *Annals of the New York Academy of Sciences*, **893**, 282-285.
- 199 Farber, D.B., Flannery, J.G. and Bowes-Rickman, C. (1994) The rd mouse story: seventy years of research on an animal model of inherited retinal degeneration. *Progress in retinal and eye research*, **13**, 31-64.
- 200 Thaung, C., Arnold, K., Jackson, I.J. and Coffey, P.J. (2002) Presence of visual head tracking differentiates normal sighted from retinal degenerate mice. *Neurosci Lett*, **325**, 21-24.
- 201 Carter-Dawson, L., LaVail, M. and Sidman, R. (1978) Differential effect of the rd mutation on rods and cones in the mouse retina. *Investigative ophthalmology & visual science*, **17**, 489-498.
- 202 Doshi, M., Voaden, M. and Arden, G. (1985) Cyclic GMP in the retinas of normal mice and those heterozygous for early-onset photoreceptor dystrophy. *Experimental eye research*, **41**, 61-65.
- 203 Voaden, M.J. and Willmott, N.J. (1990) Evidence for reduced binding of cyclic GMP to cyclic GMP phosphodiesterase in photoreceptors of mice heterozygous for the rd gene. *Current eye research*, **9**, 643-651.
- 204 Low, J. (1987) The corneal ERG of the heterozygous retinal degeneration mouse. *Graefe's archive for clinical and experimental ophthalmology*, **225**, 413-417.
- 205 Hattar, S., Lucas, R.J., Mrosovsky, N., Thompson, S., Douglas, R.H., Hankins, M.W., Lem, J., Biel, M., Hofmann, F., Foster, R.G. *et al.* (2003) Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature*, **424**, 76-81.
- 206 Guler, A.D., Ecker, J.L., Lall, G.S., Haq, S., Altimus, C.M., Liao, H.W., Barnard, A.R., Cahill, H., Badea, T.C., Zhao, H. *et al.* (2008) Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. *Nature*, **453**, 102-105.
- 207 Bi, A., Cui, J., Ma, Y.P., Olshevskaya, E., Pu, M., Dizhoor, A.M. and Pan, Z.H. (2006) Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. *Neuron*, **50**, 23-33.
- 208 Ganjawala, T.H., Lu, Q., Fenner, M.D., Abrams, G.W. and Pan, Z.-H. (2019) Improved Channelrhodopsin Variants Restore Visual Acuity and Contrast Sensitivity in A Mouse Model of Blindness under Ambient Light Conditions. *Molecular Therapy*.
- 209 Hughes, S., Rodgers, J., Hickey, D., Foster, R.G., Peirson, S.N. and Hankins, M.W. (2016) Characterisation of light responses in the retina of mice lacking principle components of rod, cone and melanopsin phototransduction signalling pathways. *Sci Rep*, **6**, 28086.
- 210 Zhang, Y., Ivanova, E., Bi, A. and Pan, Z.H. (2009) Ectopic expression of multiple microbial rhodopsins restores ON and OFF light responses in retinas with photoreceptor degeneration. *J Neurosci*, **29**, 9186-9196.
- 211 Dalkara, D., Duebel, J. and Sahel, J.A. (2015) Gene therapy for the eye focus on mutation-independent approaches. *Curr Opin Neurol*, **28**, 51-60.

- 212 Lu, Q. and Pan, Z.H. (2017) Immunohistochemical Procedures for Characterizing the Retinal Expression Patterns of Cre Driver Mouse Lines. *Methods Mol Biol*, **1642**, 181-194.
- 213 Ivanova, E., Hwang, G.S. and Pan, Z.H. (2010) Characterization of transgenic mouse lines expressing Cre recombinase in the retina. *Neuroscience*, **165**, 233-243.
- 214 Nagy, A. (2000) Cre recombinase: the universal reagent for genome tailoring. *genesis*, **26**, 99-109.
- 215 Vuong, H.E., Perez de Sevilla Muller, L., Hardi, C.N., McMahon, D.G. and Brecha, N.C. (2015) Heterogeneous transgene expression in the retinas of the TH-RFP, TH-Cre, TH-BAC-Cre and DAT-Cre mouse lines. *Neuroscience*, **307**, 319-337.
- 216 Gabriel, R., Erdelyi, F., Szabo, G., Lawrence, J.J. and Wilhelm, M. (2016) Ectopic transgene expression in the retina of four transgenic mouse lines. *Brain Struct Funct*, **221**, 3729-3741.
- 217 Ju, W. (2019) *Neuroscience: Canadian 1st Edition Open Textbook*, Ontario.
- 218 Lu, Q., Ivanova, E. and Pan, Z.H. (2009) Characterization of green fluorescent protein-expressing retinal cone bipolar cells in a 5-hydroxytryptamine receptor 2a transgenic mouse line. *Neuroscience*, **163**, 662-668.
- 219 Rowan, S. and Cepko, C.L. (2004) Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. *Dev Biol*, **271**, 388-402.
- 220 Lu, Q., Ivanova, E., Ganjawala, T.H. and Pan, Z.H. (2013) Cre-mediated recombination efficiency and transgene expression patterns of three retinal bipolar cell-expressing Cre transgenic mouse lines. *Mol Vis*, **19**, 1310-1320.
- 221 Ivanova, E., Lee, P. and Pan, Z.H. (2013) Characterization of multiple bistratified retinal ganglion cells in a purkinje cell protein 2-Cre transgenic mouse line. *J Comp Neurol*, **521**, 2165-2180.
- 222 Martersteck, E.M., Hirokawa, K.E., Evarts, M., Bernard, A., Duan, X., Li, Y., Ng, L., Oh, S.W., Ouellette, B., Royall, J.J. *et al.* (2017) Diverse Central Projection Patterns of Retinal Ganglion Cells. *Cell Rep*, **18**, 2058-2072.
- 223 Jo, A., Xu, J., Deniz, S., Cherian, S., DeVries, S.H. and Zhu, Y. (2018) Intersectional Strategies for Targeting Amacrine and Ganglion Cell Types in the Mouse Retina. *Front Neural Circuits*, **12**, 66.
- 224 Oliver, P. (2017) Personal communication
- 225 Finelli, M.J., Paramo, T., Pires, E., Ryan, B.J., Wade-Martins, R., Biggin, P.C., McCullagh, J. and Oliver, P.L. (2019) Oxidation Resistance 1 Modulates Glycolytic Pathways in the Cerebellum via an Interaction with Glucose-6-Phosphate Isomerase. *Mol Neurobiol*, **56**, 1558-1577.
- 226 Srinivas, S., Watanabe, T., Lin, C.-S., William, C.M., Tanabe, Y., Jessell, T.M. and Costantini, F. (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC developmental biology*, **1**, 4.
- 227 Peachey, N.S., Ray, T.A., Florijn, R., Rowe, L.B., Sjoerdsma, T., Contreras-Alcantara, S., Baba, K., Tosini, G., Pozdeyev, N., Iuvone, P.M. *et al.* (2012) GPR179 is required for depolarizing bipolar cell function and is mutated in autosomal-recessive complete congenital stationary night blindness. *Am J Hum Genet*, **90**, 331-339.
- 228 Nishiguchi, K.M., Carvalho, L.S., Rizzi, M., Powell, K., Holthaus, S.M., Azam, S.A., Duran, Y., Ribeiro, J., Luhmann, U.F., Bainbridge, J.W. *et al.* (2015) Gene therapy

- restores vision in rd1 mice after removal of a confounding mutation in Gpr179. *Nat Commun*, **6**, 6006.
- 229 Oberdick, J., Smeyne, R.J., Mann, J.R., Zackson, S. and Morgan, J.I. (1990) A promoter that drives transgene expression in cerebellar Purkinje and retinal bipolar neurons. *Science*, **248**, 223-226.
- 230 Oberdick, J., Levinthal, F. and Levinthal, C. (1988) A Purkinje cell differentiation marker shows a partial DNA sequence homology to the cellular sis/PDGF2 gene. *Neuron*, **1**, 367-376.
- 231 Rodgers, J. (2018), Personal communication with J Rodgers, University of Manchester.
- 232 Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R. *et al.* (2010) A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci*, **13**, 133-140.
- 233 Wong, A.A. and Brown, R.E. (2006) Visual detection, pattern discrimination and visual acuity in 14 strains of mice. *Genes Brain Behav*, **5**, 389-403.
- 234 Peirson, S.N., Brown, L.A., Pothecary, C.A., Benson, L.A. and Fisk, A.S., 2018. Light and the laboratory mouse. *Journal of neuroscience methods*, **300**, pp.26-36.
- 235 Banks, G., Heise, I., Starbuck, B., Osborne, T., Wisby, L., Potter, P., Jackson, I.J., Foster, R.G., Peirson, S.N. and Nolan, P.M. (2015) Genetic background influences age-related decline in visual and nonvisual retinal responses, circadian rhythms, and sleep. *Neurobiol Aging*, **36**, 380-393.
- 236 Lambert, R. (2009) Breeding strategies for maintaining colonies of laboratory mice. *The Jackson Laboratory*
- 237 Edwards, T.L., Cottrill, C.L., Xue, K., Simunovic, M.P., Ramsden, J.D., Zrenner, E. and MacLaren, R.E. (2018) Assessment of the Electronic Retinal Implant Alpha AMS in Restoring Vision to Blind Patients with End-Stage Retinitis Pigmentosa. *Ophthalmology*, **125**, 432-443.
- 238 Jones, B.W. and Marc, R.E. (2005) Retinal remodeling during retinal degeneration. *Exp Eye Res*, **81**, 123-137.
- 239 Varela, C., Igartua, I., De la Rosa, E.J. and De la Villa, P. (2003) Functional modifications in rod bipolar cells in a mouse model of retinitis pigmentosa. *Vision Research*, **43**, 879-885.
- 240 Gayet-Primo, J. and Puthussery, T. (2015) Alterations in Kainate Receptor and TRPM1 Localization in Bipolar Cells after Retinal Photoreceptor Degeneration. *Front Cell Neurosci*, **9**, 486.
- 241 Dorrell, M.I., Aguilar, E., Weber, C. and Friedlander, M. (2004) Global Gene Expression Analysis of the Developing Postnatal Mouse Retina. *Investigative Ophthalmology & Visual Science*, **45**, 1009-1019.
- 242 Hackam, A.S., Strom, R., Liu, D., Qian, J., Wang, C., Otteson, D., Gunatilaka, T., Farkas, R.H., Chowers, I., Kageyama, M. *et al.* (2004) Identification of gene expression changes associated with the progression of retinal degeneration in the rd1 mouse. *Invest Ophthalmol Vis Sci*, **45**, 2929-2942.
- 243 Punzo, C. and Cepko, C. (2007) Cellular responses to photoreceptor death in the rd1 mouse model of retinal degeneration. *Invest Ophthalmol Vis Sci*, **48**, 849-857.

- 244 Hornan, D.M., Peirson, S.N., Hardcastle, A.J., Molday, R.S., Cheetham, M.E. and Webster, A.R. (2007) Novel retinal and cone photoreceptor transcripts revealed by human macular expression profiling. *Invest Ophthalmol Vis Sci*, **48**, 5388-5396.
- 245 Yu, P., Pearson, C.S. and Geller, H.M. (2018) Flexible Roles for Proteoglycan Sulfation and Receptor Signaling. *Trends Neurosci*, **41**, 47-61.
- 246 de Leeuw, C.N., Dyka, F.M., Boye, S.L., Laprise, S., Zhou, M., Chou, A.Y., Borretta, L., McInerney, S.C., Banks, K.G., Portales-Casamar, E. *et al.* (2014) Targeted CNS Delivery Using Human MiniPromoters and Demonstrated Compatibility with Adeno-Associated Viral Vectors. *Mol Ther Methods Clin Dev*, **1**, 5.
- 247 Peirson, S.N. (2007), *Real-time PCR*. Taylor & Francis, pp. 135-154.
- 248 Du, P., Kibbe, W.A. and Lin, S.M. (2008) lumi: a pipeline for processing Illumina microarray. *Bioinformatics*, **24**, 1547-1548.
- 249 Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W. and Smyth, G.K. (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research*, **43**, e47-e47.
- 250 Team, R.C. (2013). R Foundation for Statistical Computing, Vienna.
- 251 Benjamini, Y.H., Yosef. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal stat soc*, **57**.
- 252 Vardi, N. (1998) Alpha subunit of Go localizes in the dendritic tips of ON bipolar cells. *Journal of Comparative Neurology*, **395**, 43-52.
- 253 Vardi, N. and Dhingra, A. (2014) Martemyanov, K.A. and Sampath, A.P. (eds.), In Mechanistic Basis for G Protein Function in ON Bipolar Cells *Spr Ser Vis Res*. Springer New York, New York, NY, pp. 81-97.
- 254 Ramakrishnan, H., Dhingra, A., Tummala, S.R., Fina, M.E., Li, J.J., Lyubarsky, A. and Vardi, N. (2015) Differential function of Ggamma13 in rod bipolar and ON cone bipolar cells. *J Physiol*, **593**, 1531-1550.
- 255 Dhingra, A., Tummala, S.R., Lyubarsky, A. and Vardi, N. (2014) PDE9A is expressed in the inner retina and contributes to the normal shape of the photopic ERG waveform. *Front Mol Neurosci*, **7**, 60.
- 256 Siegert, S., Cabuy, E., Scherf, B.G., Kohler, H., Panda, S., Le, Y.Z., Fehling, H.J., Gaidatzis, D., Stadler, M.B. and Roska, B. (2012) Transcriptional code and disease map for adult retinal cell types. *Nat Neurosci*, **15**, 487-495, S481-482.
- 257 Berg, D.J., Kartheiser, K., Leyrer, M., Saali, A. and Berson, D.M., 2019. Transcriptomic Signatures of Postnatal and Adult Intrinsically Photosensitive Ganglion Cells. *eNeuro*, 6(4).
- 258 Daiger, S., Rossiter, B., Greenberg, J., Christoffels, A. and Hide, W. (1998) Data services and software for identifying genes and mutations causing retinal degeneration. *Invest Ophthalmol Vis Sci*, **39**, S295.
- 259 Warde-Farley, D., Donaldson, S.L., Comes, O., Zuberi, K., Badrawi, R., Chao, P., Franz, M., Grouios, C., Kazi, F., Lopes, C.T. *et al.* (2010) The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res*, **38**, W214-220.
- 260 Hatori, Y. and Lutsenko, S. (2013) An expanding range of functions for the copper chaperone/antioxidant protein Atox1. *Antioxid Redox Signal*, **19**, 945-957.
- 261 Pascual, I., Larrayoz, I.M., Campos, M.M. and Rodriguez, I.R. (2010) Methionine sulfoxide reductase B2 is highly expressed in the retina and protects retinal pigmented epithelium cells from oxidative damage. *Exp Eye Res*, **90**, 420-428.

- 262 Vasak, M. (2005) Advances in metallothionein structure and functions. *J Trace Elem Med Biol*, **19**, 13-17.
- 263 Fabregat, A., Sidiropoulos, K., Garapati, P., Gillespie, M., Hausmann, K., Haw, R., Jassal, B., Jupe, S., Korninger, F., McKay, S. *et al.* (2016) The Reactome pathway Knowledgebase. *Nucleic Acids Res*, **44**, D481-487.
- 264 Fabregat, A., Sidiropoulos, K., Garapati, P., Gillespie, M., Hausmann, K., Haw, R., Jassal, B., Jupe, S., Korninger, F., McKay, S. and Matthews, L., 2015. The reactome pathway knowledgebase. *Nucleic acids research*, **44**(D1), pp.D481-D487.
- 265 Harris, M.A., Clark, J., Ireland, A., Lomax, J., Ashburner, M., Foulger, R., Eilbeck, K., Lewis, S., Marshall, B., Mungall, C. *et al.* (2004) The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Res*, **32**, D258-261.
- 266 Fairbank, P.D., Lee, C., Ellis, A., Hildebrand, J.D., Gross, J.M. and Wallingford, J.B. (2006) Shroom2 (APXL) regulates melanosome biogenesis and localization in the retinal pigment epithelium. *Development*, **133**, 4109-4118.
- 267 Morimoto-Tomita, M., Uchimura, K., Werb, Z., Hemmerich, S. and Rosen, S.D. (2002) Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans. *J Biol Chem*, **277**, 49175-49185.
- 268 Caohuy, H. and Pollard, H.B. (2002) Protein kinase C and guanosine triphosphate combine to potentiate calcium-dependent membrane fusion driven by annexin 7. *J Biol Chem*, **277**, 25217-25225.
- 269 Grewal, T., Wason, S.J., Enrich, C. and Rentero, C. (2016) Annexins - insights from knockout mice. *Biol Chem*, **397**, 1031-1053.
- 270 Hoque, M., Rentero, C., Cairns, R., Tebar, F., Enrich, C. and Grewal, T. (2014) Annexins - scaffolds modulating PKC localization and signaling. *Cell Signal*, **26**, 1213-1225.
- 271 Davisson, M.T., Bronson, R.T., Tadenev, A.L., Motley, W.W., Krishnaswamy, A., Seburn, K.L. and Burgess, R.W. (2011) A spontaneous mutation in contactin 1 in the mouse. *PLoS One*, **6**, e29538.
- 272 Masu, M. (2013) Kageyama, R. and Yamamori, T. (eds.), In *Cortical Development: Neural Diversity and Neocortical Organization*. Springer Japan, Tokyo. pp. 203-214.
- 273 Murai, K.K., Misner, D. and Ranscht, B. (2002) Contactin supports synaptic plasticity associated with hippocampal long-term depression but not potentiation. *Curr Biol*, **12**, 181-190.
- 274 Gulisano, W., Bizzoca, A., Gennarini, G., Palmeri, A. and Puzzo, D. (2017) Role of the adhesion molecule F3/Contactin in synaptic plasticity and memory. *Mol Cell Neurosci*, **81**, 64-71.
- 275 Chatterjee, M., Schild, D. and Teunissen, C. (2019) Contactins in the central nervous system: role in health and disease. *Neural Regeneration Research*, **14**, 206-216.
- 276 Etournay, R., Zwaenepoel, I., Perfettini, I., Legrain, P., Petit, C. and El-Amraoui, A. (2007) Shroom2, a myosin-VIIa- and actin-binding protein, directly interacts with ZO-1 at tight junctions. *J Cell Sci*, **120**, 2838-2850.
- 277 Watson, W.D., Srivastava, M., Leighton, X., Glasman, M., Faraday, M., Fossam, L.H., Pollard, H.B. and Verma, A. (2004) Annexin 7 mobilizes calcium from endoplasmic reticulum stores in brain. *Biochim Biophys Acta*, **1742**, 151-160.

- 278 Orlandi, C., Omori, Y., Wang, Y., Cao, Y., Ueno, A., Roux, M.J., Condomitti, G., de Wit, J., Kanagawa, M., Furukawa, T. *et al.* (2018) Transsynaptic Binding of Orphan Receptor GPR179 to Dystroglycan-Pikachurin Complex Is Essential for the Synaptic Organization of Photoreceptors. *Cell Rep*, **25**, 130-145 e135.
- 279 Fabian-Jessing, B.K., Vestergaard, E.M., Plomp, A.S., Bergen, A.A., Dreschler, W.A., Duno, M., Winiarska, B.S., Neumann, L., Gaihede, M., Vorum, H. *et al.* (2018) Ocular albinism with infertility and late-onset sensorineural hearing loss. *Am J Med Genet A*, **176**, 1587-1593.
- 280 McBride, A.N. (2016). Thesis submitted for the degree of *Ph. D* University of Edinburgh.
- 281 Schmitz, F., Augustin, I. and Brose, N. (2001) The synaptic vesicle priming protein Munc13-1 is absent from tonically active ribbon synapses of the rat retina. *Brain Research*, **895**, 258-263.
- 282 Ruether, K., Feigenspan, A., Pirngruber, J., Leitges, M., Baehr, W. and Strauss, O. (2010) PKC{alpha} is essential for the proper activation and termination of rod bipolar cell response. *Invest Ophthalmol Vis Sci*, **51**, 6051-6058.
- 283 Xiong, W.H., Pang, J.J., Pennesi, M.E., Duvoisin, R.M., Wu, S.M. and Morgans, C.W. (2015) The Effect of PKCalpha on the Light Response of Rod Bipolar Cells in the Mouse Retina. *Invest Ophthalmol Vis Sci*, **56**, 4961-4974.
- 284 Puzzo, D., Bizzoca, A., Privitera, L., Furnari, D., Giunta, S., Girolamo, F., Pinto, M., Gennarini, G. and Palmeri, A. (2013) F3/Contactin promotes hippocampal neurogenesis, synaptic plasticity, and memory in adult mice. *Hippocampus*, **23**, 1367-1382.
- 285 Shimoda, Y. and Watanabe, K. (2009) Contactins: emerging key roles in the development and function of the nervous system. *Cell Adh Migr*, **3**, 64-70.
- 286 Haenisch, C., Diekmann, H., Klinger, M., Gennarini, G., Kuwada, J.Y. and Stuermer, C.A. (2005) The neuronal growth and regeneration associated Cntn1 (F3/F11/Contactin) gene is duplicated in fish: expression during development and retinal axon regeneration. *Mol Cell Neurosci*, **28**, 361-374.
- 287 Cehajic-Kapetanovic, J., Le Goff, M.M., Allen, A., Lucas, R.J. and Bishop, P.N., 2011. Glycosidic enzymes enhance retinal transduction following intravitreal delivery of AAV2. *Molecular vision*, **17**, p.1771.
- 288 Busskamp, V., Picaud, S., Sahel, J.A. and Roska, B. (2012) Optogenetic therapy for retinitis pigmentosa. *Gene Ther*, **19**, 169-175.
- 289 van Wyk, M., Hulliger, E.C., Girod, L., Ebner, A. and Kleinlogel, S. (2017) Present molecular limitations of ON-bipolar cell targeted gene therapy. *Frontiers in Neuroscience*, **11**, 161.
- 290 Allen, A.E., Storch, R., Martial, F.P., Petersen, R.S., Montemurro, M.A., Brown, T.M. and Lucas, R.J. (2014) Melanopsin-driven light adaptation in mouse vision. *Curr Biol*, **24**, 2481-2490.
- 291 Liu, W., Liu, M., Liu, Y., Li, S., Weng, C., Fu, Y., He, J., Gong, Y., Liu, W., Zhao, C. *et al.* (2018) Validation and Safety of visual restoration by ectopic expression of human melanopsin in retinal ganglion cells. *Hum Gene Ther*.
- 292 Mure, L.S., Hatori, M., Zhu, Q., Demas, J., Kim, I.M., Nayak, S.K. and Panda, S. (2016) Melanopsin-Encoded Response Properties of Intrinsically Photosensitive Retinal Ganglion Cells. *Neuron*, **90**, 1016-1027.

- 293 Van Gelder, R.N. and Buhr, E.D. (2016) Melanopsin: The Tale of the Tail. *Neuron*, **90**, 909-911.
- 294 Blasic, J.R., Jr., Matos-Cruz, V., Ujla, D., Cameron, E.G., Hattar, S., Halpern, M.E. and Robinson, P.R. (2014) Identification of critical phosphorylation sites on the carboxy tail of melanopsin. *Biochemistry*, **53**, 2644-2649.
- 295 Somasundaram, P., Wyrick, G.R., Fernandez, D.C., Ghahari, A., Pinhal, C.M., Simmonds Richardson, M., Rupp, A.C., Cui, L., Wu, Z., Brown, R.L. *et al.* (2017) C-terminal phosphorylation regulates the kinetics of a subset of melanopsin-mediated behaviors in mice. *Proc Natl Acad Sci U S A*, **114**, 2741-2746.
- 296 Sexton, T.J. and Van Gelder, R.N. (2015) G-Protein Coupled Receptor Kinase 2 Minimally Regulates Melanopsin Activity in Intrinsically Photosensitive Retinal Ganglion Cells. *PLoS One*, **10**, e0128690.
- 297 Cameron, E.G. and Robinson, P.R. (2014) β -Arrestin-dependent deactivation of mouse melanopsin. *PloS one*, **9**, e113138.
- 298 Mure, L.S., Hatori, M., Ruda, K., Benegiamo, G., Demas, J. and Panda, S. (2018) Sustained Melanopsin Photoresponse Is Supported by Specific Roles of beta-Arrestin 1 and 2 in Deactivation and Regeneration of Photopigment. *Cell Rep*, **25**, 2497-2509 e2494.
- 299 Haverkamp, S. and Wässle, H. (2000) Immunocytochemical analysis of the mouse retina. *Journal of Comparative Neurology*, **424**, 1-23.
- 300 Sulaiman, P., Fina, M., Feddersen, R. and Vardi, N. (2010) Ret-PCP2 colocalizes with protein kinase C in a subset of primate ON cone bipolar cells. *J Comp Neurol*, **518**, 1098-1112.
- 301 Stachurska, A. and Sarna, T. (2019) Regulation of Melanopsin Signaling: Key Interactions of the Nonvisual Photopigment. *Photochem Photobiol*, **95**, 83-94.
- 302 Peirson, S.N., Oster, H., Jones, S.L., Leitges, M., Hankins, M.W. and Foster, R.G. (2007) Microarray analysis and functional genomics identify novel components of melanopsin signaling. *Curr Biol*, **17**, 1363-1372.
- 303 Bailes, H.J. and Lucas, R.J. (2013) Human melanopsin forms a pigment maximally sensitive to blue light (lambda_{max} approximately 479 nm) supporting activation of G(q/11) and G(i/o) signalling cascades. *Proc Biol Sci*, **280**, 20122987.
- 304 Spoida, K., Eickelbeck, D., Karapinar, R., Eckhardt, T., Mark, M.D., Jancke, D., Ehinger, B.V., Konig, P., Dalkara, D., Herlitze, S. *et al.* (2016) Melanopsin Variants as Intrinsic Optogenetic On and Off Switches for Transient versus Sustained Activation of G Protein Pathways. *Curr Biol*, **26**, 1206-1212.
- 305 Hughes, S., Jagannath, A., Hickey, D., Gatti, S., Wood, M., Peirson, S.N., Foster, R.G. and Hankins, M.W. (2015) Using siRNA to define functional interactions between melanopsin and multiple G Protein partners. *Cell Mol Life Sci*, **72**, 165-179.
- 306 Publio, R., Ceballos, C.C. and Roque, A.C. (2012) Dynamic range of vertebrate retina ganglion cells: importance of active dendrites and coupling by electrical synapses. *PLoS One*, **7**, e48517.
- 307 Lucas, R.J., Douglas, R.H. and Foster, R.G. (2001) Characterization of an ocular photopigment capable of driving pupillary constriction in mice. *Nat Neurosci*, **4**, 621-626.
- 308 Prusky, G.T., West, P.W. and Douglas, R.M., 2000. Behavioral assessment of visual acuity in mice and rats. *Vision research*, **40**(16), pp.2201-2209.

- 309 Lucas, R.J., Hattar, S., Takao, M., Berson, D.M., Foster, R.G. and Yau, K.W. (2003) Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. *Science*, **299**, 245-247.
- 310 Simpson, J.I. (1984) The accessory optic system. *Annual review of neuroscience*, **7**, 13-41.
- 311 Schmidt, T.M. and Kofuji, P. (2010) Differential cone pathway influence on intrinsically photosensitive retinal ganglion cell subtypes. *J Neurosci*, **30**, 16262-16271.
- 312 Sekaran, S., Lupi, D., Jones, S.L., Sheely, C.J., Hattar, S., Yau, K.W., Lucas, R.J., Foster, R.G. and Hankins, M.W. (2005) Melanopsin-dependent photoreception provides earliest light detection in the mammalian retina. *Curr Biol*, **15**, 1099-1107.
- 313 Johnson, J., Wu, V., Donovan, M., Majumdar, S., Renteria, R.C., Porco, T., Van Gelder, R.N. and Copenhagen, D.R. (2010) Melanopsin-dependent light avoidance in neonatal mice. *Proc Natl Acad Sci U S A*, **107**, 17374-17378.
- 314 Renna, J.M., Weng, S. and Berson, D.M. (2011) Light acts through melanopsin to alter retinal waves and segregation of retinogeniculate afferents. *Nat Neurosci*, **14**, 827-829.
- 315 Kirkby, L.A. and Feller, M.B. (2013) Intrinsically photosensitive ganglion cells contribute to plasticity in retinal wave circuits. *Proc Natl Acad Sci U S A*, **110**, 12090-12095.
- 316 Tufford, A.R., Onyak, J.R., Sondereker, K.B., Lucas, J.A., Earley, A.M., Mattar, P., Hattar, S., Schmidt, T.M., Renna, J.M. and Cayouette, M. (2018) Melanopsin Retinal Ganglion Cells Regulate Cone Photoreceptor Lamination in the Mouse Retina. *Cell Rep*, **23**, 2416-2428.
- 317 Rao, S., Chun, C., Fan, J., Kofron, J.M., Yang, M.B., Hegde, R.S., Ferrara, N., Copenhagen, D.R. and Lang, R.A. (2013) A direct and melanopsin-dependent fetal light response regulates mouse eye development. *Nature*, **494**, 243-246.
- 318 Hattar, S., Kumar, M., Park, A., Tong, P., Tung, J., Yau, K.W. and Berson, D.M. (2006) Central projections of melanopsin-expressing retinal ganglion cells in the mouse. *J Comp Neurol*, **497**, 326-349.
- 319 Singh, M.S., Charbel Issa, P., Butler, R., Martin, C., Lipinski, D.M., Sekaran, S., Barnard, A.R. and MacLaren, R.E. (2013) Reversal of end-stage retinal degeneration and restoration of visual function by photoreceptor transplantation. *Proc Natl Acad Sci U S A*, **110**, 1101-1106.
- 320 Fisk, A.S.G., Guillaumin, M (2019), Personal communication with A. Fish and M Guillaumin, University of Oxford
- 321 Allen, A.E., Storchi, R., Martial, F.P., Bedford, R.A. and Lucas, R.J. (2017) Melanopsin Contributions to the Representation of Images in the Early Visual System. *Curr Biol*.
- 322 Lerea, C.L., Somers, D.E., Hurley, J.B., Klock, I.B. and Bunt-Milam, A.H. (1986) Identification of specific transducin alpha subunits in retinal rod and cone photoreceptors. *Science*, **234**, 77-81.
- 323 Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T. and Stenkamp, R.E. (2000) Crystal structure of rhodopsin: AG protein-coupled receptor. *science*, **289**, 739-745.
- 324 Menon, S.T., Han, M. and Sakmar, T.P. (2001) Rhodopsin: structural basis of molecular physiology. *Physiological reviews*, **81**, 1659-1688.

- 325 Palczewski, K. (2006) G protein-coupled receptor rhodopsin. *Annu Rev Biochem*, **75**, 743-767.
- 326 Li, X., Gutierrez, D.V., Hanson, M.G., Han, J., Mark, M.D., Chiel, H., Hegemann, P., Landmesser, L.T. and Herlitze, S. (2005) Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin. *Proc Natl Acad Sci U S A*, **102**, 17816-17821.
- 327 Cao, P., Sun, W., Kramp, K., Zheng, M., Salom, D., Jastrzebska, B., Jin, H., Palczewski, K. and Feng, Z. (2012) Light-sensitive coupling of rhodopsin and melanopsin to G(i/o) and G(q) signal transduction in *Caenorhabditis elegans*. *FASEB J*, **26**, 480-491.
- 328 Gutierrez, D.V., Mark, M.D., Maseck, O., Maejima, T., Kuckelsberg, D., Hyde, R.A., Krause, M., Kruse, W. and Herlitze, S. (2011) Optogenetic control of motor coordination by Gi/o protein-coupled vertebrate rhodopsin in cerebellar Purkinje cells. *J Biol Chem*, **286**, 25848-25858.
- 329 Kaylor, J.J., Yuan, Q., Cook, J., Sarfare, S., Makshanoff, J., Miu, A., Kim, A., Kim, P., Habib, S., Roybal, C.N. *et al.* (2013) Identification of DES1 as a vitamin A isomerase in Muller glial cells of the retina. *Nat Chem Biol*, **9**, 30-36.
- 330 Polosukhina, A., Litt, J., Tochitsky, I., Nemargut, J., Sychev, Y., De Kouchkovsky, I., Huang, T., Borges, K., Trauner, D., Van Gelder, R.N. *et al.* (2012) Photochemical restoration of visual responses in blind mice. *Neuron*, **75**, 271-282.
- 331 Tochitsky, I., Polosukhina, A., Degtyar, V.E., Gallerani, N., Smith, C.M., Friedman, A., Van Gelder, R.N., Trauner, D., Kaufer, D. and Kramer, R.H. (2014) Restoring visual function to blind mice with a photoswitch that exploits electrophysiological remodeling of retinal ganglion cells. *Neuron*, **81**, 800-813.
- 332 Banghart, M.R., Volgraf, M. and Trauner, D. (2006) Engineering light-gated ion channels. *Biochemistry*, **45**, 15129-15141.
- 333 Levitz, J., Pantoja, C., Gaub, B., Janovjak, H., Reiner, A., Hoagland, A., Schoppik, D., Kane, B., Stawski, P. and Schier, A.F. (2013) Optical control of metabotropic glutamate receptors. *Nature neuroscience*, **16**, 507.
- 334 Berry, M.H., Holt, A., Levitz, J., Broichhagen, J., Gaub, B.M., Visel, M., Stanley, C., Aghi, K., Kim, Y.J., Trauner, D. *et al.* (2017) Restoration of patterned vision with an engineered photoactivatable G protein-coupled receptor. *Nat Commun*, **8**, 1862.
- 335 Busskamp, V., Duebel, J., Balya, D., Fradot, M., Viney, T.J., Siebert, S., Groner, A.C., Cabuy, E., Forster, V., Seeliger, M. *et al.* (2010) Genetic reactivation of cone photoreceptors restores visual responses in retinitis pigmentosa. *Science*, **329**, 413-417.
- 336 Greenberg, K.P., Pham, A. and Werblin, F.S. (2011) Differential targeting of optical neuromodulators to ganglion cell soma and dendrites allows dynamic control of center-surround antagonism. *Neuron*, **69**, 713-720.
- 337 Bamann, C., Kirsch, T., Nagel, G. and Bamberg, E. (2008) Spectral characteristics of the photocycle of channelrhodopsin-2 and its implication for channel function. *J Mol Biol*, **375**, 686-694.
- 338 Bergs, A., Schultheis, C., Fischer, E., Tsunoda, S.P., Erbguth, K., Husson, S.J., Govorunova, E., Spudich, J.L., Nagel, G., Gottschalk, A. *et al.* (2018) Rhodopsin optogenetic toolbox v2.0 for light-sensitive excitation and inhibition in *Caenorhabditis elegans*. *PLoS One*, **13**, e0191802.

- 339 Sugano, E., Isago, H., Wang, Z., Murayama, N., Tamai, M. and Tomita, H. (2011) Immune responses to adeno-associated virus type 2 encoding channelrhodopsin-2 in a genetically blind rat model for gene therapy. *Gene Ther*, **18**, 266-274.
- 340 Chaffiol, A., Caplette, R., Jaillard, C., Brazhnikova, E., Desrosiers, M., Dubus, E., Duhamel, L., Mace, E., Marre, O., Benoit, P. *et al.* (2017) A New Promoter Allows Optogenetic Vision Restoration with Enhanced Sensitivity in Macaque Retina. *Mol Ther*, **25**, 2546-2560.
- 341 Lu, Q., Ganjawala, T.H., Hattar, S., Abrams, G.W. and Pan, Z.H. (2018) A Robust Optomotor Assay for Assessing the Efficacy of Optogenetic Tools for Vision Restoration. *Invest Ophthalmol Vis Sci*, **59**, 1288-1294.
- 342 Bi, A., Cui, J., Ma, Y.-P., Olshevskaya, E., Pu, M., Dizhoor, A.M. and Pan, Z.-H. (2006) Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. *Neuron*, **50**, 23-33.
- 343 Tomita, H., Sugano, E., Yawo, H., Ishizuka, T., Isago, H., Narikawa, S., Kugler, S. and Tamai, M. (2007) Restoration of visual response in aged dystrophic RCS rats using AAV-mediated channelrhodopsin-2 gene transfer. *Invest Ophthalmol Vis Sci*, **48**, 3821-3826.
- 344 Farah, N., Reutsky, I. and Shoham, S. (2007), In *2007 29th Annual International Conference of the IEEE Engineering in Medicine and Biology Society*. IEEE. pp. 6368-6370.
- 345 Ivanova, E. and Pan, Z.H. (2009) Evaluation of the adeno-associated virus mediated long-term expression of channelrhodopsin-2 in the mouse retina. *Mol Vis*, **15**, 1680-1689.
- 346 Thyagarajan, S., van Wyk, M., Lehmann, K., Lowel, S., Feng, G. and Wässle, H. (2010) Visual function in mice with photoreceptor degeneration and transgenic expression of channelrhodopsin 2 in ganglion cells. *J Neurosci*, **30**, 8745-8758.
- 347 Perry, V., Morris, R. and Raisman, G. (1984) Is Thy-1 expressed only by ganglion cells and their axons in the retina and optic nerve? *Journal of neurocytology*, **13**, 809-824.
- 348 Wang, X., Archibald, M.L., Stevens, K., Baldrige, W.H. and Chauhan, B.C. (2010) Cyan fluorescent protein (CFP) expressing cells in the retina of Thy1-CFP transgenic mice before and after optic nerve injury. *Neurosci Lett*, **468**, 110-114.
- 349 ICNIRP (2013) ICNIRP guidelines on limits of exposure to incoherent visible and infrared radiation. *Health Physics*, **105**, 74-96.
- 350 Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto, T.K., Chuong, A.S., Carpenter, E.J., Tian, Z. *et al.* (2014) Independent optical excitation of distinct neural populations. *Nat Methods*, **11**, 338-346.
- 351 Kleinlogel, S., Feldbauer, K., Dempski, R.E., Fotis, H., Wood, P.G., Bamann, C. and Bamberg, E. (2011) Ultra light-sensitive and fast neuronal activation with the Ca²⁺-permeable channelrhodopsin CatCh. *Nat Neurosci*, **14**, 513-518.
- 352 Tomita, H., Sugano, E., Murayama, N., Ozaki, T., Nishiyama, F., Tabata, K., Takahashi, M., Saito, T. and Tamai, M. (2014) Restoration of the majority of the visual spectrum by using modified Volvox channelrhodopsin-1. *Molecular Therapy*, **22**, 1434-1440.
- 353 Zhang, F., Prigge, M., Beyriere, F., Tsunoda, S.P., Mattis, J., Yizhar, O., Hegemann, P. and Deisseroth, K. (2008) Red-shifted optogenetic excitation: a tool for fast neural control derived from *Volvox carteri*. *Nat Neurosci*, **11**, 631-633.

- 354 Lin, J.Y., Knutsen, P.M., Muller, A., Kleinfeld, D. and Tsien, R.Y. (2013) ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. *Nature neuroscience*, **16**, 1499-1508.
- 355 Lin, J.Y., Lin, M.Z., Steinbach, P. and Tsien, R.Y. (2009) Characterization of engineered channelrhodopsin variants with improved properties and kinetics. *Biophys J*, **96**, 1803-1814.
- 356 Wang, H., Sugiyama, Y., Hikima, T., Sugano, E., Tomita, H., Takahashi, T., Ishizuka, T. and Yawo, H. (2009) Molecular determinants differentiating photocurrent properties of two channelrhodopsins from *Chlamydomonas*. *J Biol Chem*, **284**, 5685-5696.
- 357 Sengupta, A., Chaffiol, A., Macé, E., Caplette, R., Desrosiers, M., Lampič, M., Forster, V., Marre, O., Lin, J.Y. and Sahel, J.A. (2016) Red-shifted channelrhodopsin stimulation restores light responses in blind mice, macaque retina, and human retina. *EMBO Molecular Medicine*, **8**, 1248-1264.
- 358 Feldbauer, K., Zimmermann, D., Pintschovius, V., Spitz, J., Bamann, C. and Bamberg, E. (2009) Channelrhodopsin-2 is a leaky proton pump. *Proc Natl Acad Sci U S A*, **106**, 12317-12322.
- 359 Wu, Z., Yang, H. and Colosi, P. (2010) Effect of genome size on AAV vector packaging. *Mol Ther*, **18**, 80-86.
- 360 Hickey, D.G., Edwards, T.L., Barnard, A.R., Singh, M.S., de Silva, S.R., McClements, M.E., Flannery, J.G., Hankins, M.W. and MacLaren, R.E. (2017) Tropism of engineered and evolved recombinant AAV serotypes in the rd1 mouse and ex vivo primate retina. *Gene Ther*.
- 361 Kim, D.S., Matsuda, T. and Cepko, C.L. (2008) A core paired-type and POU homeodomain-containing transcription factor program drives retinal bipolar cell gene expression. *J Neurosci*, **28**, 7748-7764.
- 362 Lu, Q., Ganjawala, T.H., Ivanova, E., Cheng, J.G., Troilo, D. and Pan, Z.H. (2016) AAV-mediated transduction and targeting of retinal bipolar cells with improved mGluR6 promoters in rodents and primates. *Gene Ther*, **23**, 680-689.
- 363 Vandaele, S., Nordquist, D., Feddersen, R., Tretjakoff, I., Peterson, A. and Orr, H. (1991) Purkinje cell protein-2 regulatory regions and transgene expression in cerebellar compartments. *Genes & Development*, **5**, 1136-1148.
- 364 Zhang, X.M., Chen, B.Y., Ng, A.H.L., Tanner, J.A., Tay, D., So, K.F., Rachel, R.A., Copeland, N.G., Jenkins, N.A. and Huang, J.D., 2005. Transgenic mice expressing Cre-recombinase specifically in retinal rod bipolar neurons. *Investigative ophthalmology & visual science*, **46**(10), pp.3515-3520.
- 365 Xu, Y., Sulaiman, P., Feddersen, R.M., Liu, J., Smith, R.G. and Vardi, N. (2008) Retinal ON bipolar cells express a new PCP2 splice variant that accelerates the light response. *J Neurosci*, **28**, 8873-8884.
- 366 Barski, J.J., Denker, B.M., Guan, J., Lauth, M., Spreafico, F., Fertala, A. and Meyer, M. (2014) Developmental upregulation of an alternative form of pcp2 with reduced GDI activity. *Cerebellum*, **13**, 207-214.
- 367 Smeyne, R.J., Chu, T., Lewin, A., Bian, F., Salih, S., Kunsch, C., Lira, S.A. and Oberdick, J. (1995) Local control of granule cell generation by cerebellar Purkinje cells. *Molecular and Cellular Neuroscience*, **6**, 230-251.

- 368 Lewis, P.M., Gritli-Linde, A., Smeyne, R., Kottmann, A. and McMahon, A.P. (2004) Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. *Dev Biol*, **270**, 393-410.
- 369 Anderson, G.W., Hagen, S.G., Larson, R.J., Strait, K.A., Schwartz, H.L., Mariash, C.N. and Oppenheimer, J.H. (1997) Purkinje cell protein-2 cis-elements mediate repression of T3-dependent transcriptional activation. *Molecular and cellular endocrinology*, **131**, 79-87.
- 370 Portales-Casamar, E., Swanson, D.J., Liu, L., de Leeuw, C.N., Banks, K.G., Ho Sui, S.J., Fulton, D.L., Ali, J., Amirabbasi, M., Arenillas, D.J. *et al.* (2010) A regulatory toolbox of MiniPromoters to drive selective expression in the brain. *Proc Natl Acad Sci U S A*, **107**, 16589-16594.
- 371 Scalabrino, M.L., Boye, S.L., Fransen, K.M., Noel, J.M., Dyka, F.M., Min, S.H., Ruan, Q., De Leeuw, C.N., Simpson, E.M., Gregg, R.G. *et al.* (2015) Intravitreal delivery of a novel AAV vector targets ON bipolar cells and restores visual function in a mouse model of complete congenital stationary night blindness. *Hum Mol Genet*, **24**, 6229-6239.
- 372 Simpson, E.M. (2019) Personal communication with E Simpson, University of British Columbia.