

Functional Characterisation of Key Residues in the Photopigment Melanopsin

Jessica Rodgers

Worcester College
Nuffield Laboratory of Ophthalmology
Nuffield Department of Clinical Neurosciences



Thesis submitted for the degree of
Doctor of Philosophy in Clinical Neurosciences

June 2016

Abstract

Melanopsin (*Opn4*) is the opsin photopigment of intrinsically photosensitive retinal ganglion cells (ipRGCs). It has a conserved opsin structure and activation mechanism, yet demonstrates unusual functional properties that suggest it will possess unique structure-function relationships. The aim of this thesis was to characterise key OPN4 residues by examining the impact of non-synonymous mutations on melanopsin function. A genotype-driven screen of a chemically-mutagenized mouse archive led to the identification of a novel *Opn4* mutant, S310A, located at a known opsin spectral tuning site. Action spectra from ipRGC and pupil light responses (PLR) of *Opn4*^{S310A} mice revealed no change in wavelength of peak sensitivity. However, *Opn4*^{S310A} PLR was significantly less sensitive at longer wavelengths, consistent with a short-wavelength shift in spectral sensitivity. This suggests S310A acts as a spectral tuning site in melanopsin. Next, the impact of naturally-occurring missense variants in human melanopsin (*hOPN4*) was examined *in vitro*. Fluorescent calcium imaging of 16 *hOPN4* variants expressed in HEK293 cells revealed four *hOPN4* variants abolished or attenuated responses to light (Y146C, R168C, G208S and S308F). These variants were located in conserved opsin motifs for chromophore binding or hydrogen-bond networks, functional roles apparently shared by melanopsin. Finally, two *hOPN4* single nucleotide polymorphisms (SNPs) P10L and T394I, associated with abnormal non-image forming behaviour in humans, were explored *in vivo*. Using targeted viral-delivery of *hOPN4* SNPs to mouse ipRGCs, a range of OPN4-driven behaviours, such as circadian photoentrainment and pupil light responses, were found to be comparable with *hOPN4* WT control. Multi-electrode array recordings of ipRGCs transduced with *hOPN4* T394I virus had significantly attenuated sensitivity and faster response offset, indicating this site may be functionally important for melanopsin activity but compensatory rod and cone input limits changes to non-image forming behaviour.

Contents

Abstract	1
Contents	2
List of Figures	8
List of Tables.....	11
Acknowledgements	12
Abbreviations	13
Declaration of Authorship.....	15
 Chapter 1 - Introduction	 16
1.1. Photoreceptors and visual pigments	17
1.1.1 <i>Structure of the mammalian retina</i>	17
1.1.2 <i>Photoreceptors in the mammalian retina.....</i>	18
1.1.3 <i>Discovery of intrinsically photosensitive retinal ganglion cells</i>	19
1.1.4 <i>ipRGCs in non-image forming responses to light</i>	21
1.1.5 <i>Functional properties of ipRGC subtypes.....</i>	24
1.2. Functional properties of melanopsin	27
1.2.1 <i>Phototransduction cascade.....</i>	27
1.2.2 <i>Spectral sensitivity</i>	31
1.2.3 <i>Bistability</i>	35
1.3. Opsin structure-function	39
1.3.1 <i>Methods for determining structure-function relationships</i>	39
1.3.2 <i>Key Opsin Residues</i>	41
1.3.3 <i>Key Residues in the Opsin Transmembrane Domain.....</i>	43
1.3.4 <i>Key Residues in the Opsin Extracellular Domain</i>	48
1.3.5 <i>Key Residues in the Opsin Intracellular Domain</i>	50
1.4. Melanopsin structure-function	53
1.4.1 <i>Retinal Binding Site.....</i>	54
1.4.2 <i>Post-translational modification sites.....</i>	54
1.4.3 <i>Alternative splicing and melanopsin isoforms.....</i>	56
1.4.4 <i>Single nucleotide polymorphisms.....</i>	57
1.5. Thesis Rationale	58
1.6. Thesis Aims	60

Chapter 2 – Materials and Methods	61
2.1. Materials	61
2.1.1 <i>Kits used</i>	61
2.1.2 <i>Reagents, Buffers and Solutions</i>	61
2.1.2.1 Plasmid Preparation	61
2.1.2.2 Gel Electrophoresis	62
2.1.2.3 PCR purification and Gel extraction	63
2.1.2.4 RNA extraction	63
2.1.2.5 Calcium Imaging	63
2.1.2.6 Immunocytochemistry	64
2.1.2.7 Multi-electrode Arrays	64
2.1.3 <i>Vector Information</i>	65
2.1.3.1 pIRES2-acGFP	65
2.1.3.2 pcDNA3.1	66
2.1.3.3 pAAV-EF1 α -DIO	67
2.2. Methods	68
2.2.1 <i>Plasmid Preparation</i>	68
2.2.1.1 Bacterial Transformation	68
2.2.1.2 Bacterial Cultures and Glycerol Stocks	68
2.2.1.3 Minipreps	69
2.2.1.4 Midipreps	70
2.2.2 <i>Sequencing</i>	71
2.2.2.1 Principles	71
2.2.2.2 Sequencing reactions	71
2.2.3 <i>Subcloning into pcDNA3.1</i>	72
2.2.3.1 Overview	72
2.2.3.2 Polymerase Chain Reaction	73
2.2.3.3 PCR to add Restriction Enzyme Sites	73
2.2.3.4 DNA Electrophoresis	75
2.2.3.5 PCR purification	75
2.2.3.6 Restriction Enzyme Digest	76
2.2.3.7 Gel Extraction	77
2.2.3.8 Ligation	78
2.2.3.9 Transformation	78
2.2.3.10 PCR Colony Screen	78

2.2.4	<i>Site-directed mutagenesis</i>	79
2.2.4.1	Principles.....	79
2.2.4.2	Mutagenesis Primer Design.....	80
2.2.4.3	Mutant Strand Synthesis (Thermal Cycling).....	80
2.2.4.4	<i>DpnI</i> digest	81
2.2.4.5	Transformation in XL10 Gold Cells	81
2.2.5	<i>Genotyping</i>	82
2.2.5.1	Genomic DNA extraction	82
2.2.5.2	Rodless coneless (<i>rd/rd cl</i>) genotyping	82
2.2.5.3	<i>Opn4</i> ^{S310A} genotyping.....	84
2.2.5.4	<i>Opn4</i> ^{-/-} genotyping	85
2.2.5.5	<i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/+} genotyping.....	86
2.2.6	<i>Reverse-transcriptase qPCR</i>	88
2.2.6.1	RNA extraction	88
2.2.6.2	cDNA synthesis	89
2.2.6.3	Quantitative PCR (qPCR).....	90
2.2.7	<i>Cell Culture</i>	91
2.2.7.1	Routine Cell culture	91
2.2.7.2	Transient Transfections	91
2.2.7.3	Fluorescent Calcium Imaging	92
2.2.7.4	Immunocytochemistry.....	93
2.2.8	<i>Behavioural Techniques</i>	94
2.2.8.1	Animal Husbandry	94
2.2.8.2	Pupillometry.....	94
2.2.9	<i>Multi-electrode Array Recordings</i>	97
2.2.10	<i>Statistical Analysis</i>	100
2.2.11	<i>List of URLs</i>	100

Chapter 3 – Characterisation of a novel chemical-induced *Opn4* missense mutation..... 101

3.1. Introduction 101

3.2. Methods 103

3.2.1	<i>Rationale for screening <i>Opn4</i> exon 6</i>	103
3.2.2	<i>Genotype-driven ENU archive screen</i>	104
3.2.3	<i>Bioinformatics</i>	107
3.2.4	<i>In vitro expression of <i>Opn4</i> S310A</i>	108

3.2.5	<i>Fluorescent calcium imaging</i>	109
3.2.6	<i>Immunocytochemistry</i>	109
3.2.7	<i>Animals</i>	110
3.2.8	<i>Pupillometry</i>	111
3.2.9	<i>Multi-electrode array recordings</i>	114
3.2.10	<i>Irradiance response curves and action spectra construction</i>	115
3.2.11	<i>Statistical Analysis</i>	121
3.3.	Results	124
3.3.1	<i>Identification of a novel ENU-induced melanopsin mutation</i>	124
3.3.2	<i>Opn4 S310A is located at a highly conserved opsin spectral tuning site</i>	126
3.3.3	<i>Functional characterisation of Opn4 S310A in vitro</i>	128
3.3.4	<i>Opn4^{S310A} mice show attenuated PLR sensitivity to 550nm light</i>	132
3.3.5	<i>MEA action spectrum shows no change in Opn4^{S310A} spectral sensitivity</i> 134	
3.3.6	<i>Pupillometry action spectrum shows specific loss of sensitivity at long wavelengths but no change in Opn4^{S310A} λ_{max}.</i>	139
3.4.	Discussion	145
Chapter 4 – Screening human OPN4 variants in vitro		149
4.1.	Introduction	149
4.2.	Methods	151
4.2.1	<i>SNP prioritisation</i>	151
4.2.2	<i>In vitro expression of hOPN4 variants</i>	154
4.2.3	<i>Fluorescent Calcium Imaging</i>	157
4.2.4	<i>Immunocytochemistry</i>	157
4.2.5	<i>Statistical Analysis</i>	158
4.3.	Results	159
4.3.1	<i>Human OPN4 variants with potential functional consequences</i>	159
4.3.2	<i>Human OPN4 variants have abnormal intracellular signalling</i>	164
4.3.3	<i>All hOPN4 variants show normal plasma membrane localisation.</i>	170
4.4.	Discussion	172

Chapter 5 – Functional assessment of <i>hOPN4</i> SNPs, P10L and T394I, using targeted AAV delivery <i>in vivo</i>	177
5.1. Introduction	177
5.2. Methods	179
5.2.1 <i>Experimental Design</i>	179
5.2.2 <i>Animals</i>	180
5.2.3 <i>Virus Production</i>	181
5.2.4 <i>Intravitreal injections</i>	187
5.2.5 <i>Pupillometry</i>	188
5.2.6 <i>Passive infra-red (PIR) circadian screen</i>	189
5.2.7 <i>Multi-electrode array recordings</i>	194
5.2.8 <i>Immunohistochemistry</i>	196
5.2.9 <i>RT-qPCR</i>	197
5.2.10 <i>Statistical Analysis</i>	198
5.3. Results	199
5.3.1 <i>Technique Validation</i>	199
5.3.1.1 ipRGC-specific AAV delivery of <i>hOPN4</i> in <i>Opn4^{-/-} Cre^{+/+}</i> mice	199
5.3.1.2 Restoration of wildtype pupil phenotype to <i>Opn4^{-/-} Cre^{+/+}</i> mice using ipRGC-specific <i>hOPN4</i> AAV delivery	201
5.3.2 <i>Titre Optimisation</i>	204
5.3.2.1 Number of virus particles injected determines restoration of pupil phenotype	204
5.3.3 <i>Effect of <i>hOPN4</i> SNPs, P10L and T394I, on OPN4 function</i>	208
5.3.3.1 AAV-delivered <i>hOPN4</i> P10L and T394I are able to restore PLR to <i>Opn4^{-/-} Cre^{+/+}</i> mice comparable to <i>hOPN4</i> WT AAV	209
5.3.3.2 PLR does not differ between <i>hOPN4</i> P10L, T394I and WT control	211
5.3.3.3 Entrainment to light-dark cycle and freerunning in constant dark are unaffected by AAV-delivered <i>hOPN4</i>	212
5.3.3.4 Timing of activity-rest rhythm relative to zeitgeber is altered by restoration of OPN4 light input	214
5.3.3.5 Activity patterns before and after restoration of OPN4 light input	217
5.3.3.6 Increased phase shifting after restoration of OPN4 light input	220
5.3.3.7 Period lengthening in LL after restoration of OPN4 light input	222
5.3.3.8 Circadian behaviour comparable for <i>hOPN4</i> WT, P10L and T394I	224

5.3.3.9 Increases in immobility-defined sleep after injection are light-phase dependent.....	226
5.3.3.10 Sleep bout distribution throughout light-dark cycle.....	229
5.3.3.11 Comparison of sleep parameters for <i>hOPN4</i> WT, P10L and T394I.....	232
5.3.3.12 AAV-restored intrinsic ipRGC responses can be recorded using multi-electrode array (MEA)	234
5.3.3.13 Higher number of ipRGC responses observed for retina treated with <i>hOPN4</i> WT AAV compared to <i>hOPN4</i> P10L or T394I AAV	236
5.3.3.14 Properties of ipRGC responses from <i>hOPN4</i> AAV treated retina.....	240
5.3.3.15 ipRGC sensitivity is significantly reduced in <i>hOPN4</i> T394I AAV-treated retina	245
5.3.3.16 Consistent ipRGC sensitivity across different AAV-treated retina	248
5.3.3.17 Light adaptation of <i>hOPN4</i> AAV-treated retina	250
5.3.3.18 Comparison of ipRGC spike firing in <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/+} retina treated with <i>hOPN4</i> WT AAV and <i>Opn4</i> ^{+/+} retina	256
5.4. Discussion	260
Chapter 6 - Discussion	266
6.1. Summary of Results	266
6.2. Thesis Outcomes	269
6.2.1 Strategies for identification of critical functional residues.....	269
6.2.2 Identification of novel mutations in the mouse <i>Opn4</i> gene	272
6.2.3 Characterisation of naturally-occurring <i>hOPN4</i> missense variants	274
6.2.4 Characterisation of <i>Opn4</i> mutants in vivo.....	276
6.2.5 Development of techniques for screening of <i>Opn4</i> mutants in vivo	278
6.3. Conclusions	280
Appendices	281
Appendix 1 - Opsin spectral tuning sites in melanopsin.	281
Appendix 2 - Human <i>OPN4</i> missense variants in dbSNP	282
Appendix 3 - Validity of net PIPR to assess <i>OPN4</i> function	286
Appendix 4 - Diversity of ipRGC responses in <i>hOPN4</i> WT AAV-treated retina _	290
References	296

List of Figures

Figure	Page
1.1. Structure of the mammalian retina.	17
1.2. Brain regions innervated by ipRGCs.	24
1.3. Connectivity and morphology of ipRGC subtypes.	25
1.4. Vertebrate rhodopsin and melanopsin phototransduction cascades.	28
1.5. Spectral sensitivity of visual pigments in the macaque retina.	31
1.6. Chromophore recycling in visual pigments.	35
1.7. Tristability of melanopsin.	38
1.8. Crystal structures of bovine and squid rhodopsin.	40
1.9. Location of the retinal chromophore in the opsin protein.	41
1.10. Key residues in the bovine rhodopsin transmembrane domain.	43
1.11. Opsin counterions.	45
1.12. Spectral tuning mechanisms.	46
1.13. Conformation change during opsin activation.	47
1.14. Key residues in the bovine rhodopsin extracellular domain.	48
1.15. Key residues in the bovine rhodopsin intracellular domain.	50
1.16. Arrestin binding for GPCR deactivation.	52
1.17. Key functional sites in mouse melanopsin.	53
2.1. pIRES2-acGFP expression vector.	65
2.2. pcDNA3.1 expression vector.	66
2.3. pAAV-EF1 α -DIO vector map.	67
2.4. Pupillometry of consensual pupil light response.	95
2.5. Pupillometry analysis for maximum constriction.	96
2.6. Preparation of retina for multi-electrode array (MEA) recordings.	99
3.1. Region screened for ENU-induced mutations.	104
3.2. High resolution melt curve analysis for mutation detection.	106
3.3. Irradiance response curves (IRCs) fit by least squares minimisation.	118
3.4. Example action spectrum construction.	120
3.5. Identification of <i>Opn4</i> mutation, S310A, in genotype-driven ENU archive screen.	125
3.6. <i>Opn4</i> S310A mutation occurs at a highly conserved residue difference between <i>Opn4X</i> and <i>Opn4M</i> genes.	128
3.7. OPN4 S310A is trafficked normally to cell membrane.	129
3.8. Fluorescent calcium imaging shows OPN4 S310A can trigger a G _{q/11} signalling cascade in response to light comparable to OPN4 WT.	131
3.9. <i>Opn4</i> ^{S310A} pupil light responses are less sensitive to 550nm light than <i>Opn4</i> ^{WT} .	133

3.10. Recording order affects sensitivity of ipRGC responses.	135
3.11. No difference in sensitivity of <i>Opn4^{WT}</i> and <i>Opn4^{S310A}</i> ipRGC responses at any wavelength.	137
3.12. MEA action spectra are comparable for <i>Opn4^{WT}</i> and <i>Opn4^{S310A}</i> .	139
3.13. <i>Opn4^{S310A}</i> mice show specific loss of sensitivity of PLR at long wavelengths.	142
3.14. <i>Opn4^{S310A}</i> mice show trend towards shorter $\lambda_{\max}$ compared to <i>Opn4^{WT}</i> .	144
3.15. Contribution of different IRC wavelengths to fitting Govardovskii template.	146
4.1. BLAST protein alignment of human melanopsin (OPN4) and bovine rhodopsin (RHO).	154
4.2. <i>hOPN4</i> genetic variants selected for in vitro testing.	160
4.3. <i>hOPN4</i> variants show abnormal intracellular calcium responses to light.	165
4.4. <i>hOPN4</i> variants have abnormal response kinetics.	168
4.5. <i>hOPN4</i> variants with abnormal response onset kinetics.	169
4.6. <i>hOPN4</i> variants show normal membrane localisation.	171
5.1. Double-floxed inverted orientation AAV (DIO-AAV) for cell-type specific expression.	182
5.2. Pupillometry analysis for post-illumination pupil response (PIPR).	189
5.3. Passive infra-red detection for motion detection.	190
5.4. Light schedules for PIR circadian screen.	192
5.5. Light stimulus protocols for MEA experiments.	195
5.6. ipRGC-specific AAV delivery of <i>hOPN4</i> in <i>Opn4^{-/-} Cre^{+/+}</i> mice.	200
5.7. Pupil light response is restored in <i>Opn4^{-/-} Cre^{+/+}</i> animals with ipRGC-specific <i>hOPN4</i> WT expression.	203
5.8. PLR restoration is dependent on virus titre.	206
5.9. Effect of titre on transduction efficiency.	207
5.10. <i>hOPN4</i> P10L and <i>hOPN4</i> T394I are able to restore PLR to <i>Opn4^{-/-} Cre^{+/+}</i> animals comparable to <i>hOPN4</i> WT AAV.	210
5.11. PLR for <i>hOPN4</i> P10L and T394I AAV is comparable with <i>hOPN4</i> WT AAV.	211
5.12. Entrainment to 12:12 LD cycle and free-running under constant dark are comparable before and after injection.	213
5.13. <i>hOpn4</i> AAV delays phase angle of entrainment and peak activity timing.	216
5.14. Daily activity is attenuated and activity rhythms are more fragmented after injection.	219
5.15. AAV-delivered <i>hOpn4</i> enhances phase shifting to nocturnal light pulse.	221
5.16. Period lengthening in constant light is similar for <i>hOPN4</i> WT, P10L and T394I.	223
5.17. Immobility-defined sleep is increased after injection during dark phase of 12:12 LD cycle.	228
5.18. Sleep bout frequency, but not duration, is altered after injection.	231

5.19. AAV-restored intrinsic ipRGC responses can be observed after pharmacological blockade of rod-cone signals.	235
5.20. Transduction efficiency is comparable between <i>hOPN4</i> SNP and WT AAV.	239
5.21. ipRGCs spike firing to a single 10s light pulse across a 5-log range of intensities.	241
5.22. Differences in kinetics of ipRGC spike firing to 10s 480nm light pulse between <i>hOPN4</i> WT control and <i>hOPN4</i> T394I, but not <i>hOPN4</i> P10L.	243
5.23. ipRGC sensitivity is significantly reduced in AAV-delivered <i>hOPN4</i> T394I compared to <i>hOPN4</i> WT control.	246
5.24. Sensitivity is consistent between retina treated with same AAV, despite different frequency of ipRGC responses.	249
5.25. <i>hOPN4</i> WT, P10L and AAV treated retina show light adaptation to repeated light pulses.	252
5.26. Light adaptation to repeated light pulses is not consistent between <i>hOPN4</i> AAV WT, P10L and T394I.	255
5.27. Comparison of ipRGC responses from retina of <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/+} treated with <i>hOPN4</i> WT AAV and <i>Opn4</i> ^{+/+} retina.	259
6.1. Key functional residues in melanopsin.	266
S1. Net rate of recovery and net PIPR is comparable between <i>Opn4</i> ^{-/-} and <i>Opn4</i> ^{+/+} mice.	289
S2. Diverse ipRGC responses from retina of <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/+} treated with <i>hOPN4</i> WT AAV.	291
S3. IpRGC subtypes classified by response latency to subsaturating light pulse.	292
S4. Histograms of ipRGC spike firing properties to identify discrete populations of ipRGC subtypes.	295

List of Tables

Table	Page
1.1. Comparison of rod, cone and ipRGC photoreceptors in mice (<i>Mus musculus</i>).	18
1.2. Non-image forming responses to light in <i>Opn4</i> ^{-/-} animals.	22
1.3. Spectral sensitivity of melanopsin.	34
1.4. Key functional residues in Rhodopsin.	42
3.1. Opsin protein sequences used for alignments.	107
3.2. Cohorts used in pupillometry and multi-electrode array studies.	111
3.3. Pupillometry action spectrum sessions.	113
3.4. Wavelength order and number of ipRGC responses.	115
3.5. Mutation of equivalent site to OPN4 S310A causes spectral tuning shifts in other opsins.	127
3.6. No difference in sensitivity of ipRGC responses between <i>Opn4</i> ^{WT} and <i>Opn4</i> ^{S310A} retina.	136
3.7. <i>Opn4</i> ^{S310A} mice show reduced pupil constriction sensitivity at longer wavelengths compared to <i>Opn4</i> ^{WT} .	141
4.1. Criteria for identifying OPN4 variants for <i>in vitro</i> screening.	153
4.2. Site-directed mutagenesis primers used to introduce the <i>hOPN4</i> genetic variants and control mutations.	156
4.3. Properties of genetic variants selected for <i>in vitro</i> screening.	161-2
4.4. Properties of equivalent residues in Rhodopsin.	163
4.5. Summary of key features and phenotype of 16 screened <i>hOPN4</i> variants.	173
5.1. Summary of the cohorts used in the 3 hOpn4-AAV studies.	180
5.2. Circadian parameters used to assess activity during PIR screen.	193
5.3. Opn4 and housekeeping gene primers used for RT-qPCR.	197
5.4. Descriptive statistics for circadian activity parameters and results of One-way Between subjects ANOVA.	225
5.5. Descriptive statistics for immobility-defined sleep and results of One-way Between subjects ANOVA.	233
5.6. Retina expressing AAV-delivered hOpn4 P10L and T394I show fewer ipRGC responses compared to hOpn4 WT control during multi-electrode array recording (MEA).	238
I. Amino acid at equivalent position of known opsin spectral tuning sites in zebrafish <i>Opn4M2</i> and <i>Opn4X1</i> , and mouse <i>Opn4</i> .	281
II. 96 <i>hOpn4</i> missense variants in dbSNP.	282-5

Acknowledgements

First, thank you to my supervisors Professors Mark Hankins and Stuart Peirson. I will always be grateful for your advice, guidance and encouragement over the last four years.

Thank you to EPSRC for funding my DPhil studentship and to our collaborators at MRC Harwell, Dr. Pat Nolan and Dr. Mike Parsons for their expertise and kind assistance performing the ENU archive screen. Thank you to the staff at the JR BMS, particularly Andrew and Jonathon, for their tireless work supporting our research.

Thank you to the many talented scientists I was lucky enough to work with, including Michelle, Violetta, Eric, Aarti, Simona, Maria and Nicola, for answering all my questions and sharing their technical knowledge.

Special thanks to Carrie, Laurence and Steve for being so incredibly generous with their time, advice and support, and to my amazing fellow DPhil students, especially Doron for being so patient and helpful, Jovi for being my fairy thesis-mother and Laura for making Oxford feel like home. The biggest brownie in the world could not begin to express my appreciation for you.

Most of all, thank you to my family, Mãe, Pai, Becky and Sam, for their unwavering support and willingness to listen to anything and everything to do with melanopsin. You are all brilliant and I could not have done it without you.

Abbreviations

AAV	Adeno-Associated Virus
ANOVA	Analysis of Variance
ARBP	Acidic ribosomal phosphoprotein P0
B2M	Beta-2-Microglobulin
B-gal	β -galactosidase
BLAST	Basic Local Alignment Search Tool
CDS	Coding DNA sequence
CG	Ciliary Ganglion
cGMP	Cyclic Guanosine Monophosphate
CMV	Cytomegalovirus
CNG	Cyclic Nucleotide-gated Channels
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAG	Diacylglycerol
d-AP5	d-2-amino-5-phosphonovalerate
DAPI	4',6-diamidino-2-phenylindole
dbSNP	Database of Single Nucleotide Polymorphisms
DD	Constant Dark
DF	Degrees of Freedom
DIO	Double-floxed inverted orientation
dLGN	Dorsal Lateral Geniculate Nucleus
DNQX	6,7-dinitroquinoxaline-2,3-dione
EC50	Intensity for half-maximum response (excitatory)
EDTA	Ethylenediaminetetraacetic Acid
EF1α	Elongation factor 1 α
EL	Extracellular Loop
ENU	<i>N</i> -ethyl- <i>N</i> -nitrosourea
ER	Endoplasmic Reticulum
EWN	Edinger-Westphal Nucleus
eYFP	Enhanced Yellow Fluorescent Protein
FAM	6-carboxyfluorescein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GCL	Ganglion Cell Layer
GFP	Green Fluorescent Protein
GMP	Guanosine monophosphate
GPCR	G protein coupled receptor
G protein	Guanine nucleotide-binding protein
GRK	G protein receptor kinase
G_t	Transducin
Gα	G protein alpha subunit
<i>hOPN4</i>	Human melanopsin (gene/RNA)
hOPN4	Human melanopsin (protein)
HSD	Honest Significance Difference
IC50	Intensity for half-maximum response (inhibitory)
IgG	Immunoglobulin G
IGL	Intergeniculate Nucleus
IL	Intracellular Loop
ILM	Inner Limiting Membrane
INL	Inner Nuclear Layer
IP₃	Inositol 1, 4, 5-trisphosphate
IPL	Inner Plexiform Layer
ipRGC	Intrinsically Photosensitive Retinal Ganglion Cell
IRC	Irradiance Response Curve

ITR	Inverted Terminal Repeat
I-AP4	I(+)-2-amino-4-phosphonobutyrate
LD	Light-Dark cycle
LED	Light Emitting Diode
LGN	Lateral Geniculate Nucleus
LL	Constant Light
LWS	Long Wavelength Sensitive
MAFFT	Multiple Alignment by Fast Fourier Transform
MAF	Minor Allele Frequency
MEA	Multi-electrode array
MGB	Minor Groove Binder
MRC	Medical Research Council
MWS	Middle Wavelength Sensitive
NCBI	National Center for Biotechnology Information
NIF	Non-image forming
OPL	Outer Plexiform Layer
OPN	Olivary Pretectal Nucleus
<i>Opn4</i>	Melanopsin (gene or mRNA)
OPN4	Melanopsin (protein)
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PDE	Phosphodiesterase
PIP₂	Phosphatidyl inositol 4, 5-bisphosphate
PIPR	Post-illumination Pupil Response
PIR	Passive Infra-Red Detector
PLC	Phospholipase C
PLR	Pupil Light Responses
PNGase	Peptide -N-Glycosidase F
PSMB2	Proteasome subunit, beta type, 2
<i>rd/rd cl</i>	Rodless coneless mouse
RGC	Retinal Ganglion Cell
<i>RH1</i>	<i>Drosophila</i> rhodopsin
<i>Rho</i>	Rhodopsin (gene or RNA)
<i>RHO</i>	Rhodopsin (protein)
RMS	Root mean square
RP	Retinitis Pigmentosa
RPE	Retinal Pigment Epithelium
RT-qPCR	Reverse-transcriptase quantitative PCR
SCN	Suprachiasmatic Nucleus
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
SNP	Single Nucleotide Polymorphism
SS	Sum of Squares
SWS	Short Wavelength Sensitive
SWS2	Ultraviolet Wavelength Sensitive
TALENS	Transcription Activator-Like Effector Nucleases
TM	Transmembrane Helix
TRP	Transient receptor potential
VIC	4,7,2-trichloro-7-phenyl-6-carboxyfluorescein
vLGN	Ventral Lateral Geniculate Nucleus
WPRE	Woodchuck Hepatitis B Virus Post-Transcriptional Regulatory Element
ZT	Zeitgeber Time
λ_{max}	Wavelength of Peak Sensitivity

Declaration of Authorship

The work contained in this thesis has not previously been submitted for a degree or diploma at the University of Oxford or any other higher education institution. All figures or tables reproduced or adapted from journal articles and other sources are referenced in the text. The contents of this thesis are all my own work, with the exception of the material described below:

1. Staff at the Mary Lyons Centre at MRC Harwell recovered the *Opn4*^{S310A} mice by *in vitro* fertilisation described in Chapter 3.
2. Prof. Gebhard Schertler created the homology model of OPN4 protein shown in Figure 3.5 in Chapter 3, shared with Prof. Mark Hankins in a personal communication.
3. Dr. Carina Potheary designed the pupillometry analysis software used in Chapter 3 and 5, and assisted in collection of pupillometry data in Chapter 5.
4. Dr. Steven Hughes and Ms. Simona di Pretoro organised breeding and carried out genotyping of *Opn4-Cre* and *Opn4 tau-Lacz* mice used in Chapter 5.
5. Dr. Doron Hickey created the pEF1α-AAV2-DIO-hOPN4-1D4 plasmid and assisted in the intravitreal injections described in Chapter 5.
6. Dr. Laurence Brown designed the passive infrared (PIR) detectors and software for PIR data collection and analysis used in Chapter 5.
7. Dr. Steven Hughes performed the immunohistochemistry and acquired images of the virus-treated *Opn4-Cre* retina shown in Figure 5.6, 4.9 and 4.20 in Chapter 5.
8. Dr. Hughes also collected MEA data from *Opn4*^{+/+} mice used in section 5.3.3.18, and assisted in the collection of MEA data described in Chapter 3 and Chapter 5.

Final word count: 59973 excluding tables, appendices and reference list.

Jessica Rodgers (June 2016)

Chapter 1

Introduction

This chapter reviews the literature on the structure and function of melanopsin, the opsin photopigment of intrinsically photosensitive retinal ganglion cells (ipRGCs). The aim of this chapter is to provide context and a rationale for the following experimental chapters, which identify and characterise key functional residues in melanopsin.

First, the photoreceptors and opsins of the mammalian retina are introduced, concentrating on ipRGCs and their role in non-image forming responses to light.

Second, the unique functional properties of melanopsin are described, including its phototransduction cascade, spectral sensitivity and potential bistability.

Third, the literature on opsin structure-function is reviewed, focusing on critical residues in each of the three protein domains – intracellular, extracellular and transmembrane.

Finally, the current literature on key functional residues and domains in melanopsin is examined, concluding with the thesis rationale, aims and objectives.

1.1 Photoreceptors and visual pigments

1.1.1 Structure of the mammalian retina

The retina consists of 6 types of neurons: photoreceptors, horizontal, bipolar, amacrine, interplexiform and ganglion cells. Photoreceptors convert light into electrical signals, which are relayed through the retina to different visual centres of the brain. As shown in **Figure 1.1**, the retina is formed of 5 layers; the inner and outer nuclear layer, the ganglion cell layer, and the inner and outer plexiform layer. Synapses between retinal neurons occur in the plexiform layers, with cell bodies located in the alternate layers. Light information flows from the outer to inner retina, predominantly from the photoreceptors, via bipolar cells, to ganglion cells (Wässle, 2004). There are also lateral interactions in the two plexiform layers, mediated by horizontal and amacrine cells, which provide preliminary processing of visual signals (Demb & Singer, 2015). The processes of Müller glial cells span all retinal layers, supporting metabolic and homeostatic requirements of retinal neurons (Newman & Reichenbach, 1996). The remainder of this chapter will focus on photoreceptor cells, particularly intrinsically photosensitive retinal ganglion cells (ipRGCs).

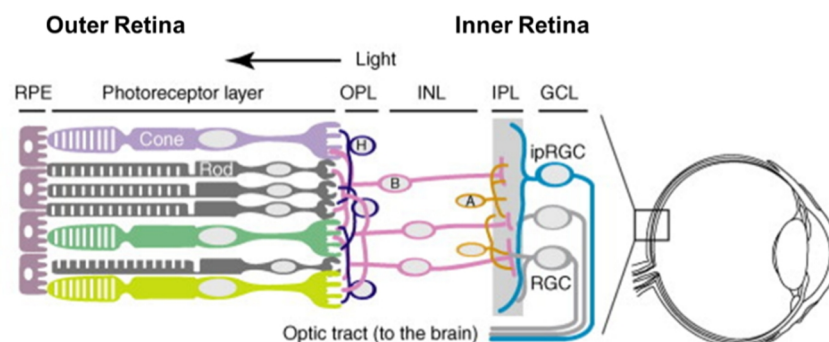


Figure 1.1. Structure of the mammalian retina. Rod and cone photoreceptors are found in the outer retina, in the outer nuclear (photoreceptor) layer. Light signals from rods and cones are processed by horizontal (H), bipolar (B) and amacrine (A) cells before reaching retinal ganglion cells (RGC) in the inner retina. RGCs then relay information via the optic tract to different brain targets. A small subset of RGCs are directly photosensitive, as well as receiving light input from rods and cones. These are ipRGCs. RPE = retinal pigment epithelium, OPL = outer plexiform layer, INL = Inner nuclear layer, IPL = inner plexiform layer. GCL = ganglion cell layer. Image reproduced from Hatori & Panda (2010) with permission from Elsevier.

1.1.2 Photoreceptors in the mammalian retina

There are three classes of photoreceptor cells in the mammalian retina: rods, cones and ipRGCs (**Figure 1.1**). Their morphology and response properties, outlined in **Table 1.1**, are adapted for different types of vision.

Table 1.1. Comparison of rod, cone and ipRGC photoreceptors in mice (*Mus musculus*). Rod and cone cell properties from Chalupa & Williams (2008), ipRGC properties from Do & Yau (2010) unless specified otherwise.

	Rods	Cones	ipRGCs
<i>Opsin</i>	Rhodopsin	Cone opsin (Ultraviolet sensitive, or co-expression of Ultraviolet and middle-wavelength sensitive)	Melanopsin
<i>Structure</i>	Inner segment containing organelles and nucleus, outer segments with internalised rhodopsin-containing membrane discs	Inner segment containing organelles and nucleus, outer segments with continuous infolding of plasma membrane containing cone photopigment	Soma, axon and long dendritic fields covering large area. Melanopsin is expressed in soma and dendrites
<i>Pigment Density</i>	High (~25000 μm^{-2})	High (~25000 μm^{-2})	Low (~3 μm^{-2}) ^a
<i>Wavelength of peak spectral sensitivity</i>	500nm	360nm (UVS) 510nm (MWS)	~480nm
<i>Phototransduction Cascade</i>	Activates transducin (G_t) signalling pathway leading to hyperpolarisation of cell	Activates transducin (G_t) signalling pathway leading to hyperpolarisation of cell	Activates $G_{q/11}$ signalling pathway leading to depolarisation of cell
<i>Location in retina</i>	Outer retina	Outer retina	Inner Retina
<i>Temporal Resolution</i>	Low	High	Very Low ^a
<i>Spatial Resolution</i>	Low	High	Very Low
<i>Sensitivity to low light levels</i>	High	Low	Low (but long temporal integration and low spike threshold increases sensitivity) ^a
<i>Number in Retina</i>	~6.4 million	~200 000	~2000 ^b
<i>Distribution throughout retina</i>	Evenly distributed throughout retina	Dorso-ventral gradient (MWS more dorsal and SWS more ventral)	Evenly distributed throughout retina ^b
<i>Input</i>	Intrinsically photosensitive	Intrinsically Photosensitive	Intrinsically Photosensitive and Rod/Cone input
<i>Behaviour</i>	Scotopic vision	Photopic and colour vision	Non-image forming responses to light

^aDo et al (2009)

^bEcker et al (2010)

Each photoreceptor cell expresses a different membrane-bound photopigment, consisting of an opsin protein bound to a vitamin A-derived 11-*cis* retinal chromophore. Isomerisation of the chromophore to all-*trans* retinal after absorbing a photon causes a conformation change in the opsin protein from inactive to active state. This triggers a signalling pathway called a phototransduction cascade. Visual pigments which bind a retinaldehyde chromophore, such as 11-*cis* retinal, are known as A₁-visual pigments to distinguish them from A₂-pigments, which bind a 3, 4-didehydroretinaldehyde chromophore. The isomerised chromophore is recycled in the retinal pigment epithelium (RPE), discussed in more detail below, which provides a continual supply of 11-*cis* retinal for phototransduction. The photopigment in rods and cones is rhodopsin and cone visual pigment, respectively. Melanopsin (*Opn4* – gene and mRNA, OPN4 – protein) is the photopigment in ipRGCs. Photopigments can be distinguished by their structure and function, discussed in more detail later in this chapter.

1.1.3 Discovery of intrinsically photosensitive retinal ganglion cells

Until relatively recently, it was believed there were only two photoreceptors in the mammalian retina - rods and cones. The first evidence for a third came from behavioural studies demonstrating the persistence of non-image forming (NIF) responses to light, such as circadian photoentrainment and melatonin suppression, in animals without rods and cones (Ebihara & Tsuji, 1980; Provencio & Foster, 1995; Yoshimura & Ebihara, 1996; Freedman et al., 1999; Lucas et al., 1999). As previous reports had demonstrated enucleation abolished these NIF responses (Nelson & Zucker, 1981, Foster et al., 1991), these studies of animals with outer retinal degeneration indicated the presence of a novel photoreceptor in the mammalian retina.

The spectral sensitivity of enduring NIF responses in retinally-degenerate animals suggested they were driven by an A₁-visual pigment with peak spectral sensitivity ($\lambda_{\max}$) at 479nm (Lucas et al., 2001). This did not correspond to $\lambda_{\max}$ for any of the known mouse visual pigments, shown in **Table 1.1**. In 1998, Provencio et al. discovered a novel opsin in the melanophores, retina and hypothalamus of *Xenopus laevis*, named melanopsin, which was predicted to form an A₁-visual pigment. Melanopsin was then found to be expressed exclusively in the human inner retina (Provencio et al., 2000), matching the proposed location of the novel photoreceptor.

Several immunohistochemistry and reporter gene studies found melanopsin in approximately 1% of retinal ganglion cells (RGC) in the retina and those forming the retino-hypothalamic tract (Gooley et al., 2001; Hannibal et al., 2002; Hattar et al., 2002; Provencio et al., 2002), which conveys light input from the eye to the suprachiasmatic nucleus (SCN), the master circadian pacemaker of the brain. This led to the hypothesis that melanopsin was the photopigment responsible for enduring NIF responses to light in rodless coneless animals.

Electrophysiology of fluorescent-labelled RGCs in the retinohypothalamic tract, surgically or pharmacologically isolated from rods and cones, demonstrated these cells were directly photosensitive (Berson et al., 2002). These results were supported by similar photosensitive responses found in a subset of RGCs during calcium imaging of retina from rodless coneless (*rd/rd cl*) mice (Sekaran et al., 2003). These intrinsically photosensitive retinal ganglion cells (ipRGCs) were also shown to express melanopsin (Hattar et al., 2002), although at the time it was still unclear whether melanopsin was the source of intrinsic phototransduction in these cells.

A series of complementary studies established melanopsin as the photopigment of ipRGCs. First, melanopsin knockout mice, *Opn4^{-/-}*, were shown to have attenuated phase shifting, PLR and negative masking (**Table 1.2**), suggesting melanopsin was necessary for normal NIF responses to light. Then, in 2003, Hattar et al. generated mice

without rod, cone and melanopsin phototransduction. These “triple knockout” mice lacked both image-forming and NIF responses to light, indicating no other photopigments in the mammalian retina contribute to visual responses.

Finally, several key studies demonstrated heterologous expression of melanopsin conferred photosensitivity to non-light responsive cells (Melyan et al., 2005; Panda et al., 2005; Qiu et al., 2005). Electrophysiology revealed these melanopsin-expressing cells were maximally sensitive to ~480nm light, matching the peak spectral sensitivity of ipRGC-mediated NIF responses in *rd/rd cl* mice (Lucas et al., 2001). Combined with earlier evidence, this confirmed melanopsin as the ipRGC photopigment.

It was later discovered non-mammalian vertebrates have 2 melanopsin genes, *Opn4X* (*Xenopus*-like, as it was first identified in *Xenopus laevis*) and *Opn4M* (Mammalian-like), whereas mammals only possess *Opn4M* (Bellingham et al., 2006). Both are functional and exhibit similar expression throughout the retina of non-mammals (Davies et al., 2011), although *Opn4M* expression is limited to ipRGCs in mammals (Hattar et al., 2002).

1.1.4 ipRGCs in non-image forming responses to light

The observation that mice without rods and cones have persistent NIF responses to light led to the identification of a third photoreceptor and its photopigment in the mammalian retina - ipRGCs and melanopsin. Characterisation of mice with genetically ablated melanopsin but normal rod-cone function (*Opn4^{-/-}*), summarised in **Table 1.2**, established melanopsin was necessary for normal NIF behaviour. A defining feature of the *Opn4^{-/-}* phenotype is that NIF responses are attenuated but not abolished, indicating melanopsin photoreception is not the sole source of light input for NIF responses.

Table 1.2. Non-image forming responses to light in *Opn4*^{-/-} animals. Phenotype of *Opn4*^{-/-} mice is described relative to animals with normal melanopsin function (*Opn4*^{+/+}).

Response to Light	Description	References	Phenotype in <i>Opn4</i> ^{-/-} mice
Pupil light responses (PLR)	Constriction of the pupil in response to light	<i>Lucas et al. (2003)</i>	Attenuated constriction at high intensities
Intrinsic PLR	Pupil constriction in eye isolated from the neuronal circuitry through brain.	<i>Xue et al (2011)</i>	No intrinsic PLR
Circadian Photoentrainment	Synchronisation of internal circadian rhythms to external changes in environmental light levels. The duration of one complete oscillation or cycle of a biological rhythm is called the period (<i>tau</i>).	<i>Ruby et al (2002)</i> <i>Panda et al (2002)</i>	Normal entrainment to 12:12 light:dark cycle No difference in <i>tau</i> in constant dark Attenuated phase-shifting Attenuated period-lengthening in constant light
Masking	Decrease in locomotor activity in response to light during active phase	<i>Mrosovsky & Hattar (2003)</i>	Suppression at start of light pulse with increased activity over time
Light aversion	Nocturnal animals demonstrate aversion to light tested using light-dark box	<i>Semo et al (2010)</i> <i>Johnson et al (2010)</i>	No difference from <i>Opn4</i> ^{+/+} Neonate <i>Opn4</i> ^{-/-} do not show negative phototaxis
Light-induced sleep	Light pulse during dark phase can induce sleep in nocturnal animals.	<i>Lupi et al (2008)</i> <i>Altimus et al (2008)</i> <i>Tsai et al (2009)</i> <i>Muindi et al (2013)</i>	No light-induced sleep Attenuated and transitory light-induced sleep Shorter duration of light-induced sleep. Increased activity in 12:12 LD
<i>c-fos</i> induction in SCN after light pulse	SCN receives light input from ipRGCs. <i>c-fos</i> induction after light pulse indicates neuronal activity.	<i>Ruby et al (2002)</i> <i>Lupi et al (2006)</i>	Attenuated <i>c-Fos</i> induction in SCN Attenuated FOS induction in SCN at P4 but not P14

Since the triple knockout mouse demonstrates no responses to light (Hattar et al., 2003), these residual responses must be supported by rods and cones. Several studies found selective ablation of ipRGCs using conditional expression of a diphtheria toxin causes near total loss of NIF behaviour (Güler et al., 2008; Hatori et al., 2008), demonstrating ipRGCs form the principal conduit for rod and cone light input to NIF centres of the brain.

Trans-synaptic viral tracing (Viney et al., 2007), electron microscopy (Belenky et al., 2003) and pharmacological methods (Wong et al., 2007) established ipRGCs form synapses with amacrine and bipolar cells, which process light information from rods and cones. This results in two types of ipRGC activity – intrinsic responses driven by melanopsin phototransduction and extrinsic responses driven by synaptic connections with rods and cones. It is important to remember this when studying melanopsin function in the intact retina, as the results in a retinally-degenerate animal could appear quite different.

The role of ipRGCs in NIF responses to light is further supported by studies of retrograde labelling of NIF brain areas (Morin et al., 2003; Sollars et al., 2003) and melanopsin knock-in mice with reporter genes, such as *tau-lacZ* (Hattar et al., 2002; 2006), alkaline phosphatase or GFP (Ecker et al., 2010), which selectively label the axons and dendrites of ipRGCs. As shown in **Figure 1.2**, ipRGCs directly innervate NIF centres of the brain, including the suprachiasmatic nucleus (SCN), olivary pretectal nucleus (OPN) and the intergeniculate nucleus (IGL). Notably, the SCN and OPN are part of circuits responsible for circadian entrainment and pupillary light responses (PLR) respectively, behaviours known to be attenuated in *Opn4^{-/-}* mice (see **Table 1.2**).

Unexpectedly, ipRGC projections were also found to ventral and dorsal regions of the lateral geniculate nucleus (LGN). The LGN is retinotopically mapped, suggesting ipRGCs may also play a role in pattern vision. This is likely to be fairly crude as ipRGCs have very low spatial resolution, being sparsely distributed with large receptive fields. However, several studies have reported *rd/rd cl* mice with intact ipRGCs can perform basic visual tasks, such as brightness discrimination (Brown et al., 2010, 2012), indicating ipRGCs may contribute to classical image-forming vision, as well as NIF responses to light.

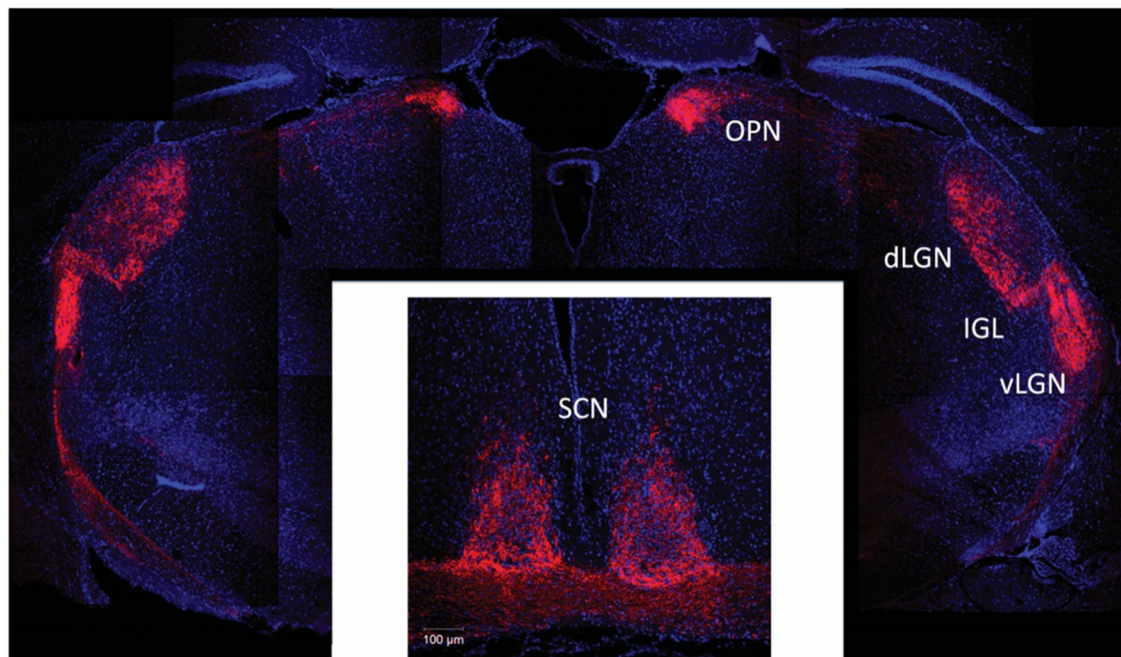


Figure 1.2. Brain regions innervated by ipRGCs. Targeted expression of mCherry fluorescent reporter in ipRGCs reveals axonal projections to the suprachiasmatic nucleus (SCN), olivary pretectal nucleus (OPN), intergeniculate leaflet (IGL) and the dorsal and ventral regions of the lateral geniculate nucleus (dLGN and vLGN respectively). Image reproduced from Hughes et al. (2016) with permission from Nature Publishing Group.

1.1.5 Functional properties of ipRGC subtypes

Originally believed to be a uniform population, it is now apparent that ipRGCs have at least 5 anatomically distinct subtypes. These can be distinguished by their morphology and dendrite stratification (see **Figure 1.3**). M1 cells are monostратified in the OFF sublamina of the inner plexiform layer (IPL); M2, M4 and M5 cells are monostратified in the ON sublamina; and M3 cells are bistratified in the ON and OFF sublaminae (Schmidt & Kofuji, 2009; 2011a; Schmidt et al., 2011). Both M4 and M5 cells express melanopsin at very low levels and cannot be identified using anti-melanopsin immunolabelling. Instead a fluorescent reporter driven by the melanopsin promoter is required to identify M4 and M5 ipRGCs (Ecker et al., 2010), which can be distinguished from M2 cells by their morphology. M4 cells have very large cell bodies and complex dendritic fields, whereas M5 cells have small, highly branched and uniform dendritic arbors.

There is growing evidence that in addition to morphological differences, ipRGC subtypes are functionally diverse. NIF centres of the brain do not appear to receive equal projections from all ipRGC subtypes. The *Opn4^{tau-lacZ}* mouse, which selectively labels M1-type ipRGCs with a β -galactosidase (β -gal) reporter, revealed M1 input to many NIF regions, including the SCN, OPN and IGL (Hattar et al., 2002). Retrograde labelling studies show the SCN is primarily innervated by M1 cells, and the OPN shell and core are predominantly innervated by M1 and M2 cells respectively (Baver et al., 2008). A similar technique demonstrated M4 cells form the majority of ipRGC input to the dLGN, with additional input from other non-M1 ipRGC subtypes (Weng et al., 2013).

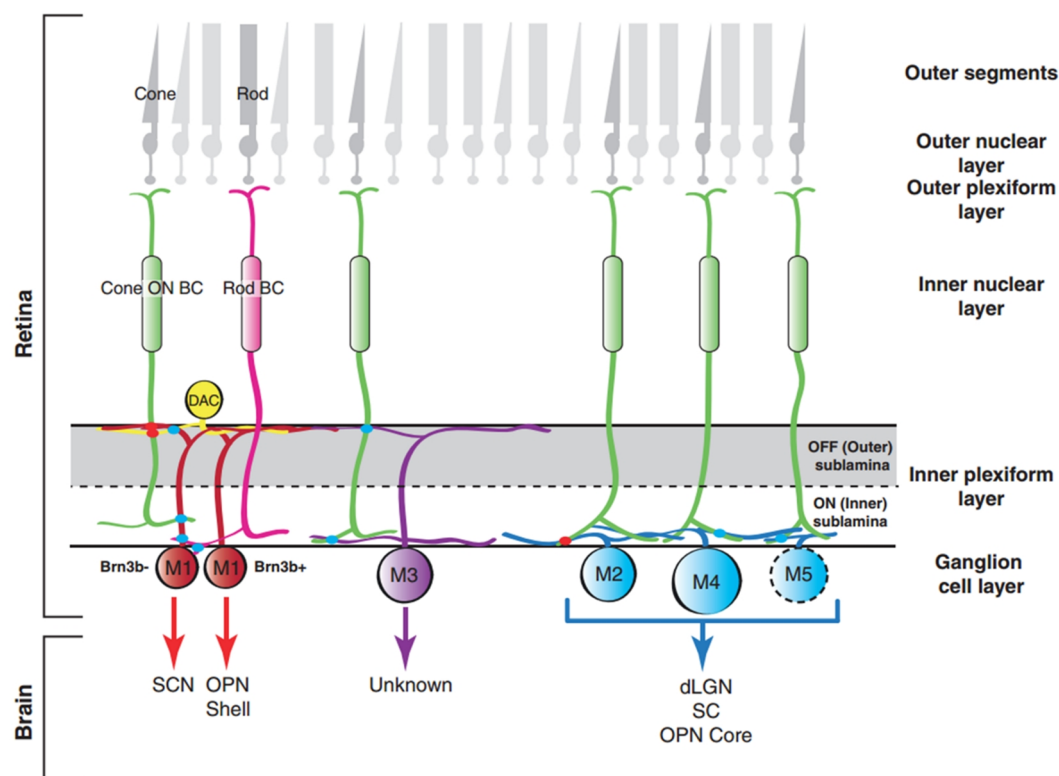


Figure 1.3. Connectivity and morphology of ipRGC subtypes. Schematic diagram of dendrite stratification of M1 cells in outer sublamina of IPL (red); M2, M4 and M5 cells in inner sublamina of IPL (blue); and M3 cells in the inner and outer sublamina (purple). M1 cells are further divided into subpopulations by expression of *Brn3b* transcription factor. Red dots indicate synaptic connections, confirmed by both anatomical and functional evidence, with cone (green) or rod (pink) bipolar cells (BC), dopaminergic amacrine cells (DAC, yellow). Blue dots indicate synaptic connections confirmed by either functional or anatomical evidence. Brain targets of different subtypes are also shown. Image reproduced from Schmidt et al. (2011) with permission from Elsevier.

All these studies suggest that ipRGC subtypes may have specific functional roles. To confirm this requires selective ablation of individual ipRGC subtypes. For example, M1 axonal projections to the OPN indicate M1 cells are involved in PLR. This hypothesis is supported by evidence that the timing of M1 innervations to the OPN matches the onset of PLR in mice (McNeill et al., 2011) and that selective ablation of β -gal labelled ipRGCs, known to be exclusively M1, abolishes PLR (Güler et al., 2008). Further investigation of the molecular characteristics of each subtype, such as expression of transcription factor *Brn3b* in subpopulations of M1 cells (Chen et al., 2011), could enable the ablation of each ipRGC subtype and establish their function.

IpRGC subtypes can also be distinguished by their response characteristics. Tu et al. (2005) described two populations of ipRGC responses in the adult mouse retina recorded using a multi-electrode array – Type II are insensitive with slow onset and slow offset, whereas Type III are very sensitive with rapid onset and slow offset. Electrophysiology of different ipRGC subtypes found similar response diversity, with Type III matching response kinetics and sensitivity of M1 cells and Type II corresponding to non-M1 cells (Hu et al., 2013; Zhao et al., 2014), although responses from all subtypes can be heterogeneous in adult retina (Sexton et al., 2015). This response diversity might represent functional specialisation of ipRGCs for different behavioural roles, reviewed in more detail in Hughes et al. (2016).

1.2 Functional properties of melanopsin

Having described the anatomical and functional characteristics of ipRGCs, and how they differ from classical rod and cone photoreceptors, the focus of this chapter shifts to the ipRGC photopigment – melanopsin. This section compares and contrasts functional properties of melanopsin with other visual pigments.

1.2.1 Phototransduction cascade

Opsins are G protein coupled receptors (GPCRs), ligand-binding membrane proteins that interact with different classes of heterotrimeric guanine nucleotide-binding proteins (G proteins). GPCRs have a shared structure and common activation mechanism (Kobilka, 2007). Depending on which alpha subunit ($G\alpha$) of the G protein is activated by the GPCR, the signalling pathway will be very different, involving distinct effectors and secondary messengers. Vertebrate and invertebrate opsins have divergent phototransduction cascades partly because they activate different $G\alpha$ proteins.

As shown in **Figure 1.4A**, vertebrate opsins, such as mammalian rhodopsin, activate transducin ($G\alpha_t$) which stimulates phosphodiesterase (PDE) to convert cyclic guanosine monophosphate (cGMP) to guanosine monophosphate (GMP). Decrease in cGMP causes the closure of cyclic nucleotide-gated (CNG) channels and hyperpolarisation of membrane potential (Hargrave & McDowell, 1992). Invertebrate opsins, such as *Drosophila RH1*, activate $G\alpha_{q/11}$, which stimulates the enzyme phospholipase C (PLC) to catalyse the hydrolysis of phosphatidyl inositol 4, 5-bisphosphate (PIP_2) into inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG), as shown in **Figure 1.4B**. This causes the opening of non-specific cation transient receptor potential (TRP) channels or TRP-like channels, leading to an intracellular influx of calcium and depolarisation of membrane potential (Montell, 1999).

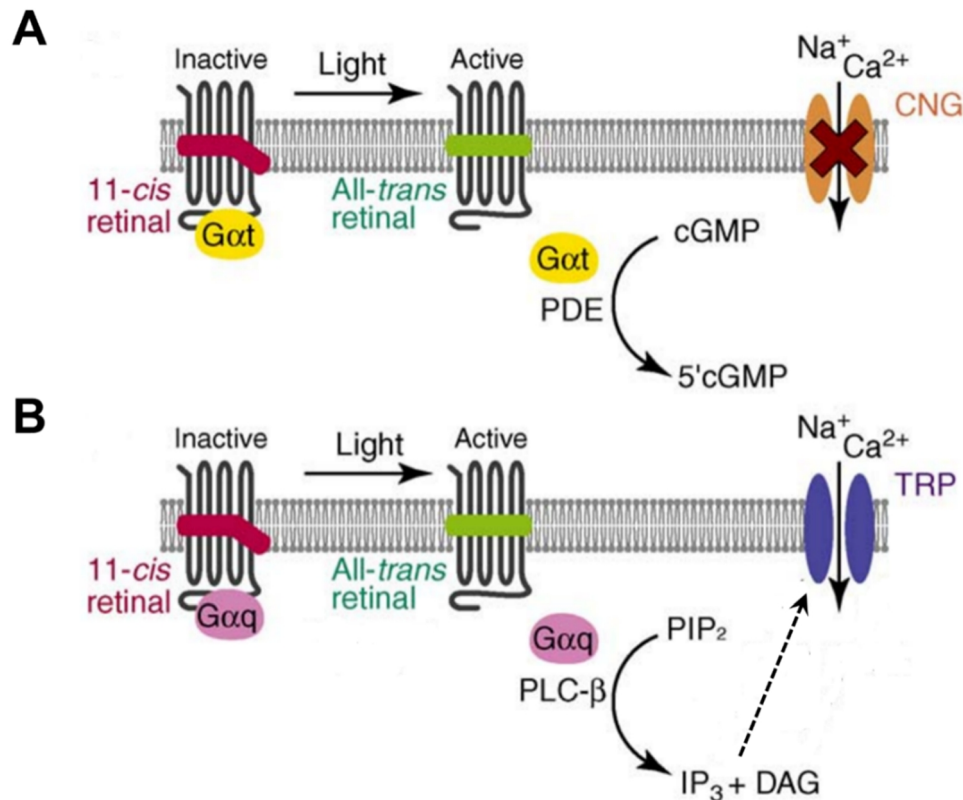


Figure 1.4. Vertebrate and invertebrate rhodopsin phototransduction cascades. (A) $G\alpha_t$ signalling in vertebrate rhodopsin and (B) proposed $G\alpha_q$ signalling in melanopsin, based on invertebrate rhodopsin phototransduction cascade. Dashed arrow indicates currently unknown TRP-channel activation mechanism. Adapted from Hatori and Panda (2010) with permission from Elsevier.

There is compelling evidence that melanopsin demonstrates invertebrate-like $G\alpha_{q/11}$ signalling. First, melanopsin phototransduction persists in *Gnat1*^{-/-} and *Cng3*^{-/-} mice, which lack necessary components for a vertebrate opsin phototransduction cascade (Barnard et al., 2004.; Hattar et al., 2003), suggesting OPN4 has an alternate signalling pathway.

Second, *in situ* hybridisation (Koyanagi et al., 2005), reverse transcriptase-PCR (Graham et al., 2008) and immunohistochemistry (Hughes et al., 2015) reveal co-localisation of melanopsin with components of the $G\alpha_{q/11}$ signalling pathway in ipRGCs, including the $G\alpha_q$ subunit and PLC β isoforms (Koyanagi et al., 2005; Graham et al., 2008;). These studies demonstrate the necessary components for $G\alpha_{q/11}$ signalling are present in ipRGCs.

Third, heterologous expression of melanopsin leads to intracellular calcium influx and depolarisation in response to light (Melyan et al., 2005; Qiu et al., 2005; Kumbalasiri et al., 2007), consistent with a $G\alpha_{q/11}$ phototransduction cascade. Electrophysiology of ipRGCs also reveals depolarisation to light, as well as light-activated currents consistent with TRP channels (Qiu et al., 2005; Warren et al., 2006), which demonstrate melanopsin possesses $G\alpha_{q/11}$ -like responses *in vitro* and *in vivo*.

Fourth, $G\alpha_q$ antagonists and PLC inhibitors significantly attenuate or abolish light responses in melanopsin-expressing HEK293 cells (Qiu et al., 2005) and *Xenopus* oocytes (Panda et al., 2005), while other $G\alpha$ subunit antagonists, such as pertussis toxin blocking $G\alpha_{i/o}$ activity, have no effect. The attenuating effects of PLC inhibitors were also found during electrophysiology of cultured (Contín et al., 2010) and *in situ* ipRGCs (Graham et al., 2008). Additionally, calcium imaging responses of cultured ipRGCs are abolished by TRP channel antagonists (Hartwick et al., 2007). These studies suggest $G\alpha_{q/11}$ phototransduction is required for normal melanopsin function.

Furthermore, these antagonist studies are supported by evidence that genetic ablation of different components of the $G\alpha_{q/11}$ signalling pathway disrupts normal melanopsin function *in vivo*. *TRPM1*^{-/-} and *TRPM3*^{-/-} mice both show deficits in PLR (Hughes et al., 2012a), as do *PKCζ*^{-/-} mice, which lack protein kinase C(ζ-type), an enzyme involved in activation of TRP channels. *PKCζ*^{-/-} mice also show attenuated phase shifting and period lengthening under constant light, phenocopying *Opn4*^{-/-} mice (Peirson et al., 2007). *PLCβ*^{-/-} mice show no intrinsic PLR and M1 ipRGCs in *TRPC6,7*^{-/-} double knockout mice lose intrinsic photosensitivity (Xue et al., 2011). Perez-Leighton et al. (2011) found genetic ablation of TRPC3, TRPC6 or TRPC7 ion channels individually did not interrupt melanopsin-driven behaviours, indicating there may be some redundancy in the melanopsin phototransduction pathway. Although the precise composition of this pathway is still unclear, the collective evidence suggests melanopsin uses invertebrate-like $G\alpha_{q/11}$ signalling.

A recent study challenged this consensus by examining the effect of single and double knockouts of different members of the $G\alpha_{q/11}$ family on NIF responses to light. Chew et al. (2014) found genetic ablation of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15}$ individually, or $G\alpha_{11}$ and $G\alpha_{14}$, or $G\alpha_q$ and $G\alpha_{11}$ had no effect on PLR and circadian photoentrainment. There are two possible reasons for this disagreement. First, is the choice of double knockouts. The $G\alpha_q$ and $G\alpha_{14}$ compound knockout is noticeably absent, as is the triple knockout of $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$, which may be required to produce PLR at *Opn4*^{-/-} levels, if $G\alpha$ subunits from the same family can compensate for each other. Secondly, this study used mixed strain C57BL6/SV129 mice, which typically have normal outer retinal function. As discussed previously, any rod-cone compensation makes it harder to detect subtle changes in NIF responses to light caused by loss of OPN4 function.

These issues were addressed in a subsequent study by Hughes et al. (2015), who used RNAi-based silencing to explore the G protein interactions of melanopsin in *rd/rd cl* mice. Silencing $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$ simultaneously produced the largest deficit in PLR, more than combined silencing of $G\alpha_q$ and $G\alpha_{11}$, suggesting all three subunits support melanopsin phototransduction *in vivo*. It is worth noting, that melanopsin and other GPCRs can be promiscuous and are capable of coupling to multiple $G\alpha$ subunits, including transducin, in heterologous expression in cell lines (Newman et al., 2003; Bailes & Lucas, 2013). This is a potential issue for studies examining melanopsin *in vitro*. Nevertheless, the current consensus is that melanopsin has an invertebrate-like $G\alpha_{q/11}$ phototransduction cascade.

1.2.2 Spectral sensitivity

All opsins have a distinctive spectral sensitivity profile, known as a visual pigment template, characterised by the wavelength of peak sensitivity or absorption, $\lambda_{\max}$. In humans, $\lambda_{\max}$ for rhodopsin is ~498nm. $\lambda_{\max}$ for human LWS, MWS and SWS cone visual pigments is ~563nm, ~534nm and ~420nm, respectively (Bowmaker & Dartnell, 1980). The consensus $\lambda_{\max}$ for melanopsin is ~480nm, discussed in more detail below.

As shown in **Figure 1.5**, the spectral sensitivity of different opsins overlaps, so that more than one pigment will respond to each wavelength. For example, 480nm light will, per quanta, maximally stimulate melanopsin, but it will also cause moderate rod and small cone responses. Therefore, monochromatic light cannot be used to isolate activity of a specific pigment, unless the activity of other pigments has been controlled for.

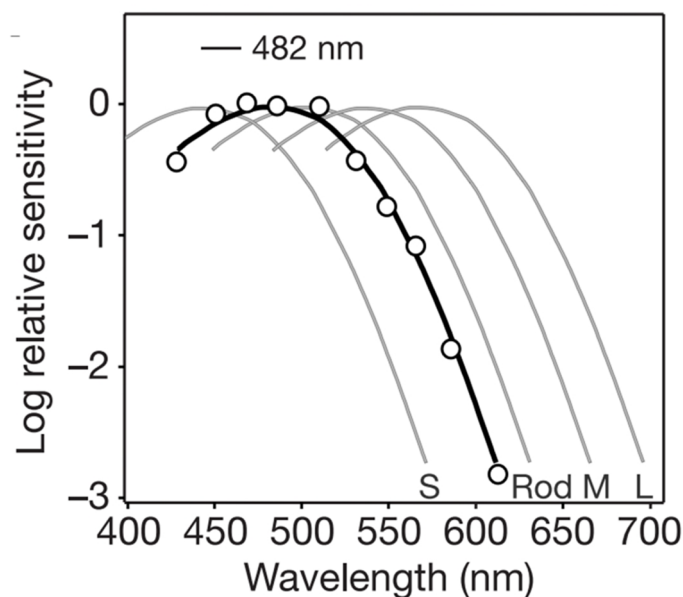


Figure 1.5. Spectral sensitivity of visual pigments in the macaque retina. Relative sensitivity of short, medium and long wavelength (S, M and L) cones, rods and ipRGCs (shown in bold) fit to an A_1 visual pigment template. $\lambda_{\max}$ for rods, S, M and L cones is ~510nm, ~450nm, ~540nm and ~570nm respectively. ipRGCs have $\lambda_{\max}$ at 482nm. Based on action spectra of ipRGCs in the macaque retina. Image reproduced from Dacey et al. (2005) with permission from Nature Publishing Group.

$\lambda_{\max}$ can be identified by two approaches. The first, an absorbance spectrum, is the relative absorption of light by a protein across a range of wavelengths and represents the most direct method for obtaining $\lambda_{\max}$. It requires large quantities of purified opsin, isolated from retina or heterologous expression in cell lines. ipRGCs are comparatively rare in the retina, accounting for only 1-3% of retinal ganglion cells and have low pigment density as they lack specialised morphology to increase opsin abundance (Do et al., 2009), such as the outer segments of rod and cone cells. This makes it difficult to purify sufficient melanopsin protein from ipRGCs to generate good quality absorption spectra and there are currently no published examples using this approach (although see Walker et al., 2008, for absorption spectrum from solubilised ipRGCs).

Alternatively, protein can be purified from heterologous expression of recombinant opsin. Large amounts of protein can be produced relatively easily, compared to the effort required to extract the equivalent amount from retinae. The recombinant protein is translated and folded in a non-native environment, potentially affecting the quality of the absorbance spectrum. Protein folding can be improved by supplementing substrates, such as the 11-*cis* retinal chromophore (Matsuyama et al., 2012), to mimic a native environment. There appears to be a particular issue purifying mammalian melanopsin from heterologous expression, compared to other visual pigments. Although good quality absorption spectra have been published for amphioxus and zebrafish melanopsin, mouse melanopsin absorption spectra are highly variable with $\lambda_{\max}$ reported from 424-500nm (**Table 1.3**). In comparison, rhodopsin or cone visual pigments purified by similar methods produce highly consistent absorption spectra (Oprian et al., 1987;1991 ; Merbs & Nathans, 1993). This suggests melanopsin produced by heterologous expression requires additional chaperones for correct protein folding. As these have not yet been identified (see Shirzad-Wasei et al., 2013 for potential chaperones), absorbance spectra have proved problematic and an unreliable method for determining mammalian melanopsin spectral sensitivity.

A more indirect approach to determine $\lambda_{\max}$ of an opsin is an action spectrum, which measures the spectral sensitivity of a physiological response. Melanopsin action spectra have been produced from a wide range of OPN4-driven responses, both *in vivo* and *in vitro* (**Table 1.3**) and are much more consistent with $\lambda_{\max}$ between 475 and 484nm. The general consensus is that peak spectral sensitivity of OPN4 is at 480nm.

When using action spectra to estimate melanopsin $\lambda_{\max}$, the choice of physiological response and model system is very important as, ideally, it should isolate OPN4-driven activity from rod-cone signalling to ipRGCs. This might be by pharmacological blockade (Gamlin et al., 2007), or genetic/disease ablation of rod and cone photoreceptors (Lucas et al., 2001; Hattar et al., 2003; Zaidi et al., 2007). Provided care is taken to minimise outer retina signalling, such as performing action spectra under rod-cone saturating conditions (Hankins & Lucas, 2002), behaviours partially driven by melanopsin can also be used to generate accurate estimates of $\lambda_{\max}$.

It is also important to consider factors, such as lens transmission, which may affect the intensity of light reaching the retina and whether this differs significantly from the measured value. Lens transmission of short wavelength light differs between species and, in primates and rodents, can decrease over time, due to age-related changes in lens size and accumulation of yellow pigmentation in the lens (Douglas & Jeffrey, 2014). This can lead the short wavelength sensitivity of a pigment to appear lower than it actually is, distorting the data available to fit to a visual pigment template and estimate $\lambda_{\max}$.

Table 1.3. Spectral sensitivity of melanopsin. Methods shown for absorbance spectra are those used to produce purified melanopsin protein. Methods shown for action spectra are the biological responses recorded to fit an A1-visual pigment template to estimate wavelength of peak spectral sensitivity ($\lambda_{\max}$)

^a Data corrected for lens transmission.

^b Derived from post-illumination pupil response. All other PLR action spectra are from maximum constriction data.

Method	Reference	Species	$\lambda_{\max}$
Absorbance spectra			
Purified from heterologous expression in COS cell	<i>Newman et al (2003)</i>	Mouse	424nm
Purified from heterologous expression in Hek293 cells	<i>Matsuyama et al (2012)</i>	Mouse	467nm
	<i>Davies et al (2011)</i>	Zebrafish X1	470nm
		Zebrafish M2	484nm
	<i>Matos-Cruz et al (2011)</i>	Zebrafish M2	403nm
	<i>Torii et al (2007)</i>	Chicken X	476nm
		Chicken M	484nm
	<i>Koyanagi et al (2005)</i>	Amphioxus	485nm
Purified from heterologous expression in SF9 cells	<i>Shirzad-Wasei et al (2013)</i>	Mouse	480-500nm
Isolated ipRGCs	<i>Walker et al (2008)</i>	Mouse	500nm
In vivo action spectra			
Pupillary light responses	<i>Lucas et al (2001)</i>	Mouse	479nm
	<i>Gamlin et al (2007)</i>	Human	482nm ^{a,b}
		Macaque	476nm
			483nm ^b
	<i>Zaidi et al (2007)</i>	Human	480nm ^a
Phase Shifting	<i>Hattar et al (2003)</i>	Mouse	481nm ^a
Melatonin suppression	<i>Thapan et al (2001)</i>	Human	459nm ^a
	<i>Brainard et al (2001)</i>	Human	464nm
Electroretinogram	<i>Hankins & Lucas (2002)</i>	Human	483nm ^a
In vitro action spectra			
Whole cell current clamp of isolated ipRGCs	<i>Berson et al (2002)</i>	Rat	484nm
	<i>Dacey et al (2005)</i>	Macaque	482nm
Whole cell patch clamp of <i>OPN4</i> expressing Hek293 cells	<i>Qiu et al (2005)</i>	Mouse	479.2nm
Whole cell patch clamp of <i>OPN4</i> expressing <i>Xenopus</i> oocytes	<i>Panda et al (2005)</i>	Mouse	479.8nm
Multi-electrode array recording of post-natal P8 retina flatmount	<i>Tu et al (2005)</i>	Mouse	479.5-481nm
Iris sphincter muscle contraction	<i>Xue et al (2011)</i>	Mouse	480nm
Bioluminescent Ca ²⁺ imaging of <i>OPN4</i> expressing Hek293 cells	<i>Bailes & Lucas (2013)</i>	Human	479nm
		Mouse	482nm

1.2.3 Bistability

A final unusual functional property of melanopsin is chromophore bistability. After photoisomerisation, the all-*trans* chromophore must be replaced by a new 11-*cis* retinal molecule before the opsin protein can respond again. As shown in **Figure 1.6**, in vertebrate visual pigments, this is accomplished by chromophore recycling in the RPE, as part of the visual retinoid cycle. In comparison, invertebrate opsins are bistable and have the ability to endogenously regenerate chromophore by exposure to a specific wavelength of light, without the need for external retinal recycling mechanisms (Montell, 1999). There is growing evidence that melanopsin is also a bistable pigment.

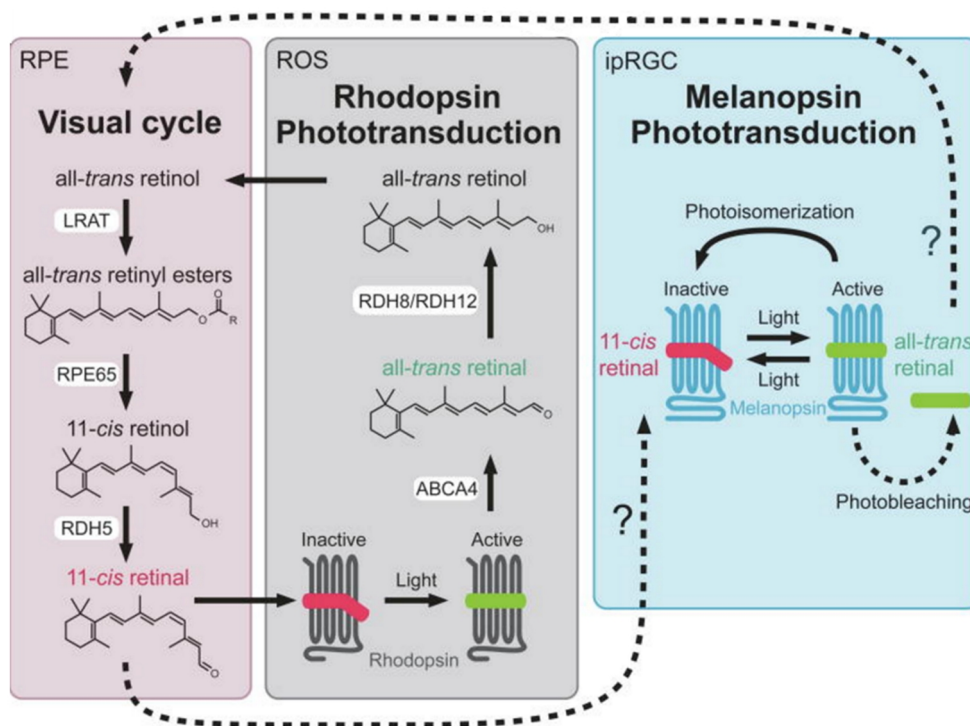


Figure 1.6. Chromophore recycling in visual pigments. Schematic of vertebrate visual cycle, where 11-*cis* retinal (shown in red) is regenerated in a multistep enzymatic process in the retinal pigment epithelium (RPE) after isomerisation to all-*trans* retinal (shown in green) in rod outer segments (ROS). Enzymes such as *LRAT*, *RPE65* and *RDH5* are critical in this pathway. In invertebrate visual pigments, the opsin can bind both *cis*- and *trans*- retinaldehyde. Photoregeneration of chromophore occurs after exposure to different wavelengths of light. There is evidence melanopsin uses both vertebrate and invertebrate-like chromophore regeneration (shown as dashed lines). Image reproduced from Hatori & Panda (2010) with permission from Elsevier.

For vertebrate visual pigments, like rhodopsin and cone visual pigments, prolonged light exposure leads to photobleaching, a decrease in response over time as more 11-*cis* retinal is isomerised to all-*trans* and dissociates from the opsin protein, leaving the opsin unable to activate further. In comparison, melanopsin exhibits sustained responses during prolonged light exposure and seems resistant to photobleaching (Warren et al., 2003; Melyan et al., 2005; Graham et al., 2008; Sexton et al., 2012) suggesting it does not require external chromophore regeneration necessary in rod and cone visual pigments.

OPN4-driven behaviour persists attenuated in *Rpe65*^{-/-} and *Lrat*^{-/-} knockout mice, which lack enzymes necessary for chromophore regeneration in the RPE (Redmond et al., 1998; Batten et al., 2004), demonstrating this external chromophore recycling is not necessary for OPN4 signalling, but may be required for normal activity. A recent study supported this, finding pharmacological inhibition of the RPE causes less sustained pupil constriction (Zhao et al., 2016).

Follow-up studies suggest this attenuation may be the result of decreased chromophore availability due to vast amounts required for rod phototransduction, rather than OPN4 phototransduction requiring 11-*cis* retinal from the RPE. PLR is less severely attenuated in *Rpe65*^{-/-} and *Lrat*^{-/-} mice with outer retina degeneration (Fu et al., 2005; Doyle et al., 2006) and pharmacological inhibition of the visual retinoid cycle causes no change in PLR sensitivity of *rd/rd* mice (Tu et al., 2006). In the absence of these interactions with the outer retina, melanopsin can apparently function independent of chromophore regeneration in the RPE. Given the physical distance between ipRGCs and the RPE, it is unlikely melanopsin would receive exogenously regenerated chromophore, suggesting melanopsin either uses an alternate chromophore regeneration mechanism, such as the role played by Müller glial cells in cones (Wang & Kefalov, 2009), or an endogenous invertebrate-like recycling process.

Whether melanopsin has an invertebrate-like bistable mechanism that requires long-wavelength light to regenerate its chromophore, is unclear. Melyan et al. (2005) found that melanopsin-expressing HEK293 cells were photosensitive when supplemented with all-*trans* retinal provided cells were previously exposed to long-wavelength light. There is also behavioural evidence that PLR, SCN spike firing and negative masking are more sensitive after exposure to red light, an effect not seen in *Opn4*^{-/-} mice (Mure et al., 2009), arguably because more all-*trans* retinal is converted back to 11-*cis* retinal, increasing the amount of melanopsin available for activation. The evidence for bistability in ipRGC activity is less clear. Some studies report no effect of long-wavelength light on sensitivity of ipRGC spike firing (Mawad & Van Gelder, 2008), while others find that high-intensity 580nm light can cut short prolonged depolarising after-potentials triggered by high-intensity 480nm light (Qiu & Berson, 2007), a classic property of bistable photopigments.

Recent evidence has suggested melanopsin may be tristable. By examining the spectral sensitivity of MEA-recorded ipRGCs under different lighting conditions, Emanuel and Do (2015) found melanopsin exists in two inactive states, termed “blue” and “violet” for their spectral sensitivity, and a third active “meta” state. These three states are maintained in equilibrium and exposure to different wavelengths of light shifts the amount of melanopsin in each state. The predominant state absorbs the wavelength of light least effectively. For example, short wavelengths cause preferential isomerisation of “violet” ($\lambda_{\text{max}} = 453\text{nm}$) and “blue” states ($\lambda_{\text{max}} = 471\text{nm}$), meaning the “meta” active state is most abundant. For long-wavelength light, the silent violet state is most abundant as it absorbs these wavelengths least effectively, explaining why ipRGC signalling appears to cease after exposure to long-wavelengths. This equilibrium between the silent and active states enables melanopsin to maintain long temporal integration, as it ensures there is always sufficient silent state melanopsin to allow activation.

Spectroscopy will be required to confirm the tristability hypothesis, but, as shown in **Figure 1.7**, the spectral sensitivity of these violet, blue and meta states match the extramelanopsin ($\lambda_{\max} = 446\text{nm}$, 7-*cis* retinal), melanopsin ($\lambda_{\max} = 467\text{nm}$, 11-*cis* retinal) and metamelanopsin ($\lambda_{\max} = 476\text{nm}$, all-*trans* retinal) states found by Matsuyama et al. (2012) when they performed absorption spectra of purified mouse melanopsin under different lighting conditions. Binding of a 7-*cis* retinal chromophore is extremely unusual and could only be confirmed by purification of OPN4 bound to 7-*cis* retinal from ipRGCs, which as previously discussed is extremely difficult due to low pigment abundance. These studies suggest melanopsin possesses a highly unusual activation and chromophore regeneration mechanisms, likely unique amongst vertebrate visual pigments.

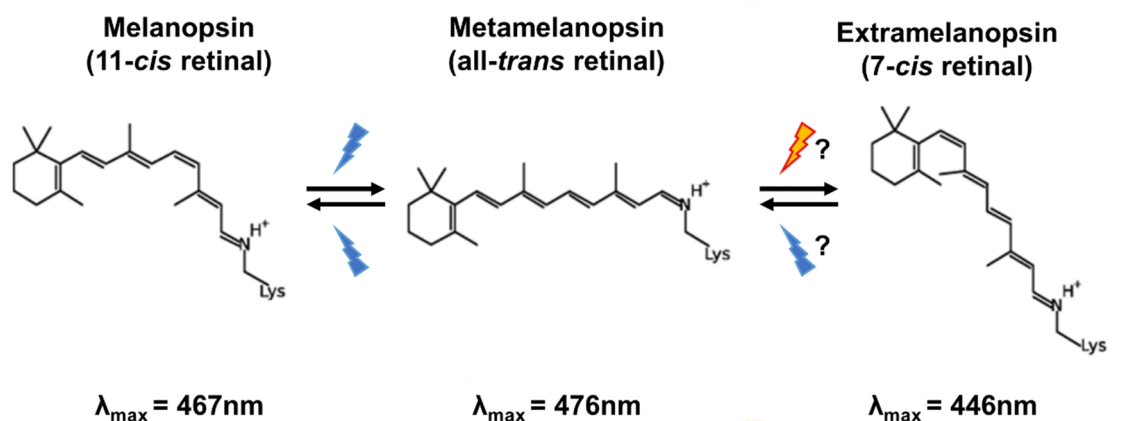


Figure 1.7. Tristability of melanopsin. Isomerisation of either 11-*cis* retinal (melanopsin) or 7-*cis* retinal (extramelanopsin) to all-*trans* retinal (metamelanopsin). The transition of melanopsin from inactive (*cis* retinal) to active (*trans* retinal) and vice versa is driven by exposure to different wavelengths of light. Adapted from Matsuyama et al. (2012) with permission from American Chemical Society.

1.3 Opsin structure-function

The functional properties of melanopsin and other visual pigments are determined by the structure of the opsin protein. In this section, the structure-function relationships of opsins are discussed, based on the rhodopsin crystal structure and mutagenesis studies of rhodopsin and cone visual pigments, and how these are relevant to melanopsin.

1.3.1 Methods for determining structure-function relationships

The gold standard for structure-function research is the protein crystal structure, a high-resolution visualisation of the individual atoms in a protein, which can be used to determine the relative arrangement of amino acids and their side chains, and therefore the 3D structure of the protein (Hargrave, 2001). Protein crystallisation requires large quantities of purified protein. As ipRGCs are relatively scarce in the retina and have low pigment density (Do et al., 2009), there is currently no melanopsin crystal structure. However, there are crystal structures for bovine (Palczewski et al., 2000) and squid rhodopsin (Murakami & Kouyama, 2008), shown in **Figure 1.8**, purified from the opsin-rich rod outer segments. For bovine rhodopsin, multiple structures have been published, including rhodopsin in the inactive conformation (Okada et al., 2004), without an 11-*cis* chromophore (Park et al., 2008) and during G protein activation (Scheerer et al., 2008).

For proteins without crystal structures, structure-function relationships can still be explored indirectly using sequence homology and mutagenesis. Sequence homology relies on conserved motifs (shared amino acid sequences) to make predictions about structure-function based on properties of a better-described member of the same protein family. Many assumptions about melanopsin structure-function are based on structural or sequence homology with rhodopsin and its crystal structures. Sequence homology is less useful for exploring poorly conserved regions, such as the intracellular loops, or where the functional properties differ significantly between members of the same protein class.

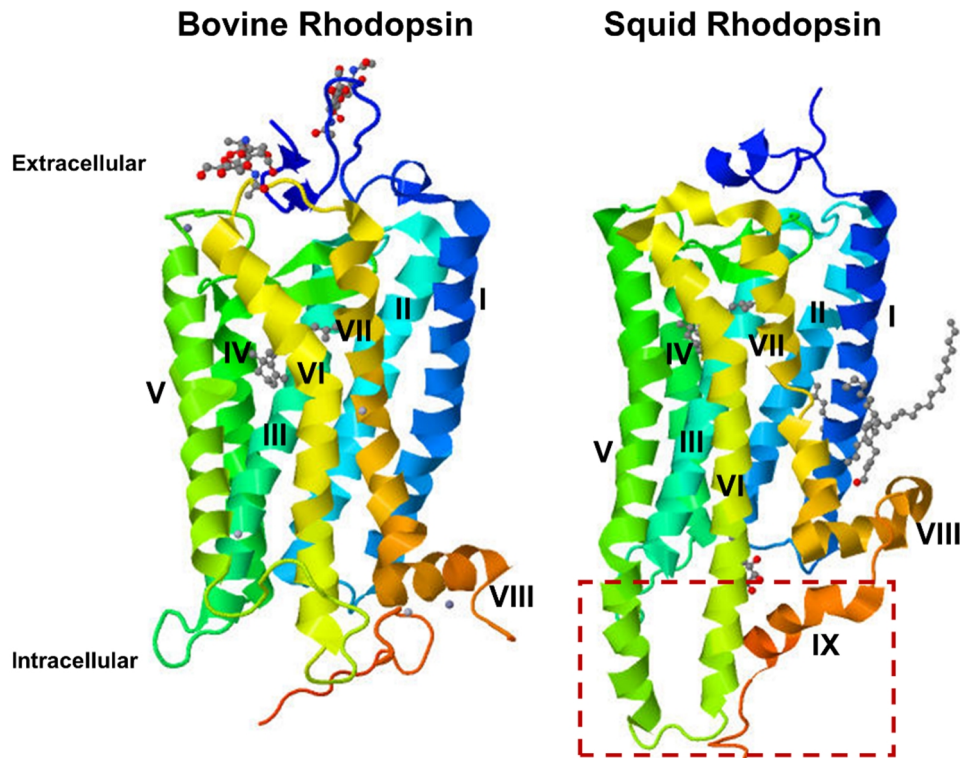


Figure 1.8. Crystal structures of bovine and squid rhodopsin. Ribbon representation of crystal structure of Bovine (PDB 1F88) and Squid rhodopsin (PDB 2Z73). Transmembrane helices are labelled I-VII, with C-terminus helix labelled VIII. Squid rhodopsin has an extended fifth and sixth transmembrane helix, as well as the additional ninth helix formed by the C terminus in the cytoplasm (dashed red line). Image acquired from Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB, <http://www.rcsb.org/pdb/home/home.do>), generated using Jmol; an open-source Java viewer for chemical structures in 3D (<http://www.jmol.org>).

Mutagenesis studies are valuable for identifying critical residues in a protein. Assessing the impact of amino acid substitutions on protein function can help build a clear picture of which sites and amino acid properties are necessary for specific functional roles. These mutations might be induced or can occur naturally, potentially causing disease. For example over 100 different missense mutations in the rhodopsin gene (*Rho*) have been associated with retinitis pigmentosa (RP, Okada et al., 2001), a heterogeneous group of retinal degenerative disorders characterised by progressive loss of photoreceptors (Mendes et al., 2005). A cluster of RP-linked mutations in the C-terminus led to the identification of key residues for normal outer segment localisation (Tai et al., 1999), demonstrating the contribution pathogenic variants can bring to the study of protein structure-function.

1.3.2 Key Opsin Residues

Opsins have a typical GPCR structure, consisting of 7 transmembrane helices (TM1-7) connected by 3 intracellular (cytoplasmic, IL1-3) loops, 3 extracellular loops (EL1-3), an extracellular N-terminus and an intracellular C-terminus. This heptahelical structure is likely shared by melanopsin (Provencio et al., 1998), as suggested by the presence of conserved sequence motifs found in all GPCRs. In the tertiary structure, the membrane-embedded helices are arranged circularly forming a central cavity, occupied by the retinal chromophore in opsins, as shown in **Figure 1.9**.

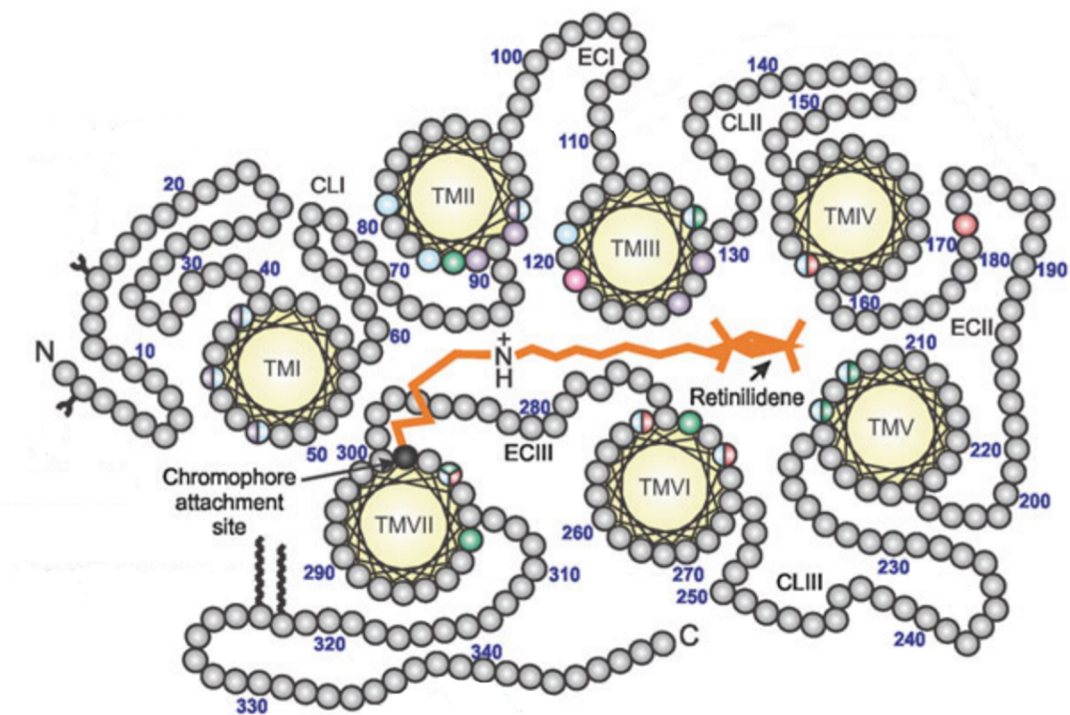


Figure 1.9. Location of the retinal chromophore in the opsin protein. Schematic of the retinal chromophore (orange) bound to the chromophore attachment site in TM7. EC = Extracellular Loop and CL = Intracellular (Cytoplasmic) Loop. Image adapted from Hunt et al. (2009) with permission from The Royal Society.

The three transmembrane, extracellular and intracellular domains can broadly be divided by their functional roles. **Table 1.4** summarises the key functional sites in each opsin domain, discussed in more detail in the following section.

Table 1.4. Key functional residues in Rhodopsin. Rhodopsin residues are numbered according to Bovine Rhodopsin sequence. Equivalent amino acid and position in human melanopsin (hOPN4) is also shown, with conserved amino acids highlighted in bold. Poor sequence homology in N and C-termini means key sites in these regions have no hOPN4 equivalent.

Key Rhodopsin Residue	Location	Functional Role in Rhodopsin	Equivalent hOPN4 Residues
Transmembrane			
K296	TM7	Retinal Binding Site	K340
E113	TM3	Schiff Base Counterion	Y146
G90	TM2	Interacts with Schiff Base linkage Mutated in Retinitis Pigmentosa	Q123
A164	TM4	Spectral tuning sites for long wavelength shifts	A198
F261, A269	TM6		F305 , S313
M86, G90,	TM2	Spectral tuning sites for short wavelength shifts	M119 , Q123,
A117, E122, A124	TM3		G150, I155, S157
W265	TM6		W309
A292, A295, A299	TM7		A336, A339, A343
E134, R135, Y146	TM3	Ionic lock (E/DRY motif)	D167, R168, Y169
C264, W265, L266, P267, Y268	TM6	Rotamer Toggle Switch (xWxPY motif)	S308, W309 , A310, P311, Y312
N302, P303, V304, I305, Y306	TM7	Hydrogen bonds between TM6 and TM7 (NPxxY motif)	N346, P347 , I348, I349, Y350
Extracellular			
P23	NT	Mutated in Retinitis Pigmentosa Necessary for normal protein folding	No equivalent
C110	TM3	Disulphide bridge stabilises tertiary structure	C143
C187	EL2		C221
N2, N15	NT	Glycosylation sites	No equivalent
R177, D190	EL2	Necessary for thermal stability in inactive state	A211, D224
E181	EL2	Invertebrate counterion Interacts with retinal chromophore	E215
Intracellular			
S334, S338, S343	CT	Phosphorylation sites for arrestin binding and opsin deactivation	No equivalent
C322, C323	CT	Palmitoylation sites	P366, C367

1.3.3 Key Residues in the Opsin Transmembrane Domain

The transmembrane domain consists of 7 hydrophobic α -helices embedded in the lipid bilayer of the plasma membrane. These transmembrane helices possess a number of invariable residues critical for opsin activation and response characteristics, such as spectral sensitivity. Key functional residues in this region are highlighted in **Figure 1.10**, and described in more detail below.

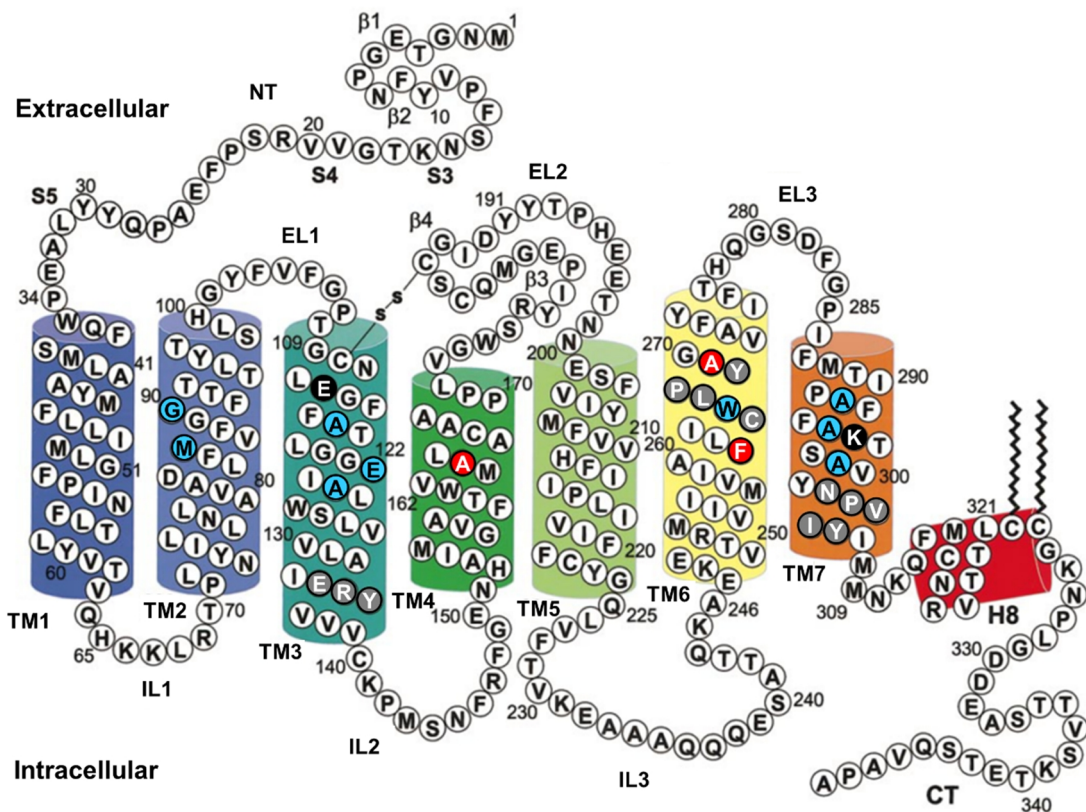


Figure 1.10. Key residues in the bovine rhodopsin transmembrane domain. Schematic secondary structure of bovine rhodopsin, with transmembrane helices (TM1-7) shown as coloured cylinders. The retinal binding site in TM6 (K296) and vertebrate Schiff base counterion in TM3 (E113) are shown in black. Conserved GPCR motifs (ERY in TM3, xWxPY in TM6 and NPxxY in TM7) are shown in grey. LWS and MWS cone visual pigment spectral tuning sites are shown in red, while 9 sites required to short-wavelength shift rhodopsin spectral sensitivity to SWS cone visual pigment $\lambda_{\max}$ (Lin et al., 1998) are shown in blue. Image adapted from Menon et al. (2001) with permission from the American Physiological Society.

1.3.3.1 Retinal binding site and counterion

All opsins are covalently linked to the retinal chromophore by Schiff base linkage at a lysine residue on the seventh transmembrane helix. This is also true for melanopsin as mutation of the equivalent lysine abolishes melanopsin responses to light (Kumbalasiri et al., 2007). The retinal molecule lies parallel with the plasma membrane surface and interacts with residues of surrounding helices, known as the chromophore binding pocket (Palczewski et al., 2000). This chromophore binding pocket includes a negatively charged counterion, which neutralises the protonated Schiff base and allows activation of the photopigment in the visual wavelength range. Mutation of the counterion leads to constitutive activity of the opsin protein (Sakmar et al., 1989), as do mutations disrupting the salt-bridge between the counterion and retinal-binding site, such as G90D in TM2 of rhodopsin, which causes congenital stationary night-blindness (Rao et al., 1994).

The location of this counterion is a highly conserved difference between vertebrate and invertebrate opsins (**Figure 1.11**). In vertebrate rhodopsin, the counterion is E113 in TM3 (bovine rhodopsin numbering, Sakmar et al., 1989). In invertebrate rhodopsin, E113 is replaced by a polar amino acid, usually tyrosine, and the counterion is displaced to E181 in EL2 (Terakita et al., 2004). The consequences of this are unclear, although an E181 counterion appears to be a highly conserved property of bistable visual pigments (Tsukamoto & Terakita, 2010). Melanopsin has the equivalent of Y113 and E181, similar to invertebrate-visual pigments. It should be noted that even though E181 is not the vertebrate counterion, it still interacts with the retinal chromophore to ensure photoisomerisation is restricted to the C11-C12 bond (Menon et al., 2001), suggesting both position 113 and 181 play important functional roles in both vertebrate and invertebrate opsins.

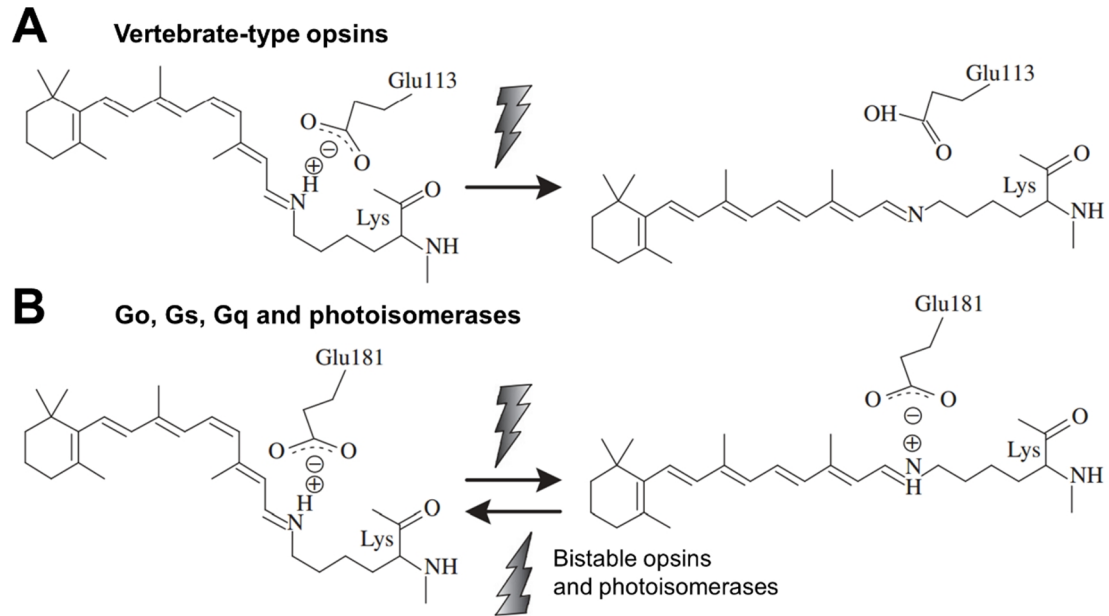


Figure 1.11. Opsin counterions. (A) Vertebrate opsins have a negatively charged glutamic acid at position 113 in TM3, which acts as counterion for the protonated retinal Schiff base binding retinal to Lysine binding site. (B) The counterion of invertebrate opsins, and those binding to $G\alpha_q$, $G\alpha_{i/o}$ or $G\alpha_s$ signalling pathways, is at position 181 in EL2. The E181 counterion is a feature of bistable pigments and photoisomerases. Adapted from Shichida & Matsuyama (2009) with permission from the Royal Society.

1.3.3.2 Chromophore binding pocket and spectral sensitivity

In addition to the counterion, the chromophore binding pocket contains several residues which determine the spectral sensitivity of the opsin photopigments. Interactions between the side chains of spectral tuning sites and 11-*cis* retinal affect the stability of the ground (S_0) and excited (S_i) state of the retinal chromophore. As shown in **Figure 1.12**, the distance between these states affects the amount of energy required for photoisomerisation. As different wavelengths of light have different levels of energy, with shorter wavelengths possessing more energy than longer wavelengths, this energy gap determines the wavelength of peak absorption (Kochendoerfer et al., 1999). As the distance between S_0 and S_i increases, more energy is required for isomerisation so shorter wavelengths produce peak absorption. Decreasing the distance between the excited and ground state means less energy is required so spectral sensitivity shifts to longer wavelengths.

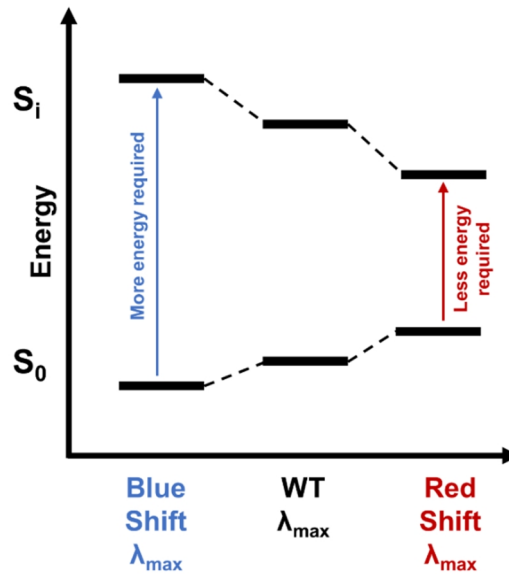


Figure 1.12. Spectral tuning mechanisms. Spectral sensitivity is determined by difference between ground (S_0) and excited (S_i) states. Lowering S_i or increasing S_0 will lead to long-wavelength shifts in spectral sensitivity, while increasing S_i or stabilising S_0 will cause short wavelength shifts. These effects are mediated by interactions between chromophore and amino acid side chains (spectral tuning sites).

There are distinct mechanisms controlling spectral tuning in long and short-wavelength sensitive opsins. For example, the 30nm increase in $\lambda_{\max}$ in LWS cone visual pigment from MWS cone visual pigment is predominantly due to addition of polar amino acids at 3 sites, position 277 and 285 in TM6 and 180 in TM4 (human LWS cone visual pigment numbering, Merbs & Nathans, 1993; Asenjo et al., 1994; Sun et al., 1997). These polar residues alter electrostatic interactions with the β -ionone ring and polyene chain of 11-*cis* retinal, decreasing distance between excited and ground states to produce long-wavelength spectral tuning shifts (Yokoyama & Radlwimmer, 2001). In comparison, 9 simultaneous amino acid substitutions are required to short-wavelength shift bovine rhodopsin $\lambda_{\max}$ by 80nm towards the peak spectral sensitivity of SWS cone visual pigment (Lin et al., 1998). Most of these sites interact with the protonated Schiff base linkage at TM7 (Chang et al., 1995), to increase distance between excited and ground state. With a $\lambda_{\max}$ of 480nm, melanopsin spectral sensitivity is between rhodopsin and SWS cone visual pigment, suggesting it might possess a combination of long and short-wavelength spectral tuning sites.

1.3.3.3 Hydrogen-bonding networks and opsin activation

The rhodopsin crystal structure revealed networks of hydrogen-bonds (H-bonds), which maintain correct alignment of the transmembrane helices in the inactive ground state and are critical for normal opsin activation (Palczewski et al., 2000). Isomerisation of 11-*cis* retinal causes change in relative orientation of TM3 and 6, disrupting these H-bonds and triggering opsin activation (Okada et al., 2001, **Figure 1.13**).

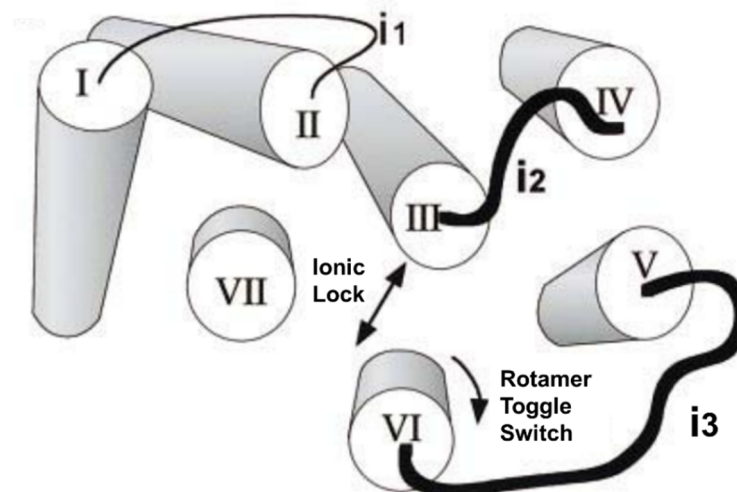


Figure 1.13. Conformation change during opsin activation. Intracellular view of the active opsin conformation. Retinal isomerisation leads to changes in rotameric states of xWxPY motif in causing rotation of TM6 (known as the rotamer toggle switch). This disrupts ionic lock between DRY motif in TM3 and glutamic acid TM6 causing movement of TM3 and TM6 away from each other. These conformation changes allow G protein activation by IL2 and IL3 (thick black lines). Adapted from Bockaert & Pin (1999) with permission from John Wiley and Sons, and Bourne et al (1997) with permission from Elsevier.

Several highly GPCR conserved motifs are involved in opsin activation, including NPxxY in TM7, which mediates H-bonds between TM6 and 7 (Fritze et al., 2003); xWxPY, the “rotamer toggle switch” which maintains the kink in TM6 necessary to cause rotation of this helix during activation (Deupi & Kobilka, 2007); and E/DRY in TM3, which mediates the “ionic lock” between TM3 and TM6 (Palczewski et al., 2000). Several rhodopsin mutations associated with autosomal dominant RP are located in these highly conserved motifs (Angel et al., 2009), demonstrating they are critical for normal function. Melanopsin possesses all these motifs and likely shares the hydrogen-bond networks and activation mechanisms found in rhodopsin.

1.3.4 Key Residues in the Opsin Extracellular Domain

The extracellular domain consists of the N terminus and 3 hydrophilic extracellular loops. It primarily reinforces the tertiary structure of the opsin, and has less influence over response characteristics. Key functional residues in this region are highlighted in **Figure 1.14**, and described in more detail below.

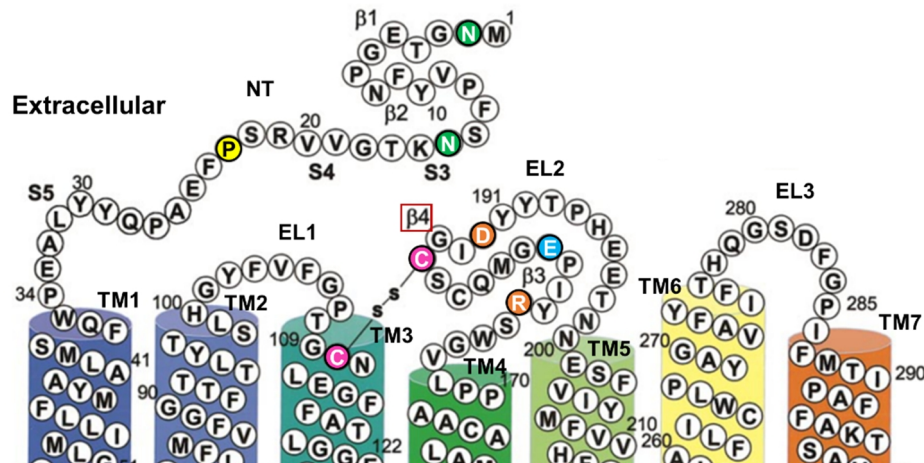


Figure 1.14. Key residues in the bovine rhodopsin extracellular domain. Schematic diagram shows disease-linked P23H mutation in N-terminus highlighted in yellow. Cysteine pair (C110 in TM3 and C187 in EL2, shown in pink) form disulphide bridge stabilising tertiary structure. Glycosylation sites in the N-terminus shown in green. β 4-pleated sheet in EL2 (red box) folds into transmembrane region. Residues D190 and R177 are necessary for thermal stability. Invertebrate counterion E181 is shown in blue. Adapted from Menon et al. (2001).

In bovine rhodopsin, interactions between residues on the N-terminus and the extracellular loops maintain correct orientation of α -helices relative to each other (Palczewski et al., 2000). Disruption of these interactions results in misfolding and retention of the protein in the endoplasmic reticulum, which can lead to visual disorders. For example, an N-terminus rhodopsin mutation, P23H, is the most common cause of autosomal dominant RP (Mendes et al., 2005). In melanopsin, this region is poorly conserved between species and has low sequence homology with rhodopsin. This suggests key rhodopsin residues in this domain will not necessarily perform the same role in melanopsin, and that extracellular mutations might be better tolerated in melanopsin.

1.3.4.1 Structural Stability and Disulphide Bridge

A pair of conserved cysteine residues, C110 in TM3 and C187 in EL2, form a disulphide-bridge that further stabilises the tertiary structure of rhodopsin. Substitution of either cysteine causes misfolding and prevents proper translocation to the plasma membrane (Karnik et al., 1988), leading to retinal degeneration *in vivo* (Hwa et al., 1999). These cysteines are conserved in the melanopsin protein sequence (C142 and C220 in mouse OPN4).

1.3.4.2 Glycosylation Sites

In rhodopsin, N2 and N15 in the N-terminus are glycosylated, meaning a short oligosaccharide (carbohydrate molecule) is attached to the asparagine side chains post-translation. The attached oligosaccharide chain extends away from the rhodopsin molecule and does not appear to affect protein folding, although mutation of this site in rhodopsin does lead to decreased activation of transducin (Kaushal et al., 1994). Melanopsin also appears to possess glycosylation sites in N-terminus, discussed in more detail later in this chapter.

1.3.4.3 Extracellular Loop 2

In both bovine and squid rhodopsin, EL2 forms 2 β -pleated strands, β 3 and β 4. β 4 is folded into the transmembrane domain, securing retinal in the chromophore binding pocket (Murakami & Kouyama, 2008; Palczewski et al., 2000). The position of these β -pleated strands is altered upon activation (Ahuja et al., 2009), controlling access to the chromophore binding site and activation of GPCR. Two residues on this loop, R177 and D190, are necessary for thermal stability of rhodopsin in the dark state (Janz et al., 2003) and the side chains of several amino acids in β 4 interact with the retinal chromophore to influence spectral sensitivity, including the invertebrate counterion E181 (Menon et al., 2001). Given that EL2 folding is found in multiple GPCRs (Shi & Javitch, 2004; Shimamura et al., 2011) and several EL2 residues are conserved in melanopsin, it is highly probable EL2 in melanopsin is also folded into the transmembrane domain.

1.3.5 Key Residues in the Opsin Intracellular Domain

The opsin intracellular domain consists of 3 hydrophilic intracellular loops and the C-terminus. This region is functionally important in opsins for G protein coupling and opsin deactivation. Key functional residues in this region are highlighted in **Figure 1.15**, and described in more detail below.

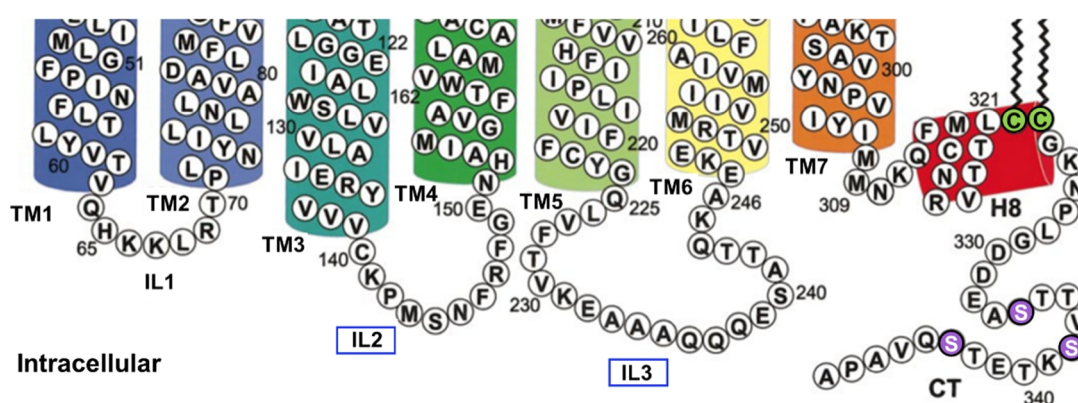


Figure 1.15. Key residues in the bovine rhodopsin intracellular domain. Schematic shows intracellular loops 2 and 3 (highlighted in blue box) involved in G protein specificity. Phosphorylation sites in the C-terminus (CT) necessary for arrestin binding are shown in purple. C-terminus Palmitoylation sites (in green, with zigzag lines) and the 8th alpha helix (H8 in red) are also shown. Adapted from Menon et al. (2001).

1.3.5.1 G protein specificity

In rhodopsin, the intracellular loops, particularly IL2 and IL3, determine which class of G protein the opsin activates. The rhodopsin IL2 sequence appears specific for transducin activation, as deletion or substitution of IL2 residues disrupts rhodopsin signalling (Franke et al., 1992). In comparison, IL3 is much more tolerable of substitutions. Replacing the entire rhodopsin IL3 with the corresponding sequence from another $G\alpha_{i/o}$ -coupled GPCR does not affect rhodopsin activation or transducin signalling (Yamashita et al., 2000). Furthermore, substitution of rhodopsin IL3 with IL3 from β 2-adrenergic receptor, a $G\alpha_s$ – coupled GPCR, leads to $G\alpha_s$ – signalling in response to light (Kim et al., 2005), suggesting the IL3 is particularly important for G protein specificity.

As IL2 and IL3 have highly flexible 3D structures that vary between GPCRs of the same class, their role in G protein coupling must be related to amino acid sequence. However, the specific residues or sequence motifs associated with coupling to each class of $G\alpha$ subunit are currently unknown. One structural feature, which may determine G protein specificity is IL3 length. Invertebrate opsins coupled to $G\alpha_q$, such as squid rhodopsin, have an insertion in IL3 (Murakami & Kouyama, 2008) not seen in vertebrate opsins, which couple to $G\alpha_{i/o}$. Melanopsin also has an IL3 insertion and a $G\alpha_q$ phototransduction cascade, suggesting it may share this invertebrate-like structure-function relationship.

1.3.5.2 C-terminus and phosphorylation sites

The C-terminus in rhodopsin is critical for rhodopsin deactivation. As shown in **Figure 1.16**, serines and threonines in the C-terminus of the activated rhodopsin are first phosphorylated by rhodopsin kinase, *GRK1*. Arrestin then binds to the phosphorylated protein to halt rhodopsin activity (Kühn & Wilden, 1987; Wilden et al., 1986). Mice with mutated rhodopsin phosphorylation sites demonstrate prolonged responses to light because the phototransduction cascade is not deactivated normally (Chen et al., 1995). However, phosphorylation is not required for deactivation in *Drosophila RH1* (Montell, 1999) suggesting arrestin-mediated deactivation is specific to vertebrate opsins.

The C-terminus in melanopsin does not appear to be functionally important for G protein binding, as melanopsin with a truncated C-terminus can still respond to light (Terakita et al., 2008). This region does contain potential protein kinase phosphorylation sites (Provencio et al., 2000). Whether these bind arrestin is unclear. β -arrestin 1 and β -arrestin 2 co-localise with melanopsin in ipRGCs, and co-expression of melanopsin and arrestin decreases the duration of light responses *in vitro* (Cameron & Robinson, 2014b), suggesting melanopsin can use arrestin for deactivation. The functional implications of melanopsin phosphorylation sites are discussed in more detail below.

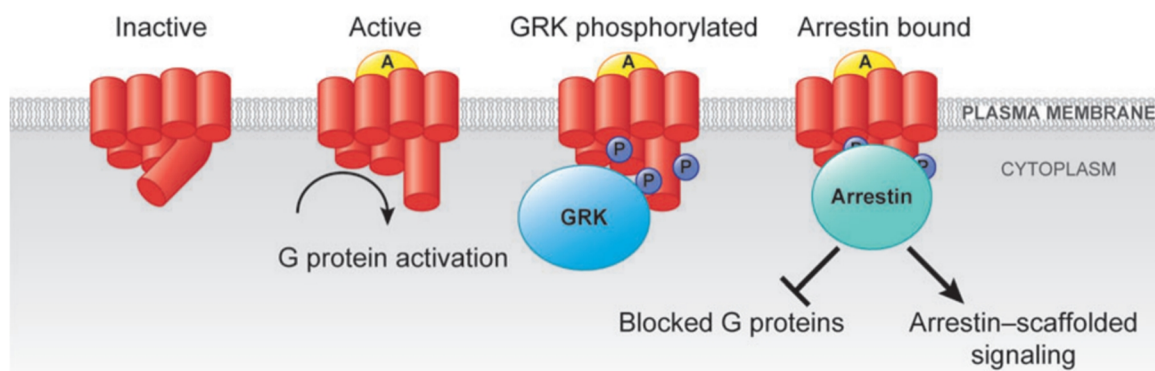


Figure 1.16. Arrestin binding for GPCR deactivation. Binding of ligand (A) or retinal isomerisation leads to GPCR conformation change and G protein activation. G protein coupled receptor kinase (GRK) binds to the active GPCR, phosphorylates (P) the intracellular domain and is then released. The phosphorylated active state GPCR then binds an arrestin protein, which halts G protein activation and triggers G protein independent arrestin signalling. Image reproduced from Premont & Gainetdinov (2007) with permission from Annual Reviews.

1.3.5.3 Eighth α -helix and palmitoylation sites

Both bovine and squid rhodopsin crystal structures reveal an amphipathic 8th α -helix, part of the C-terminus proximal to TM7 (Palczewski et al., 2000; Murakami & Kouyama, 2008). This 8th helix is secured in position by palmitoylation sites, located at C322 and C323 in bovine rhodopsin, and at C337 in squid rhodopsin. This alpha helix is also found in *Drosophila RH1*, suggesting it is a conserved opsin structure. In bovine rhodopsin, this helix has been associated with transducin activation (Marin et al., 2000), while in *Drosophila RH1* it is necessary for correct localisation of protein in plasma membrane (Kock et al., 2009). Melanopsin possesses a proposed palmitoylation site at C364 and the PxYR motif found in *Drosophila RH1*, suggesting it does contain an 8th α -helix.

1.4 Melanopsin structure-function

The structure of rhodopsin has been explored in detail, aided by multiple crystal structures. There has been comparatively little research on melanopsin structure-function. Without a melanopsin crystal structure, critical domains must be explored using site-directed mutagenesis or naturally-occurring sequence changes. This section examines the literature on key functional domains in melanopsin, summarised in **Figure 1.17**.

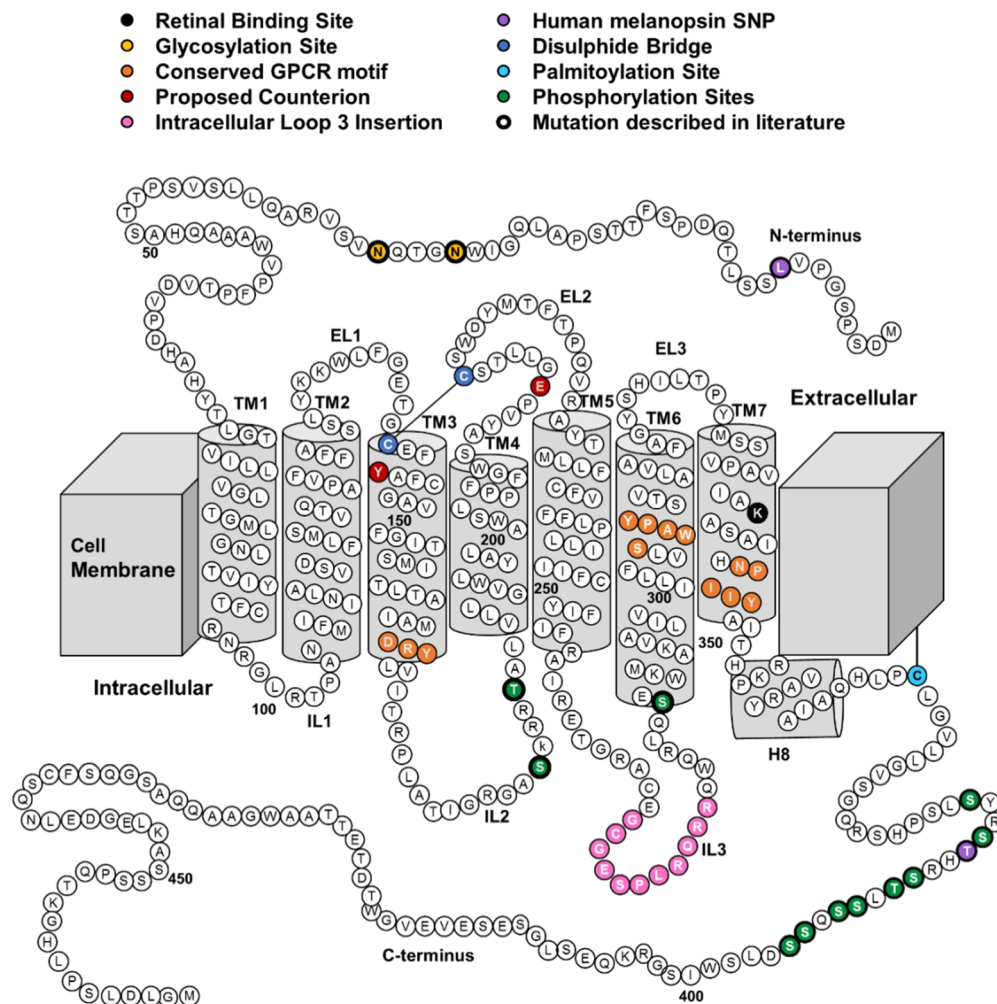


Figure 1.17. Key functional sites in mouse melanopsin. Conserved opsin sites and GPCR motifs in motifs are highlighted, but apart from retinal binding site, none have been investigated in melanopsin. Post-translational modification sites and equivalent position of human melanopsin SNPs described in literature are outlined with thick black lines. Adapted from Peirson et al. (2009) under Creative Commons licence Attribution 4.0 International.

1.4.1 Retinal Binding Site

Mutagenesis studies confirmed melanopsin has a lysine retinal binding site in TM7, highly conserved between opsins. Human OPN4 with K340A mutation, expressed in HEK293 cells, showed no intracellular influx of calcium in response to light, measured using fluorescent calcium imaging (Kumbalasiri et al., 2007). This demonstrated retinal attachment to K340 was necessary for melanopsin-driven responses to light.

1.4.2 Post-translational modification sites

The majority of site-directed mutagenesis studies of melanopsin have focused on post-translational modification sites. These are sites modified by enzymes after protein synthesis, for example the addition of functional groups, such as phosphoryl groups (phosphorylation) or oligosaccharides (glycosylation), which contribute to structural and functional properties of the protein. Their location can sometimes be predicted based on consensus sequences, amino acid motifs that act as enzyme binding sites.

1.4.2.1 Glycosylation sites

Fahrenkrug et al. (2009) explored glycosylation in rat melanopsin at two sites in the N-terminus, N31 and N35. Immunoblotting revealed two melanopsin bands at 49kDa (predicted molecular mass) and 69kDa. Treatment with tunicamycin or PNGase F to prevent N-linked glycosylation, or mutating both N31 and N35 to isoleucine, produced a band at 55kDa, suggesting melanopsin is N-linked glycosylated at one or both of these sites. Further treatment with O-glycosidase and neuraminidase to stop O-linked glycosylation produced 49kDa band matching molecular mass of unmodified melanopsin, indicating melanopsin is both N- and O-linked glycosylated. Deglycosylated melanopsin had same level of *Fos* induction upon light exposure as wildtype melanopsin *in vitro*, suggesting N-linked glycosylation is not necessary for normal melanopsin function.

1.4.2.2 Phosphorylation sites

There is also evidence that melanopsin is phosphorylated both *in vitro* and *in vivo*. This phosphorylation appears to be light-dependent, although phosphorylation has been observed both in dark (Fahrenkrug et al., 2014) and light-adapted retina (Blasic et al., 2012a). The identity of the phosphorylation enzyme is unclear, with reports melanopsin phosphorylation can be blocked by protein kinase A and protein kinase C inhibitors (Fahrenkrug et al., 2014), as well as RNAi based silencing of *GRK2*, a receptor kinase that co-localises with melanopsin in ipRGCs (Blasic et al., 2012a). However, ipRGC-specific ablation of *GRK2* has limited effect on ipRGC responses to light (Sexton & Van Gelder, 2015), suggesting there may be multiple sources of melanopsin phosphorylation *in vivo*.

Site-directed mutagenesis has explored several potential phosphorylation sites *in vitro*. These include T186 in IL2 and S287 in IL3 (Blasic et al., 2012b), and 6 sites (S388, T389, S391, S392, S394 and S395) in the C-terminus of mouse melanopsin (Blasic et al., 2014), and S381 and S394 in the C-terminus of rat melanopsin (Fahrenkrug et al., 2014). Mutation of these sites to alanine disrupts normal melanopsin function *in vitro*. For example, HEK293 cells expressing rat melanopsin with S381A or S394A mutations have attenuated intracellular calcium influx in response to light compared to wildtype melanopsin (Fahrenkrug et al., 2014). Heterologous expression of mouse *Opn4* triple mutant, S381A, S388A and S394A, results in normal response amplitude but delayed deactivation kinetics *in vitro*, not seen by mutation of each site in isolation (Blasic et al., 2012b). A recent *in vivo* study by Mure et al. (2016), published during completion of this thesis, also finds a cluster of C terminus phosphorylation sites are necessary for normal deactivation and is discussed further in Chapter 6. These studies suggest the loss of phosphorylation sites prevents arrestin binding, necessary for normal deactivation of melanopsin. How a rhodopsin-like deactivation mechanism fits with other properties of the melanopsin signalling pathway, long temporal integration, is currently unclear.

1.4.3 Alternative splicing and melanopsin isoforms

One of the most detailed explorations of melanopsin structure-function has been the effect of alternate splicing on the C-terminus of melanopsin. Alternate splicing occurs when exons are included or excluded during transcription to produce multiple RNA products from the same gene. The melanopsin gene is alternatively spliced to produce proteins with different C-terminus lengths, with isoforms found in mice (Pires et al., 2009) and chickens (Torii et al., 2007).

In mouse melanopsin, the two isoforms have identical amino acid sequences up to position 455. The long isoform, OPN4L, has a longer C-terminus tail (521aa total), while the short isoform, OPN4S, has a shorter C-terminus tail (466aa total, Pires et al., 2009). No difference is observed in the responses of *Opn4L* and *Opn4S* expressed in HEK293 cells using fluorescent calcium imaging (Blasic et al., 2014) or patch-clamping of Neuro-2A cells (Pires et al., 2009). *In vivo*, the two isoforms are differentially expressed in ipRGC subtypes, with *Opn4L* preferentially expressed in M2 cells and predominant expression of *Opn4S* in M1 cells (Hughes et al., 2012b). M1 and M2 subtypes project to separate brain targets and may exhibit different functional properties (Schmidt & Kofuji, 2009; Ecker et al., 2010; Estevez et al., 2012), raising the possibility C-terminus elongation may be functionally significant for melanopsin light sensitivity.

A recent study by Jagannath et al. (2015) used RNAi-based silencing to knockdown the long and short isoforms *in vivo* and measure their effects on melanopsin-driven behaviour in *rd/rd cl* mice. A double dissociation was observed with *Opn4L* knockdown attenuating negative masking but not PLR, and *Opn4S* siRNA attenuating PLR but not negative masking. Knockdown of either isoform disrupted phase-shifting and sleep-induction to nocturnal light pulse, suggesting both isoforms contribute to these behaviours. Whether the different behaviours mediated by the two isoforms are a consequence of ipRGC subtype and projection, or melanopsin function is not yet known.

1.4.4 Single nucleotide polymorphisms

In addition to site-directed mutagenesis *in vitro*, two single nucleotide polymorphisms (SNPs) in the human melanopsin gene have been associated with abnormal NIF responses to light. The first of these, P10L, occurs when cytosine (C) is replaced by thymine (T). Roecklein et al. (2009) found 7 individuals possessed the TT genotype (Leucine at position 10) out of 132 patients with seasonal affective disorder (SAD), while 90 healthy controls, matched for demographic variables, only had CC or CT genotype. SAD is a mood disorder where decreased daylength during winter months is associated with increased frequency of depressive episodes (Partonen & Lönngqvist, 1998). Using self-report questionnaires conducted at different times of year, the same group found an interaction of the TT genotype with daylength on sleep onset and chronotype, not seen in CC and CT genotypes (Roecklein et al., 2012). The studies hypothesise this interaction is because P10L alters NIF light input to become more susceptible to seasonal variation in light levels.

The second SNP, T394I, also occurs when cytosine is replaced by thymine. People with the TT genotype (Isoleucine at position 394) appear to have attenuated pupil constriction to a range of white light intensities (Higuchi et al., 2013), as well as high intensity blue and green, but not red, monochromatic light (Lee et al., 2013). Roecklein et al. (2013) reported attenuated post-illumination pupil response to blue, but not red, monochromatic light in individuals with the TT genotype. For all three studies, these pupil deficits were not seen in the CT and CC genotypes. Finally, Lee et al. (2014) found Japanese students with a CC genotype showed later wake times on weekdays, and later sleep midpoint and bed times on weekends, compared to students with CT or TT genotypes. The pupillometry studies of T394I indicate the TT genotype may result in a deficit in melanopsin function, given that it attenuates behaviour partially driven by melanopsin. Collectively, the four papers suggest T394I may be associated with an abnormal melanopsin phenotype.

1.5 Thesis Rationale

The studies discussed in this chapter demonstrate the complex structure-function relationships found in opsin photopigments. It is tempting to generalise many of the findings from crystal structures and site-directed mutagenesis of rhodopsin to melanopsin. Although this approach may be valid for conserved opsin and GPCR motifs, such as the retinal binding site and DRY motifs, it is limited for a number of reasons. First, sequence homology between melanopsin and rhodopsin is low, under 30% (Hankins et al., 2008). Melanopsin also demonstrates a number of structural features not observed in other vertebrate opsins, such as the IL3 insertion and extended C-terminus. Second, melanopsin has a number of unusual functional properties for a vertebrate visual pigment. There is evidence to suggest it uses an invertebrate-like phototransduction cascade, is potentially bistable and capable of prolonged signalling and temporal integration over long durations. The structural features that underlie these characteristics are likely different from regions that define the equivalent properties in rhodopsin.

There is currently very limited data on the unique structure-function relationships in melanopsin. Very few mutagenesis studies have been conducted, with most focusing on post-translational modifications. The few melanopsin SNPs that have been associated with disease or abnormal NIF responses to light have not been investigated *in vitro*, to establish a causal link between mutation and phenotype. With the exception of the retinal binding site (Kumbalasiri et al., 2007), there has been no investigation of critical transmembrane residues in melanopsin and there is currently only one study examining the effect of point mutations on whole-animal physiology (Mure et al., 2016). As the study of melanopsin isoforms demonstrated, the effects of structural changes may only be apparent once tested *in vivo* and it will be crucial to investigate melanopsin structure-function in its native environment.

There are also practical reasons to investigate the key functional domains of melanopsin. Several studies have proposed the use of melanopsin as an optogenetic tool for visual restoration (Lin et al., 2008; Liu et al., 2016), control of neuronal or cellular activity (Tsunematsu et al., 2013; Beiert et al., 2014) and other synthetic biology systems (Ye et al., 2011). Melanopsin is more sensitive than microbial opsins, such as channelrhodopsin (Koizumi et al., 2013), and as a native mammalian photopigment is suitable for therapeutic applications in humans. Maximising the efficacy of melanopsin as an optogenetic tool will require the ability to adapt melanopsin a wide-range of applications, for example by changing the response kinetics or G protein signalling pathway.

Without a melanopsin crystal structure, the best approach to investigate its structure-function relationships is using non-synonymous point mutations. A mutagenesis approach provides greater relevance to human disease, by identifying locations where naturally occurring melanopsin variants may be pathogenic. This approach also allows for more subtle phenotypes, in an attempt to understand the biochemical properties and location of residues that define critical functional properties.

1.6 Thesis Aims

The overall aim of this thesis was to identify and characterise key functional residues in melanopsin. The following objectives were established to accomplish this.

1. Develop strategies for identifying potential critical functional residues

Limited resources mean a targeted approach is required, so only the best candidates for key amino acids are investigated fully *in vivo*. Chapter 3 and 3 describe methods for prioritising mutants for further study.

2. Identify novel mutations in the mouse *Opn4* gene

Melanopsin mutations in the transmembrane region have not yet been studied. Chapter 3 describes a genotype-driven screen of a large mutant mouse archive for new mutants with unusual phenotypes.

3. Characterise naturally-occurring Human *OPN4* genetic variants

Potentially the most important mutations to characterise, as they could be relevant for human disease. Chapter 4 and 4 describe bioinformatics, *in vitro* and *in vivo* characterisation of naturally-occurring human melanopsin mutations.

4. Develop methods to allow high-throughput screening of *OPN4* mutants *in vivo*

The ability to test different mutants in the native ipRGC environment is required to avoid recovery of multiple animal models. Chapter 5 describes a targeted-viral technique developed to accomplish this.

5. Characterise human and mouse *OPN4* mutants *in vivo*

In vivo characterisation is necessary to understand the effect of mutations both on melanopsin function specifically and NIF behaviours as a whole, particularly in the intact retina. Chapter 3 and 4 describe two different mouse models used for behavioural and cellular phenotyping of melanopsin mutants.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Kits used

GenElute Plasmid Miniprep Kit (Sigma-Aldrich)

Quikchange II XL site-directed mutagenesis kit (Stratagene).

QiaQuick PCR purification kit (Qiagen)

QiaQuick Gel Extraction kit (Qiagen)

RNeasy Mini Kit (Qiagen)

qScript cDNA synthesis kit (QuantaBio)

2.1.2 Reagents, Buffers and Solutions

2.1.2.1 Plasmid Preparation

LB Broth

Luria-Bertani (LB) Broth was prepared by dissolving 25g of LB powder (Sigma) in 1L of distilled water, which was then autoclaved. Each litre of LB broth contains 10g Bacto-tryptone, 10g NaCl and 5g Yeast Extract. Appropriate volume of antibiotic was added to the autoclaved LB Broth once cooled.

LB-agar plates

LB-agar plates were prepared by dissolving 7.5g of bacteriological agar (Sigma) in 500ml of LB Broth, which was then autoclaved. The LB-agar was then cooled to 55°C before adding the appropriate volume of antibiotic. Approximately 20ml of LB-agar was then poured into 10cm petri dishes. Plates were allowed to cool and solidify for 30-60mins before inverting and storing in sealed plastic bags at 4°C until needed.

Ampicillin

Ampicillin sodium salt (Sigma) was dissolved in distilled water at 100mg/ml, then filter sterilised through a 0.22µm filter. Ampicillin was diluted 1:1000 when used to give final concentration of 100µg/ml.

Kanamycin

Kanamycin sulphate (Sigma) was dissolved in distilled water at 50mg/ml, then filter sterilised through a 0.22µm filter. Kanamycin was diluted 1:1000 when used to give final concentration of 50µg/ml.

Glycerol stocks

Glycerol stocks were prepared by mixing equal volumes of ≥99% Glycerol (Sigma) with LB broth. The resulting 50% glycerol in LB broth was then aliquoted into cryovials (500µl each). Cryovials were positioned upright with lids partially closed and autoclaved. Once autoclaved, the lids were sealed, ready for 500µl of starter culture to be added to create a glycerol stock.

Resuspension and Wash Solutions

Before either the GenElute miniprep or midiprep kits (Sigma) could be used, 13µl of RNase A solution (supplied as part of kit) had to be added for every 2.5ml of Resuspension solution. 10ml of 100% ethanol also had to be added to for every 2.5ml of Wash solution concentrate.

2.1.2.2 Gel Electrophoresis

TAE buffer

TAE (Tris-acetate-EDTA) buffer was prepared by diluting TAE Buffer 50X (Thermo Fischer) in double distilled H₂O using 1:50 dilution. Final concentration of 1x TAE is 40 mM Tris, 20 mM acetic acid, 1 mM EDTA.

2.1.2.3 PCR purification and Gel extraction

Buffer PE

Before either the QiaQuick PCR purification or QiaQuick Gel extraction kits could be used, 48ml of 100% ethanol was added for every 6ml of Buffer PE concentrate.

2.1.2.4 RNA extraction

On-Column DNase Digest

During RNA extraction, an on-column DNase digest step was performed using the RNase-Free DNase set (Qiagen). This DNase stock solution was prepared by dissolving the lyophilized *DNaseI* (1500 Kunitz units) in 550µl of RNase free water. Since the *DNaseI* is sealed in a glass vial, an RNase-free needle and syringe was used to add water to enzyme. The solution was mixed by inverting gently. The DNase stock solution was then removed from vial using a sterile needle and syringe and divided into 50µl aliquots and stored at -20°C.

2.1.2.5 Calcium Imaging

Probenecid

Probenecid solution was prepared by dissolving 77mg of desiccated Probenecid (Invitrogen) in 1ml of Hanks Buffered Saline Solution (HBSS). The solution was vortexed before use and stored at 4°C until required.

Pluronic F-127

Pluronic F-127 was purchased as a 20% solution in Dimethyl Sulfoxide (DMSO) from Invitrogen. Pluronic F-127 was stored at room temperature and vortexed before use.

9-cis retinal

9-*cis* retinal (Sigma) was dissolved in 100% ethanol to a concentration of 40nM. Preparation of 9-*cis* retinal was carried out in dim red light to prevent photoisomerisation. Aliquots of 9-*cis* retinal were stored wrapped in foil at -20°C.

2.1.2.6 Immunocytochemistry

PBS with 0.02% (v/v) Triton

PBS (pH 7.45) was prepared by dissolving 5g of PBS tablets (Gibco) in a litre of distilled water, which was then autoclaved. 2ml of Triton X-100 (Sigma) was then added per litre of PBS. The solution was mixed using a magnetic stirrer, then stored at room temperature until needed.

PBS with 0.05% Tween-20

0.5ml of Tween-20 (Sigma) was added to 1L PBS (prepared as above). The solution was mixed using a magnetic stirrer, then stored at room temperature until needed.

2.1.2.7 Multi-electrode Arrays

Ames Media

Ames media powder was kept at 4°C until needed. 1L of water was gassed with 95% O₂, 5% CO₂ before adding 1.9g of sodium bicarbonate (Sigma). 8.8g of Ames media powder was then added and stirred until fully dissolved. 2ml of solution was used to rinse any traces of powder from the original container. Ames media was prepared fresh for each experiment.

2.1.3 Vector Information

2.1.3.1 pIRES2-acGFP

pIRES2-acGFP (BD Biosciences) possesses an internal ribosome entry site (IRES), which allows simultaneous but separate expression of a GFP reporter derived from jellyfish *Aequorea coerulescens* (acGFP) with gene-of-interest (cloned into multiple cloning site) from a single bicistronic RNA transcript. As shown in **Figure 2.1**, Expression of gene-of-interest is driven by the immediate early promoter of human cytomegalovirus (CMV). This vector also contains a neomycin-kanamycin resistance gene for positive selection, SV40 polyadenylation signals downstream of acGFP for proper processing of bicistronic mRNA and an SV40 origin for replication in mammalian cells expressing SV40 T antigen.

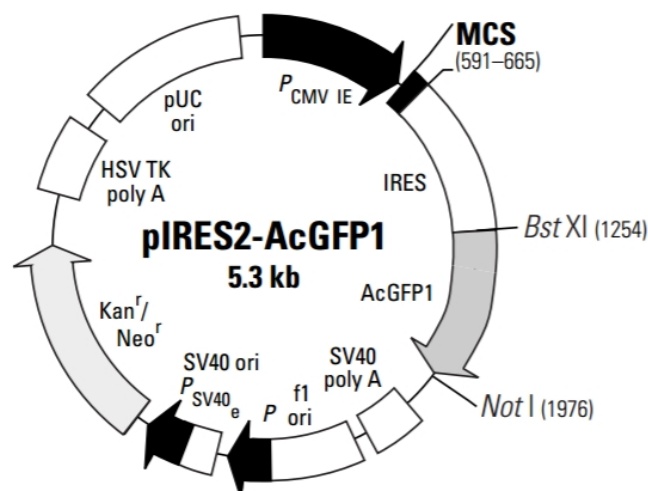


Figure 2.1. pIRES2-acGFP expression vector from Clontech (formerly BD Biosciences). Obtained from http://www.clontech.com/NL/Products/Fluorescent_Proteins_and_Reporters/Coexpression/IRES_Bicistronic?sitex=μ23:22372:US#

2.1.3.2 pcDNA3.1

pcDNA3.1 (Invitrogen) is a mammalian expression vector. As shown in **Figure 2.2**, it possesses a Human cytomegalovirus immediate-early (CMV) promoter that drives constitutive expression of the gene-of-interest at high-levels. This vector also contains a neomycin-ampicillin resistance gene for positive selection, an SV40 origin for replication in mammalian cells expressing SV40 T antigen and a Bovine Growth Hormone (BGH) polyadenylation signal and transcription termination sequence for enhanced mRNA stability.

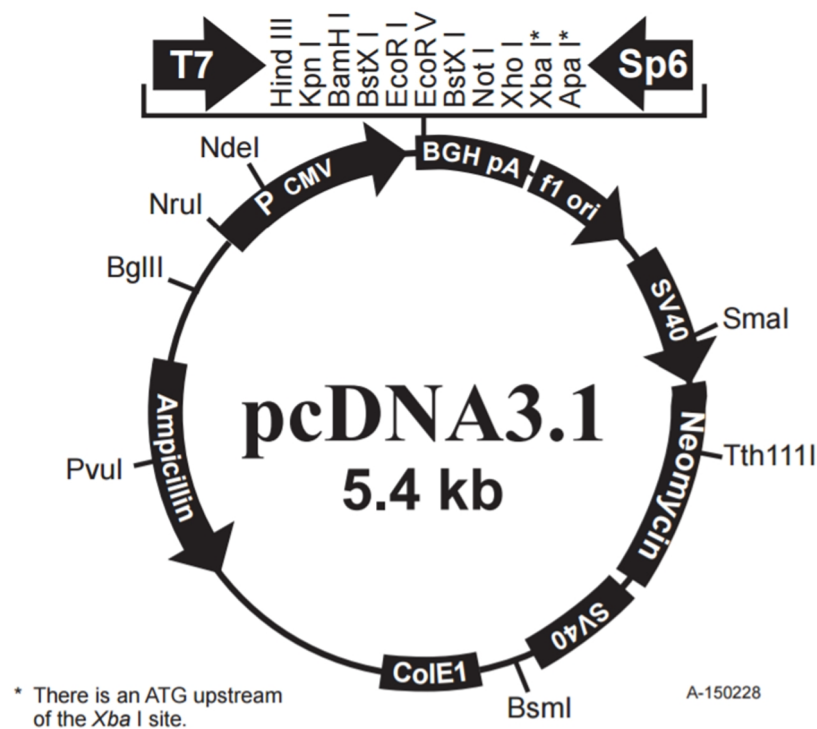


Figure 2.2. pcDNA3.1 vector map from Invitrogen. Obtained from <https://tools.thermofisher.com/content/sfs/vectors/pcdna3.pdf>.

2.1.3.3 pAAV-EF1 α -DIO

pAAV-EF1 α -DIO (Addgene) is an adeno-associated viral vector. As shown in **Figure 2.3**, Expression of double-floxed inverted ORF (DIO) is driven by ubiquitous EF1 α promoter in presence of Cre-recombinase. This vector also includes a woodchuck hepatitis B virus post-transcriptional regulatory element (WPRE), which facilitates nuclear export of viral RNA and increases expression of transgene (Zufferey et al., 1999) and poly(A) sequence between two inverted terminal repeats (ITRs). The two ITRs contain the necessary *cis*-acting elements for viral replication and packaging.

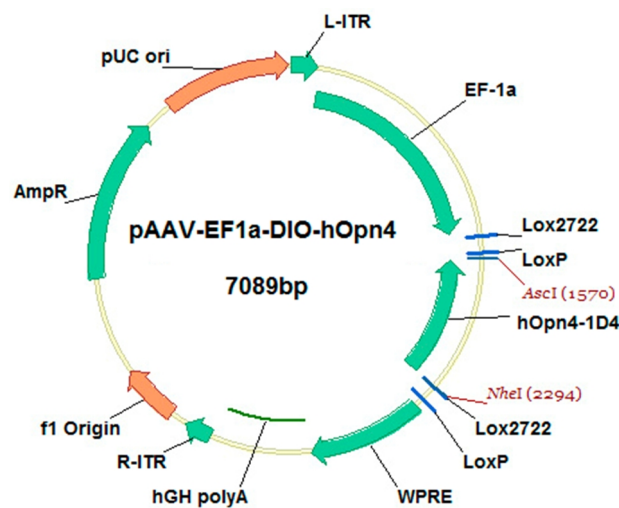


Figure 2.3. pAAV-EF1 α -DIO vector map from Addgene. Adapted from <https://www.addgene.org/50462/>.

2.2 Methods

This section describes the principles and protocols for techniques used throughout this thesis. Specific protocol details are provided in the relevant Methods section of each experimental chapter.

2.2.1 Plasmid Preparation

2.2.1.1 Bacterial Transformation

DH5 α competent cells (Invitrogen) were used for transformation. After thawing cells on ice, 50 μ l was aliquoted into pre-chilled 1.5ml microcentrifuge tubes. 10ng of DNA (1-5 μ l) was added to cells and mixed gently with a pipette tip. Transformation reactions were incubated on ice for 30mins, then heat shocked in a 42°C water bath for 20s. Transformation reactions were then cooled on ice for 2mins, before 950 μ l of SOC media (pre-warmed to 37°C) was added to each reaction. Tubes were then incubated for 1 hour at 37°C shaking at 225rpm. Between 50-100 μ l was spread on LB-agar plates containing appropriate antibiotic (100 μ g/ml for ampicillin, 50 μ g/ml for kanamycin). Plates were then incubated inverted overnight at 37°C.

2.2.1.2 Bacterial Cultures and Glycerol Stocks

Single colonies were then picked from LB-agar plates to produce starter cultures. A sterile pipette tip was used to transfer an individual bacterial colony to 6ml of sterile LB broth with appropriate antibiotic (100 μ g/ml for ampicillin, 50 μ g/ml for kanamycin) and grown overnight at 37°C shaking at 225rpm. The next day, 500 μ l of bacterial culture was added to 500 μ l autoclaved LB broth with 50% glycerol (Sigma Aldrich) in cryovials to create a 25% glycerol stock, which were stored at -80°C. If more bacterial culture was necessary, a small chunk of frozen glycerol stock was scraped from inside the cryovial using a sterile pipette tip and added to 6ml sterile LB-broth with antibiotic, avoiding the need for an additional transformation reaction.

If only a small amount of plasmid was required, i.e. minipreps, the remaining 5.5ml of starter culture was centrifuged for 1min at $\geq 12\,000 \times g$. The supernatant was discarded and cell pellets were stored at -20°C until required.

If a larger amount of plasmid was required, i.e. for midipreps, then 75 μl of starter culture was used to inoculate 50ml of sterile LB broth containing appropriate antibiotic for selection (100 $\mu\text{g}/\text{ml}$ for ampicillin, 50 $\mu\text{g}/\text{ml}$ for kanamycin). This large volume of bacterial culture was then incubated overnight at 37°C shaking at 225rpm. The bacterial culture was then transferred to a 50ml Falcon tube and spun down at $3000 \times g$ for 20mins. The supernatant was discarded and cell pellets were stored at -20°C until required.

2.2.1.3 Minipreps

Minipreps were conducted using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) according to manufacturer's instructions. Cell pellets from 5ml of starter culture were first resuspended in 200 μl Resuspension solution (with RNase A) and vortexed until homogenous. Cells were then lysed in 200 μl Lysis buffer, immediately mixed by inverting 6-8 times and left for up to 5mins. 350 μl of Neutralisation buffer was then added and mixed by inverting 4-6 times. The tubes were then centrifuged for 10mins at $\geq 12000 \times g$.

Binding columns were prepared by adding 500 μl Column Preparation solution to each column and centrifuging at $\geq 12000 \times g$ for 1min. The clear lysate was then transferred to each prepared miniprep column (up to 750 μl at a time) and centrifuged at $12000 \times g$ for 1min. Flow-through was discarded and more lysate loaded and centrifuged, if necessary. 500 μl of Optional Wash solution was then applied to each column and centrifuged at $12000 \times g$ for 1min. Flow-through was discarded, then 750 μl of Wash solution (with ethanol) was applied to each column and centrifuged at $12000 \times g$ for 1min. Flow-through was again discarded, then each column was centrifuged at $12000 \times g$ for 1min to dry the column membrane and remove excess alcohol.

The column was transferred to new sterile 1.5ml microcentrifuge tube and 50µl of Elution solution was applied directly to the column membrane and left at room temperature for 1min before being centrifuged at 12000 x *g* for 1min to elute plasmid. The full volume of flow-through containing plasmid DNA was then reappplied to the column membrane and centrifuged at 12000 x *g* for 1min to increase the plasmid concentration. DNA concentration was then quantified using a UV-Vis spectrophotometer (Nanodrop). Plasmids were stored at -20°C until needed.

2.2.1.4 Midipreps

Midipreps were conducted using the GenElute HP Plasmid midiprep kit (Sigma-Aldrich) according to manufacturer's instructions. Cell pellets from 50ml of starter culture were resuspended in 4ml of Resuspension solution (with RNase A) and vortexed until homogenous. Bacterial cells were lysed using 4ml of Lysis solution and inverted 6-8 times to mix, then left for up to 5mins. Lysis was neutralised by adding 4ml of pre-chilled Neutralisation buffer (4°C) and inverting the mixture 4-6 times. 3ml of Binding solution was then added and mixed by inversion, the entire mixture was then poured into the barrel of an upright filter syringe with plunger removed. The lysate was then left for 5mins at room temperature. The midiprep columns were prepared by adding 4ml of Column preparation solution to column and centrifuging at 3000 x *g* for 2mins, then discarding the flow-through. The filter syringe was then positioned over the midiprep column and the plunger used to expel half of the cleared lysate into the midiprep column, withdrawing the plunger gently to stop the flow of liquid and prevent over-filling.

The column was then centrifuged at 3000 x *g* for 2mins and flow-through was discarded. The column was then filled with the remaining cleared lysate and centrifuged for 3000 x *g* for 2mins, again discarding the flow-through. 4ml of Wash solution 1 was then applied to the column, which was centrifuged for 2mins at 3000 x *g* and flow-through discarded. 4ml of Wash solution 2 (with ethanol) was then applied to the column, which was centrifuged for 2mins at 3000 x *g* and flow-through was discarded.

The midiprep column was then transferred to a new sterile 15ml Falcon tube and 1ml of Elution solution was applied directly to the column membrane and left at room temperature for 1min before being centrifuged at 3000 x *g* for 5mins to elute plasmid. The full volume of flow-through containing plasmid DNA was then reapplied to the column membrane and centrifuged at 3000 x *g* for 5min a second time to increase plasmid concentration. The plasmid was then transferred to a sterile 1.5ml microcentrifuge tube and stored at -20°C until needed.

2.2.2 Sequencing

2.2.2.1 Principles

Presence of mutations was confirmed using Sanger Sequencing, a chain termination sequencing method. Using this approach, DNA is replicated in the presence of a mix of nucleotide and dideoxynucleotide bases. Dideoxynucleotide bases have a hydrogen instead of a hydroxyl (OH) group attached to the 3' carbon, which prevents the formation of a phosphodiester bond between nucleotides. When dideoxynucleotides are included in a DNA sequence, DNA polymerase can no longer extend the DNA strand. During Sanger sequencing, dideoxynucleotides labelled with different fluorescent dyes and unlabelled nucleotides are randomly incorporated into the DNA sequence, resulting in multiple copies of the target DNA sequence of different lengths. Eventually all possible chain-termination molecules are produced, so that once ordered from shortest to longest using capillary electrophoresis, analysing the fluorescence of the final dideoxynucleotide base of each copy reveals the original DNA sequence.

2.2.2.2 Sequencing reactions

Sequencing was performed by Source Biosciences. For each sequencing reaction, 5µl of template DNA (100ng/µl) and 5µl of sequencing primer (3.2µM) were provided. Sequencing primers were designed according to the following criteria:

- 1) Primers should be 18-22bp long to increase binding specificity
- 2) Primers should have GC content of 40-60%
- 3) Presence of G or C base within last 5 bases from 3' end of primer to improve binding.
- 4) Minimal secondary structure

The specific primers used for sequencing reactions are detailed in the methods section of each Chapter. Results of each sequencing reaction were analysed using Geneious software version 6.1 (Biomatters Ltd).

2.2.3 Subcloning into pcDNA3.1

2.2.3.1 Overview

Occasionally it was necessary to modify a gene-of-interest and/or move it into the pcDNA3.1 vector (Invitrogen). This was accomplished by subcloning using restriction enzyme digests. To briefly summarise the full method outlined below, the entire gene-of-interest was first amplified from the donor plasmid using oligonucleotide primers containing restriction enzymes sites absent from the gene-of-interest. This produced a PCR product flanked by restriction enzyme sites necessary for ligating the insert into the linearized recipient vector. The PCR product was then purified to isolate it from the rest of PCR reaction. Both purified PCR product and the target vector were digested using the same restriction enzymes. The linearized target vector and digested insert were then isolated by gel purification and ligated to fuse the two together. The ligated plasmids were then transformed in XL10 Gold competent cells and grown overnight on LB-agar plates with ampicillin (100µg/ml). Individual colonies were then screened using colony PCR to confirm the presence of insert in the pcDNA3.1 vector, before plasmid purification (Section 2.2.1) and sequencing (Section 2.2.2).

2.2.3.2 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a method for amplifying and isolating specific DNA sequences. The region amplified can be controlled using oligonucleotide primers that hybridise to specific sequences on opposite strands of DNA and flank the target region. PCR consists of three temperature stages. First, a high temperature stage (95°C) which causes the double-stranded DNA molecule to denature and separate into single strands. In the second stage, the oligonucleotide primers anneal to the corresponding regions on each DNA strand. The temperature of this stage is specific to the primers, with lower temperatures increasing the likelihood of non-specific binding outside the target region and higher temperatures decreasing probability of primer-annealing. The final stage is an elongation step, where the DNA sequence is extended from the primer in a 5' to 3' direction by a DNA polymerase enzyme using nucleoside triphosphates present in the reaction mixture. The temperature of this stage is specific to the DNA polymerase enzyme used. The result of these three temperature stages is a double-stranded copy of the target DNA sequence. After the first round of thermal cycling, there is double the amount of target DNA (the original plus the copy), both of which will then be denatured and undergo primer annealing and elongation leading to four times the original quantity of target DNA. The amount of target DNA increases exponentially with each round of thermal cycling, with the target amplified 2^n -fold, where n = number of cycles.

2.2.3.3 PCR to add Restriction Enzyme Sites

In order to clone the target gene-of-interest into the pcDNA3.1 vector, restriction enzyme sites need to be added to the start and end of the DNA sequence. When cloning an open-reading frame (ORF), this means adding restriction enzyme sites just before the start codon (ATG) and just after the stop codon (eg: TGA). For cloning modified genes-of-interest, e.g. creating a C-terminal truncation, a stop codon and restriction enzyme site need to be added after the chosen truncation point.

The addition of restriction enzyme sites to the ORF is accomplished by amplifying the products using oligonucleotide primers that partially anneal to the ends of the target sequence (18-22bp), but also contain the sequence for a restriction enzyme site of choice (usually 6-8bp) followed by a short leader sequence (3-6bp) to assist with restriction enzyme digest. It is important that the chosen restriction enzyme sites do not also occur within the gene-of-interest insert.

For cloning into pcDNA3.1, *EcoRI* and *NotI* restriction enzymes were used. An *EcoRI* site was added to the 5' end of the target sequence by designing a forward primer containing the leader sequence (italics), then the *EcoRI* site (bold) before the first 18-21bp of the target ORF (underlined), eg: 5' – **AACGAATTC**ATGGACTCTCCTTCAGGACCA – 3'. A *NotI* site was added to the 3' end of the target sequence by designing a reverse primer that is the reverse complement of the last 18-21bp of the ORF (underlined), followed by the *NotI* site (bold) and finally the leader sequence (italics), eg: 5' – **TATGCGGCCG**CCTACATCCCGAGATCCAGACT.

The following reaction conditions were used for the PCR to add restriction enzyme sites: 10ng of plasmid template, 5µl of 10x Reaction buffer, 3µl of MgSO₄ (25mM), 5µl dNTPs (2mM each), 1.5µl of Forward primer (containing *EcoRI* site, 10µM), 1.5µl of Reverse primer (containing *NotI* site, 10µM), 1µl of *KOD* Hot-Start polymerase (Novagen) and ddH₂O to final volume of 50µl.

The thermal cycling conditions were 95°C for 2mins, then 35 cycles of 95°C for 20s, 60°C for 10s and 70°C for 35s. All thermal cycling was carried out using a T100 Thermal cycler (Bio-Rad).

2.2.3.4 DNA Electrophoresis

To confirm amplification of the target region, PCR products were size-sorted using agarose gel electrophoresis. A 1% (w/v) agarose gel was prepared by microwaving 2g of agarose (Thermo Scientific) in 200ml of 1 x TAE buffer in a conical flask until the agarose completely dissolved and the solution was transparent. 4µl of ethidium bromide (final concentration 0.05µg/ml, Sigma-Aldrich) was added to the agarose-TAE solution once cooled for 10mins at room temperature and mixed by gently swirling the conical flask. Ethidium bromide is a fluorescent dye that intercalates between DNA bases, allowing visualisation of DNA using an ultraviolet transilluminator. The agarose solution was then poured in a gel tray with well comb in place and allowed to solidify for 30mins at room temperature. The gel was submerged in 1xTAE in a gel tank and the well comb removed before use. 10µl of PCR product was mixed with 2µl of OrangeG loading dye (New England Bioscience) and pipetted into each well. 10µl of a HighRanger 1KB ladder (Norgen Biotek Corp.) was pipetted into a well next to the PCR products for size reference. An electric field was then applied to the gel (100mV) that caused the negatively charged DNA to be drawn towards the anode, and different size DNA fragments to be separated as they move at different speeds through the agarose gel. The gel was imaged using a digital camera attached to a UV gel documentation system.

2.2.3.5 PCR purification

PCR products with added restriction enzyme sites were then purified using QiaQuick PCR purification kit (Qiagen). This kit binds up to 10µg DNA (100bp to 10kb) to a silica-membrane, allowing removal of other components of the PCR reaction, such as primers and nucleotides. 5 volumes of Buffer PB were added to each volume of PCR reaction and mixed by pipetting up and down. The sample was then applied to the QiaQuick spin column (up to 750µl at a time) and centrifuged for 1min at 13000rpm.

The flow-through was discarded, and 750µl of wash Buffer PE (with ethanol) was added to column, then centrifuged for 1min at 13000rpm. Flow-through was discarded and the column spun for a further 1min at 13000rpm to remove residual wash buffer. Each column was then transferred to a new sterile 1.5ml microcentrifuge tube, 40µl of Buffer EB was pipetted directly onto the membrane of each column and left for 1min at room temperature. Purified PCR product was then eluted by centrifuging at 13000rpm for 1min. Purified samples were stored at -20°C until required.

2.2.3.6 Restriction Enzyme Digest

Restriction endonucleases are enzymes which digest double-stranded DNA at specific recognition sequences (typically 4-8bp). The fragments of cleaved DNA can then be separated using DNA electrophoresis (Section 2.2.3.4.) for visualisation or gel extraction. The *EcoRI* and *NotI* digests were performed simultaneously using the following reaction conditions: 40µl purified PCR product or 1µg of recipient plasmid (pcDNA3.1), 1µl of *EcoRI* High Fidelity enzyme (NEB), 1µl *NotI* High Fidelity enzyme (NEB), 5µl CutSmart buffer (NEB) and ddH₂O to final volume of 50µl.

A large volume of restriction digest reaction was set up to ensure sufficient DNA was available after gel extraction for the ligation reaction. The reactions were mixed by pipetting gently up and down, then incubated at 37°C for 1hour. The restriction enzymes were then heat-inactivated by incubating at 65°C for 20mins. The digested fragments were then separated using gel electrophoresis (see Section 2.2.3.4.). Digested samples were stored at -20°C until required.

2.2.3.7 Gel Extraction

The digested insert and linearized pcDNA3.1 vector were isolated for ligation using the QiaQuick Gel Extraction kit (Qiagen). The entire 50µl of restriction digest reaction was mixed with 10µl of loading dye, then loaded into wells formed using a wide well comb. Where possible, alternate wells were loaded to leave space for cutting out appropriate bands. The gel was then run as described in Section 2.2.3.4., until the bands were sufficiently separate for isolation. Using a UV transilluminator, the bands were visualised and the correct-sized bands corresponding to the digested insert and linearized vector were excised from the gel using a sterile scalpel blade.

Each block of gel containing a band-of-interest was then placed in a 1.5ml microcentrifuge tube and labelled appropriately. The weight of the gel fragment was then calculated by subtracting the weight of an empty microcentrifuge tube from the total weight of the gel fragment plus microcentrifuge tube.

3 volumes of Buffer QG were added for each volume of gel (100mg of gel ~ 100µl), then incubated at 50°C for 10mins in a heat block. The tube was vortexed every 2mins to aid dissolving of the gel. 1 volume of isopropanol was then added to the tube for every volume of sample and mixed (if necessary, reaction was transferred to a 15ml Falcon tube). Up to 750µl of each reaction was then transferred to a QiaQuick spin column and centrifuged at 13000rpm for 1min. Flow-through was discarded and if necessary, loading of column repeated until no sample remained. 750µl of Buffer PE was added to the column, left for 5mins at room temperature, and then centrifuged for 1min at 13000rpm. Flow-through was discarded. The column was then transferred to a new sterile 1.5ml microcentrifuge tube. 50µl of Buffer EB was pipetted directly onto the membrane of each column and left for 5mins at room temperature, then centrifuged at 13000rpm for 1min. Purified digested samples were stored at -20°C until required.

2.2.3.8 Ligation

The purified digested insert and linearized pcDNA3.1 vector were then ligated in a 10µl reaction. The following reaction conditions were used: 1µl of T4 DNA Ligase enzyme, 1µl of 10x T4 Ligase Buffer, 6µl of digested insert, 1µl of linearized vector and 1µl of ddH₂O. Two negative control reactions were also setup using the same conditions but without insert or vector to confirm there was no contamination of reaction with intact plasmid or re-circularised vector. Reactions were mixed by gently pipetting up and down, then spun down and incubated overnight at room temperature. Ligation reactions were then stored at -20°C until required.

2.2.3.9 Transformation

The ligation reaction was transformed in 50µl of XL10 Gold competent cells (Agilent) thawed on ice. 2µl of β-mercaptoethanol (Sigma-Aldrich) was added to each aliquot of XL10 Gold cells, which were left on ice for 10mins before adding 2-4µl of the ligation reaction. The transformation reaction was then incubated on ice for 30mins, before being heat-shocked at 42°C for 30s and placed on ice for 2mins. 500µl of pre-warmed SOC media (37-42°C) was then added and each transformation reaction was incubated for 1hour at 37 °C shaking at 225rpm. 100µl of each transformation reaction was then spread on LB-agar plates containing ampicillin (100µg/µl) and incubated inverted overnight at 37°C.

2.2.3.10 PCR Colony Screen

To confirm presence of the insert in the ligated plasmid before plasmid purification, a PCR colony screen was conducted. Individual bacterial colonies were picked using a sterile pipette tip and used to inoculate 50µl of sterile ddH₂O to create a small bacterial culture. The colony screen PCR reaction was then set up using following conditions: 10µl of ImmoMix reaction mix (Bioline), 0.6µl of Dimethyl sulfoxide (DMSO), 1µl of Forward primer used in Section 2.2.3.3 (10µM), 1µl of Reverse primer used in Section 2.2.3.3 (10µM), 1µl of bacterial culture and 6.4µl of ddH₂O.

The thermal cycling conditions were 95°C for 10mins, then 25 cycles of 95°C for 30s, 60°C for 30s and 72°C for 30s and finally 72 °C for 10mins, carried out using a T100 Thermal cycler (Bio-Rad). Presence of insert within ligated plasmid was confirmed by presence of bands corresponding to correct insert size after gel electrophoresis. If appropriate bands were identified after colony PCR screen, the remaining 49µl of bacterial culture was used to create a 6ml starter culture (Section 2.2.1.2.) for plasmid purification (Section 2.2.1.3) and sequencing (Section 2.2.2.).

2.2.4 Site-directed mutagenesis

2.2.4.1 Principles

Site-directed mutagenesis was performed using a Quikchange II XL site-directed mutagenesis kit (Stratagene). Point mutations in a double-stranded plasmid were introduced using two synthetic oligonucleotide primers, complementary to each strand of the vector. Each primer possesses the desired base pair change or changes. During mutant strand synthesis, the template DNA was first denatured, allowing mutagenesis primers to anneal. The primers were then extended using high-fidelity *PfuUltra* DNA polymerase to create a copy of the entire template plasmid with required mutation. This mutant copy also contains staggered nicks, where the phosphodiester bond is missing between neighbouring nucleotides of a single strand. Once thermal cycling was completed, the sample was treated with *Dpn1* endonuclease, which specifically digests methylated and hemi-methylated DNA and removes the parental DNA template, which is methylated after isolation from *E.coli*. Finally, the nicked DNA vector was transformed into XL10 Gold competent cells, which repair the nicks in the mutant plasmid.

2.2.4.2 Mutagenesis Primer Design

Primers were designed using Quikchange Primer Design Program, <http://www.genomics.agilent.com/primerDesignProgram.jsp>, according to the following criteria.

- 1) The two primers must both contain the desired mutation and anneal to the same sequence of opposite plasmid strands.
- 2) Primers should be between 25 and 40 base pairs long, with melting temperature (T_m) $\geq 78^\circ\text{C}$, where $T_m = 81.5 + 0.41(\%GC) - (675/N) - \%mismatch$, and N = primer length and values for $\%GC$ and $\%mismatch$ are whole numbers
- 3) The desired mutation is positioned in the middle of the primer with a minimum of 10 bases of unaltered sequence either side
- 4) Primers should have minimum GC content of 40% and should terminate in at least one G or C base.

Oligonucleotide primers were PAGE (polyacrylamide gel electrophoresis) purified and obtained at a 0.05 μmole scale from Sigma Aldrich.

2.2.4.3 Mutant Strand Synthesis (Thermal Cycling)

The conditions for the Mutant strand synthesis reaction were: 5 μl of 10 $\times$ reaction buffer, 10ng of DNA template, 125ng of mutagenesis primer 1, 125ng of oligonucleotide primer 2, 1 μl of dNTP mix, 3 μl of QuikSolution, 1 μl of *PfuUltra* DNA polymerase (2.5 U/ μl) and double distilled H_2O (dd H_2O) to a final volume of 50 μl .

The thermal cycling conditions were 95 $^\circ\text{C}$ for 1min, then 18 cycles of 95 $^\circ\text{C}$ for 50s, 60 $^\circ\text{C}$ for 50s and 68 $^\circ\text{C}$ for 1min/kb of plasmid length (eg: 7min for 6886bp template plasmid), and finally 68 $^\circ\text{C}$ for 7mins. Thermal cycling was carried out using a T100 thermal cycler (Bio-Rad).

2.2.4.4 *DpnI* digest

After mutant strand synthesis, 1µl of *DpnI* enzyme (10 U/µl, provided with Quikchange kit) was added directly to each 50µl PCR reaction. The enzyme was mixed with the reaction by pipetting up and down repeatedly. Reactions were then spun for 1min in a microcentrifuge, and incubated at 37°C for 1 hour to digest the methylated template plasmid.

2.2.4.5 Transformation in XL10 Gold Cells

Aliquots of XL10-Gold competent cells (provided with Quikchange kit) were thawed on ice, then 45µl of cells per transformation reaction were aliquoted into pre-chilled 15ml Falcon tubes on ice. 2µl of β-mercaptoethanol (provided with Quikchange kit) was added to the 45µl of cells, mixed gently with a pipette tip and then incubated on ice for 10mins. 2µl of *DpnI* treated DNA was added to each aliquot of competent cells. Transformation efficiency of cells was confirmed by adding 1µl of pUC18 control plasmid (0.01ng/µl) to another 45µl aliquot of cells.

Transformation reactions were mixed gently with a pipette tip, then incubated on ice for 30mins. Each reaction was then heat shocked in a 42°C water bath for 30s, followed by a 2min incubation on ice. 500µl of SOC media (preheated to 37°C) was added to each reaction, then incubated at 37°C for 1 hour at 225–250 rpm. 100µl of each transformation reaction was then spread on LB-agar plates containing ampicillin (100µg/ml) or kanamycin (50µg/ml). Plates were incubated inverted at 37°C overnight (~15hrs) and colonies were selected the next day. Plasmids were then isolated (Section 2.2.1) and sequenced (Section 2.2.2), as described previously.

2.2.5 Genotyping

2.2.5.1 Genomic DNA extraction

Ear notches or tail tips were transferred to a sterile 1.5ml microcentrifuge tube. Tissue was then covered with 100µl of NaOH (50mM, pH12) and incubated at 95 °C for 1hour in a heat block. Each tube was then vortexed for ~10s, then centrifuged at 13000rpm for 2mins. 10µl of Tris-HCl (pH6) was then added to each tube, which were vortexed for ~10s, then centrifuged at 13000rpm for 2mins. 500µl of sterile ddH₂O was then added to each tube. Genomic DNA was stored at -20 °C until required.

2.2.5.2 Rodless coneless (*rd/rd cl*) genotyping

Genomic DNA was extracted as described in section 2.2.5.1. Rodless coneless (*rd/rd cl*) mice were genotyped for the *Pde6b* rd1 mutation (*rd*) and a diphtheria toxin transgene under control of human LWS cone opsin promotor (*cl*). For *rd* genotyping, a multiplex PCR was performed, using one forward primer (R3, 5'-TGACAATTACTCCTTTTCCCTCAGTCT) and two reverse primers (RD4, 5' – GTAAACAGC AAGAGGCTTTATTGGGAA and RD6, 5' - GCATTAATTCTGGGGCGCATG). The RD6 primer binds to a region present only in the wild-type *Pde6* allele, while the RD4 primer binds to a sequence present only in the mutant *Pde6b*^{rd1} allele.

The following reaction conditions were used for *rd* genotyping: 6.25µl ImmoMix reaction mix (Bioline), 3.75µl ddH₂O, 0.5µl Primer RD3 (20µM), 0.5µl Primer RD4 (20µM), 0.5µl Primer RD6 (20µM) and 1µl genomic DNA. Thermal cycling conditions were: 95°C for 10mins, then 35 cycles of 94°C for 45s, 60°C for 1min and 72°C for 15s, followed by 72°C for 10mins, carried out using a T100 Thermal cycler (Bio-Rad).

The product amplified by RD3/RD4 primer pair is 0.55Kb long, while the product amplified by the RD3/RD6 primer pair is 0.4Kb long. Therefore, multiplex-PCR of genomic DNA from mice with genotype of *Pde6b*^{rd1/rd1} will produce a single band of 0.55Kb, *Pde6b*^{WT/WT} will produce a single band of 0.4Kb, and *Pde6b*^{WT/rd1} will produce two bands of 0.55Kb and 0.4Kb. The number and size of PCR products can be visualised using gel electrophoresis, as described in Section 2.2.3.4., and used to genotype animals for the *rd1* allele.

The presence of *c/* transgene is also determined using PCR and gel electrophoresis. Instead of using a multiplex PCR, the *c/* genotyping uses primers (Forward, 5' – GTACCACGGGACTAAACC, and Reverse, 5' – ATACTCATACATCGCATCTTG) that amplify a 486bp region of the diphtheria toxin transgene. The following reaction conditions were used for *rd* genotyping: 10µl SYBR Green master mix (Qiagen), 7µl ddH₂O, 1µl Forward Primer (20µM), 1µl Reverse Primer (20µM), and 1µl genomic DNA. Thermal cycling conditions were: 95°C for 10mins, then 39 cycles of 95°C for 15s and 60°C for 1min, followed by 4°C for 15mins, carried out using a T100 Thermal cycler (Bio-Rad).

The number and size of PCR products can be visualised using gel electrophoresis, as described in Section 2.2.3.4. As the *c/* animals are maintained as a heterozygous colony, there are no mice homozygous for the *c/* transgene. Instead, presence of a single band of 486bp size indicates mice are heterozygous for *c/* transgene and absence of a band indicates mice are homozygous wildtypes.

2.2.5.3 *Opn4*^{S310A} genotyping

Genomic DNA was extracted as described in section 2.2.5.1. *Opn4*^{S310A} animals were genotyped using a TaqMan MGB probe assay (Shen et al., 2009). Custom MGB-TaqMan probes (Applied Biosystems) were designed for wildtype (5'-CTCCCTACTCCACTG) and S310A (5'-CCCTACGCCACTGT) *Opn4* alleles. Each probe contains a quenched fluorescent dye label, FAM (wildtype) or VIC (S310A). A short region of the *Opn4* gene containing the mutation was amplified using forward (5'-CACTGATTGTCATTCTTCTCTTCGT) and reverse (5'-CTGCTCACCCAGCAAAGG) primers. The probe hybridises to corresponding DNA in the amplified region. During PCR, Taq polymerase extends the primers until it reaches the probe and cleaves it, unquenching the dye. The intensity of FAM or VIC fluorescence is used for allelic discrimination. Genotyping results were analysed using StepOnePlus software (Applied Biosystems).

The following reaction conditions were used: 10µl Taqman Universal PCR MasterMix (with ROX reference dye, Applied Biosystems), 0.05µl WT Probe, 0.05µl S310A probe, 1µl Forward primer (20µM), 1µl Reverse Primer (20µM), 5.9µl of ddH₂O and 2µl genomic DNA. Thermal cycling conditions were: 50°C for 2mins, 95°C for 10mins then 40 cycles of 95°C for 15s and 60°C for 1min, followed by 504°C for 30s. Thermal cycling was conducted using a StepOne plus machine (Applied Biosystems).

2.2.5.4 *Opn4*^{-/-} genotyping

Genomic DNA was extracted as described in section 2.2.5.1. Genotyping of *Opn4*^{-/-} mice was performed according to method described in Hattar et al. (2003). In *Opn4*^{-/-} the *Opn4* ORF is disrupted by insertion of a cassette containing *tau-LacZ* and floxed PGK-neomycin immediately downstream of *Opn4* ORF start codon. *Opn4* animals are genotyped using a multiplex PCR. Two sets of primers were added to the reaction, the first primer pair (*Opn4* F, 5' - AGAAGTGGCTCTTTGGGGAGAC and *Opn4* R, 5' - GCAGAAGGCATAGAACTCGCAAC) amplify a 200bp sequence of the wild-type *Opn4* gene, the second primer pair amplify a 300bp region of the *LacZ* gene (*LacZ* F, 5' - AAGCAGTCA GCAGCCCAAAG and *LacZ* R, 5' - TGTCACCCTCCTGGTCTTGC).

The following reaction conditions were used: 10µl SYBR Green Master Mix, 0.5µl *Opn4* F primer (10µM), 0.5µl *Opn4* R primer (10µM), 0.5µl *LacZ* F primer (10µM), 0.5µl *LacZ* R primer (10µM), 7µl ddH₂O and 1µl genomic DNA. Thermal cycling conditions were: 95°C for 10mins, then 35 cycles of 95°C for 15s and 60°C for 60s, then 95°C for 15mins, 60°C for 60s, followed by a +0.5°C ramp to 95°C for 15s to produce a melt curve. All thermal cycling was performed using a StepOne Plus machine (Applied Biosystems).

Melt curve analysis was used to genotype *Opn4* animals. The amplification product of wildtype *Opn4* has a melting temperature of 82°C, whereas the amplification product of *LacZ* has a melting temperature of 85°C. The presence of melt curves with single peaks at 82°C and 85°C were used to genotype *Opn4*^{+/+} and *Opn4*^{-/-} mice, respectively. Heterozygous mice carry both alleles so possess two melt curve peaks at 82°C and 85°C (the second peak at 85°C is usually smaller than the first peak at 82°C).

2.2.5.5 *Opn4*^{-/-} *Cre*^{+/+} genotyping

Opn4.Cre.eYFP mice were genotyped using PCR and gel electrophoresis. In these mice, Cre recombinase expression is driven by the endogenous *Opn4* promoter. These mice also have a lox.STOP.lox.EYFP. sequence inserted at the Rosa26 locus. In *Opn4*^{Cre/Cre} mice, Cre expression replaces *Opn4* expression in ipRGCs (Ecker et al., 2010). Expression of Cre leads to excision of the floxed STOP sequence resulting in co-expression of EYFP in ipRGCs (Srinivas et al., 2001). These mice therefore need to be genotyped for *Opn4*, Cre recombinase and eYFP.

The *Opn4* genotyping relies on a pair of primers, (Melanopsin F, 5' - AGAAGTGGCTCTTTGGGGAGAC, and Melanopsin R, 5' - GCAGAAGGCATAGAACTCGCAAC) to amplify a 201bp region in the *Opn4* gene. The following reaction conditions were used: The following reaction conditions were used: 10µl SYBR Green Master Mix, 0.25µl Melanopsin F primer (10µM), 0.25µl Melanopsin R primer (10µM), 7.5µl ddH₂O and 2µl genomic DNA.

Thermal cycling conditions were: 94°C for 10mins, then 40 cycles of 94°C for 30s, 60°C for 30s and 72°C for 15s, followed by 72°C for 5min. The presence of PCR products was visualised using gel electrophoresis, as described in Section 2.2.3.4., and used to genotype animals. Presence of a single band indicated the animal was either homozygous (*Opn4*^{+/+}) or heterozygous (*Opn4*^{+/-}) for *Opn4*. If no band was present, this indicated animal was *Opn4*^{-/-}.

Cre recombinase genotyping used a pair of primers (Cre F, 5' - AAACAGCATTGCTGTCACTT and Cre R, 5' - TCCAATTTACTGACCGTACA) that amplified a 337bp region in the Cre recombinase gene. The following reaction conditions were used: The following reaction conditions were used: 10µl SYBR Green Master Mix, 0.25µl Cre F primer (10µM), 0.25µl Cre R primer (10µM), 7.5µl ddH₂O and 2µl genomic DNA. Thermal cycling conditions were: 94°C for 10mins, then 40 cycles of 94°C for 30s, 60°C for 30s and 72°C for 15s, followed by 72°C for 5min.

The presence of PCR products was visualised using gel electrophoresis, as described in Section 2.2.3.4., and used to genotype animals. Presence of a single band indicated the animal was either homozygous ($Cre^{+/+}$) or heterozygous ($Cre^{+/-}$) for Cre recombinase. If no band was present, this indicated animal was $Cre^{-/-}$, and therefore $Opn4^{+/+}$. Although individually the *Opn4* and *Cre* genotyping protocols could not distinguish heterozygous from homozygous transgenic animals, given that *Opn4* is replaced by Cre recombinase, if genomic DNA from animals revealed bands after both *Opn4* and *Cre* genotyping, this demonstrated the animal was heterozygous ($Opn4^{+/-} Cre^{+/-}$).

Genotyping of eYFP was performed using multiplex PCR, using one forward and two reverse primers. The first primer pair amplified a 500bp region of the Rosa26/EYFP locus containing EYFP (EYFP F, 5' – AAAGTCGCTCTGAGTTGTTAT, EYFP Mutant R, 5' – GCGAAGAGTTTGTCTCAACC) while the second primer pair amplified a 250bp region of the WT Rosa26 locus (EYFP F, 5' – AAAGTCGCTCTGAGTTGTTAT, EYFP WT R, 5' – GGAGCGGGAGAAATGGATATG).

The following reaction conditions were used: 10µl SYBR Green Master Mix, 0.25µl EYFP F primer (10µM), 0.25µl EYFP Mutant R primer (10µM), 0.25µl EYFP WT R primer (10µM), 7.25µl ddH₂O and 2µl genomic DNA. Thermal cycling conditions were: 94°C for 10mins, then 40 cycles of 94°C for 30s, 60°C for 30s and 72°C for 15s, followed by 72°C for 5min. The presence of PCR products was visualised using gel electrophoresis, as described in Section 2.2.3.4., and used to genotype animals. Presence of a single band at 500bp indicated the animal was homozygous ($eYFP^{+/+}$) for eYFP, while a single band at 250bp indicated animal was a homozygous wildtype ($eYFP^{-/-}$). Presence of two bands indicated animal was heterozygous ($eYFP^{+/-}$).

2.2.6 Reverse-transcriptase qPCR

2.2.6.1 RNA extraction

RNA extraction was performed using the RNeasy Mini Kit (Qiagen). Prior to beginning the RNA extraction, all work surfaces, pipettes and tube racks were cleaned using ddH₂O and 96-100% ethanol. Microcentrifuge tubes and pipette tips were sterilised by autoclaving. An appropriate volume of RLT buffer (600µl per sample if ≥30mg, 300µl per sample if ≤30mg) was prepared by adding 10µl of β-mercaptoethanol for every 1ml of RLT buffer. Tissue for RNA extraction was frozen on dry ice, then disrupted using a pestle. 600µl of RLT buffer was added to the sample, which was further disrupted using a pestle. The sample was then homogenised using a 20-gauge needle and syringe, by aspirating and expelling the sample at least 10 times.

Homogenised samples were then centrifuged for 3mins at 13000rpm, then the supernatant was removed and mixed with 600µl of 70% ethanol by pipetting. Up to 700µl of the supernatant-ethanol mixture was applied to an RNeasy spin column, then centrifuged at 8000 x *g* for 15s. The flow-through was discarded and the previous step repeated until all remaining sample was loaded onto the spin column.

An on-column DNase digest (Qiagen) was then performed to remove potential genomic DNA contamination. 350µl of Buffer RW1 was added to the column, centrifuged for 15s at 8000 x *g* and flow-through was discarded. For each sample, 10µl *DNase I* stock solution was added to 70µl Buffer RDD and mixed gently by inverting the tube to produce a *DNase I* incubation mix. 80µl of *DNase I* incubation mix was applied directly to the column membrane, and left at room temperature for 15mins. 350µl of Buffer RW1 was then added to the column, centrifuged for 15s at 8000 x *g* and flow-through was discarded.

Continuing with the RNA extraction, 500µl of Buffer RPE (with ethanol) was then added, centrifuged for 15s at 8000 x *g* and flow-through was discarded. This step was repeated using an additional 500µl of Buffer RPE (with ethanol). Once complete, the empty spin column was centrifuged for 1min at 8000 x *g* to dry the membrane. The spin column was placed in a new sterile 1.5ml microcentrifuge tube and 30µl of RNase-free water was applied directly to the column membrane. After 1min at room temperature, the column was centrifuged for 1min at 8000 x *g* to elute the RNA. The eluate was then re-applied to the membrane and centrifuged for 1min at 8000 x *g* to increase the concentration of RNA. RNA was then immediately used for cDNA synthesis or temporarily stored at -20°C. RNA was stored long term at -80 °C.

2.2.6.2 cDNA synthesis

cDNA synthesis was performed using the qScript cDNA synthesis kit (QuantaBio). cDNA synthesis is the production of single stranded complementary DNA (cDNA) from single stranded RNA molecules by a Reverse Transcriptase (RT) enzyme. A combination of random hexamers and Oligo(dT) primers, present in excess, bind to the RNA template and are then extended by RT enzyme using dNTPs supplied during reaction to create cDNA. The following reaction conditions were used: up to 1µg of isolated RNA, 4µl of qScript 5x Reaction Mix, 1µl of qScript Reverse Transcriptase enzyme and ddH₂O to a final volume of 20µl. Reaction was mixed gently by pipetting, then incubated at 22°C for 5mins, then 42°C for 30mins and finally 85°C for 5min. cDNA was stored at -20°C until required.

2.2.6.3 Quantitative PCR (qPCR)

RT-qPCR uses cDNA in order to assess the amount of a specific mRNA transcript in a particular tissue. qPCR uses a dye (e.g. SYBR Green) that is fluorescent when bound to double stranded DNA, in order to measure the amount of amplified product after each round of thermal cycling (described in Section 2.2.3.2.). The threshold cycle, C_t value, is the point at which sufficient product has been amplified to produce a statistically significant change in fluorescence relative to background. As fluorescence signal is directly proportional to the DNA concentration, the C_t value allows the initial quantity of template in the reaction to be calculated. qPCR reactions were performed in triplicate using the following reaction conditions: 10 μ l Quantifast SYBR Green PCR MasterMix (Qiagen), 7 μ l ddH₂O, 1 μ l Forward qPCR primer (20 μ M), 1 μ l Reverse qPCR primer (20 μ M), and 1 μ l of cDNA template. A negative control was included by replacing cDNA template with an additional 1 μ l of ddH₂O.

The following thermal cycling conditions were used: 95°C for 10mins, followed by 40 cycles of 95°C for 15s, 60°C for 60s, 76°C for 15s, and then 95°C for 15mins, 60°C for 60s then a +0.5°C ramp to 95°C for 15s. All qPCR reactions were run on a StepOnePlus Real Time qPCR machine (Applied Biosystems) using the StepOne software (version 2.1).

Relative gene expression was calculated using the $2^{-(\Delta\Delta C_t)}$ relative gene expression method described in Peirson et al. (2003) with assumed efficiency = 2, after normalising to geometric mean of 3-4 housekeeping genes using method described in Vandesompele et al. (2002).

2.2.7 Cell Culture and *in vitro* assays

2.2.7.1 Routine Cell culture

Neuro-2A cells (ECACC) or HEK293 cells (ATCC) were cultured in DMEM with 4500mg/L glucose (Sigma) with 10% foetal bovine serum (Life Technologies), 2mM l-glutamine (Sigma) and 1% (v/v) penicillin/streptomycin (Sigma). The DMEM provides necessary glucose, minerals and vitamins necessary to support and maintain cell survival and growth, l-glutamine supports the high energy demand and protein synthesis of cells, while the antibiotics prevent bacterial infection. Cells were kept in T75 flasks (Corning) in a humidified chamber at 37°C with 5% CO₂, fed fresh media every 2-3 days and passaged before reaching 80% confluence. Cells were passaged by replacing media with 3ml PBS containing 1xTrypsin solution (Sigma-Aldrich) and incubating cells for 2-3mins at 37°C. This caused the adherent cells to detach from the T75 flask. 6ml DMEM was then added to cells, pipetted over the walls of the flask to ensure all cells were detached. The entire 9ml volume of DMEM plus Trypsin/PBS was then pipetted into a 15ml Falcon tube and centrifuged at 500 x g for 15mins. The supernatant was poured off the cell pellet, which was then resuspended in 5ml fresh DMEM. Between 0.25 – 0.5ml of resuspended cell solution was added to a new T75 flask containing 10-15ml of culture medium. All media or reagents added to cells were pre-warmed to 37°C. All cell culture was performed according to aseptic technique to avoid contamination of cells.

2.2.7.2 Transient Transfections

For calcium imaging, cells were seeded into 24-well plates at $\sim 4 \times 10^4$ cells/well after being counted using a haemocytometer and diluted to correct concentration in culture medium. After 24hrs, cells were placed in 250µl antibiotic-free DMEM media (Sigma) with 10% foetal bovine serum and 2mM l-glutamine and transiently transfected using Genejuice transfection reagent (Merck Millipore) according to manufacturer's

instructions. For each well of the 24-well plate, 0.75µl of Genejuice transfection reagent was added drop-wise to 25µl of sterile RPMI (Sigma) and mixed by vortexing.

The RPMI-Genejuice mixture was then incubated at room temperature for 5mins before 0.25µg of plasmid DNA was added for every well to be transfected and mixed gently using a pipette tip. The RPMI/Genejuice/DNA mix was then left to incubate at room temperature for 15mins, before 27µl was added drop-wise to each well containing cells in antibiotic-free media. For immunocytochemistry, cells were seeded onto glass coverslips in a 6-well plate at a density of $\sim 1.5 \times 10^5$ cells/well. For each well of the 6-well plate, 100µl of RPMI, 3µl of Genejuice transfection reagent and 1µg of plasmid DNA were used, combined as described above. For both 24-well and 6-well plate transfections, cells were cultured in transfection reagent overnight, before transfection media was replaced with regular culture media containing antibiotics. Cells were cultured for 48hrs after transfection before being used for calcium imaging or fixed for immunocytochemistry.

2.2.7.3 Fluorescent Calcium Imaging

Intracellular calcium in transiently transfected cells was monitored using a fluorescent calcium indicator, Fluo-4 AM (Molecular Probes). All steps were performed in dim red light. Forty-eight hours after transient transfection with plasmid, cells were loaded with Fluo-4AM dye. Each aliquot of Fluo-4AM contains 50µg of dried Fluo-4AM powder, which was dissolved in 90µl of probenecid (Invitrogen) and 18µl of Pluronic F-127 (Invitrogen). 12µl of this dye solution and 1µl of 9-*cis* retinal (40nM, Sigma) was then added for every 1ml of DMEM (Sigma), with volumes scaled depending on number of wells to be imaged (250µl of DMEM required per well of a 24-well plate). Probenecid improves dye loading and retention, by preventing efflux of dye from cells during imaging, while the Pluronic F-127 ensures the dye is evenly distributed throughout the cell. The culture media in each well of the 24-well plate was replaced with 250µl of DMEM with dye-loading solution, to give final concentration of 5µM Fluo-4 AM, 0.015%

Pluronic F-127, 2.5 μ M probenecid (Life Technologies) and 20 μ M 9-*cis* retinal (Sigma). Cells were incubated in dye loading solution for 20mins at 37°C.

The dye-loading media was then replaced with 250 μ l of DMEM with 2.7 μ M probenecid (10 μ l probenecid, 270 μ M, for every 1ml DMEM) and cells were incubated for a further 10mins at 37°C. This DMEM-probenecid media was then replaced with 250 μ l of Hank's buffered saline solution without additional calcium (Gibco) for imaging. Although 11-*cis* retinal is the principal native chromophore, melanopsin can also bind 9-*cis* retinal, without any change to photoactivation (Melyan et al., 2005). As 9-*cis* is commercially available, it was used for calcium imaging experiments.

Calcium imaging was performed using a FLUOstar Omega plate reader (BMG Labtech) at room temperature. Fluorescence was excited using a 485nm (12nm bandwidth) filter and collected with a 520nm (30nm bandwidth) emission filter. Total fluorescence from each well was recorded every 2s for a total of 120s, before imaging the next well. Light for fluorescence excitation (200 flashes) was also used as the stimulus for melanopsin phototransduction, with intracellular calcium increasing in response to the first light exposure (Blasic et al., 2012b; Qiu et al., 2005). Responses were normalised to baseline (first data point, $\Delta F/F$). Each 24-well plate contained 4 technical replicates of each plasmid (one column per plasmid), which were averaged to give a single biological replicate value. Six plates were imaged in total, each transfected separately, to provide 6 biological replicates per plasmid.

2.2.7.4 Immunocytochemistry

Forty-eight hours after transfection, cells were fixed for 10mins at room temperature using 4% paraformaldehyde in phosphate buffered saline (PBS). Cells were then permeabilised in 0.2% Triton-X (Sigma) in PBS for 5mins, then blocked with 5% Donkey serum in PBS with 0.05% Tween-20. Cells were incubated in primary antibody diluted in PBS with 0.05% Tween-20 (Sigma) and 1% Donkey serum for 1hr at room temperature.

Cells were washed 5 times in PBS with 0.05% Tween-20, then incubated in a 1:200 dilution of secondary antibody raised in donkey conjugated to an Alexa fluorophore (Molecular Probes, Life Technologies) in PBS with 0.05% Tween-20 and 1% Donkey serum for 30mins at room temperature. The secondary antibody must be raised against the host species of the primary antibody. Coverslips with cells were mounted onto poly-lysine coated slides using Prolong Anti-Fade Gold media with DAPI (Molecular Probes, Life Technologies). Images were acquired using a x63 oil objective of a LSM710 laser-scanning confocal microscope (Zeiss) and Zen2009 software (Zeiss) with excitation at 405nm, 488nm and 561nm and emission at 440-480nm, 505-550nm and 600-700nm for DAPI, green and red fluorescence, respectively. Brightness and contrast enhancements carried out using ImageJ software (<http://rsbweb.nih.gov/ij>).

2.2.8 Behavioural Techniques

2.2.8.1 Animal Husbandry

All animals were housed under a 12:12 light:dark (LD) cycle with food and water *ad libitum*. All experiments were approved by the University of Oxford Animal Welfare and Ethical Review Board and all procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 (PPL 30/2812, PIL I72E6A362) and the University of Oxford Policy on the Use of Animals in Scientific Research. All procedures were carried out in a designated establishment.

2.2.8.2 Pupillometry

All pupillometry testing took place between ZT4-8, during the light phase, since a circadian rhythm in pupil light response has previously been identified (C. Potheary, unpublished data). Animals were dark-adapted for a minimum of 1hr prior to each light exposure. During pupillometry, mice were manually restrained by scruffing and were unanaesthetised.

The consensual pupil response was recorded from the contralateral unexposed eye. As shown in **Figure 2.4**, the contralateral eye was illuminated by an infrared light emitting diodes (LED, 850nm, 10nm half-bandwidth) and recorded using a Prosilica near infrared sensitive charge coupled device camera (BRSL) perpendicular to the eye. Images were collected at a rate of 5 frames/second during dark and 10 frames/second during light exposure. All recordings started with 2s of dark, followed by 10s of light exposure and then up to 10s of dark. Light source was a xenon arc lamp (150W solar simulator, Lot-Oriel) filtered using monochromatic bandpass (480, 500, 550 or 570nm, 10nm half-bandwidth, Andover) and neutral density filters (0.5, 1, 1.5, 2, 2.5 and 3ND, Lot-Oriel). Using a liquid light-guide, light was conveyed to a Ganzfeld integrating sphere (5cm diameter, Pro-lite Technology) to provide uniform whole-field illumination. A shutter (LSZ160, Lot Oriel) controlled by custom software (BRSL) regulated light exposure.

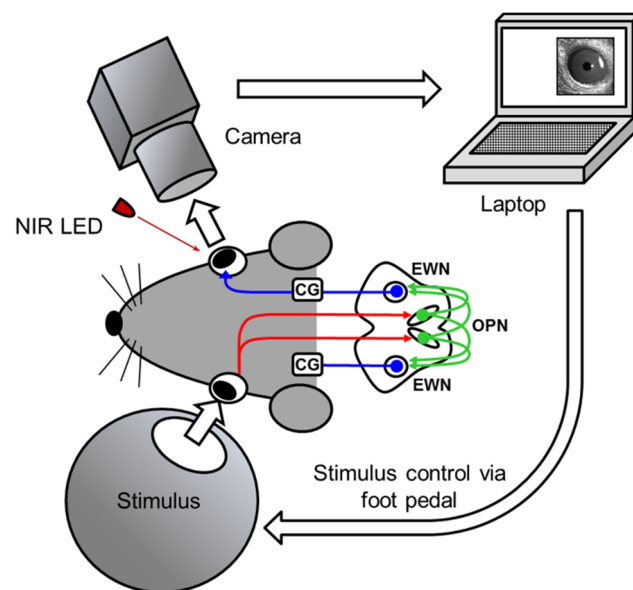


Figure 2.4. Pupillometry of consensual pupil light response. Consensual pupil response is recorded from contralateral eye illuminated by near-infra red light emitting diode (NIR LED). Schematic of consensual pupil response is shown by coloured lines. ipRGCs in stimulated eye carry light signal to olivary pretectal nucleus (OPN, red lines), where it is transferred to the Edinger-Westphal nuclei (EWN, green lines) and, via ciliary ganglion (CG), to contralateral eye causing iris sphincter muscles, which contract leading to pupil constriction (blue lines). Stimulus and camera are controlled using custom software. Image from Dr. Carina Potheary and Prof. Stuart Peirson.

If necessary, a 1% tropicamide solution (Bausch & Lomb) was applied to the exposed eye at the start of each session, approximately 10-15mins before first recording. All animals received at least 2 training sessions before collecting experimental data. This acclimatised animals to handling and light exposure, reducing stress-related confounds. Each animal could be exposed to light up to 3 times per pupillometry session, with at least 48hrs between each session. A maximum of 10 pupillometry sessions were conducted using each animal.

Images were analysed using ImageJ (<http://rsbweb.nih.gov/ij/>). Minimum pupil area was calculated by manually fitting a circle to the dark-adapted pupil in the first frame of recording and the constricted pupil in the last frame of light exposure (**Figure 2.5**). The area of the constricted pupil was normalised to dark-adapted pupil area to give minimum pupil size (maximum constriction).

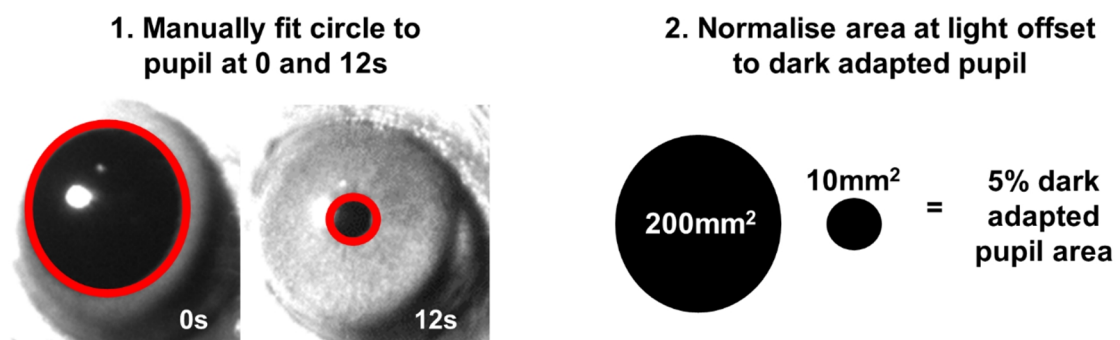


Figure 2.5. Pupillometry analysis for maximum constriction.

2.2.9 Multi-electrode Array Recordings

Animals were removed from home cage at ZT5-7, dark-adapted for 1hr, culled by cervical dislocation (approved Schedule 1 method) and enucleated. As shown in **Figure 2.6**, retinae were dissected at room temperature under dim red light at room temperature in Ames media gassed with 95% O₂, 5% CO₂ (pH 7.3) (Sigma). Retinae were positioned ganglion-side down onto a glass-based MEA with 60 electrodes (30µm Ø, 200µm apart, Multi-Channel Systems) and secured by glass-coated metal harps (ALA Scientific Instruments). Once placed into the MEA amplifier (MEA2100-1x60 system, Multi-Channel Systems), the MEA was attached to a gas-permeable perfusion manifold (ALA Scientific Instruments). The MEA amplifier and MEA were positioned onto the stage of an Olympus-IX71 inverted microscope so MEA electrodes were located in the microscope light path for light stimulation. Retinae were perfused continuously with Ames media gassed with 95% O₂, 5% CO₂ (pH 7.3) and heated to 34°C using a water bath heater (36°C), in-line perfusion heater (35°C), and base plate heater incorporated into the MEA system (34°C) to minimise temperature variations. Recordings were started at ZT7-9. Recorded signals were collected, amplified and digitized at 25kHz using MC Rack software (Multi-Channel Systems).

Light stimuli were generated by an X-cite 120W metal halide light source (EXFO) and delivered via x10 objective beneath the MEA chamber. Light stimuli duration was manually controlled by a high-speed shutter (Prior). Intensity and wavelength were adjusted using neutral density filters (0-5log units, Thor Labs) and bandpass monochromatic filters (450-570nm in 30nm steps, each ±10nm, Thor Labs), respectively. Filters were controlled by an automated filter wheel placed into the microscope light path (Prior Scientific). Power of light stimuli (µW/cm²/s) was measured at the sample focal plane using an in-line power meter (PM160T, Thor Labs).

Conversion of power measurements to irradiance (photons/cm²/s) was performed using an irradiance conversion toolbox (<http://www.ndcn.ox.ac.uk/team/stuart-peirson/downloadstile-14/filetile-21>).

Prior to all recordings, retinae were stimulated with 480nm light (10^{14} photons/cm²/s) for 10s, then dark-adapted for 15mins to produce an equal dark-adaptation baseline for all retina. For irradiance response curve recordings, activity was recorded for 30s prior to 10s light stimulation (baseline), then for up to 2mins after light exposure (response). Retinae were first exposed to the lowest light intensity of each wavelength (eg: 480nm 10^{11} photons/cm²/s), dark-adapted for 2mins, then stimulated with the next lowest light intensity (eg: 480nm 10^{12} photons/cm²/s) and dark-adapted for 5mins, and so on, with light intensity increased in 1log unit steps up to brightest light intensity (eg: 480nm 10^{15} photons/cm²/s). Interstimulus interval was also increased depending on intensity of preceding light stimulation (from 2-15mins).

Spike data was extracted using MC rack software (Multi-Channel Systems) using amplitude thresholding of 3 standard deviations from baseline. Spikes were then sorted into 1 second bins (spikes/s).

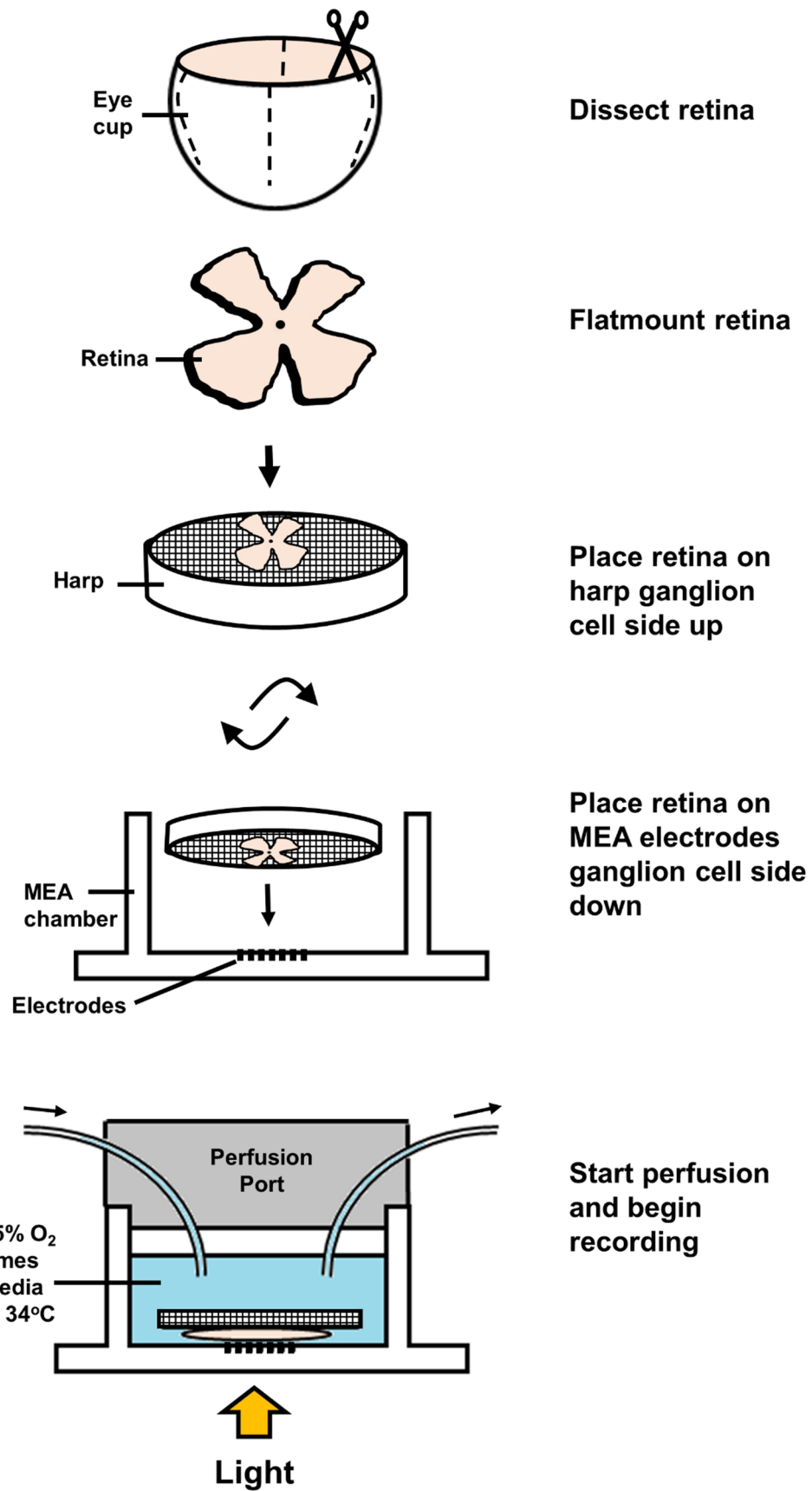


Figure 2.6. Preparation of retina for multi-electrode array (MEA) recordings.

2.2.10 Statistical Analysis

Statistical tests were conducted using SPSS 22.0 (IBM). All comparisons between two groups were tested using Student's t-tests. All comparisons between more than 2 groups were tested using One-way analysis of variance (ANOVA). For One-way between-subjects ANOVAs, a post-hoc Dunnett's test was used to explore significant main effects provided there were clear control and experimental groups. If there was no control group or comparisons were required between all conditions, a Tukey's HSD post-hoc test was used to explore main effects. Where there were 2 independent variables, a Two-way ANOVA was used. This allowed the main effects of each variable to be explored, as well as their potential interaction. Any significant main effects were explored using the post-hoc tests described above. If a significant interaction was identified, any significant main effects were deemed unreliable as they may be driven by a single group rather than an overall trend. Significant interactions were explored using Sidak corrected Student's t-test. The Sidak corrected alpha is calculated as $1 - (1 - \alpha)^{1/m}$ where α = original alpha (0.05) and m = number of tests. A significance threshold of $p \leq 0.05$ was used. In all figures * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Error bars show standard error of mean.

2.2.11 List of URLs

<http://genetics.bwh.harvard.edu/pph2/>
<http://mafft.cbrc.jp/alignment/software/>
<http://rsbweb.nih.gov/ij>
<http://sift.jcvi.org/>
<http://www.genomics.agilent.com/primerDesignProgram.jsp>
<http://www.jmol.org/>
<http://www.ncbi.nlm.nih.gov/pubmed/>
<http://www.ndcn.ox.ac.uk/team/stuart-peirson/downloadstile-14/filetile-21>
<http://www.rcsb.org/pdb/home/home.do>
<https://blast.ncbi.nlm.nih.gov/Blast.cgi>
<https://scholar.google.co.uk/>
<https://www.ncbi.nlm.nih.gov/gene/>
<http://cels.uri.edu/gsc/cndna.html>
https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi

Chapter 3

Characterisation of a novel chemical-induced *Opn4* missense mutation

Chapter 1 reviewed the literature on opsin structure-function, including the use of mutagenesis to identify critical functional residues. In this chapter, a novel missense mutation in melanopsin (*Opn4*) is identified and characterised.

3.1 Introduction

Forward and reverse genetics are two approaches for identifying critical amino acids. In forward genetics, phenotypes are linked to mutations in a particular gene. This can be accomplished by genome-wide association studies (Bush & Moore, 2012) or phenotype-screening of large numbers of animals with random chemical-induced mutations (Nolan et al., 2000). The phenotype of *Opn4* mutations may be subtle in individuals with intact retina, as rods and cones can partially sustain NIF behaviour, using ipRGCs as a conduit to NIF centres of the brain (Güler et al., 2008). Furthermore, the majority of naturally occurring missense SNPs in the human *OPN4* gene are rare, which limits the practicality of forward genetics for identification of key functional OPN4 residues.

The second approach, reverse genetics, investigates specific mutations in a gene of interest to determine their effect on behaviour. Targets for mutation can be identified based on homology with other well-described proteins, especially those with crystal structures, or residues conserved between different species. Until recent advances in genome editing using site-specific nucleases, such as CRISPR or TALENs (for review see Gaj et al., 2013), reverse genetics was considerably more difficult *in vivo* than *in vitro*. Chemical mutagens, such as *N*-ethyl-*N*-nitrosourea (ENU) offered a viable alternative.

ENU is an alkylating agent, which generates multiple point mutations at random throughout the genome (Nolan et al., 2002). Typically, ENU-treated mice are screened for phenotypes of interest before identifying the underlying mutation. However, the DNA of mutagenized animals can also be screened to identify mutations in target genes. The inability to control the type and position of mutation can be ameliorated by creating DNA archives of thousands of mutagenized individuals, which improves the probability of finding a non-silent mutation in a given region of interest (Coghill et al., 2002). MRC Harwell have created two parallel archives of genomic DNA and sperm from ENU-mutagenized mice (Quwailid et al., 2004). This allows the screening and subsequent recovery of interesting mutants for further *in vivo* testing. This approach is ideal for melanopsin, where comparatively few naturally-occurring mutants have been identified and characterised *in vivo*.

A genotype-driven screen of the ENU archive at MRC Harwell was conducted with the aim of identifying novel melanopsin mutations. These were characterised *in vitro* and assessed using bioinformatics methods to determine whether the *Opn4* mutant potentially possessed interesting functional properties. If so, mouse models of the ENU mutant were recovered and characterised *in vivo* using assays of cellular and whole animal physiology.

3.2 Methods

3.2.1 Rationale for screening *Opn4* exon 6

Genotype-driven screens of the Harwell ENU archive are limited to regions under 450bp. Due to large introns in the *Opn4* gene, each screen is effectively restricted to a single *Opn4* exon. Therefore, the exon most likely to affect function if mutated was selected for initial screen of the ENU archive. The protein sequences of mouse melanopsin (short isoform OPN4S, NP_001122071.1) and bovine rhodopsin (NP_001014890.1) were aligned using BLAST (Altschul et al., 1990) to identify the OPN4 residues in each protein domain, based on the bovine rhodopsin crystal structure (Palczewski et al., 2000). The location of amino acids in OPN4S encoded by each exon of the mouse *Opn4* gene (gene ID: 30044) were then identified. These were M1 - T47 (exon 1), T48 – N97 (exon 2), R98 – G141 (exon 3), C142 – S209 (exon 4), A210 – G265 (exon 5), R266 – A318 (exon 6), G319 – R355 (exon 7), V356 – V415 (exon 8), E416 – Q454 (exon 9), T455 – M466 (exon 10).

Exon 6 was chosen for screening, as it encoded the entire 6th transmembrane helix (TM6) and the majority of the 3rd intracellular loop (IL3, **Figure 3.1**), two protein domains which possess critical functional properties in other opsins. IL3 is involved in G-protein interaction (Scheerer et al., 2008) and TM6 rotation is crucial in the conformational change necessary for GPCR activation (Kobilka & Deupi, 2007; Katritch et al., 2013). Furthermore, some residues encoded by exon 6 form part of the chromophore binding pocket in rhodopsin, influencing spectral tuning and chromophore stability (Palczewski et al., 2000). It was concluded missense mutations in exon 6 were more likely to cause interesting phenotypes compared to mutations in other exons, making it a suitable target for the first ENU archive screen.

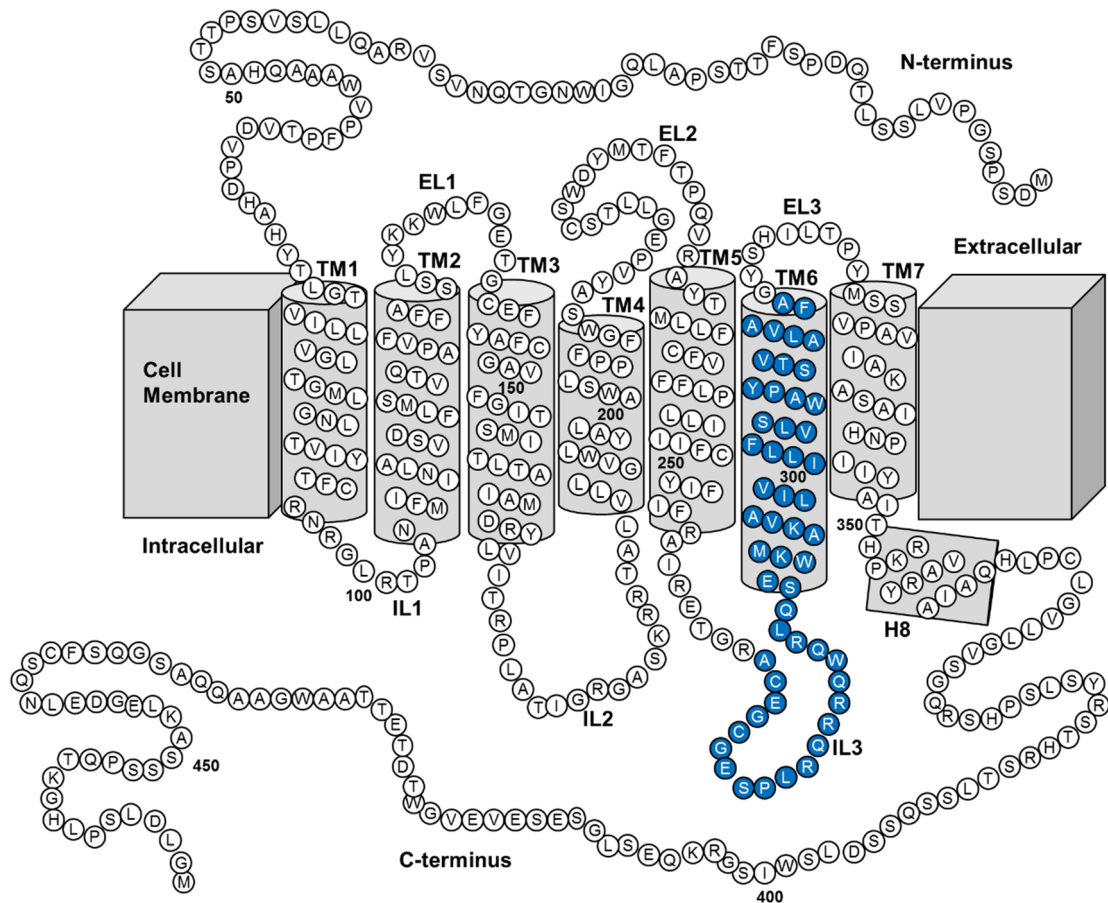


Figure 3.1. Region screened for ENU-induced mutations. Residues encoded by exon 6 (highlighted in blue) of the mouse melanopsin short isoform. Secondary structure based on homology with bovine rhodopsin. Figure adapted from Peirson et al. (2009) under Creative Commons licence Attribution 4.0 International.

3.2.2 Genotype-driven ENU archive screen

The ENU archive at MRC Harwell contains the genomic DNA of 11323 mice. These mice are the F1 mutant progeny of ENU-treated males and untreated females (Nolan et al., 2000), so inherited mutations are heterozygous. This DNA is from a mix of tail, liver, kidney, spleen and heart tissue (Quwailid et al., 2004). To allow fast screening of the archive, DNA samples are initially pooled so each well holds DNA samples from 4 individual mice (final concentration 5ng/μl). Thirty 96-well plates of pooled DNA were screened for mutations.

Primers for amplifying a 250bp region, including the *Opn4* exon 6 coding region (159bp) and 40-50bp of the surrounding introns, were designed using Lightscanner primer design software (Idaho Technologies). The forward primer bound to the intron between exon 5 and 6 (5'–CCTGCAGTGAACCTGGT) and the reverse primer bound to the intron between exon 6 and 7 (5'–TAGCCCTCGTACAGCGT). The conditions for the PCR amplification reaction were; 2µl of pooled DNA at 5ng/µl, 0.1µl of Forward primer (20µM), 0.1µl Reverse primer (20µM), 1µl of LC Green dye (Idaho Technologies) and 1.8µl of ddH₂O. The thermal cycling steps for amplification were 95°C for 2mins, then 44 cycles of 95 °C for 30s, 57.8°C for 30s and 72°C for 30s using a GS4 thermal cycler (G-storm).

Amplification was followed immediately by a hybridisation step: 95°C for 30s, 25°C for 30s and 15°C for 30s. This step causes the amplification products to denature and rapidly reanneal. As shown in **Figure 3.2**, if mutations are present in the amplified region of a sample, this can lead to heteroduplexes – double stands of DNA with base pair differences. A heteroduplex is a strong indicator of an ENU induced mutation or naturally-occurring SNP.

Heteroduplexes were detected by high-resolution melt curve analysis using a Lightscanner (Idaho Technologies). PCR products after hybridisation were melted over a gradient of 80-98°C. Fluorescence caused by binding of LC Green dye to double stranded DNA tracked when melting occurred. Since the bonding between the two strands of a heteroduplex is relatively weak due to mismatch between base pairs, heteroduplexes melt at lower temperatures than homoduplexes. This produces a leftwards shift in the melt curve of heteroduplexes used to identify possible mutants (**Figure 3.2B**). Results of pooled plate screen were analysed independently by two scorers to ensure accuracy in identification of interesting samples.

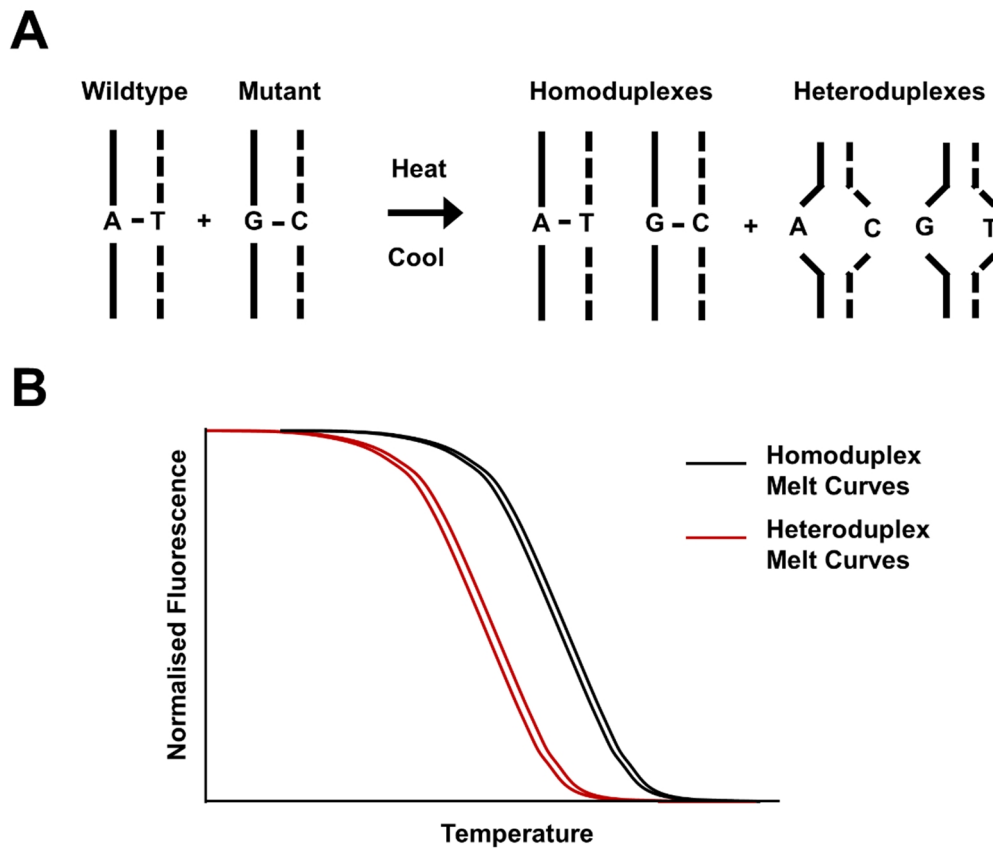


Figure 3.2. High resolution melt curve analysis for mutation detection (A) Hybridisation step (heating then rapid cooling) leads to formation of heteroduplexes if a mutation is present in amplified region. **(B)** Heteroduplexes have lower melting points because of weak bonds between mismatched strands. A left-ward shift in melt curve is used to identify mutations.

Any potentially interesting pooled samples were repeated to reduce false positive results. If a pooled sample consistently demonstrated a lower melting point, then the 4 individual DNA samples were screened as described above, to identify which sample possessed the mutation. Location and type of mutation was confirmed by Sanger sequencing (Source Biosciences), as described in Section 2.2.2, using the following primers: 5'-CCTGCAGTGAACCTGGT and 5'-TAGCCCTCGTACAGCGT.

3.2.3 Bioinformatics

Opsin amino acid alignments were completed using MAFFT software (Katoh et al., 2002, <http://mafft.cbrc.jp/alignment/software/>). Sequences used are shown in **Table 3.1**.

Table 3.1. Opsin protein sequences used for alignments. Protein sequences are from NCBI Genbank (<https://www.ncbi.nlm.nih.gov/gene/>). LWS = long wavelength sensitive, MWS = middle wavelength sensitive and SWS = short-wavelength sensitive. Where applicable, the protein sequences for different melanopsin genes in the same species are given, eg: OPN4M (mammalian-like) and OPN4X (xenopus – like).

Opsin	Species	Protein Sequence
<i>Melanopsin</i>	Chimpanzee (<i>Pan troglodytes</i>)	XP_001135445.1
	Human (<i>Homo sapiens</i>)	NP_150598.1
	Dog (<i>Canis canis</i>)	XP_853735.2
	Cow (<i>Bos taurus</i>)	NP_001179328.1
	Cat (<i>Felis catus</i>)	AAR36861.1
	Mouse (<i>Mus musculus</i>)	NP_038915.1
	Rat (<i>Rattus norvegicus</i>)	NP_620215.1
	Hamster (<i>Phodopus sungorus</i>)	AAU11506.1
	Mole Rat (<i>Nannospalax ehrenbergi</i>)	CAO02487.1
	Fat-tailed Dunnart (<i>Sminthopsis crassicaudata</i>)	ABD38715.1
	Chicken (<i>Gallus gallus</i>)	
	OPN4M	NP_001038118.1
	OPN4X	NP_989956.1
	Zebrafish (<i>Danio rerio</i>)	
	OPN4 M1	ADN39430.1
	OPN4 M2	NP_840074.1
	OPN4 M3	NP_001245153.1
	OPN4 X1	NP_001243006.1
	OPN4 X2	ADN39434.1
	Amphioxus (<i>Branchiostoma lanceolatum</i>)	BAE00065.1
	Lizard (<i>Podarcis siculus</i>)	AAY34941.2
	Clawed frog (<i>Xenopus laevis</i>)	NP_001079143.1
<i>Rhodopsin</i>	Human (<i>Homo sapiens</i>)	NP_000530.1
	Cow (<i>Bos taurus</i>)	NP_001014890.1
<i>LWS cone opsin</i>	Human (<i>Homo sapiens</i>)	NP_064445.2
<i>MWS cone opsin</i>	Human (<i>Homo sapiens</i>)	NP_000504.1
<i>SWS cone opsin</i>	Human (<i>Homo sapiens</i>)	NP_001699.1

3.2.4 *In vitro* expression of *Opn4* S310A

The S310A point mutation was introduced to the long, *Opn4L* (NP_001122071.1), or short isoform, *Opn4S* (NP_038915.1) of mouse melanopsin (Pires et al., 2009) in a pIRES2-acGFP plasmid (BD Biosciences), using a Quikchange II Site-directed mutagenesis kit (Stratagene), as described in Section 2.2.4. A full description of pIRES2-acGFP vector is provided in Section 2.1.3.1. The following mutagenesis primers were used (altered nucleotide highlighted in bold): 5'-CTGGGCTCCCTAC**G**CCACTGTGGCTCTG (S310A forward) and 5'-CAGAGCCACAGTGG**C**GTAGGGAGCCCAG (S310A reverse). Presence of the mutation was confirmed by Sanger sequencing (see Section 2.2.2) using following sequencing primers: CMV F, 5' – CAACGGGACTTTCCAAAATG, *Opn4* ex4F, 5' – GCCACCTTTCTTTGGTTGGA, *Opn4* ex9aR, 5' – CTACAGATGTCTGAGAGTCAC (for *Opn4L*) and *Opn4* ex10R, 5' – CTACATCCCGAGATCCAGACT (for *Opn4S*).

As the pIRES2-acGFP plasmid expresses a GFP reporter, it could not be used for calcium imaging with the calcium indicator Fluo-4, which has similar excitation and emission spectra to GFP. Therefore, for calcium imaging the full-length coding regions of *Opn4L* and *Opn4S* were cloned into the expression vector pcDNA3.1 (Invitrogen) by restriction enzyme cloning (See Section 2.2.3). A full description of the pcDNA3.1 vector is provided in section 2.1.3.2. Primers were used to add *EcoRI* sites before the start codon, 5' – AACGAATTCATGGACTCTCCTTCAGGACCA, and to add *NotI* sites after stop codon of *Opn4L*, 5' – TATGCGGCCGCCTACATCCCGAGATCCAGACT, and *Opn4S*, 5' - TATGCGGCCGCCTACAGATGTCTGAGAGT. The S310A mutation was then introduced into the pcDNA3.1 *Opn4* plasmid and confirmed with sequencing as above.

Neuro-2A cells (ECACC) were cultured as described in Section 2.2.7.1. Cells were seeded into 24-well plates for calcium imaging and transiently transfected with

pcDNA3.1 *Opn4L* and *Opn4S* WT and S310A as described in Section 2.2.7.2. Cells were seeded onto coverslips in 6-well plates for immunocytochemistry and transiently transfected with pIRES2-acGFP *Opn4L* and *Opn4S* WT and S310A as described in Section 2.2.7.2.

3.2.5 Fluorescent calcium imaging

Intracellular calcium in Neuro-2A cells transiently transfected with pIRES2-acGFP-*Opn4* WT or S310A was monitored using a fluorescent calcium indicator, Fluo-4 AM (Molecular Probes). Cells were prepared for calcium imaging and imaged using FLUOstar Omega plate reader, as described in Section 2.2.7.3.

Each 24-well plate contained 4 technical replicates of each plasmid: pcDNA3.1 *Opn4L* WT, *Opn4L* S310A, *Opn4S* WT *Opn4S* S310A, and a no-transfection control. These technical replicates were averaged to give a single biological replicate value. Six plates were imaged in total, each transfected separately, to provide 6 biological replicates per plasmid.

3.2.6 Immunocytochemistry

Neuro-2A cells were seeded onto glass coverslips in a 6-well plate at a density of $\sim 1.5 \times 10^5$ cells/well and transfected with pIRES2-acGFP-*Opn4* WT or S310A as described previously. Forty-eight hours after transfection, cells were fixed for 10mins at room temperature using 4% paraformaldehyde in phosphate buffered saline (PBS). Cells were then permeabilised in 0.2% Triton-X (Sigma) in PBS for 5mins, then blocked with 5% Donkey serum in PBS with 0.05% Tween-20. Cells were incubated in 1:2500 dilution of primary rabbit anti-melanopsin antibody (UF006, Advanced Targeting Systems) in PBS with 0.05% Tween-20 (Sigma) for 1hr at room temperature. Cells were washed 5 times in PBS with 0.05% Tween-20, then incubated in a 1:200 dilution of secondary donkey anti-rabbit IgG conjugated to Alexa 568 (Molecular Probes, Life Technologies) in PBS with 0.05% Tween-20 for 30mins at room temperature. Coverslips

with cells were mounted onto poly-lysine coated slides using Prolong Anti-Fade Gold media with DAPI (Molecular Probes, Life Technologies).

Images were taken using a x63 oil objective of a LSM710 laser-scanning confocal microscope (Zeiss) and Zen2009 image acquisition software (Zeiss) with excitation at 405nm, 488nm and 561nm and emission at 440-480nm, 505-550nm and 600-700nm for DAPI, green and red fluorescence, respectively. Brightness and contrast enhancements carried out using ImageJ software (<http://rsbweb.nih.gov/ij>).

3.2.7 Animals

Mice possessing the *Opn4* S310A mutation (*Opn4*^{S310A}) were recovered by *in vitro* fertilisation (IVF) performed at the Mary Lyon Centre, MRC Harwell. Cryopreserved sperm from the *Opn4*^{S310A} F1 male mouse was used for IVF with C3H/He oocytes. Resulting two-cell stage embryos were then implanted into pseudo-pregnant C3H/He females, resulting in 21 animals. The heterozygote progeny, 3 male and 5 female, were used as founders for the *Opn4*^{S310A} colony at Oxford.

This *Opn4*^{S310A} line was backcrossed onto a C3H/He strain for four generations. The C3H/He strain has the *Pde6b*^{rd1} mutation, which causes degeneration of the outer retina (Pittler & Baehr, 1991). A fifth backcross was carried out with C3H/He *rd/rd cl* mice (Lucas et al., 1999). In addition to the *Pde6b*^{rd1} mutation, *rd/rd cl* carry an attenuated diphtheria toxin-transgene driven by a human LWS cone opsin promoter that targets cones for cytotoxic lesion (*cl*, Soucy et al., 1998; Wang et al., 1992). Full genotyping protocol for *rd/rd cl* animals can be found in section 2.2.5.2. *Opn4*^{S310A} animals were genotyped using a TaqMan MGB probe assay, described in full in Section 2.2.5.1. Details of animal housing conditions and ethical approval can be found in Section 2.2.8.1. *Opn4*^{S310A} mice were backcrossed onto a retinally degenerate strain and tested at 80 days or older when outer retina degeneration was complete, in order to isolate

melanopsin function from confounding light input from rod or cone photoreceptors. The different cohorts used in this study are described in **Table 3.2**.

Table 3.2. Cohorts used in pupillometry and multi-electrode array studies. Animals used for MEA action spectrum includes all mice used for 550nm Pupillometry IRC and some additional animals too young for pupillometry at start of testing. All *Opn4^{WT}* animals were littermates of *Opn4^{S310A}* animals. IRC = irradiance response curve. MEA = multi-electrode array.

Backcrosses (Strain)	Test	Number
3 Backcrosses (C3H/He <i>Pde6b^{rd1}</i>)	Pupillometry IRC at 550nm	4 <i>Opn4^{WT}</i> 5 <i>Opn4^{S310A}</i>
	MEA action spectrum	11 <i>Opn4^{WT}</i> 10 <i>Opn4^{S310A}</i>
5 Backcrosses (C3H/He <i>rd/rd cl</i>)	Pupillometry action spectrum	7 <i>Opn4^{WT}</i> 6 <i>Opn4^{S310A}</i>

3.2.8 Pupillometry

The first pupillometry study represented an initial screen for a shift in spectral sensitivity of the pupil light responses (PLR) of the novel mutant, whereas the second study provided a detailed characterisation of spectral sensitivity (see below for details on why specific wavelengths were chosen). Pupillometry was conducted as described in Section 2.2.8.2.

For the first pupillometry experiment, few mice achieved full pupil constriction even at brightest intensities. Constriction of the exposed eye reduces light input, which affects magnitude of consensual pupil response. To improve PLR dynamic range for the second pupillometry study, a 1% tropicamide solution (Bausch & Lomb) was applied to the exposed eye at the start of each session, approximately 10-15mins before first recording. For the second pupillometry study, data collection and analysis was conducted blind to genotype to prevent subjective bias. This was not controlled during the first pupillometry experiments.

All animals received at least 2 training sessions before collecting experimental data. This acclimatised animals to handling and light exposure, reducing stress-related confounds. For the first pupillometry experiment, a single irradiance response curve (IRC) was generated at 550nm. For the second pupillometry study where an action spectrum was produced, IRCs were generated for 4 wavelengths - 480, 500, 550 and 570nm. Each irradiance response curve was constructed from PLR to 6 intensities over a 4-log quanta range, in 1 or 0.5log steps. PLR to 3 intensities of a given wavelength were recorded from all animals during each session. If an animal moved during a light exposure, the recording was repeated at the end of the session. If the repeated recording was also unsuccessful, the IRC was constructed without that intensity. A range of intensities was tested in each session to prevent variation between sessions skewing IRC. The order of wavelength and light intensities tested for the PLR action spectrum is shown in **Table 3.3**. Pupil images were analysed as described in Section 2.2.8.2.

Table 3.3. Pupillometry action spectrum sessions. Animals were given two training sessions to acclimatise to handling. Eight sessions were required to construct action spectrum. In each session pupil light responses to 3 different intensities of a given wavelength were recorded (shown in order of presentation) with 1hr interstimulus interval. HMBW = half-maximum bandwidth of bandpass filter.

Session Number	Wavelength (10nm HMBW)	Intensity (Log Quanta)
1	480nm	14.67
Training		14.67
2	480nm	14.67
Training		14.67
3	480nm	13.67
		12.67
		11.67
4	480nm	13.17
		14.67
		12.17
5	550nm	13.68
		12.68
		14.18
6	550nm	14.68
		13.18
		11.68
7	500nm	13.60
		12.60
		11.60
8	500nm	13.10
		14.60
		12.10
9	570nm	14.67
		13.67
		12.67
10	570nm	13.17
		14.17
		11.67

3.2.9 Multi-electrode array recordings

Multi-electrode array recordings were conducted as described in Section 2.2.9. For all IRC recordings, activity was recorded for 30s prior to 10s light stimulation (baseline), then for up to 2mins after light exposure (response). Responses for each wavelength were recorded at 5-6 different intensities over 4-5log quanta range, from lowest to highest intensity with increasing interstimulus intervals as described in Section 2.2.9. Where possible, IRCs were recorded for five wavelengths (450nm, 480nm, 510nm, 540nm and 570nm) from each retina. If ipRGCs stopped responding to light before all wavelengths were tested, the action spectrum for that retina was constructed from completed IRCs (minimum 3 wavelengths). The wavelengths tested for each retina and number of ipRGC responses recorded are shown in **Table 3.4**.

Spike data was extracted and sorted using MC rack software (Multi-Channel Systems) as described in Section 2.2.9. For each recording, response amplitude was defined as the difference in average number of spikes in the 10s before and after light onset. Only electrodes with a difference in spikes/s greater than 15 at the brightest intensity tested of each wavelength were used in further analysis, to ensure the electrodes were responsive across the full range of intensities. Any responses which did not demonstrate an intensity-dependent increase in response amplitude were removed as outliers. The response amplitude at different intensities was normalised to maximum response amplitude for each wavelength. Normalised responses from different ipRGCs were then averaged to give mean normalised response amplitude for each retina and used to fit IRC.

Table 3.4. Wavelength order and number of ipRGC responses. MEA action spectra were constructed from up to 5 IRCs. The order of wavelengths tested was counterbalanced across retina to prevent order effects. The number of ipRGC responses used to construct IRCs at each wavelength is shown on the right. The normalised ipRGC responses were averaged to produce a single IRC for each retina at each wavelength.

Genotype	Retina	Wavelength Recording Order (nm)					Number of ipRGC responses				
		1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th
<i>Opn4^{WT}</i>	1	480	540	570	510	450	15	11	7	3	1
	2	510	570	450			15	8	2		
	3	540	570	450			17	10	12		
	4	480	510	450	570	540	14	11	12	10	8
	5	510	540	480	570		6	8	8	1	
	6	450	480	510			7	7	3		
	7	570	480	540	510		33	36	40	30	
	8	450	480	540			3	3	7		
<i>Opn4^{S310A}</i>	1	510	480	570	540	450	6	9	10	9	10
	2	510	570	450	480	540	8	7	10	7	3
	3	570	450	540	480	510	9	9	8	10	4
	4	480	540	510	450	570	15	15	15	10	7
	5	540	450	480	510	570	16	15	15	11	13
	6	450	510	540	570	480	5	8	7	6	5

3.2.10 Irradiance response curves and action spectra construction

Given the hypothesis that *Opn4* S310A led to a spectral tuning mutation, the majority of experiments using the *Opn4^{S310A}* mouse model focused on testing spectral sensitivity. This was accomplished by comparing sensitivity at individual wavelengths, as well as the wavelength of peak sensitivity ($\lambda_{\max}$) of *Opn4^{S310A}* and *Opn4^{WT}* mice.

$\lambda_{\max}$ can be determined by fitting an opsin template to an action spectra. An action spectrum is the spectral sensitivity of a physiological response and the opsin template provides a model of the relationship between wavelength (x-axis) and relative sensitivity (y-axis) for visual pigments. Different templates exist for opsins that bind a vitamin A1-

derived or A2-derived chromophore. A2-pigment templates are broader and red shifted relative to A1-pigment templates (Govardovskii et al., 2000).

On a wavelength scale, the shape of the opsin template is the same for all A1 visual pigments and can be described by a single parameter, $\lambda_{\max}$, which determines its absorption at all wavelengths (Peirson et al., 2005).

Action spectra were created using the following method.

1. OPN4-driven behaviours were recorded under a range of light intensities for multiple monochromatic wavelengths. For this study, these were minimum pupil area or ipRGC response amplitude.
2. A sigmoid curve was fitted to normalised irradiance-response data by nonlinear regression to generate an irradiance-response curve (IRC).
3. The point of half-maximal sensitivity (EC50 or IC50) was calculated for each wavelength tested. This was compared between groups to assay sensitivity at specific wavelengths.
4. EC50 or IC50 values were used to generate relative sensitivity values, fitted to an A₁-visual pigment template by nonlinear regression. The best fitting template provides an estimate of $\lambda_{\max}$.

IRCs were defined using the following equation:

$$Response = \frac{1}{1 + 10^{Slope(EC50 - \log X)}}$$

Equation 3.1

Where EC50 (or IC50) = intensity in log quanta which elicits half-maximum response, slope = steepness of the curve, X = intensity in photons/cm²/s and log X = intensity in log quanta (Motulsky & Christopoulos, 2003). The effects of changing EC50 and slope on the IRC are shown in **Figure 3.3A-B**.

The pupillometry IRCs were fit with negative slope values as the effect of light is inhibitory on minimum pupil area; increasing light intensity causes smaller minimum pupil area. The MEA IRCs were fit with positive slope values, as the effect of light is excitatory on ipRGC response amplitude; increasing light intensity leads to larger response amplitude.

IRCs were fit to normalised responses using nonlinear least-squares minimisation. This is an iterative approach that identifies the best possible fit of IRC model to data by adjusting parameters controlling shape of IRC. The fit of the curve (R^2) was calculated using the following equation:

$$R^2 = 1 - \frac{SS_{residuals}}{SS_{total}}$$

Equation 3.2

Where $SS_{residuals}$ is the sum of squares of the vertical difference between the IRC model and data (**Figure 3.3C**) and SS_{total} is the sum of squares of the vertical difference between the IRC model and the mean of the data (**Figure 3.3D**). Least squares minimisation was conducted using the Excel solver plugin, as described in Kemmer & Keller (2010). The minimum possible $SS_{residual}$ value was obtained by changing EC50. Slope was fixed at 0.5 (550nm pupillometry IRC), 1 (MEA action spectrum IRCs) or -1 (pupillometry action spectrum IRCs).

Action spectra were constructed from IRCs at multiple wavelengths, using method described in Peirson et al. (2005). EC50 values in log quanta from the best-fit IRCs (**Figure 3.4A**) were first converted to photons/cm²/s ($10^{\log \text{ quanta}}$), then converted to relative sensitivity by dividing each EC50 value by the minimum EC50 value. Log relative sensitivity values were used for fitting the opsin template, with λ_{max} having a log relative sensitivity value of 0 (**Figure 3.4B**).

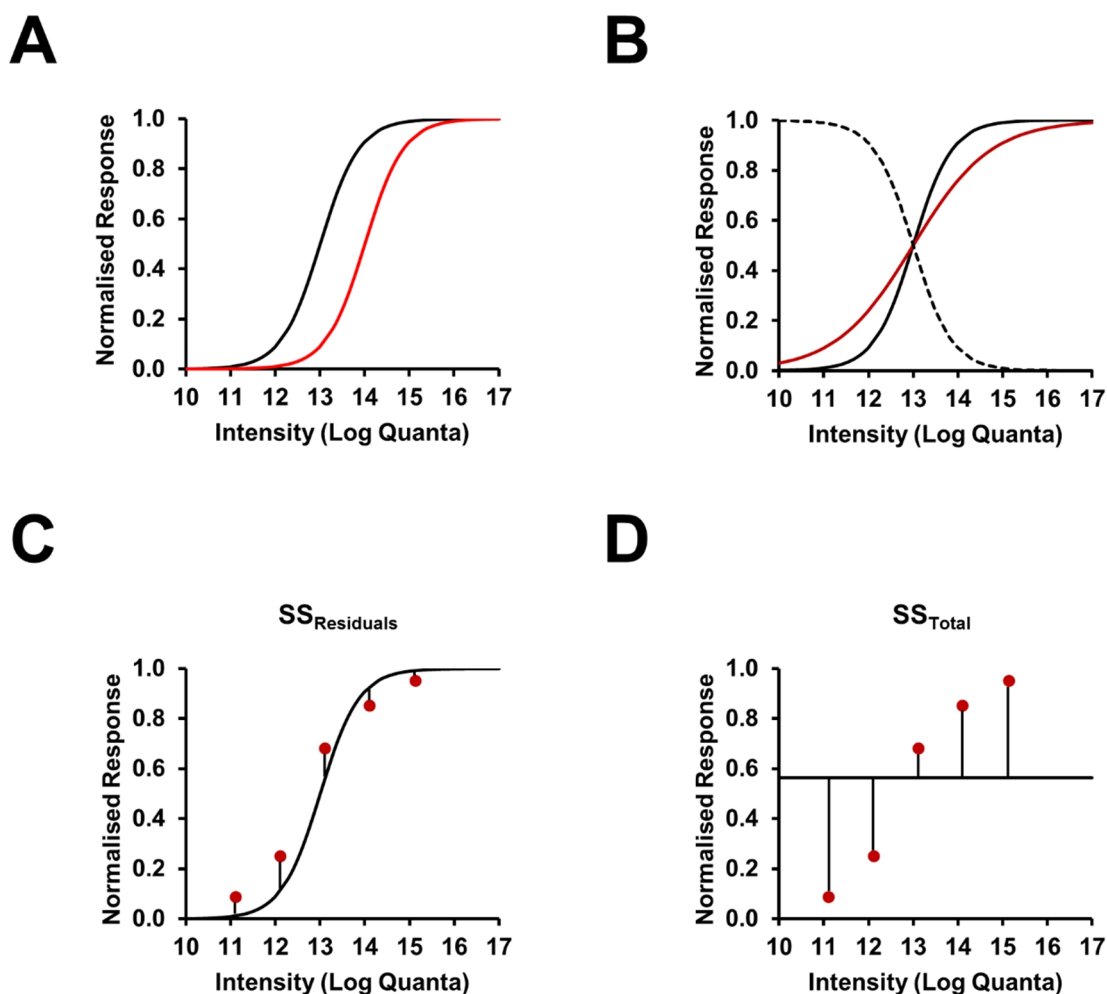


Figure 3.3. Irradiance response curves (IRCs) fit by least squares minimisation. Shape of IRC is controlled by 2 parameters – EC50 and Slope **(A)** Increasing EC50 from 13 (black curve) to 14log quanta (red curve) causes rightwards shift along X axis. Slope = 1 for both curves **(B)** Decreasing slope from 1 (black curve) to 0.5 (red curve) causes a shallower IRC and broader dynamic range. Negative slope values (dashed lines) are used for inhibitory IRCs, where response decreases with increasing light intensity. EC50 = 13log quanta for all curves. **(C)** $SS_{Residuals}$ is the sum of squared difference between data and IRC, shown as vertical black lines. EC50 is adjusted until minimum $SS_{Residuals}$ is reached. **(D)** SS_{Total} is the sum of squared difference between data and mean of data (horizontal line), shown as vertical black lines. It is used with $SS_{Residuals}$ to calculate goodness of fit of IRC to data (R^2).

The opsin template was calculated using the following equations (Govadovskii et al., 2000). First the template for α -band of the A1 visual pigment template was defined as:

$$S_{\alpha}(x) = \frac{1}{\exp[A(a - x)] + \exp[B(b - x)] + \exp[C(c - x)] + D}$$

Equation 3.3

Where $x = \lambda_{\max}/\lambda$, $A = 67.9$, $a = 0.8795 + 0.0459 \cdot \exp[-\frac{(\lambda_m - 3)^2}{1}]$, $B = 28$, $b = 0.92$, $C = -14.9$, $c = 1.104$ and $D = 0.675$.

The template for the β -band of the A1 visual pigment template was defined as:

$$S_{\beta}(\lambda) = A_{\beta} \cdot \exp\left\{-\left[\frac{\lambda - \lambda_{m\beta}}{b}\right]^2\right\}$$

Equation 3.4

Where $A = 0.26$, $\lambda_m = 189 + 0.315 (\lambda_{\max})$ and $b = -40.5 + 0.195(\lambda_{\max})$.

The α -band was added to the β -band to produce the final relative sensitivity value of the A1-pigment template at each wavelength. The opsin template was fit to the log relative sensitivity data using the same nonlinear regression method described above, with the minimum SS_{residual} achieved by changing $\lambda_{\max}$.

Comparing visual pigment templates with different $\lambda_{\max}$ allows prediction of changes in sensitivity anticipated at specific wavelengths. Shifts in $\lambda_{\max}$ will affect sensitivity across all wavelengths that cause responses, but will be especially noticeable at longer wavelengths due to the shape of the opsin template (**Figure 3.4C**). If the S310A mutation causes a spectral tuning effect, differences in EC50 may only be apparent at longer wavelengths. However, responses also become smaller the further the wavelength of light stimulus is from $\lambda_{\max}$. This reduces signal to noise ratio, meaning even large differences in sensitivity may not reach significance. Therefore, testing around 550nm provides a good balance between measurable response and sufficient sensitivity difference.

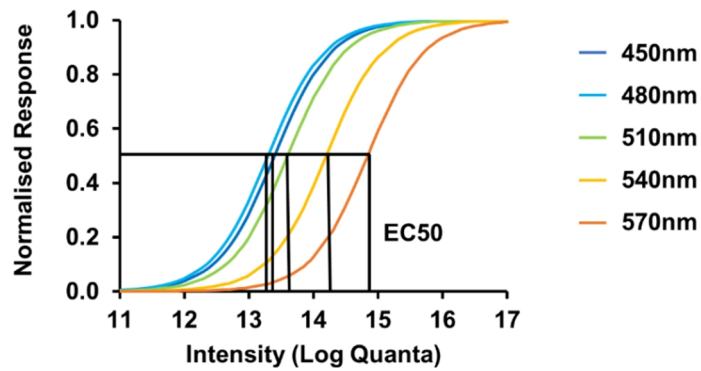
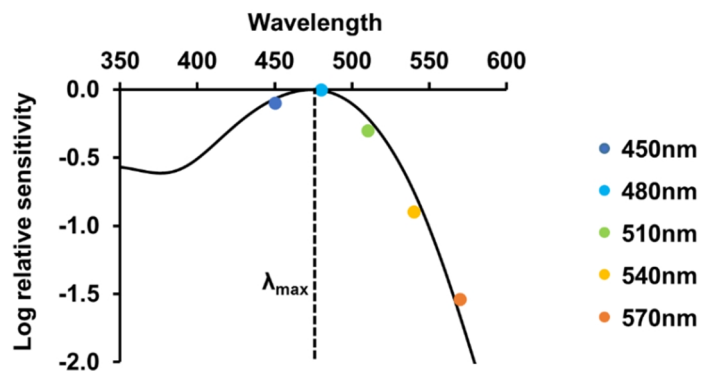
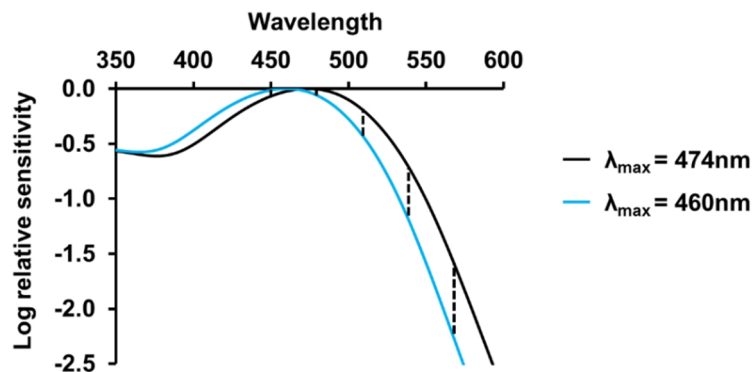
A**B****C**

Figure 3.4. Example action spectrum construction. (A) EC50, intensity in log quanta that produces half-maximum response (black lines), are calculated from IRCs over a range of wavelengths (coloured lines) **(B)** EC50 values at each wavelength are converted to photons/cm²/s and used to calculate log relative sensitivity by normalising to most sensitive wavelength (lowest EC50). An A1-visual pigment template can be fit to this data by least squares minimisation to find wavelength of peak sensitivity, $\lambda_{\max}$ **(C)** A 14nm short-wavelength shifts in spectral tuning causes no difference in sensitivity at 480nm but ~0.5log decrease in sensitivity at 550nm and ~1log decrease at 570nm (dashed lines).

3.2.11 Statistical Analysis

To assess the accuracy of the action spectrum, the 95% confidence intervals were calculated for $\lambda_{\max}$ of visual pigment templates fit to *Opn4*^{S310A} and *Opn4*^{WT} using a Monte Carlo simulation (Motulsky & Christopoulos, 2003; Peirson et al., 2005). A large number of simulated datasets were generated and fit with opsin templates. The distribution of best-fit $\lambda_{\max}$ for the simulated datasets was then used to estimate the 95% confidence intervals, which provide a measure of how precise the opsin template fits are to the actual log relative sensitivity data.

To generate simulated datasets, a Govardovskii template was first fitted to the average log relative sensitivity data by least squares minimisation. The standard deviation of the SS_{residual} or Root Mean Square (RMS) was calculated for the best-fit opsin template as:

$$RMS = \sqrt{\frac{SS_{\text{residuals}}}{DF}}$$

Equation 3.5

Where SS_{residual} = sum of squared difference between data and model, DF = degrees of freedom calculated as number of data points (wavelengths with IRCs) minus number of model parameters (1, $\lambda_{\max}$). For MEA action spectrum, DF = 4. For PLR action spectrum, DF = 3.

Random Gaussian noise was added to the average log relative sensitivity data (mean = 0, Standard Deviation = RMS calculated above) to generate 500 datasets. An A1-visual pigment template was fitted to each of the 500 datasets using the non-linear regression method described above. The 95% confidence intervals were calculated as the 2.5% and 97.5% percentiles of $\lambda_{\max}$.

Opn4^{S310A} and *Opn4*^{WT} action spectra were compared using two approaches. The first involved fitting a Govardovskii template to each individual's data, then comparing the best-fit parameter ($\lambda_{\max}$) between individuals of different groups using an independent samples Student's t-test. This approach specifically tests whether $\lambda_{\max}$ differs between individuals, as it is dependent upon the fit of the IRCs and the accuracy of the best-fit $\lambda_{\max}$.

By contrast, the second approach uses an F-test to test whether opsin templates fit to average *Opn4*^{S310A} and *Opn4*^{WT} data are statistically different. By calculating the overall improvement in fit produced by fitting templates separately to each genotype, compared to fitting a single template to pooled data, it can be determined if there is sufficient evidence to reject the null hypothesis and conclude *Opn4*^{S310A} and *Opn4*^{WT} opsin templates are significantly different.

The F-test was conducted using the following method. First, an opsin template was fitted to average *Opn4*^{S310A} and *Opn4*^{WT} log relative sensitivity data. SS_{residual} from *Opn4*^{S310A} and *Opn4*^{WT} best-fit models was summed to give the total SS_{residual} from fitting curves separately, referred to as SS_{separate} . This was repeated with degrees of freedom (DF) to obtain a DF_{separate} value. The average data from *Opn4*^{S310A} and *Opn4*^{WT} was combined to create one dataset and fitted an opsin template to this pooled data. The SS_{residual} and DF from the best-fit template for pooled data were labelled SS_{combined} and DF_{combined} . An F ratio was calculated using the following equation:

$$F = \frac{(SS_{\text{combined}} - SS_{\text{separate}}) / (DF_{\text{combined}} - DF_{\text{separate}})}{SS_{\text{separate}} / DF_{\text{separate}}}$$

Equation 3.6

A P value was calculated from the F ratio, the degrees of freedom of the numerator ($DF_{\text{combined}} - DF_{\text{separate}}$) and degrees of freedom of the denominator (DF_{separate}), using the Excel FDist function. If $P < 0.05$, the null hypothesis (*Opn4*^{S310A} and *Opn4*^{WT} opsin templates are not different) is rejected, ie: they are significantly different.

These two approaches, comparing individuals using Student's t-test and using an F-test to compare curves fit to average data, were also used to compare sensitivity at individual wavelengths. EC50 was used as a measure of sensitivity, calculated from best-fit IRCs.

Statistical comparisons were made using SPSS 22.0 (IBM). For all statistical tests a significance threshold of $p \leq 0.05$ was used. In all figures * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Error bars show standard error of the mean (SEM). Mean values in text are given $\pm$ SEM.

3.3 Results

3.3.1 Identification of a novel ENU-induced melanopsin mutation

The Mammalian Genetics Unit of MRC Harwell have created an archive of genomic DNA from 11328 mice possessing random point mutations induced by the chemical mutagen, *N*-ethyl-*N*-nitrosourea (ENU, Nolan et al., 2000). A genotype-driven screen of this archive was conducted to identify novel *Opn4* point mutations. *Opn4* exon 6 was screened because it encodes IL3 and TM6, and mutations identified in this region are likely to affect protein function (see Methods).

A heteroduplex screen of the full archive showed a possible point mutation in the coding region of the *Opn4* gene (**Figure 3.5A**). Sanger sequencing revealed this mutation was a thymine to guanine substitution at base pair 928 (c.928 T>G), leading to a non-synonymous amino acid change from serine (TCC) to alanine (GCC) at position 310 (**Figure 3.5B**). This mutation is subsequently referred to as S310A. This mutation was not found in wildtype C3H/He mice (data not shown), confirming it is not a SNP.

A structural homology model of melanopsin based on bovine rhodopsin shows position 310 is located in part of TM6 oriented towards the central cavity (**Figure 3.5C**), suggesting it may form part of the chromophore binding pocket. As the ENU-induced mutation identified is non-synonymous and occurs in a key functional protein domain, it may lead to a novel phenotype.

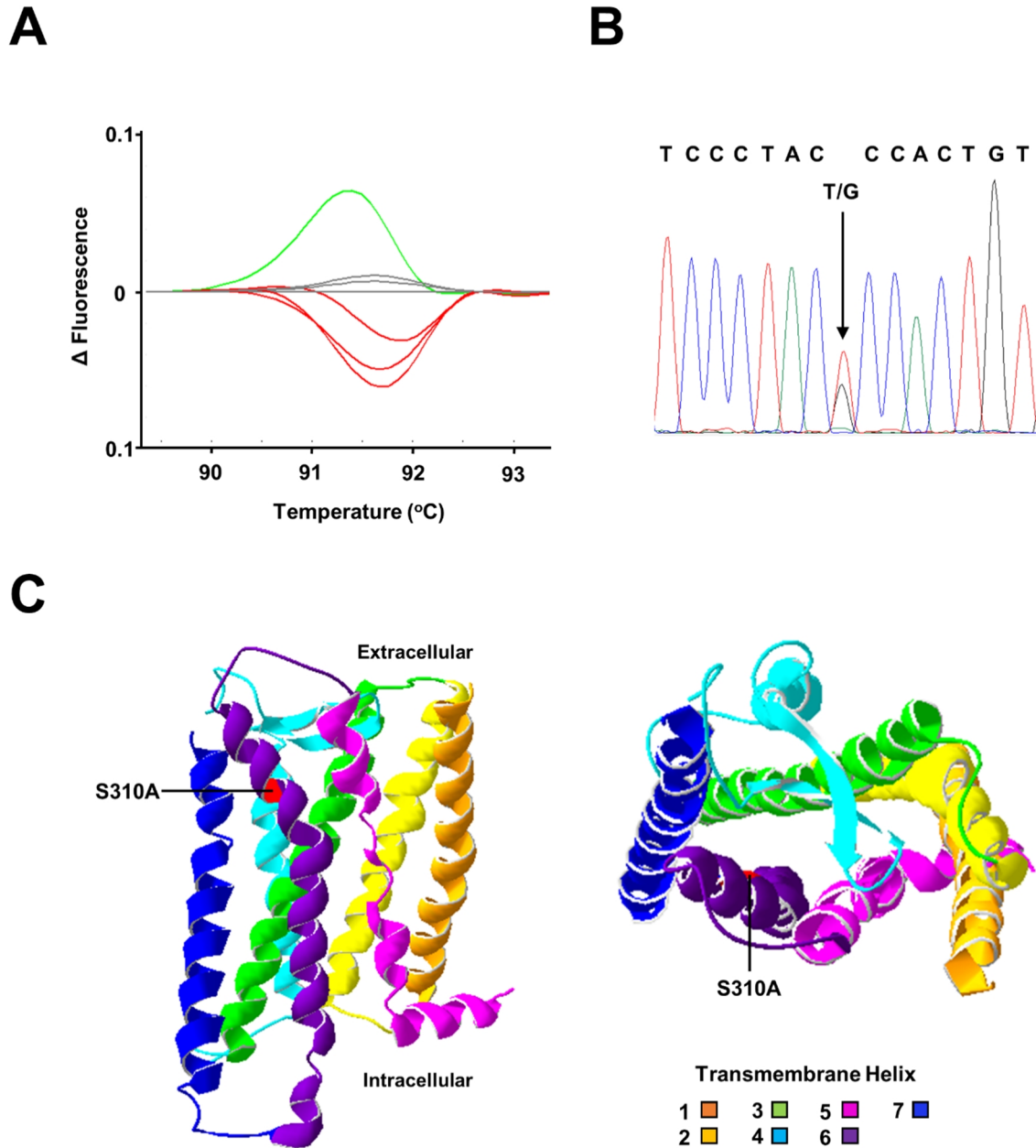


Figure 3.5. Identification of *Opn4* mutation, S310A, in genotype-driven ENU archive screen. (A) High resolution melt curve analysis revealed possible mutation (highlighted in green) in ENU archive sample. Mutation causes heteroduplex formation, lower melting point manifests as a positive amplitude on difference curve (B) Sanger sequencing confirmed presence of substitution at base pair 928 (CDS numbering) in *Opn4* exon 6. Arrow indicates heterozygous T/G genotype. (C) Homology model of OPN4 protein based on crystal structure of Bovine Rhodopsin (1GZM; Personal communication from G. Schertler). Position of S310A mutation is highlighted in red. View from extracellular surface (right) shows S310A is oriented towards the central cavity. In rhodopsin, this position is within 4.5Å of β -ionone ring of 11-*cis* retinal (Palczewski et al., 2000). Transmembrane helices are colour coded (key for helix number shown bottom right).

3.3.2 *Opn4* S310A is located at a highly conserved opsin spectral tuning site

Bioinformatics was used to predict potential functional consequences of the S310A mutation. First, mouse OPN4 was aligned with the protein sequences of human rhodopsin and cone opsins to investigate whether the equivalent position has been mutated in other opsins. OPN4 S310A corresponds to position 285 in long-wavelength (LWS) and middle-wavelength sensitive (MWS) cone opsins. As discussed in Section 1.3.3.2, this is one of three highly-conserved amino acids responsible for the ~30nm spectral tuning difference between LWS and MWS visual pigments (Neitz et al., 1991; Yokoyama & Radlwimmer, 1998). Of these 3 spectral tuning sites, 285 appears to cause the largest shift in $\lambda_{\max}$ when mutated in isolation.

As shown in **Table 3.5**, amino acid substitutions at the equivalent position to OPN4 S310A cause $\lambda_{\max}$ shifts in several opsins. This suggests it is a highly conserved opsin spectral tuning site. Furthermore, there is a consistent relationship between the type of substitution and direction of the spectral tuning shift. Addition of polar hydroxyl-bearing amino acids, such as serine, tyrosine or threonine, at this site leads to long-wavelength shifts in $\lambda_{\max}$ and their removal causes short-wavelength shifts in $\lambda_{\max}$. However, mutation of this site in short-wavelength sensitive (SWS) cone visual pigment appears to have a limited effect, suggesting it only acts as a spectral tuning site under particular conditions.

Table 3.5. Mutation of equivalent site to OPN4 S310A causes spectral tuning shifts in other opsins. Mutations are numbered according to position in protein mutated. LWS = long-wavelength sensitive, MWS = middle-wavelength sensitive, SWS2 = short-wavelength sensitive (blue).

Opsin ($\lambda_{\max}$)	Reference	Mutation	$\lambda_{\max}$	$\Delta\lambda_{\max}$
Human MWS cone opsin (530nm)	<i>Neitz, Neitz & Jacobs (1991)</i>	A285T	545	+15
	<i>Asenjo, Rim & Oprian (1994)</i>		540	+10
Human LWS cone opsin (560nm)	<i>Merbs & Nathans (1993)</i>	T285A	545	-15
	<i>Asenjo, Rim & Oprian (1994)</i>		544	-16
Bovine Rhodopsin (500nm)	<i>Chan, Lee & Sakmar (1992)</i>	A269T	514	+14
Pigeon SWS2 cone opsin (448nm)	<i>Yokoyama & Tada (2003)</i>	S277A	448	0
		S277C	450	+2
		S277T	453	+5
Goldfish SWS2 cone opsin (443nm)	<i>Yokoyama & Tada (2003)</i>	A273C	444	+1
		A273S	448	+5
		A273T	449	+6

In addition, conservation of serine at position 310 was examined by aligning the OPN4 protein sequences of multiple species (**Figure 3.6**). Interestingly, serine 310 is highly conserved in *Opn4M* genes (found in all species), whereas alanine 310 is highly conserved in *Opn4X* genes (found in non-mammals only). This serine/alanine conservation within *Opn4* genes is intriguing, as two studies have found that OPN4X is blue-shifted relative to OPN4M, by approximately 14nm in zebrafish (Davies et al., 2011) and 8nm in chickens (Torii et al., 2007). The direction and magnitude of this spectral tuning shift are similar to the effect of removing hydroxyl-bearing amino acids at the equivalent position in LWS cone visual pigment.. Zebrafish OPN4M2 and OPN4X1 do not differ at the other LWS/MWS cone visual pigment spectral tuning sites or other sites known to contribute to spectral tuning in rhodopsin and SWS cone visual pigment (**Appendix 1**), suggesting S310A may represent a highly conserved OPN4 spectral tuning site. Based on these bioinformatics results, it was hypothesised the OPN4 S310A mutation would cause a short-wavelength shift in $\lambda_{\max}$.

	OPN4M	Intracellular Loop 3	Transmembrane Helix 6	
Chimpanzee	GNGESL----	WQRQLQSE	CKMAKIMLLVILLFVLSWAPY	S AVALVAFAGYA
Human	GNGESL----	WQRQLQSE	CKMAKIMLLVILLFVLSWAPY	S AVALVAFAGYA
Dog	GGARSP----	RQRQLQRE	WKMAKMELLVILLFVLSWAPY	S AVALTAFAGYS
Cow	GGSECP----	RQRQLQNE	WKMAKIELLVILLFVLSWAPY	S TVALMGFAGYA
Cat	GGGRSP----	RQRQLQNE	WKMAKIELLVILLFVLSWAPY	S IVALMAFAGYA
Mouse	GCGESPLRQRRQWQRLQSE		WKMAKVALIVILLFVLSWAPY	S TVALVAFAGYS
Rat	GCGESPLR-RRQWQRLQSE		WKMAKVALIVILLFVLSWAPY	S TVALVGFAGYS
Hamster	GWSESPQR-RRQWHRLQSE		WKMAKVALIVILLFVLSWAPY	S TVALVAFAGYS
Mole Rat	GCGESPQR-RRQWQRLQNE		WKMAKVALIVIFLVLSWAPY	S TVALVAFAGYS
Chicken	GNRELQ----	KQYHRMKNE	WKLAKIALIVILLYVISWSPY	S VVALVAFAGYS
Dunnart	HTPSL----	RHFQRMKNE	WKMAKIALVVILLFVLSWAPY	S TVALVAFAGYS
Zebrafish M1	-KRDSM----	KRFQRLKNE	WKMAKIALIVILMYVISWSPY	S TVALTAFAGYS
Zebrafish M2	--GETH----	KVYERMQNE	WKMAKVALVVILLFIISWSPY	S VVALTATAGYS
Zebrafish M3	GPRDSI----	KKIHRMKNE	WKMAKIALIVILLYVISWSPY	S CVALTAFAGYA
	OPN4X			
Chicken	SRKSFL-----	SQSMKNE	WKLAKIAFVVIIVYVLSWSPY	A CVTLIAWAGRG
Lizard	NRKSNV-----	SQSVKSE	WKLAKIAFVAIVFVLSWSPY	A CVTLIAWAGYA
African Clawed Frog	GRQSFL-----	SQSMKNE	WKMAKIAFVIIIVFVLSWSPY	A CVTLIAWAGHG
Zebrafish X1	VRKTTL-----	IQQQSIKTE	WKLAKIAFVVIIVFVLSWSPY	A CVTLIAWAGYS
Zebrafish X2	-QKSSF-----	VKQQSMRSE	WKLAKIAAVVIVVYVLSWAPY	A CVTLVAVAGHQ
Amphioxus	VKNKA-----	QQRQRKNE	IKTAKIAFIVISLFMSAWTPY	A VVSALGTLGYQ

Figure 3.6. OPN4 S310A mutation occurs at a highly conserved residue difference between *Opn4X* and *Opn4M* genes. Alignment of melanopsin protein sequences for multiple species performed using MAFFT software. Equivalent position of S310A mutation in OPN4M (red) and OPN4X (blue) is highlighted. Vertical line shows boundary between IL3 and TM6.

3.3.3 Functional characterisation of *Opn4* S310A *in vitro*

The conserved difference between *Opn4X* and *Opn4M* genes suggests substitution of serine for alanine at position 310 in OPN4 would be well tolerated. As the phenotype of *Opn4*^{-/-} mice has already been characterised, the aim of this study was to recover an *Opn4* mutant animal with altered response properties. To confirm the S310A mutation does not abolish OPN4-driven light responses, the function of OPN4 S310A was examined *in vitro*.

First, OPN4 S310A was examined using immunocytochemistry to assess if it affected membrane trafficking in Neuro-2A cells expressing long and short isoforms of *Opn4* WT and S310A (Figure 3.7).

The pattern of anti-OPN4 staining in cells transfected with *Opn4* S310A matches those transfected with *Opn4* WT, with immunofluorescence labelling present in the plasma membrane but absent from the cytoplasm and nucleus. No difference in protein localisation was found between the two isoforms. OPN4-labelling with anti-melanopsin UF006 antibody is found in the same cells as the pIRES2 GFP plasmid reporter. Weak GFP signal in UF006-negative cells is likely due to auto-fluorescence.

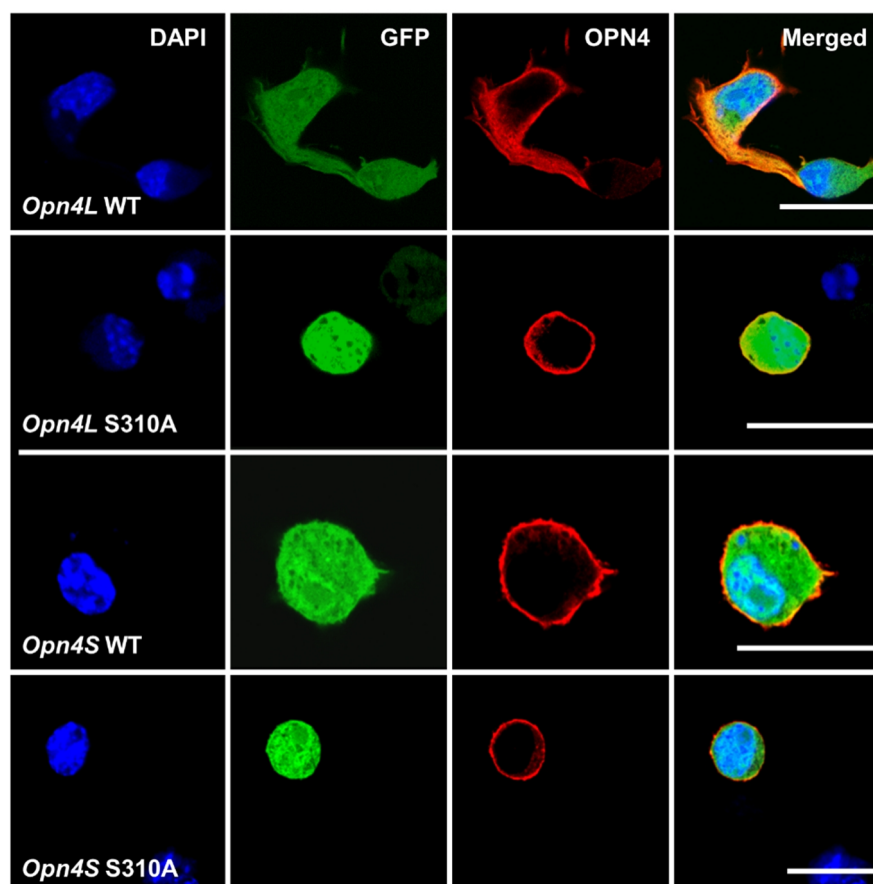


Figure 3.7. OPN4 S310A is trafficked normally to cell membrane. Confocal photomicrographs of Neuro-2A cells transiently transfected with pIRES2-acGFP *Opn4L* and *Opn4S* WT and S310A stained against OPN4 (red; UF006 anti-mouse melanopsin). DAPI nuclear stain (blue) and pIRES2 GFP reporter (green) are also shown. Scale bar = 25µm.

Opn4 expression confers photosensitivity to cell lines, resulting in $G_{q/11}$ signalling leading to increase in intracellular calcium levels (Kumbalasiri et al., 2007). Calcium imaging of melanopsin expressed in cell lines has previously been used to study its signalling pathways (Melyan et al., 2005; Qiu et al., 2005; Kumbalasiri et al., 2007). Fluorescent calcium imaging was used to confirm OPN4 S310A can act as a functional photopigment when reconstituted with 9-*cis* retinal (**Figure 3.8**). Expression of *Opn4* S310A produced an increase in intracellular calcium in transfected cells after light exposure, consistent with activation of a $G_{q/11}$ signalling pathway.

As shown in **Figure 3.8A**, the magnitude and kinetics of intracellular calcium influx were comparable between cells expressing *Opn4* S310A and WT. A two-way between-subjects ANOVA, with genotype (2 levels – WT and S310A) and isoform (2 levels – *Opn4L* and *Opn4S*) as independent variables, revealed no significant main effects of genotype on maximum normalised fluorescence, $F(1,20) = 0.08$, $p = 0.780$ (**Figure 3.8B**), time to maximum, $F(1,20) = 1.99$, $p = 0.173$ (**Figure 3.8C**), or time to half-maximum fluorescence, $F(1,20) = 1.58$, $p = 0.224$ (**Figure 3.8D**). No significant main effect of isoform on response size or kinetics was found ($p > 0.05$ for all variables tested).

As expected, no isoform by genotype interaction was found for maximum normalised fluorescence, $F(1,20) = 2.70$, $p = 0.116$, time to maximum, $F(1,20) = 0.22$, $p = 0.643$, or time to half-maximum, $F(1,20) = 0.175$, $p = 0.680$. Given the location of the S310A mutation in TM6, it was not anticipated that OPN4 S310A would be affected by the variable C-terminus lengths caused by alternative splicing. The absence of a significant interaction confirmed this *in vitro*, suggesting both isoforms of *Opn4* S310A will be functional *in vivo*.

The combined evidence from calcium imaging and immunocytochemistry suggests OPN4 S310A forms a functional photopigment, which is trafficked normally and capable of activating the $G_{q/11}$ phototransduction cascade comparable to OPN4 WT.

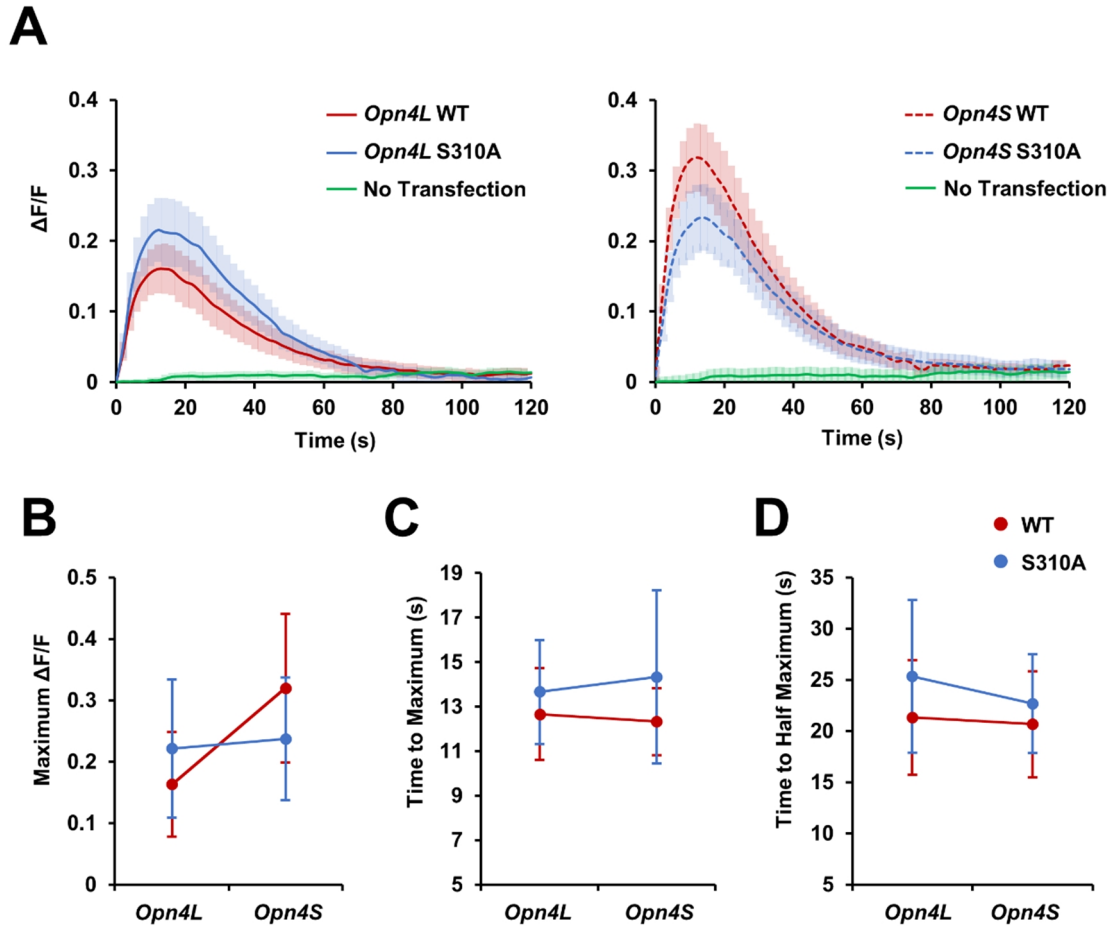


Figure 3.8. Fluorescent calcium imaging shows OPN4 S310A can trigger a $G_{q/11}$ signalling cascade in response to light comparable to OPN4 WT. (A) Intracellular calcium levels of Neuro-2A cells transiently transfected with pcDNA3.1 *Opn4L* (left) and *Opn4S* (right) WT and S310A were monitored using fluorescent calcium indicator Fluo4-AM. Melanopsin response was triggered by first light exposure for fluorescent imaging ($485\text{nm} \pm 6\text{nm}$) and measured every 2s. Untransfected Neuro-2A cells showed no change in intracellular calcium. Cells were supplemented with $20\mu\text{M}$ 9-*cis* retinal. Responses are normalised to first light exposure (B) Mean maximum normalised fluorescence. (C) Mean time to peak (s) (D) Mean time to half-maximum (s), calculated from time of peak fluorescence (s). For all groups, $n = 6$ biological replicates (each biological replicate consists of 4 technical replicates). Error bars = SEM.

3.3.4 *Opn4*^{S310A} mice show attenuated PLR sensitivity to 550nm light

The *in vitro* experiments confirmed OPN4 S310A formed a functional photopigment. Based on this, and the bioinformatics-driven hypothesis that the S310A mutation may affect spectral sensitivity, the *Opn4*^{S310A} mouse was recovered for further testing *in vivo*. These mice were backcrossed onto a retinally degenerate C3H/He background to limit outer retinal input to ipRGCs and isolate melanopsin-driven responses. Using the first cohort of homozygous *Opn4*^{S310A} and littermate *Opn4*^{WT} controls, initial experiments compared sensitivity of pupil constriction at 550nm (**Figure 3.9**). This wavelength was chosen because small changes in $\lambda_{\max}$ are easier to detect at longer wavelengths of light. 550nm is also close enough to peak sensitivity that OPN4-driven responses should not be too small (see Section 3.2.10, **Figure 3.4C**).

As shown in **Figure 3.9A**, *Opn4*^{S310A} mice showed intensity-dependent pupil constriction similar to *Opn4*^{WT} controls. However, *Opn4*^{S310A} constriction appeared to be attenuated relative to *Opn4*^{WT} mice across the range of intensities tested. When this was quantified by constructing an irradiance response curve (IRC) to average minimum pupil size for each genotype (**Figure 3.9B**), the *Opn4*^{S310A} IRC was significantly different from the *Opn4*^{WT} IRC, $F(1,10) = 6.46$, $p = 0.029$ (F-test). This was likely due to the rightwards shift of the *Opn4*^{S310A} IRC, indicating a decrease in sensitivity. Confirming this, *Opn4*^{S310A} mice individually had significantly higher IC50 values than *Opn4*^{WT}, 13.64 ± 0.11 and 13.15 ± 0.06 log quanta respectively, $t(7) = -3.59$, $p = 0.009$ (Independent samples Student's t-test). A greater intensity of light was required to produce half-maximal response in *Opn4*^{S310A} mice, suggesting they are less sensitive to 550nm than *Opn4*^{WT} mice.

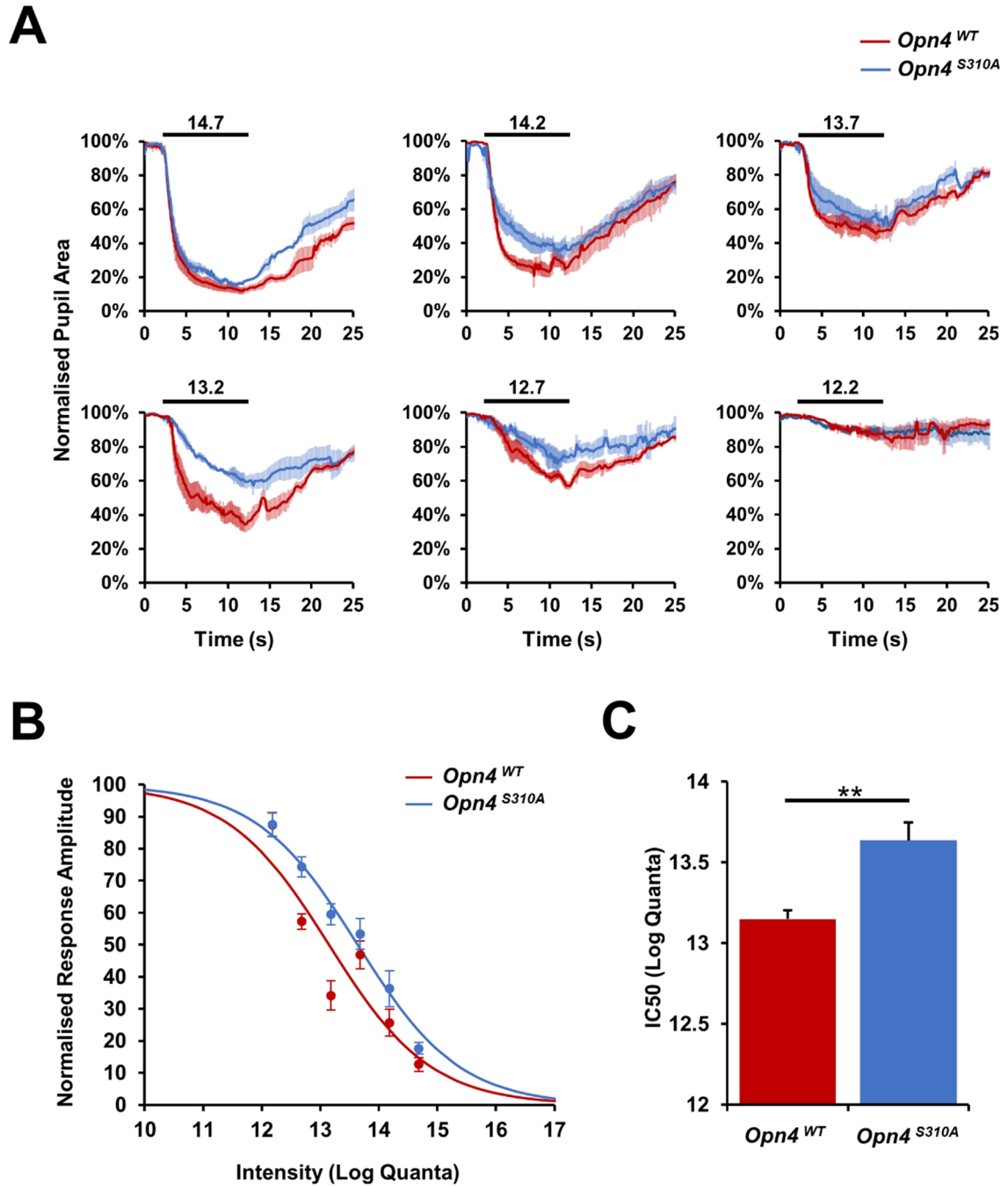


Figure 3.9. *Opn4*^{S310A} pupil light responses are less sensitive to 550nm light than *Opn4*^{WT}. (A) Average kinetics of pupil constriction to a 10s 550nm light pulse (shown as black bar above traces). Intensity of light (in log quanta) is shown above black bar. (B) Irradiance response curves fit to minimum constriction data using non-linear regression. $R^2 = 0.80$ for *Opn4*^{WT} and 0.95 for *Opn4*^{S310A}. (C) Average IC50- the intensity (log quanta) which produced half-maximal response. *Opn4*^{WT} (n = 4) and *Opn4*^{S310A} (n = 5). Error bars = SEM. ** p < 0.01, Student's t-test.

The ~0.5 log quanta decrease in sensitivity seen in *Opn4*^{S310A} is consistent with a 13nm blue shift in OPN4 $\lambda_{\max}$, based on predicted log relative sensitivity of Govardovskii templates with $\lambda_{\max}$ at 480nm or 467nm. The magnitude and direction of the sensitivity shift observed in pupil constriction of *Opn4*^{S310A} mice matches the blue-shifting effect of removing polar amino acids at this position in rhodopsin or LWS cone visual pigment. This suggests S310A may act as a melanopsin spectral tuning site through similar mechanisms.

3.3.5 MEA action spectrum shows no change in *Opn4*^{S310A} spectral sensitivity

The attenuated pupil constriction at 550nm demonstrated by *Opn4*^{S310A} mice suggests this mutation alters spectral sensitivity, as predicted. However, it is still possible this difference is due to general decreased sensitivity across the visible range of wavelengths. To confirm this, an action spectrum was generated from the same cohort of homozygous *Opn4*^{S310A} mice and littermate *Opn4*^{WT} controls, by recording the ipRGC responses from flatmount retina using a multi-electrode array (MEA), which provides a high throughput method for testing differences in spectral sensitivity. This approach allowed the comparison of *Opn4*^{S310A} and *Opn4*^{WT} sensitivity at multiple wavelengths, to determine if loss of sensitivity was specific to 550nm, and estimate the $\lambda_{\max}$ of *Opn4*^{S310A} compared to *Opn4*^{WT}.

IpRGC response amplitude, measured as an increase in spike firing rate from baseline after a 10s light pulse, was used to construct IRCs for the MEA action spectra. It was observed that the number of ipRGC responses decreased over time (see Methods **Table 3.4**), probably due to the long recording duration (approx. 6hrs) and repeated light stimulation required to generate 5 IRCs.

To confirm these factors did not bias the sensitivity of the retina, a 480nm IRC was repeated 5 times on the same retina (**Figure 3.10**). The first recording tended to be more sensitive, but subsequent IRCs had comparable sensitivity. When the EC50 from IRCs fit to individual ipRGCs was compared, a significant main effect of order on sensitivity was found, $F(4, 80) = 6.12$, $p < 0.001$ (One-way within subjects ANOVA). Post-hoc Sidak-corrected pairwise comparisons revealed this was due to a significant difference between the first and fourth IRC ($p = 0.01$). This suggests duration of recording or previous light stimulation can affect sensitivity of the retina. This variable was controlled by counterbalancing the order in which wavelengths were presented.

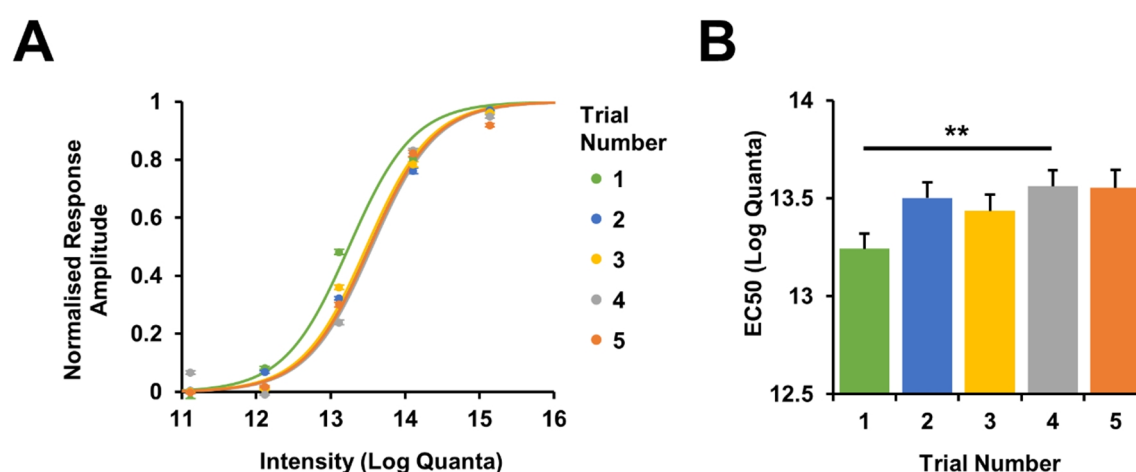


Figure 3.10. Recording order affects sensitivity of ipRGC responses. (A) 480nm IRCs were generated from 10s light pulse from 11.3 – 15.1 log quanta. Five repeats of 480nm IRC were conducted to test sensitivity over time. IRCs were fit to individual ipRGCs that remained responsive throughout the entire recording period. (B) Average EC50 values from individual ipRGCs ($n = 21$) for each of the five repeats. Error bars = SEM. ** $p < 0.01$, Sidak-corrected pairwise comparison.

IRCs were generated at 450, 480, 510, 540 and 570nm. Given the small hypothesised spectral tuning shift, differences in EC50 were only anticipated at 540nm and 570nm, and maybe 510nm if ipRGC responses were not too variable. Instead, the average sensitivity of individual *Opn4*^{S310A} and *Opn4*^{WT} retina was found to be very similar (**Table 3.6**), with no significant differences in EC50 at any of the wavelengths tested.

Table 3.6. No difference in sensitivity of ipRGC responses between *Opn4*^{WT} and *Opn4*^{S310A} retina. IRCs were fit to average ipRGC responses from each retina (n = 6 for all wavelengths and genotypes). The average EC50 values from these IRCs are shown below. Average EC50 of *Opn4*^{WT} and *Opn4*^{S310A} were compared using an independent samples Student's t-test.

Wavelength (nm)	<i>Opn4</i> ^{WT} (n = 6) Mean EC50 (SEM)	<i>Opn4</i> ^{S310A} (n = 6) Mean EC50 (SEM)	Independent Samples Student's t-test
450	13.69 (0.12)	13.63 (0.13)	t(10) = 0.35 p = 0.735
480	13.41 (0.13)	13.49 (0.12)	t(10) = -0.50 p = 0.627
510	13.73 (0.15)	13.71 (0.12)	t(10) = -0.06 p = 0.947
540	14.20 (0.09)	14.20 (0.07)	t(10) = 0.05 p = 0.955
570	14.81 (0.16)	14.94 (0.07)	t(10) = -0.75 p = 0.471

When IRCs fit to the average responses of all retinæ were compared, an F-test revealed the two genotypes were not significantly different (**Figure 3.11**). This suggests there was no change in spectral sensitivity of ipRGC responses, and that there was no overall change in sensitivity after introduction of the *Opn4* S310A mutation.

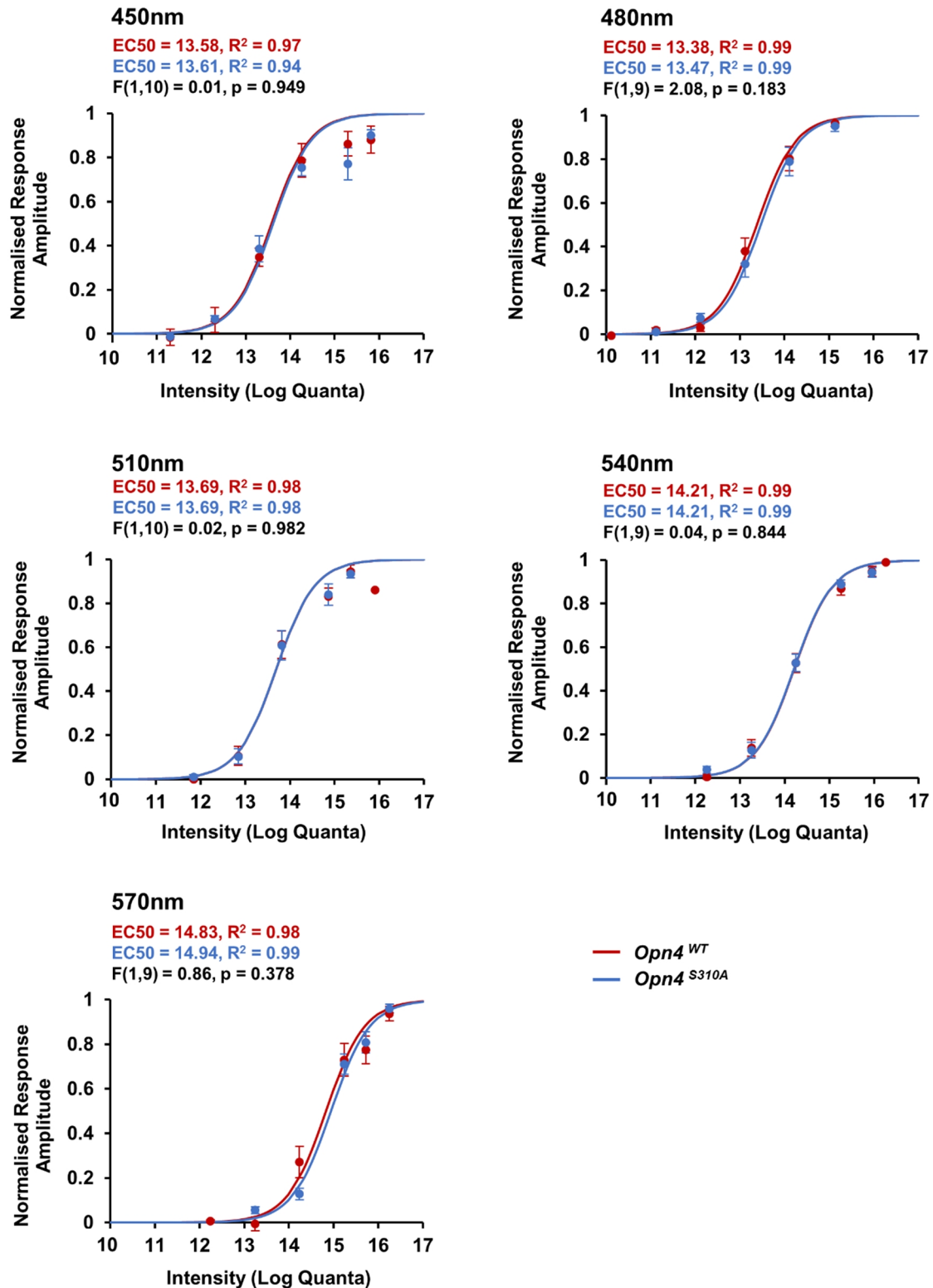


Figure 3.11. No difference in sensitivity of $Opn4^{WT}$ and $Opn4^{S310A}$ ipRGC responses at any wavelength. IRCs were fit to the average normalised responses from $Opn4^{WT}$ (red) or $Opn4^{S310A}$ (blue) retina by non-linear regression. Slope was kept constant at 1. The EC_{50} and R^2 of the best-fit IRC for each genotype is shown above each graph. The IRCs were compared using an F-test, results shown in black above each graph. Five wavelengths were tested (450-570nm in 30nm steps). Error bars = SEM.

An action spectrum was constructed from the EC50 values generated for each of the retina tested. The average $\lambda_{\max}$ of *Opn4*^{S310A} retina was slightly shorter than *Opn4*^{WT}, 473.0nm $\pm$ 2.5 and 476.7nm $\pm$ 4.4 respectively, but this difference was not significant, $t(12) = 0.66$, $p = 0.521$ (Independent samples Student's t-test). Action spectra for individual retina, particularly *Opn4*^{WT}, were quite variable, with several showing poor fits of the Govardovskii template. Mean R^2 was 0.75 ± 0.12 for *Opn4*^{WT} and 0.76 ± 0.07 for *Opn4*^{S310A}. Since the $\lambda_{\max}$ of all these retina should be the same, this variation reflects noise accumulated during the MEA recording and template fitting.

Attempting to reduce this noise, a Govardovskii template was fitted to the average retina data from *Opn4*^{S310A} and *Opn4*^{WT} (**Figure 3.12**). This method produced better fits, $R^2 = 0.99$ for *Opn4*^{S310A} and 0.95 for *Opn4*^{WT}, and a slightly longer $\lambda_{\max}$ of 477.2nm for *Opn4*^{S310A} and similar $\lambda_{\max}$ of 476.4nm for *Opn4*^{WT}. An F-test confirmed these were not significantly different from each other, $F(1,8) = 0.09$, $p = 0.768$. As shown in **Figure 3.12C**, templates with $\lambda_{\max}$ several nanometres either side of best-fit $\lambda_{\max}$ had nearly equivalent fits to the data. The 95% confidence intervals were broader for *Opn4*^{WT} than *Opn4*^{S310A}, 471.8-481.6nm and 475.0-479.7nm respectively.

These results contradict the previous pupillometry data showing *Opn4*^{S310A} mice have a robust reduction in EC50 at 550nm *in vivo*. Both pupil constriction and ipRGC responses should have similar spectral sensitivity as they are exclusively OPN4-driven in retinally degenerate mice tested at ages where there should be little to no outer retina function remaining. The confidence intervals of the MEA action spectrum suggest the fit of the Govardovskii templates may not be precise enough to reliably detect small changes in $\lambda_{\max}$. However, no changes in sensitivity were observed at the IRC level either, even at longer wavelengths, which might have been expected given the previous pupillometry results. Overall no difference was found in the spectral sensitivity of ipRGC responses from *Opn4*^{WT} and *Opn4*^{S310A} retina recorded using MEA.

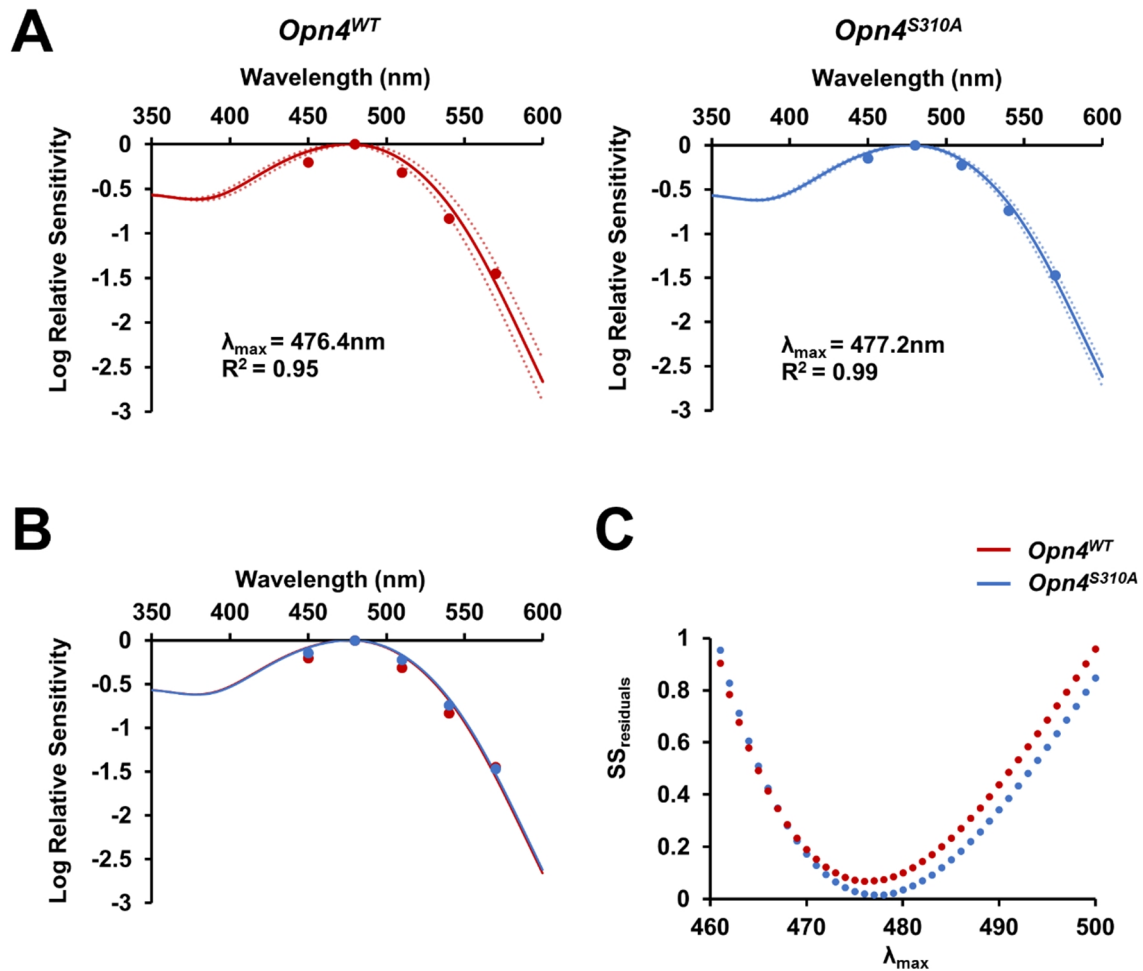


Figure 3.12. MEA action spectra are comparable for *Opn4^{WT}* and *Opn4^{S310A}*. (A) Govardovskii template (solid line) fit to relative sensitivity of ipRGC responses (circles). Relative sensitivity is derived from EC50 values of 5 IRCs fit to the average retina data from *Opn4^{WT}* and *Opn4^{S310A}* ($n = 6$ for both). The 95% confidence intervals generated from Monte Carlo simulations are shown as dotted lines. (B) Comparison of best-fit A_1 visual pigment template for *Opn4^{WT}* (red) and *Opn4^{S310A}* (blue) (C) Comparison of sum of squares of difference between average log relative sensitivity and visual templates with $\lambda_{\max}$ from 460-500nm for *Opn4^{WT}* and *Opn4^{S310A}*. Error bars = SEM.

3.3.6 Pupillometry action spectrum shows specific loss of sensitivity at long wavelengths but no change in *Opn4^{S310A}* $\lambda_{\max}$.

In vivo testing of *Opn4^{S310A}* mice revealed a significant reduction in sensitivity of pupil constriction at 550nm compared to *Opn4^{WT}* mice (Section 3.3.4). However, no difference in sensitivity of ipRGC cellular responses was identified between littermate *Opn4^{S310A}* and *Opn4^{WT}* mice using MEAs, at any wavelength tested (Section 3.3.5). There was also no change in $\lambda_{\max}$ based on action spectra constructed from these ipRGC responses.

To confirm the initial pupillometry result was genuine, the 550nm PLR IRC was repeated using homozygous *Opn4^{S310A}* mice backcrossed onto an *rd/rd cl* background and *Opn4^{WT}* littermate controls. The *rd/rd cl* strain has no outer-retinal function (Lucas et al., 1999) and was used to eliminate residual light input from cones, which might have affected the initial pupillometry data. The initial experiments (MEA action spectrum and 550nm pupillometry IRC) had been conducted on *rd/rd* mice, which are retinally degenerate but occasionally possess surviving cones. Pupillometry was performed under highly-controlled conditions, with researchers blind to genotype for data collection and analysis, to prevent experimenter bias. In addition to 550nm, IRCs were also generated at 480nm, 500nm and 570nm in order to construct action spectra for pupil light responses and compare $\lambda_{\max}$ of *Opn4^{S310A}* and *Opn4^{WT}*.

IRC's were fitted to normalised minimum pupil area data for each mouse. Unlike the MEA data, the average sensitivity of individual *Opn4^{S310A}* and *Opn4^{WT}* mice tended to differ at longer wavelengths of light (**Table 3.7**). A significant reduction in sensitivity was identified, measured as an increase in IC50, at 550nm. The magnitude and direction of this difference, ~0.5 log quanta, is consistent with the loss of sensitivity observed in the PLR of the first cohort of homozygous *rd/rd Opn4^{S310A}* mice and the hypothesised short-wavelength shift in spectral sensitivity. *Opn4^{S310A}* mice also showed a trend towards lower sensitivity at 500nm and 570nm, although these did not reach significance. *Opn4^{S310A}* and *Opn4^{WT}* mice had comparable mean IC50 at 480nm, which would suggest that the decrease in sensitivity at longer wavelengths was due to a short-wavelength shift in spectral sensitivity, rather than a non-specific loss of sensitivity.

Table 3.7. *Opn4*^{S310A} mice show reduced pupil constriction sensitivity at longer wavelengths compared to *Opn4*^{WT}. IRCs were fit to pupil constriction data from each mouse (n = 7 for *Opn4*^{WT} and n = 6 for *Opn4*^{S310A}). The mean IC50 values from these IRCs are shown below. Average IC50 were compared using an independent samples Student's t-test.

Wavelength (nm)	<i>Opn4</i> ^{WT} (n = 7) Mean IC50 (SEM)	<i>Opn4</i> ^{S310A} (n = 6) Mean IC50 (SEM)	Independent Samples Student's t-test
480	12.56 (0.09)	12.61 (0.05)	t(11) = -0.44 p = 0.672
500	12.29 (0.09)	12.61 (0.10)	t(11) = -1.91 p = 0.083
550	13.16 (0.14)	13.59 (0.05)	t(11) = -2.41 p = 0.034
570	13.65 (0.08)	13.97 (0.14)	t(11) = -1.84 p = 0.093

Next, IRCs fit to the average pupil area of all mice tested were compared. The IRCs of each genotype differed significantly at 550nm and 570nm when assessed using an F-test (**Figure 3.13**). As the F-test compares the entire IRC curve between genotypes, rather than IC50 values, it is potentially a more sensitive and accurate method for detecting differences. No difference was found in IRCs of the two genotypes at 480nm but the difference between IRCs at 500nm approached significance. At 500nm, 550nm and 570nm, the IRC fit to average *Opn4*^{S310A} data were right-shifted relative to IRCs fit to average *Opn4*^{WT} data, indicating a reduction in sensitivity. The ~0.5log quanta decrease in *Opn4*^{S310A} pupil constriction sensitivity at 550nm matches the attenuation anticipated for a 14nm short-wavelength shift in $\lambda_{\max}$ from 480nm, whereas the 0.27log quanta decrease at 570nm is consistent with a smaller short-wavelength shift of 6nm after introduction of the S310A mutation.

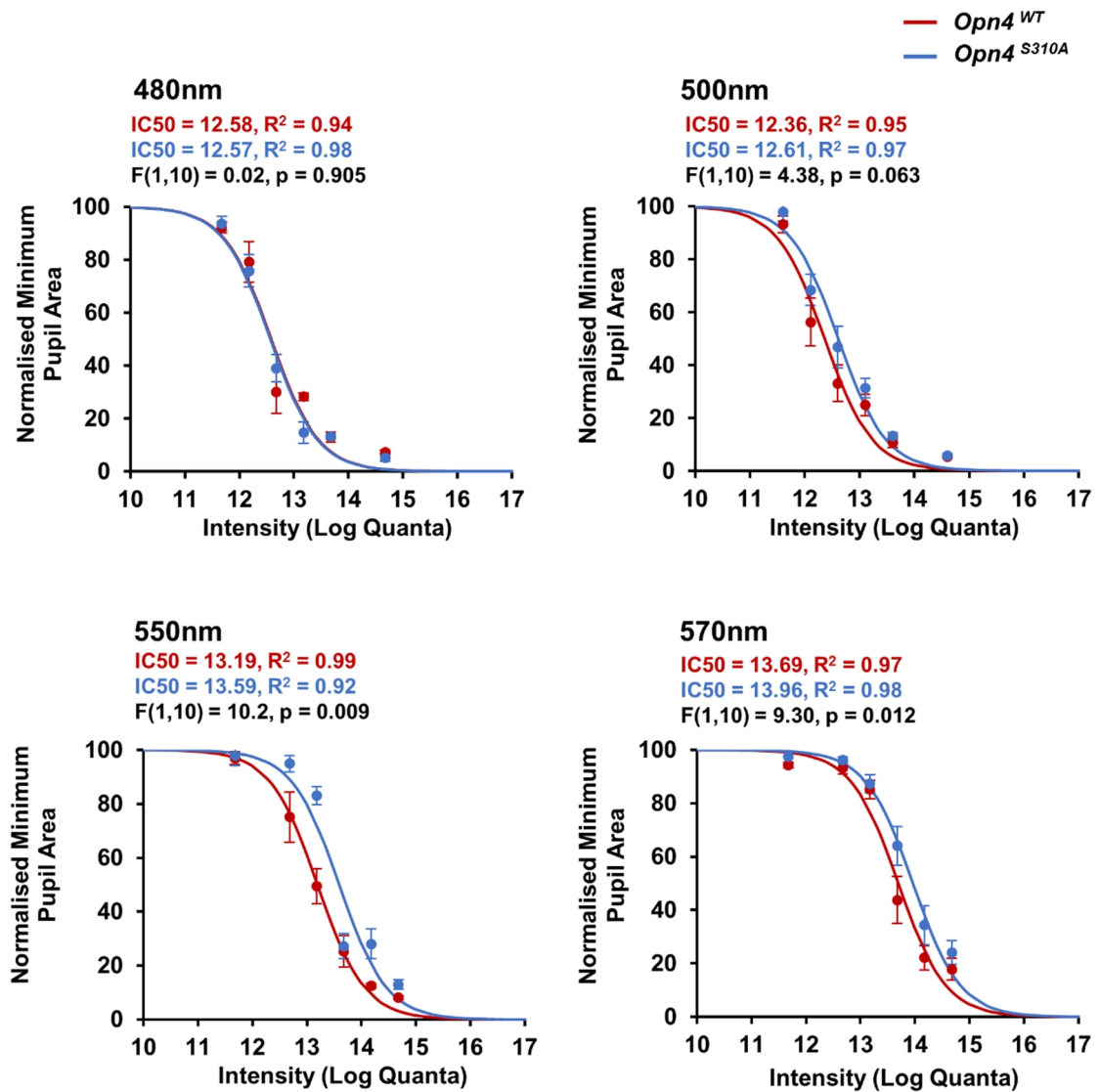


Figure 3.13. *Opn4*^{S310A} mice show specific loss of sensitivity of PLR at long wavelengths. IRCs were fit to the average normalised minimum pupil area from *Opn4*^{WT} (red) or *Opn4*^{S310A} (blue) mice by non-linear regression. Slope was kept constant at -1. The IC_{50} and R^2 of the best-fit IRC for each genotype is shown above each graph. The IRCs were compared using an F-test, shown as black text on each graph. Four wavelengths were tested (480, 500, 550 and 570nm). Error bars = SEM.

Using the IC50 values calculated for each mouse at the 4 wavelengths tested, an action spectrum was constructed for individual *Opn4*^{S310A} and *Opn4*^{WT} mice. As with the MEA action spectra, the average $\lambda_{\max}$ of *Opn4*^{S310A} retina was shorter than *Opn4*^{WT}, 477.6nm $\pm$ 1.58 and 480.5nm $\pm$ 0.96 respectively. This difference was not significant, $t(11) = 1.62$, $p = 0.135$ (Independent samples Student's t-test). The average $\lambda_{\max}$ for *Opn4*^{WT} mice is consistent with a published pupillometry action spectrum for *rd/rd cl* mice (Lucas et al., 2001), which found a $\lambda_{\max}$ of 479nm. In general, the action spectra from pupil constriction data were less variable than those from ipRGC responses, and demonstrated better fits to the Govardovskii template, mean R^2 was 0.91 ± 0.03 for *Opn4*^{WT} and 0.96 ± 0.05 for *Opn4*^{S310A}.

Fitting a Govardovskii template to the average data from *Opn4*^{S310A} and *Opn4*^{WT} mice (**Figure 3.14**), slightly improved the goodness of fit, $R^2 = 0.95$ for *Opn4*^{S310A} and 0.98 for *Opn4*^{WT}. These action spectra had a slightly longer $\lambda_{\max}$ of 478.4nm for *Opn4*^{S310A} and 481.3nm for *Opn4*^{WT}. An F-test confirmed these were not significantly different, $F(1,6) = 0.79$, $p = 0.410$. A narrow range of templates provided equally good fits as the best-fitting templates (**Figure 3.14C**). Despite the decreased variability of the pupillometry data, the 95% confidence intervals calculated from Monte Carlo simulations were similar width to those from MEA data, 476.4-486.9nm for *Opn4*^{WT} and 475.2-481.9nm for *Opn4*^{S310A}.

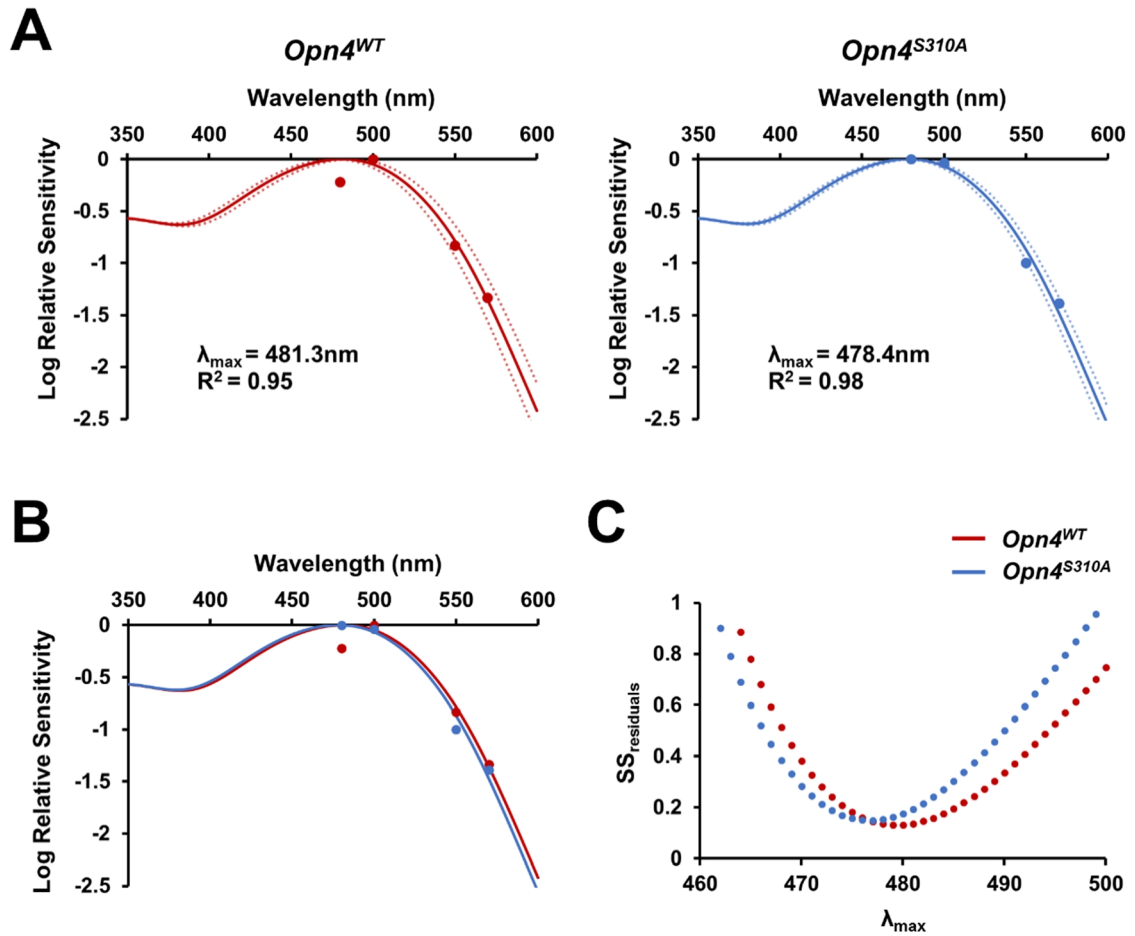


Figure 3.14. *Opn4^{S310A}* mice show trend towards shorter $\lambda_{\max}$ compared to *Opn4^{WT}*. (A) Govardovskii template (solid line) fit to relative sensitivity of minimum pupil size (circles). Relative sensitivity is derived from EC50 values of 4 IRCs fit to the average data from *Opn4^{WT}* (n = 7) and *Opn4^{S310A}* mice (n = 6). The 95% confidence intervals generated from Monte Carlo simulations are shown as dotted lines. (B) Comparison of best-fit pigment template for *Opn4^{WT}* (red) and *Opn4^{S310A}* (blue) (C) Comparison of sum of squares of difference between average log relative sensitivity and visual templates with $\lambda_{\max}$ from 460-500nm for *Opn4^{WT}* and *Opn4^{S310A}*.

In summary, a significant attenuation in *Opn4^{S310A}* pupil constriction sensitivity was identified at long (550nm and 570nm) but not short wavelengths (480nm and 500nm). These findings are consistent with the short-wavelength shift predicted from bioinformatics analysis of the S310A mutant, although no difference was detected in $\lambda_{\max}$ of pupillometry action spectra of *Opn4^{S310A}* and *Opn4^{WT}* mice.

3.4 Discussion

A genotype-driven screen of a large chemically-mutagenized mouse archive identified a single *Opn4* missense mutation, S310A. Alignment with other opsins revealed this mutation occurs at a highly conserved spectral tuning site. OPN4 S310A is found in the plasma membrane and capable of coupling to a G_{q/11} signalling cascade *in vitro*. Pupil constriction, but not *in vitro*-recorded ipRGC response amplitude, of *Opn4*^{S310A} mice was specifically less sensitive at longer wavelengths of light, consistent with the predicted short-wavelength shift in spectral tuning. However, the $\lambda_{\max}$ of *Opn4*^{S310A} mice estimated from action spectra of pupil constriction or ipRGC response amplitude was not significantly different from *Opn4*^{WT}.

The $\lambda_{\max}$ of *Opn4*^{WT} action spectra from both pupil and MEA data was consistent with previous literature (see Section 1.2.2 **Table 1.3**). The reproducibility of $\lambda_{\max}$ from action spectra fit to a wide range of OPN4-driven behaviours confirms this is a suitable approach for measuring spectral sensitivity, especially given the difficulties producing purified OPN4 protein for absorption spectra, discussed in Section 1.2.2. Nevertheless, the 95% confidence intervals of both action spectra suggest they may not be precise enough to reliably identify spectral tuning mutants, particularly those causing $\lambda_{\max}$ shifts smaller than 10nm.

The number and position of relative sensitivity values used to fit the Govardovskii template may have contributed to this. As shown in **Figure 3.15**, log relative sensitivity values on long-wavelength limb of the template are more affected by fit than those closer to peak sensitivity. Although PLR sensitivity of *Opn4*^{S310A} mice was significantly attenuated at 550nm and 570nm, this did not translate into a significant change in $\lambda_{\max}$ because only half the data points used to fit the template possessed relevant information about sensitivity.

Increasing the number of data points along the long wavelength limb of the template, even at the expense of shorter wavelengths, could increase precision of fit and allow detection of smaller shifts in $\lambda_{\max}$.

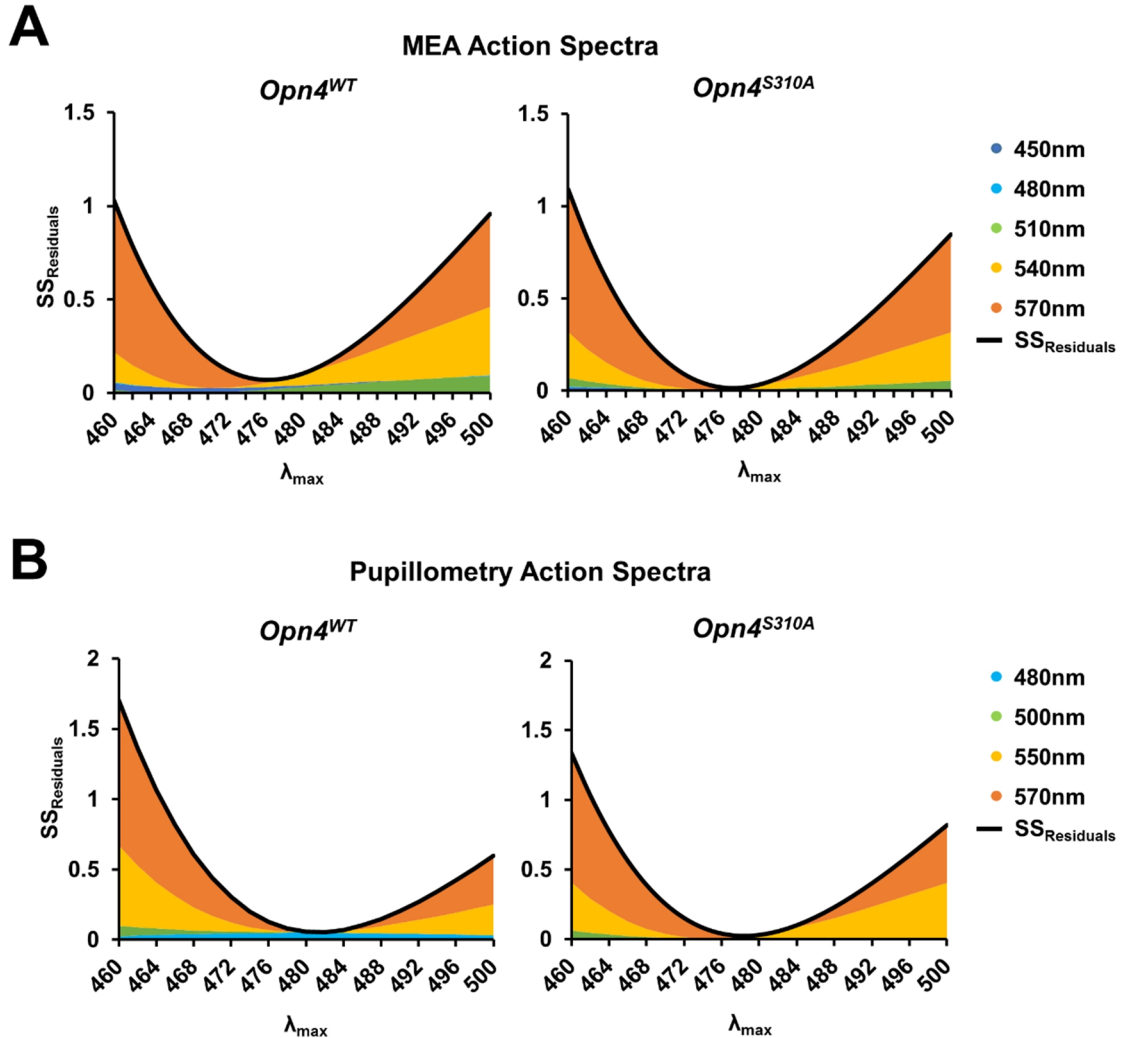


Figure 3.15. Contribution of different IRC wavelengths to fitting Govardovskii template. Stacked area graph shows the squared difference between each relative sensitivity value (colour coded by wavelength of IRC used to derive EC₅₀ value) and Govardovskii templates with different $\lambda_{\max}$ for **(A)** MEA and **(B)** Pupillometry action spectra. The squared difference between template and the normalised EC₅₀ at 480nm (blue) and 500nm (green) is very small regardless of template $\lambda_{\max}$. In comparison, the squared difference between the template and the normalised EC₅₀ at 550nm (yellow) and 570nm (orange) varies dramatically with $\lambda_{\max}$. Because least squares minimisation aims to find smallest cumulative squared differences, SS_{Residuals} (shown as thick black line), normalised EC₅₀ values from the long wavelength limb of Govardovskii template contribute more than normalised EC₅₀ values at shorter wavelengths, and are therefore more useful in resolving $\lambda_{\max}$ differences.

It is unclear why no shift in sensitivity of MEA-recorded ipRGC responses was detected at longer wavelengths of light, when this was replicated in the pupil constriction of two cohorts of *Opn4*^{S310A} and *Opn4*^{WT} mice. One obvious difference between these techniques is pre-retinal absorption of light during pupillometry, which does not occur during MEA recording of retinal flatmounts. However, as discussed in Section 1.2.2, the lens predominantly filters short wavelengths of light, which is unlikely to affect sensitivity at 550-570nm. Furthermore, pre-retinal absorption would only explain the difference in sensitivity between *Opn4*^{S310A} and *Opn4*^{WT} mice if the two genotypes possessed different lens transmission spectra. Since littermate controls were used for all experiments, this seems unlikely.

A second possibility is that the MEA records from the full range of ipRGC subtypes, whereas PLR is predominantly driven by M1-type ipRGCs (Chen et al., 2011). As discussed in Section 1.1.5, *Opn4* expression levels and sensitivity vary greatly between subtypes (Schmidt et al., 2011). Averaging responses from high- and low-sensitivity subtypes may decrease the signal to noise ratio, making it harder to detect small changes due to spectral tuning shifts. This may explain why a significant decrease in PLR sensitivity *in vivo*, but not MEA recorded ipRGC responses *in vitro*, was detected at long wavelengths.

The magnitude of the PLR sensitivity decrease at 550nm and 570nm is consistent with a 6-14nm blue-shift in spectral tuning after introduction of the S310A mutation. This short-wavelength shift is likely due to electrostatic interactions of S310A side chains with retinal chromophore. In rhodopsin and MWS cone visual pigment, polar amino acids added at this position interact with the β -ionone ring of 11-*cis* retinal, stabilising the excited-state and decreasing the energy gap, causing absorption maximum to shift to longer wavelengths (Kochendoerfer et al., 1999). *Opn4* S310A is likely to have the opposite effect, destabilising excited state and increasing energy gap to cause short-wavelength shift.

The magnitude of the shift after substitution of serine in *Opn4* S310A is much smaller than the shifts seen after mutation of the equivalent site in other opsins. This may be because conditions in the OPN4 chromophore binding pocket cannot sustain further blue shifts. Although the interaction between the residue at position 310 and the retinal chromophore may have changed, this may have a limited effect if the network of other amino acids around the retinal chromophore were already exerting similar effects as the substituted amino acid. When the residues in OPN4 at the 3 LWS/MWS cone visual pigment spectral tuning sites were examined, it was found most were in a “blue state”, possessing amino acids that contribute towards blue shifts in $\lambda_{\max}$ (see **Appendix 1**).

This is consistent with the limited effect of mutations at the equivalent position to OPN4 S310A in SWS cone visual pigment (Yokoyama & Tada, 2003) and suggests it is crucial to consider the interactions between different amino acids of the chromophore binding site. For example, Lin et al. (1998) found 9 simultaneous mutations were required to produce substantial short-wavelength shifts in rhodopsin $\lambda_{\max}$. Many of the equivalent sites in OPN4 could potentially be mutated to cause further blue shifts (**Appendix 1**). If melanopsin spectral tuning is similar to rhodopsin, single mutations may have limited ability to blue-shift OPN4 spectral tuning, but a much more substantial effect if combined.

In conclusion, a novel ENU-induced *Opn4* mutation was identified, which based on homology with visual pigments, was predicted to produce a short wavelength shift in spectral sensitivity. Consistent with this prediction, a reduction in PLR sensitivity at long, but not short, wavelengths was observed. However, when action spectra were constructed from PLR data, no difference in $\lambda_{\max}$ from *Opn4*^{WT} was apparent. Surprisingly, analysis of ipRGC responses using MEAs show no difference in spectral tuning of cellular responses. These apparently contradictory findings appear to be due to limitations in the resolution of action spectroscopy methods, as well as variability in ipRGC-subtype response properties.

Chapter 4

Screening Human *OPN4* variants *in vitro*

Chapter 3 demonstrated how a single chemical-induced point mutation could affect the functional properties of melanopsin. In this chapter, the functional consequences of genetic variation in the Human *OPN4* gene (*hOPN4*) are examined.

4.1 Introduction

The most common form of genetic variation is the single nucleotide polymorphism (SNPs), where an individual nucleotide base differs between individual genomes of a species in more than 1% of the population (1000 Genomes Project Consortium, 2010). Substitutions that occur less frequently than this are classified as rare variants (Bush & Moore, 2012). The majority of SNPs occur in non-coding regions or lead to synonymous amino acid substitutions (Kitts & Sherry, 2002). However, SNPs can also cause frameshift, nonsense and missense mutations, which alter or truncate the protein sequence. This can disrupt or eliminate protein function, and potentially cause disease (Mendes et al., 2005).

Disease-causing SNPs can be identified by a number of approaches. In genome-wide association studies, thousands of SNPs are compared between disease and healthy control populations to detect increased frequency of specific SNPs, which may be genetic risk factors. This technique allows identification of novel genes or SNPs involved in common multifactorial disorders, such as complement factor H in age-related macular degeneration (Klein et al., 2005).

For detecting genetic variants in rare disorders, linkage disequilibrium analysis can be more appropriate. Families affected by a specific heritable condition are genotyped for a series of genetic markers throughout the genome. These markers are then examined to determine how they segregate with the disease and find the location of disease-linked mutations (Bush & Moore, 2012).

Alternatively, a candidate gene approach can be adopted, where *a priori* knowledge about the biological basis of a behaviour or disease means variation in a specific gene can be compared between groups to identify variants associated with the abnormal phenotype (Tabor et al., 2002).

This candidate gene approach has already been adopted to study the effects of sequence variation in *hOPN4*. In public databases of genetic variation, 96 missense mutations have been reported for *hOPN4*. Only 5 of these (P10L, T394I, G444D, R406W and L365V) occur in more than 1% of the population and can be considered polymorphic. *hOPN4* SNPs P10L and T394I have been described in a series of studies on abnormal melanopsin-associated behaviour exhibited by individuals homozygous for L10 and I394 (Higuchi et al., 2013; Lee et al., 2013; 2014; Roecklein et al., 2009;2013). The findings of these papers and the impact of these two SNPs on melanopsin function, described in more detail in Chapters 1 and 4, suggest *hOPN4* variants have the potential to affect NIF behaviour, even in individuals with normal rod and cone function. If so, people with non-functional or severely impaired melanopsin could be found, enabling further clarification of the role of melanopsin in human NIF vision. Studying these variants could also improve understanding of the structure-function relationships in the melanopsin protein.

The aim of this study was to identify naturally-occurring *hOPN4* missense mutations with unusual functional properties. A variety of bioinformatics approaches were used to examine *hOPN4* sequence variants collated in the NCBI database of single nucleotide polymorphisms (dbSNP, Sherry et al., 2001) and select those most likely to possess abnormal phenotypes for further investigation. The functional properties of the 16 chosen variants, such as intracellular localisation and G-protein activation, were then examined *in vitro*. Several *hOPN4* variants possessed unusual functional properties, including attenuated or abolished responses, as well as unusual response kinetics, providing valuable insight into the key functional residues of the melanopsin protein.

4.2 Methods

4.2.1 SNP prioritisation

Human *OPN4* SNPs were identified from the NCBI database of single nucleotide polymorphisms (dbSNP, Sherry et al., 2001). The dbSNP is a large public database that collates simple genetic variations, such as single nucleotide substitutions, deletions, insertions or short tandem repeats. This includes both common SNPs and rare genetic variants. Each genetic variant in dbSNP is assigned a reference SNP ID (rs#), and information on the position, alleles and validation status.

Single nucleotide variants in the *hOPN4* gene (transcript NM_033282.3) in build 140 of dbSNP were used in this analysis. There were 1242 variants reported for *hOPN4*. Analysis was restricted to those causing non-synonymous amino-acid substitutions, which narrowed the list to 96 (**Appendix 2**). Variants were then prioritised for *in vitro* screening using 6 criteria described in **Table 4.1**. Variants did not have to meet all criteria to be included, with preference given to variants that altered amino acid biochemical properties and those occurring at highly conserved sites, considered most likely to affect function.

In addition to the criteria described in **Table 4.1**, the equivalent position of the *hOPN4* variant in rhodopsin was also examined, using a BLAST protein alignment (Altschul et al., 1990) of human melanopsin (NP_150598.1) and bovine rhodopsin (NP_001014890.1), as shown in **Figure 4.1**. A literature search was performed to determine whether the equivalent residue was functionally important in rhodopsin. Variations on the name of the residue and its position in rhodopsin were searched in NCBI PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>) and Google Scholar (<https://scholar.google.co.uk/>) for example: “Rhodopsin” AND “Proline*23” OR “P*23” OR “Pro*23”. Papers describing the crystal structure of bovine rhodopsin (Okada et al., 2001; Palczewski et al., 2000; Scheerer et al., 2008), and lists of rhodopsin mutations associated with retinitis pigmentosa (Mendes et al., 2005; Rakoczy et al., 2011) were also examined.

Table 4.1. Criteria for identifying *hOPN4* variants for *in vitro* screening.

Variant Property	Reasoning	Method	Criteria for Inclusion
<i>Validation Status</i>	dbSNP accepts submissions from a variety of sources of varying quality and rare variants could be false positives.	Restricted analysis to “validated” variants obtained by non-computational methods and those with frequency data attached.	Validated by multiple submissions and preferably by 1000 Genomes project (1000 Genomes Consortium, 2015)
<i>Conservation between species</i>	Highly conserved amino acids are likely to be critical to function, whereas poorly conserved regions should tolerate substitutions	Aligned 20 <i>OPN4</i> amino acid sequences from 15 different species using MAAFT software (described in Chapter 1 in more detail)	Amino acid substitutions that occur at sites highly conserved in all species
<i>Amino acid substitution properties</i>	Substitutions that significantly change residue biochemical properties, particularly hydrophobicity (Cai et al, 2004) are more likely to affect protein function.	Compared charge, hydrophobicity, possible bond formation, polarity and side chain size using NCBI amino acid explorer (Bulka et al, 2006) https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi	Change in hydrophobicity, charge or polarity.
<i>Location in Protein</i>	Mutations located in key functional domains, such as transmembrane helices or intracellular loops, are more likely to disrupt function (Small et al, 2003)	Aligned human melanopsin and bovine rhodopsin sequences using MAAFT and BLAST software and defined <i>OPN4</i> domains based on rhodopsin crystal structure (Palczewski et al, 2000)	Substitutions that occur in transmembrane helices or intracellular loops.
<i>Predicted Effect of Mutation</i>	Computational tools can be used to predict potential effect of missense mutation on protein function from sequence- and structure-based features	Used SIFT (Kumar et al, 2009) based on conserved sequence homology and POLY-PHEN 2 (Adzuhebai et al, 2010) based on structural features.	Substitutions with SIFT score < 0.05 or POLY-PHEN score > 0.85
<i>Minor Allele Frequency (MAF)</i>	SNPs typically have 2 alleles – a frequent ancestral allele and a less common (minor) allele. The more frequent the minor allele, the more individuals homozygous for minor allele can be identified, making it more relevant if damaging.	Frequency data is reported in the NCBI dbSNP. MAF is expressed as a value from 0 – 1. A MAF score of 0.4 means 40% of the population possess the minor allele.	SNPs with higher MAF scores (> 0.01).

		TM1		IL1		TM2			
<i>RHO</i>	40	LAAYMFL	LIM	LGFPINFLTLYVT	QHKKLRTPLN	YILLNLAVADLFMVFGGFTTTTLYTSLH		100	
<i>hOPN4</i>	73	LGTVILLVGL	TGMLGNLTVIYTFC	RSRSLRTPAN	MFIINLAVSDFLMSFTQAPVFFTSSLY		133		
		EL1		TM3		IL2		TM4	
<i>RHO</i>	101	GYFVFGPT	GCNLEGFFATLGGEIALWVSLVLAIER	VVVC	KPM	SNFRFGENH----	AIM	155	
<i>hOPN4</i>	134	KQWLFGET	GCEFYAF	CGALFGISSMITLTAIALDRY	LVITRPLATFGVASKRRAAF	VLL	192		
				EL2		TM5			
<i>RHO</i>	156	GVAF	TWVMALACAAPPLVGW	SRYI	PEGMQ	CSGIDYYTPHEETN	NESFVIYMFVVHFIPL	216	
<i>hOPN4</i>	193	GV---	WLYALAWSLPPFFGW	SAYV	PEGLTSCSWDYMSTPAVR	AYTMLLCCFV--FFLPL	248		
				IL3		TM6			
<i>RHO</i>	217	LIVIFFCYGQLV	FTVKEAAAQQQESATTQ-----KAE	KEVTRMVIIMVIAFLIC		264			
<i>hOPN4</i>	249	LLIIICYIIF	RAIRETGRALQTFGACKNGESLWQRQLQSE	CKMAKIMLLVILLFVLS		308			
				EL3		TM7		C Terminus	
<i>RHO</i>	265	WLPYAGVAFYIF	THQGSDFGP	IFMTIPAFFAKTSAVINPVIY	IMMNKQFRNCMVTTLCC	323			
<i>hOPN4</i>	309	WAPYSAVALVAF	AGYAHVLT	YMSSVPAVIAKASAIHNPIY	AITHPKYRVAIAQHLPC	367			

Figure 4.1. BLAST protein alignment of human melanopsin (*hOPN4*) and bovine rhodopsin (*RHO*). Protein domain boundaries (shown as vertical lines) are defined based on crystal structure of bovine rhodopsin reported in Palczewski et al. (2000). TM = transmembrane helix, IL = intracellular loop, EL = extracellular loop. Highly variable N and C termini are excluded from alignment. *hOPN4* variants selected for *in vitro* screening with equivalent rhodopsin residue are highlighted in yellow. Numbers show position of first and last residue of each row.

4.2.2 *In vitro* expression of *hOPN4* variants

Point mutations for the 16 selected variants and 2 control mutations were introduced to pcDNA3.1 plasmid (Invitrogen) containing human melanopsin, *hOPN4* (NP_150598.1) with a 1D4 tag (Bailes & Lucas, 2013), using a Quikchange II XL Site-directed mutagenesis kit (Stratagene), as described in Section 2.2.4. Mutagenesis primers are shown in **Table 4.2**. Presence of mutations was confirmed by Sanger sequencing (Source Biosciences), as described in Section 2.2.2, using the following sequencing primers:

CMV F, 5' – CAACGGGACTTTCCAAAATG,

hOPN4 midF, 5' – TGGGCACAGTGATCTTGCTGGT,

hOPN4 midR, 5' – AGGCATAGAACTCGCAGCCTGT and

BGH R, 5' – TAGAAGGCACAGTCGAGG.

A full description of the pcDNA3.1 vector is provided in section 2.1.3.2.

Mutation of 2 conserved sites known to be critical for opsin function were used as negative controls for calcium imaging experiments. The first was *hOPN4* K340A –the retinal chromophore binding site renders melanopsin non-functional when mutated (Kumbalasiri et al., 2007).

The second was *hOPN4* C143A –one of two conserved cysteines, which form disulphide bridges necessary for maintaining opsin tertiary structure (Karnik et al., 1988). Mutation of this site to alanine in rhodopsin (C110A) leads to misfolding and decreased retinal binding without total loss of function (Hwa et al., 1999). *hOPN4* C143A was used as a control for attenuated response amplitude.

A C-terminus truncation mutant was generated as a control for abnormal response kinetics. Blasic et al. (2014) found mouse OPN4 truncated at position 396 had slower deactivation kinetics, which the authors argued was due to loss of phosphorylation on the C-terminus. An equivalent C-terminus truncation mutant was created, *hOPN4* Δ 389 and cloned into pcDNA3.1 vector (without the 1D4 tag), as described in Section 2.2.3. The following primers were used to add an *EcoRI* site just before the start codon of *hOPN4*, 5' – AACGAATTCATGAACCCTCCTTCGGGGCCAA, and to truncate the *hOPN4* sequence by adding a stop codon (TAG) and *NotI* site after position 389, 5' – CCAGCTACCGCTCCACCCACTAGGCGGCCGCATA.

HEK293T cells (ATCC) were cultured as described in Section 2.2.7.1. For calcium imaging, HEK293T cells were seeded into 24-well plates (Sigma-Aldrich) at $\sim 4 \times 10^4$ cells/well and transfected with pcDNA3.1 *hOPN4* WT or variant plasmid 24hrs later as described in Section 2.2.7.2. For immunocytochemistry experiments, cells were seeded into 48-well plates at $\sim 1 \times 10^4$ cells/well and transfected with pcDNA3.1 *hOPN4* WT or variant plasmid as described in Section 2.2.7.2.

Table 4.2. Site-directed mutagenesis primers used to introduce the *hOPN4* genetic variants and control mutations. Base pair change from template sequence is shown in bold and underlined.

Mutation	Forward Primer Sequence (5' → 3')	Reverse Primer Sequence (5' → 3')
<i>P10L</i>	CCAAGAGTCC <u>T</u> GCCCAGCCCAACCC	TTGGGCTGGGC <u>A</u> GGACTCTTGGC
<i>T83M</i>	CAGCATCCCC <u>A</u> TGAGTCCCACCAGCAAGAT	ATCTTGCTGGTGGGACTCA <u>T</u> GGGGATGCTG
<i>F95L</i>	GACGGTCATCTATACC <u>C</u> TCTGCAGGAGCAG AAG	CTTCTGCTCCTGCAG <u>A</u> GGTATAGATGACCGTC
<i>Y146C</i>	CCACAGAAGGC <u>C</u> AGAACTCGCAGCCTGTC	GACAGGCTGCGAGTTCT <u>G</u> TGCCTTCTGTGG
<i>R168C</i>	CCATCGCCCTGGAC <u>T</u> GCTACCTGGTAATC	GATTACCAGGTAGC <u>A</u> GTCCAGGGCGATGG
<i>S183P</i>	GCACGCCTCTTGG <u>G</u> CGCCACACCAAAG	CTTTGGTGTGGCG <u>C</u> CCAAGAGGCGTGC
<i>R186H</i>	GGACAAATGCCGCA <u>T</u> GCCTCTTGGACGCC	GGCGTCCAAGAGGC <u>A</u> TGCGGCATTGTCC
<i>G208S</i>	TAGGCGCTCCAGC <u>T</u> GAAGAAGGGTGGC	GCCACCCTTCTC <u>A</u> GTGGAGCGCCTA
<i>V213M</i>	CCCCTCGGGCA <u>T</u> GTAGGCGCTCC	GGAGCGCCTAC <u>A</u> TGCCCCGAGGGG
<i>G216R</i>	GGATGTCAGCAACC <u>T</u> CTCGGGCAGTAGG	CCTACGTGCCCAG <u>A</u> GGTTGCTGACATCC
<i>G266E</i>	CTGGAGAGCCCGT <u>T</u> CTGTCTCCCGGAT	ATCCGGGAGACAG <u>A</u> ACGGGCTCTCCAG
<i>W283C</i>	CGAGTCCCTGTG <u>T</u> CAGCGGCAGCGG	CCGCTGCCGCTG <u>A</u> CACAGGGACTCG
<i>S308F</i>	GAATAGGGAGCCCAG <u>A</u> AGAGCACGAAGA GGA	TCCTCTTCGTGCTCT <u>T</u> CTGGGCTCCCTATTC
<i>H345Q</i>	CGTAAATGATGGGGT <u>C</u> TGGATTGCAGA GGCCT	AGGCCTCTGCAATCC <u>A</u> AACCCCATCATTTACG
<i>T394I</i>	CTCTCCACGCTGA <u>T</u> CAGCCACACCTCC	GGAGGTGTGGCTG <u>A</u> TCAGCGTGGAGCG
<i>R408C</i>	AGGGACTCCTGGC <u>A</u> CCTCCGTATGGAG	CTCCATACGGAGG <u>T</u> GCCAGGAGTCCCT
<i>C143A</i>	GAAGGCATAGAACTCG <u>G</u> CGCCTGTCTCC CCAAAG	CTTTGGGAGACAGGC <u>G</u> CGAGTTCTATGCCT TC
<i>K340A</i>	GGATTGCAGAGGCC <u>G</u> CGGCGATGACGG CTG	CAGCCGTCATCGCC <u>G</u> CGGCCTCTGCAATCC

4.2.3 Fluorescent Calcium Imaging

Forty-eight hours after transfection, calcium imaging was conducted using Fluo-4 AM (Molecular Probes) in a FLUOstar Omega plate reader, as described in Section 2.2.7.3. Each plate contained 4 technical replicates of each plasmid, which were averaged to produce a single biological replicate. Six variants were tested per plate, one was always the *hOPN4* wildtype (WT) positive control. Four 24-well plates were required to test all 16 variants and 5 controls. Variants were always allocated to the same plate. These were:

Plate 1: WT, P10L, T394I, R168C, T83M, F95L

Plate 2: WT, Y146C, S183P, R186H, G208S, G216R

Plate 3: WT, G266E, W283C, S308F, H345Q, R408C

Plate 4: WT, C143A, K340A, V213M, Δ 389, No transfection control

Each plate was tested 6 times to give a total of 6 biological replicates per variant. Biological replicates were normalised to baseline (first recorded fluorescence value) for response amplitude comparisons (Baseline = 0, $\Delta F/F$), then normalised to maximum for comparing response kinetics (Baseline = 0, Maximum = 1, $\Delta F/F_{\max}$).

4.2.4 Immunocytochemistry

Forty-eight hours after transfection, cells were fixed and immunostained as described in Section 2.2.7.4. Cells were double-labelled using chicken anti-GFP (GFP-1020, AVES Labs, 1:1000) and rabbit anti-human melanopsin antibody (H-300, Santa Cruz, 1:250) for 1hr at room temperature. Secondary antibodies were donkey anti-rabbit IgG conjugated to Alexa 568 and donkey anti-chicken IgG conjugated to Alexa 488 (Molecular Probes, Life Technologies, 1:200). Cells were washed 5 times in PBS with 0.05% Tween-20 (Sigma) after incubation in primary and secondary antibody. After the final wash, cells were incubated in PBS with 0.05% Tween-20 (Sigma) with DAPI (1:2000) for 10mins at room temperature then stored in PBS.

Images were acquired using a x63 oil objective of a LSM710 laser-scanning confocal microscope (Zeiss) and Zen2009 image acquisition software (Zeiss), with excitation at 405nm and 561nm and emission at 440-480nm, and 600-700nm for DAPI and red fluorescence, respectively. Brightness and contrast was adjusted using ImageJ software (<http://rsbweb.nih.gov/ij>).

4.2.5 Statistical Analysis

Statistical tests were conducted using SPSS 22.0 (IBM). Unless stated otherwise, all comparisons between groups were tested using One-way between-subjects analysis of variance (ANOVA) with genotype as the independent variable. For One-way ANOVA, a post-hoc Dunnett's test was used to explore significant main effects of genotype, using *hOPN4* WT as the control group. A significance threshold of $p \leq 0.05$ was used. In all figures * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Error bars show standard error of mean.

4.3 Results

4.3.1 Human *OPN4* variants with potential functional consequences

NCBI dbSNP contains 96 *hOPN4* variants that cause non-synonymous amino acid substitutions (**Appendix 2**). Several properties of each missense variant were examined to prioritise a subset for *in vitro* screening. As shown in **Figure 4.2**, 16 variants were predicted to possess abnormal functional characteristics. These are located in a range of melanopsin protein domains, each associated with distinct functional roles, in an effort to examine different types of critical *OPN4* residues.

Details of the 16 missense variants are shown in **Table 4.3**. They were all validated by multiple independent submissions, suggesting they are genuine germline variations in the human genome, rather than sequencing errors (Jiang et al., 2003). The majority of missense variants were rejected because they occurred at poorly conserved positions in the *OPN4* protein, which suggests variation at this site would be tolerated. Many variants were also excluded if the substitution caused minor changes in residue charge, hydrophobicity or polarity, which would be unlikely to disrupt interactions with other residues or the retinal chromophore, and alter function. The computational tools for predicting effects of missense mutations, SIFT (<http://sift.jcvi.org/>, Kumar et al., 2009), and POLY-PHEN2 (<http://genetics.bwh.harvard.edu/pph2/>, Adzuhebai et al., 2010) were fairly non-specific, with many variants classed as “damaging”, and were less useful for selecting variants for *in vitro* screening.

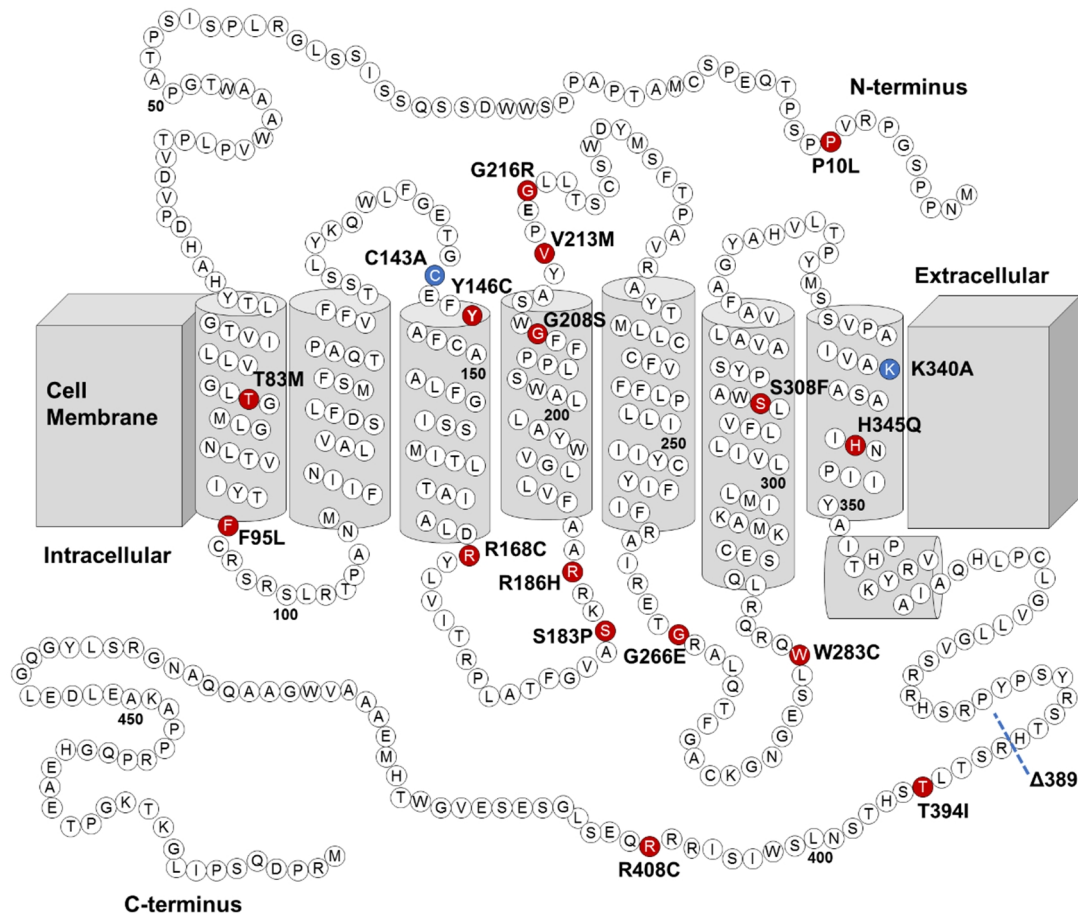


Figure 4.2. *hOPN4* genetic variants selected for *in vitro* testing. Genetic variants (red), control mutations (blue) and location of C terminus truncation ($\Delta 389$, blue dashed line). Secondary structure based on homology with bovine rhodopsin. Figure adapted from Peirson et al. (2009) under Creative Commons licence Attribution 4.0 International.

The most common SNPs tended to have “neutral” SIFT and POLY-PHEN2 scores, were located at poorly conserved sites or resulted in minor changes in biochemical properties, which suggests they are unlikely to affect function. However, abnormal NIF behaviour has been reported in individuals with *hOPN4* SNPs P10L and T394I (Higuchi et al., 2013; Lee et al., 2013; 2014; Roecklein et al., 2009; 2012; 2013). Therefore, these 2 SNPs were included in the *in vitro* screen because their functional consequences are more relevant to the general population. Minor allele frequency (MAF) suggests most potentially deleterious mutations are rare. These have the greatest potential to inform knowledge of melanopsin structure-function relationships, even if identifying individuals homozygous for the minor allele would require sequencing a very large population.

Table 4.3. Properties of genetic variants selected for *in vitro* screening. Highlighted in green are properties of each variant that met criteria for further investigation. Minor allele given in brackets beneath MAF score. 1K Genomes = 1000 Genomes Project (1000 Genomes Project Consortium, 2015).

Mutation	Verification	Conservation between species	Amino Acid Properties	Protein Position	SIFT <i>PolyPhen</i>	MAF
P10L rs2675703	1K Genomes, Multiple Submission, Published Data	Not conserved	Both non-polar and hydrophobic	NT	1 0	0.08 (T)
T83M rs202029105	1K Genomes, Multiple Submissions	Conserved in <i>Opn4M</i> , I/V/L in <i>Opn4X</i>	Polar to non-polar	TM1	0.02 0.588	0.0002 (T)
F95L rs573102269	1K Genomes, Multiple Submissions	Conserved in all species	Loss of large side-chain. Both hydrophobic and non-polar	TM1	0 0.999	0.0002 (C)
Y146C rs200099863	1K Genomes, Multiple Submissions	Conserved in all species	Both polar and similar hydrophobicity	TM3	0 0.992	0.0002 (G)
R168C rs143641898	Multiple Submissions	Conserved in all species	Positive charge to polar. Increased hydrophobicity	TM3 IL2	0.01 0.929	0.0005 (T)
S183P rs151123640	1K Genomes, Multiple Submissions	Conserved in most species (T in <i>Amphioxus</i>)	Polar to non-polar.	IL2	0 0.998	0.0002 (C)
R186H rs141089672	Multiple Submissions	Conserved in most species (K in Chicken & Zebrafish)	Both positive charge, increase in hydrophobicity	IL2	0.01 0.934	0.0005 (A)
G208S rs549998450	1K Genomes, Multiple Submissions	Conserved in all species	Nonpolar to polar. Introduce large side chain	TM4 EL2	0 0.999	0.0004 (A)
V213M rs202171086	1K Genomes, Multiple Submissions	Conserved in <i>Opn4M</i> , I/V in <i>Opn4X</i>	Both hydrophobic and non polar	EL2	0 0.997	0.0002 (A)
G216R rs201432667	1K Genomes, Multiple Submissions	Conserved in all species	Non-polar to polar, loss of hydrophobicity	EL2	0 1	0.0002 (A)
G266E rs576858032	1K Genomes, Multiple Submissions	Conserved in mammals, S/G/N in non-mammals	Non-polar to negative charge. Introduce large side chain and decrease hydrophobicity	IL3	0.07 0.845	0.0002 (A)
W283C rs145634412	1K Genomes, Multiple Submissions	Not conserved	Non-polar to polar, loss of large side chain	IL3	0.16 0.295	0.001 (T)

Table 4.3. Properties of genetic variants selected for *in vitro* screening (continued)

Mutation	Verification	Conservation between species	Amino Acid Properties	Protein Position	SIFT <i>PolyPhen</i>	MAF
S308F rs559392371	1K Genomes, Multiple Submissions	Conserved in all species	Positive charge to polar. Similar hydrophobicity	TM6	0 0.972	0.001 (T)
H345Q rs184720512	1K Genomes, Multiple Submissions	Conserved in <i>Opn4M</i> , Y in <i>Opn4X</i>	Polar to non-polar, increase hydrophobicity	TM7	0 0.976	0.0002 (G)
T394I rs1079610	1K Genomes, Multiple Submission, Published Data	Conserved as S or T in all species	Polar to non-polar, increase hydrophobicity	CT	0.28 0.003	0.25 (C)
R408C rs199878852	1K Genomes, Multiple Submissions	Conserved in mammals, not conserved in non-mammals	Positive charge to polar.	CT	0 0.985	0.0002 (T)

Six criteria were used to identify variants with potentially abnormal functional properties. This provided a fairly rapid method for reducing the 96 missense variants in dbSNP to a manageable number for screening. To predict the mechanisms by which substitutions may disrupt OPN4 function, the role of the equivalent residue in rhodopsin was examined. Critical residues in rhodopsin have been extensively documented, from crystal structures to over 100 rhodopsin mutations associated with retinal disease (Okada et al., 2001). Several *hOPN4* variants occur at sites critical for normal opsin and GPCR function, including the highly conserved E/DRY motif, the vertebrate opsin counterion in TM3, and the conserved xWxPY opsin motif in TM6 (**Table 4.4**), described in more detail in Chapter 1. Mutation of these sites in rhodopsin severely disrupts protein function. *hOPN4* variants affecting these conserved positions may also cause loss-of-function if they perform similar roles in melanopsin.

Table 4.4. Properties of equivalent residues in Rhodopsin. Alignment of hOPN4 with Bovine Rhodopsin was used to assess several properties; whether equivalent residue to hOPN4 variant was conserved in rhodopsin of different species (Rakoczy et al., 2011), mutated in cases of retinitis pigmentosa (RP), and if it had a known functional role. hOPN4 mutations that occur in motifs conserved in rhodopsin (highlighted in blue) are likely to share the functional properties described in far right column.

hOPN4 Variant	Equivalent Residue in Rhodopsin	Invariant in Rhodopsin Family	Mutated in RP	Functional Role of Equivalent Residue in Rhodopsin
T83M	L50	No	No	Forms part of entry site for a chromophore channel through opsin protein (Schädel et al., 2003).
F95L	T62	Yes	No	Binding site for conserved water molecule in H-bond network between TM1 and TM2. (Okada et al, 2002)
Y146C	E113	Yes	No	Schiff base counterion (Sakmar et al, 1989)
R168C	R135	Yes	Yes ^{a,b,c}	Part of highly conserved GPCR motif E/DRY. Forms ionic lock between TM3 and TM6, necessary for G protein activation (Palczewski et al, 2000)
S183P	E150	No	Yes ^{d,e}	Affects local electrostatic interactions necessary for normal membrane trafficking (Pulagam & Palczewski, 2010)
G208S	G174	Yes	Yes ^f	Unknown
V213M	I179	Yes	No	Unknown
G216R	G182	Yes	Yes ^{c,g,h}	Binding site for conserved water molecule in H-bond network between residues of EL2 (Sun et al, 2014). Located next to E181, counterion in invertebrate rhodopsins (Terakita et al, 2004)
G266E	A234	Yes	No	Arrestin binding site (Raman et al, 1999)
S308F	C264	No	No	Part of conserved xWxPY opsin motif. Binding site for conserved water molecule mediating H-bond between TM6 and TM7 (Angel et al, 2009)
H345Q	Y301	Yes	No	Binding site for conserved water molecule in H-bond network between TM7 and TM2 (Okada et al, 2002)

^a Macke et al (1993)

^b Bareil et al (1999)

^c Sung et al (1991)

^d Kumaramanickavel et al (1994)

^e Azam et al (2009)

^f Fujiki et al (1995)

^g Sheffield et al (1991)

^h Colley et al (1995)

Rhodopsin sequence homology can be useful for predicting the impact of *hOPN4* variants. However, this approach is only valid for variants occurring in amino acid sequences conserved between melanopsin and rhodopsin. While the sequence homology in the transmembrane region is better than other protein domains, overall, vertebrate rhodopsin and melanopsin only have 28% of residues in common (Provencio et al., 2000). The *hOPN4* N- and C-termini are particularly variable, which is why they were excluded from the alignment, and there are several insertions in melanopsin intracellular loops not seen in rhodopsin. *hOPN4* mutations in these regions, such as P10L, R186H, W283C, T394I and R408C without an analogous rhodopsin residue, are not necessarily neutral but their functional role may be specific to melanopsin. Similarly, where the *hOPN4* variant and its surrounding sequence differ from the equivalent rhodopsin residue, for example T83M, F95L, S183P, G266E, the purpose of the site is unlikely to be the same in both proteins.

4.3.2 Human *OPN4* variants have abnormal intracellular signalling

The functional properties of each of the 16 *hOPN4* variants were screened *in vitro*. The ability of *hOPN4* variants expressed in HEK293T cells to trigger a normal $G_{q/11}$ phototransduction cascade was assessed by fluorescent calcium imaging, as described in Section 2.2.7.3. The amplitude and kinetics of the intracellular calcium response in *hOPN4* variants was compared to pooled *hOPN4* WT data after no main effect of plate was found on *hOPN4* WT response amplitude, $F(3,23) = 0.54$, $p = 0.660$, time to maximum response, $F(3,23) = 0.79$, $p = 0.513$, or response amplitude at end of recording, $F(3,23) = 0.99$, $p = 0.416$ (One-way between-subjects ANOVA).

First, the response amplitude was examined (**Figure 4.3A**), measured as maximum fluorescence normalised to baseline. Several *hOPN4* variants showed no change in intracellular calcium after light exposure (**Figure 4.3B**), including K340A, as anticipated, and *hOPN4* variants, Y146C and R168C. The response amplitude of these variants was indistinguishable from untransfected cells, $F(2, 23) = 1.098$, $p = 0.373$ (One-way Between subjects ANOVA), suggesting these mutations render *hOPN4* non-functional.

The remaining *hOPN4* variants appeared functional and capable of coupling to a $G_{q/11}$ signalling pathway, demonstrated by an increase in intracellular calcium after light exposure. Their response amplitudes formed a continuum where the majority were similar to the *hOPN4* WT control, with some attenuated and elevated responses at the extremes (**Figure 4.3A**). There was a significant main effect of genotype on response amplitude, $F(16,119) = 8.82$, $p < 0.001$ (One-way between-subjects ANOVA).

A Dunnett's post-hoc test revealed this was driven by 5 variants with significantly attenuated or elevated responses compared to *hOPN4* WT control. As shown in **Figure 4.3C**, these were G208S ($p = 0.013$), S308F ($p < 0.001$) and the control mutants C143A ($p < 0.001$) and $\Delta 389$ ($p = 0.042$). Only one variant, P10L, demonstrated significantly increased response amplitude ($p = 0.010$). When the responses from each session were examined, it was found *hOPN4* G208S, S308F and C143A had consistently attenuated responses relative to the *hOPN4* WT control, suggesting they reliably disrupt normal melanopsin function. In contrast, *hOPN4* P10L and $\Delta 389$ showed highly variable response amplitudes, suggesting their respective elevated and attenuated average responses may have been confounded by transfection or dye loading efficiency between sessions.

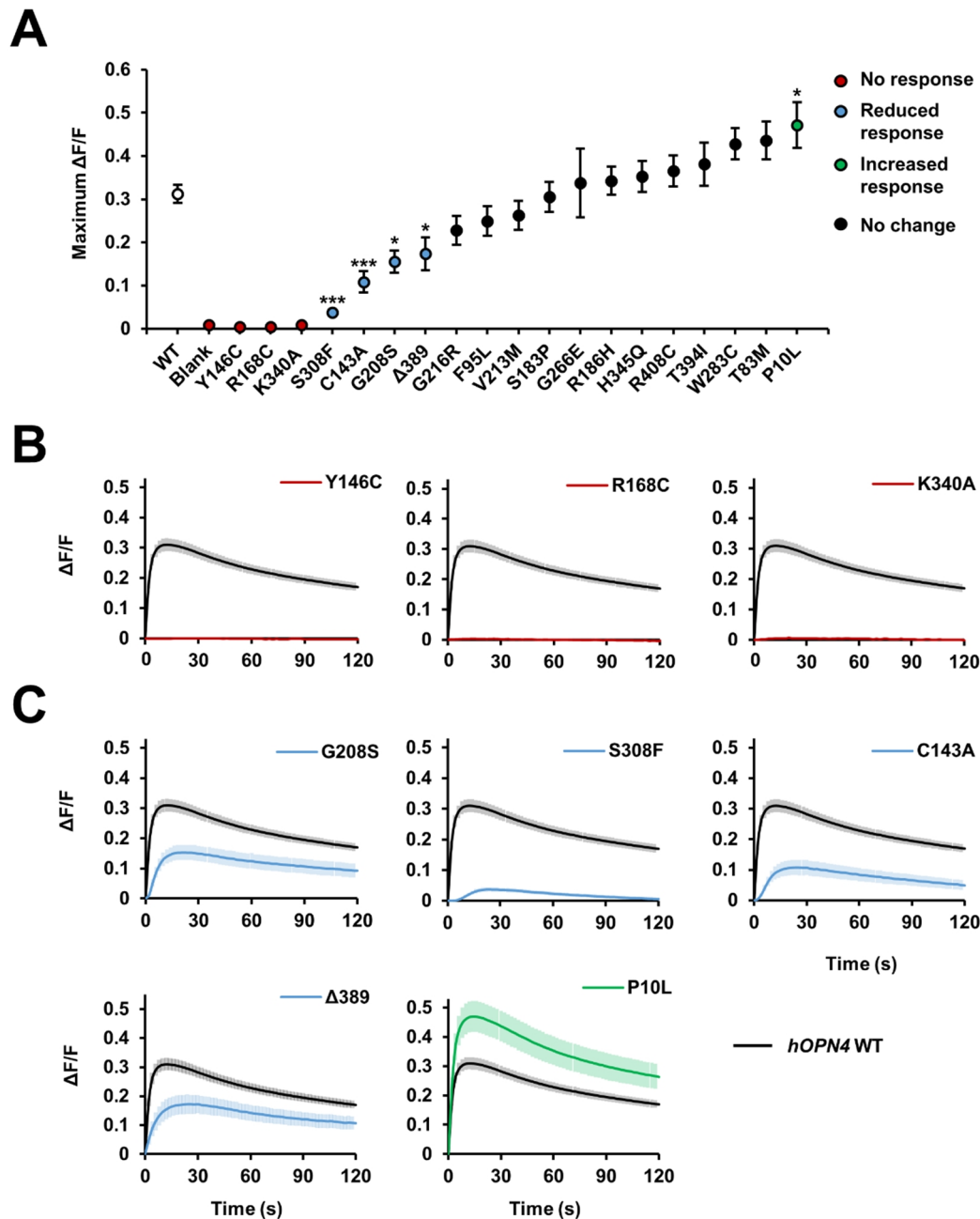


Figure 4.3. *hOPN4* variants show abnormal intracellular calcium responses to light. Intracellular calcium levels of HEK293T cells transiently transfected with pcDNA3.1 *hOPN4* WT and variants were monitored using Fluo4-AM. *hOPN4* response was triggered by first light exposure for fluorescent imaging ($485\text{nm} \pm 6\text{nm}$) and was measured every 2s. Cells were supplemented with $20\mu\text{M}$ 9-*cis* retinal. **(A)** Mean response amplitude measured as maximum normalised fluorescence ($\Delta F/F$). Dashed grey line shows *hOPN4* WT response. **(B-C)** Kinetics of intracellular calcium normalised to baseline (first data point) for **(B)** non-functional variants (red) and **(C)** variants with significantly attenuated (blue) or elevated (green) intracellular calcium influx compared to *hOPN4* WT (black). $N = 6$ biological replicates for all groups except *hOPN4* WT ($N = 24$). Error bars = SEM. * $p < 0.05$, Dunnett's Post-Hoc Test compared to *hOPN4* WT.

Next, the response kinetics of functional *hOPN4* variants were examined. To control for response amplitude, each response was normalised to maximum fluorescence ($\Delta F/F_{\max}$, **Figure 4.4A**). There was a significant main effect of genotype on response onset, measured as time to peak fluorescence, $F(16,119) = 6.47$, $p < 0.001$ (One-way Between Subjects ANOVA). A post-hoc Dunnett's test revealed this was due to significantly slower response onset in 5 *hOPN4* variants, G208S ($p < 0.001$), G216R ($p < 0.001$), S308F ($p < 0.001$), V213M ($p = 0.050$) and H345Q ($p = 0.032$), and 2 control mutations, C143A ($p < 0.001$) and $\Delta 389$ ($p < 0.001$) compared to *hOPN4* WT control (**Figure 4.4B**).

For all *hOPN4* variants, as well as WT positive control, fluorescence remained elevated after a relatively rapid increase from initial light exposure. This prevented use of time to half-maximum fluorescence as a measure of response offset. Instead normalised fluorescence at the end of the recordings was compared 2 minutes after first light stimulus (**Figure 4.4A**), with lower normalised fluorescence at 120s implying faster deactivation kinetics. There was a significant main effect of genotype on response offset, $F(16,119) = 7.80$, $p < 0.001$ (One-way Between Subjects ANOVA). A post-hoc Dunnett's test revealed this was driven by a significantly faster offset in *hOPN4* S308F ($p < 0.001$) compared to *hOPN4* WT control (**Figure 4.4C**). Intriguingly, the offset kinetics of *hOPN4* $\Delta 389$ appear normal, despite published reports suggesting it should be slower than WT control (Blasic et al., 2014).

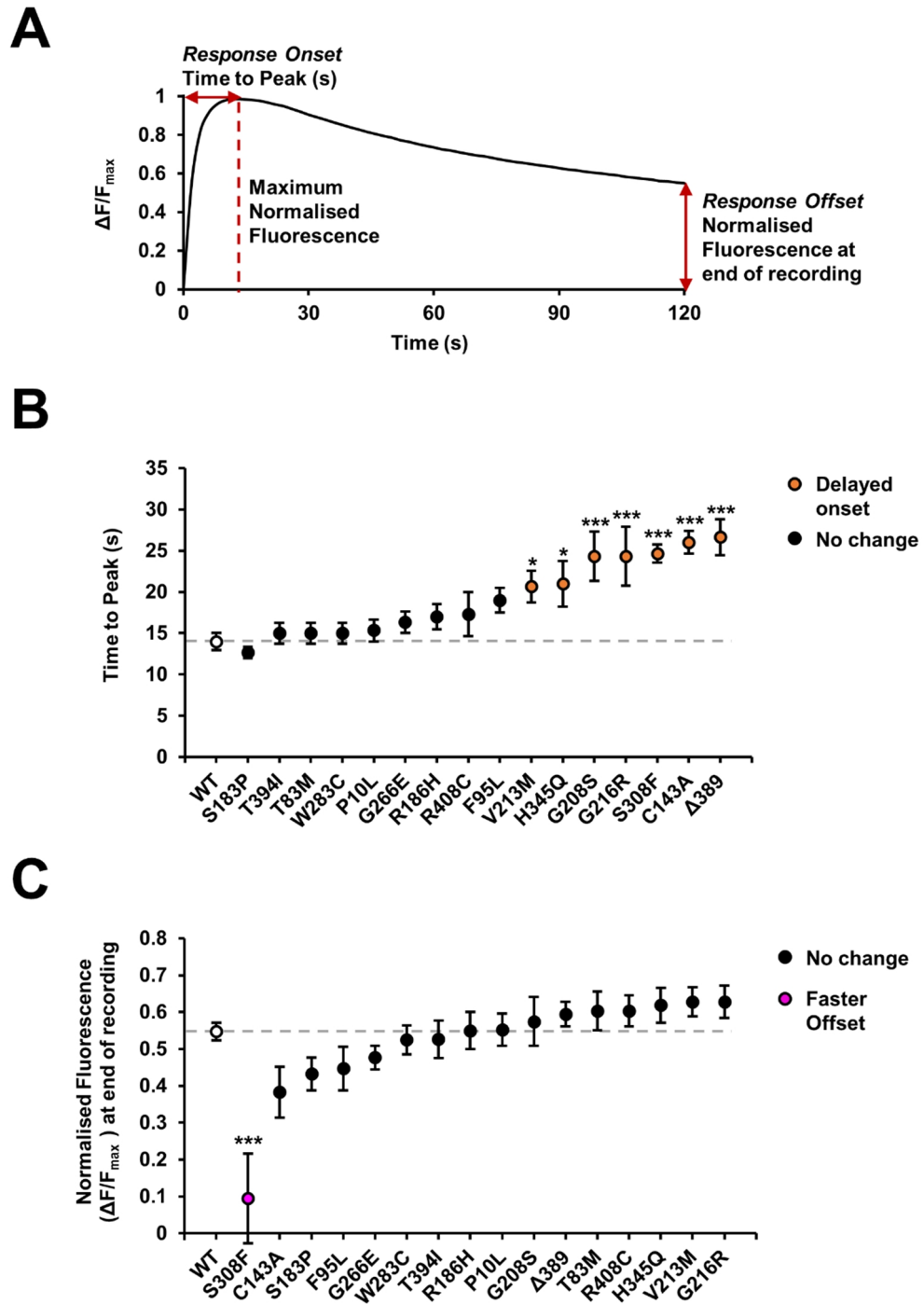


Figure 4.4. *hOPN4* variants have abnormal response kinetics. (A) Definition of response kinetics parameters. Response onset measures as time to normalised peak fluorescence (s). Response offset measured as fluorescence at end of recording (120s after first light exposure). (B) Mean response onset (C) Mean response offset. Dashed grey line shows *hOPN4* WT response. N = 6 biological replicates for all *hOPN4* variants and N = 24 biological replicates for *hOPN4* WT control. Error bars = SEM. * p < 0.05 and *** p < 0.001, Post-Hoc Dunnett's test compared to *hOPN4* WT.

As shown in **Figure 4.5**, the delayed onset is more subtle in variants with normal response amplitude, eg. H345Q and V213M, than in variants with attenuated responses, eg. S308F and G208S. This is consistent with MEA recordings of ipRGCs (Reifler et al., 2015; Tu et al., 2005; Walch et al., 2015) and data in Chapter 5, showing ipRGC responses are small with slower onset at lower light intensities. Decreasing OPN4 sensitivity has a similar effect on responses, with more light required to attain the original response amplitude and kinetics. Therefore, it is likely that *hOPN4* variants with both attenuated responses and delayed onset, such as S308F and G208S, have caused a loss of sensitivity.

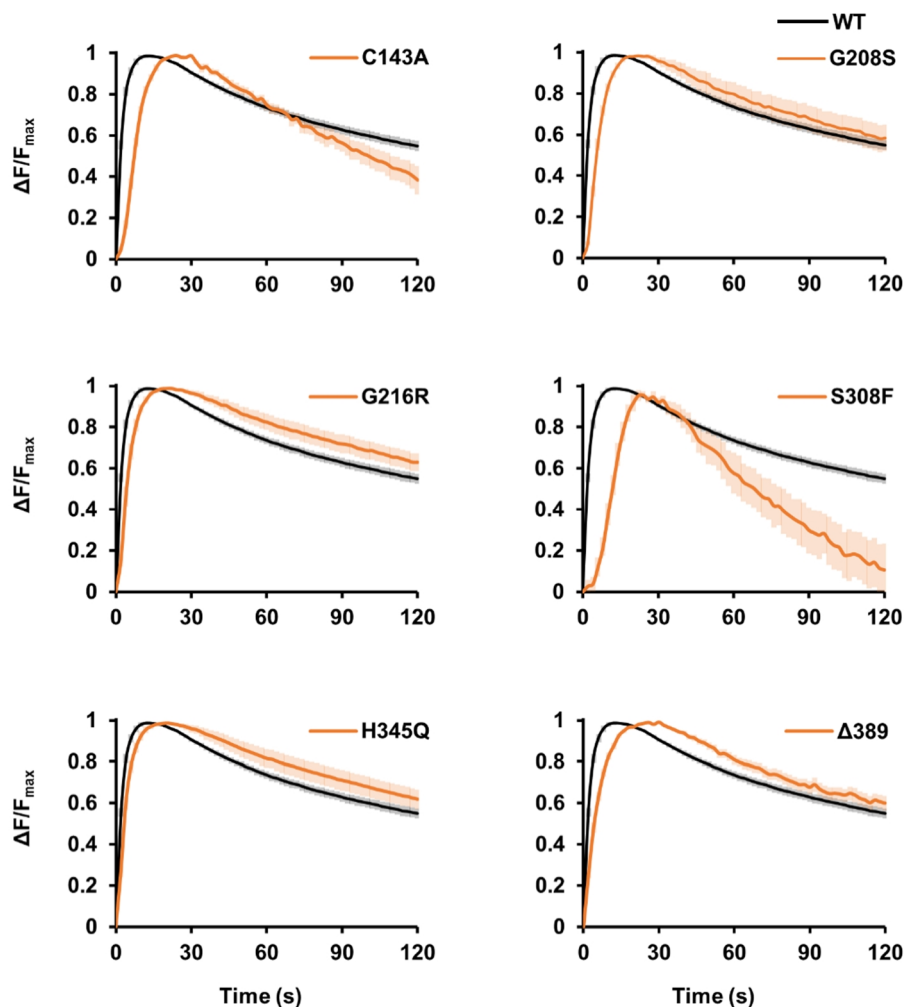


Figure 4.5. *hOPN4* variants with abnormal response onset kinetics. Responses are normalised to baseline (0) and maximum (1). Traces show mean of *hOPN4* variants (orange, n = 6 biological replicates) and WT control (black, n = 24 biological replicates). Error bars = SEM.

4.3.3 All *hOPN4* variants show normal plasma membrane localisation.

Finally, the subcellular localisation of hOPN4 variants was examined. Many pathogenic mutations affect the tertiary protein structure. The misfolded protein may no longer be recognised by intracellular transport mechanisms and trafficked to the plasma membrane. Instead, it accumulates in the endoplasmic reticulum (ER, Mendes et al., 2005), which can disrupt homeostasis, triggering an ER stress response (Rutkowski & Kaufman, 2004) or causing formation of ubiquitinated protein aggregates (Saliba et al., 2002). Both responses can lead to apoptosis, making the consequences of incorrect localisation potentially severe. Using immunocytochemistry, the plasma membrane trafficking of 16 hOPN4 variants expressed in HEK293T cells was examined (**Figure 4.6**).

First, it was confirmed transient transfection of all 16 hOPN4 variants led to OPN4 protein synthesis in HEK293T cells. It is reassuring that all transfections worked, especially for variants without a calcium response, such as R168C or Y146C, as it confirms this was due to non-functional melanopsin. The only variant without anti-hOPN4 staining was the $\Delta 389$ control mutant. The human melanopsin antibody used in these ICC experiments binds to a C-terminus epitope from residues 379-478. Although the $\Delta 389$ mutant possesses 10 residues of this epitope, this is clearly insufficient for antibody labelling. The levels of OPN4 staining appear similar between the different variants, suggesting that there were no substantial difference in expression level, which could account for the changes to intracellular signalling seen in some variants.

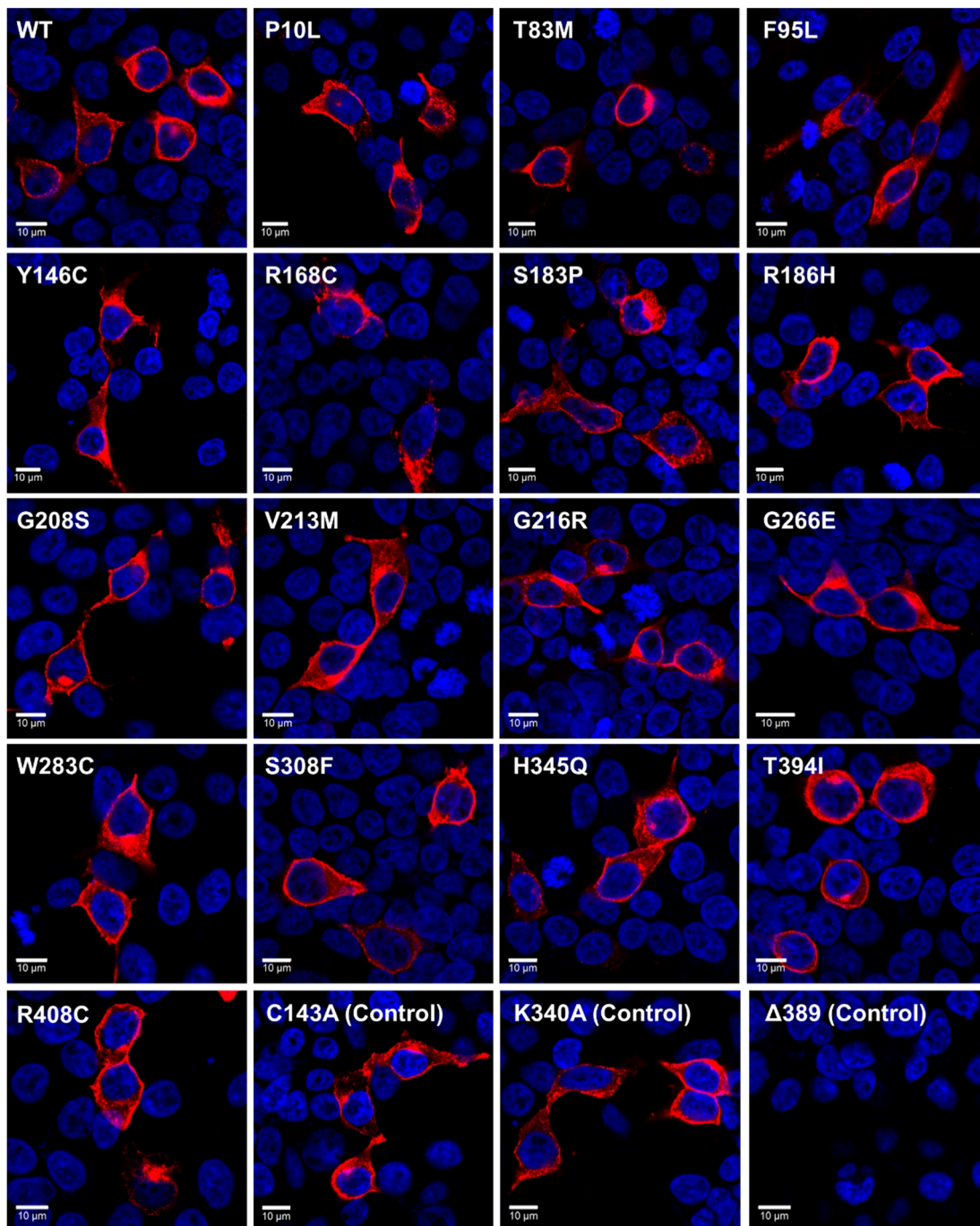


Figure 4.6. hOPN4 variants show normal membrane localisation. Heterologous expression of pcDNA3.1 *hOPN4* WT and variants in HEK293T cells labelled with anti-hOPN4 antibody (red) and DAPI nuclear stain (blue). Scale bar = 10μm.

Second, all variants appeared to be trafficked normally and were expressed in the plasma membrane. This suggests that none of the substitutions sufficiently disrupted protein folding and intracellular transport, and implies that residues with abnormal functional properties, such as Y146C, R168C and S308F, occur at critical functional sites rather than key structural positions. Even the control C143A mutation, known to cause misfolding in rhodopsin by removing one of the disulphide bonds linking TM3 and EL2, appeared to reach the membrane normally.

The presence of all hOPN4 variants in the plasma membrane may be also be an artefact of the heterologous expression used to screen the cells. Overexpression of *hOPN4* driven by the CMV (cytomegalovirus) promoter leads to excess protein synthesised in each transfected cell, which may overwhelm intracellular transport and explain why anti-melanopsin staining sometimes occurs in the cytoplasm. However, immunolabelling of misfolded rhodopsin mutant P23H, expressed in HEK293T cells using similar constitutive promoters cells, revealed distinct protein aggregates in the ER (Noorwez et al., 2003). This suggests immunocytochemistry of transfected cells can be a valid approach for detecting severely misfolded variants. None of the 16 hOPN4 variants appear to cause sufficient disruption of 3D protein structure to demonstrate abnormal localisation *in vitro*.

4.4 Discussion

This study evaluated the potential of naturally-occurring non-synonymous hOPN4 mutations to cause abnormal functional properties. By focusing on variants at highly conserved sites or those leading to a change in amino acid biochemical properties, 16 *hOPN4* genetic variants were prioritised for further *in vitro* screening. Of these, several *hOPN4* variants possessed unusual intracellular signalling pathways, summarised in **Table 4.5**. Immunocytochemistry demonstrated all 16 hOPN4 variants were correctly located in the plasma membrane, indicating variants did not affect the protein structure.

Table 4.5. Summary of key features and phenotype of 16 screened *hOPN4* variants.

Variant	Key Features	Observed Phenotype (relative to <i>hOPN4</i> WT)		
Normal				
T83M	Change in polarity	No difference		
F95L	Conserved in all species	No difference		
S183P	Conserved in most species Change in polarity	No difference		
R186H	Change in hydrophobicity	No difference		
V213M	Conserved in all species as I/V	No difference		
G266E	Change in charge and hydrophobicity	No difference		
W283C	Change in polarity	No difference		
T394I	SNP associated with abnormal NIF behaviour in humans. Conserved in all species as S/T	No difference		
R408C	Change in charge	No difference		
Non-functional				
Y146C	Conserved in all species	No response		
R168C	Conserved in all GPCRs Change in charge and hydrophobicity	No response		
Abnormal function		Amplitude	Onset	Offset
P10L	SNP associated with abnormal NIF behaviour in humans.	Elevated	No difference	No difference
G208S	Conserved in all species Change in polarity	Attenuated	Delayed	No difference
G216R	Conserved in all species Change in polarity and hydrophobicity	No difference	Delayed	No difference
S308F	Conserved in all species Change in charge	Attenuated	Delayed	Faster
H345Q	Change in polarity and hydrophobicity	No difference	Delayed	No difference

Of the 8 variants with unusual calcium responses, only 4 are strong candidates for key functional residues in the melanopsin protein. Introduction of Y146C and R168C to hOPN4 abolished responses to light and two variants, S308F and G208S, reliably demonstrated attenuated response amplitude and delayed onset compared to hOPN4 WT positive control, consistent with decreased sensitivity to light. The remaining variants showed trends towards elevated response amplitude (P10L) or delayed onset kinetics (V213M, G216R and H345Q), but as these phenotypes were not observed across multiple transfections, they are more likely artefacts of transfection or dye-loading efficiency between sessions.

Several variants with damaging phenotypes occur at conserved opsin sites, providing strong hypotheses for how these mutations disrupt melanopsin function. For example, R168C is part of the DRY motif in TM3 found in nearly all GPCRs. As described in Chapter 1, the “ionic lock” between this site and residues in TM6 is necessary for maintaining the inactive conformation (Palczewski et al., 2000). S308F is part of the xWxPY motif in TM6, acting as the “rotamer toggle switch” (Kobilka & Deupi, 2007). Photoisomerisation of the retinal chromophore changes the rotameric states of residues in this motif, causing TM6 to rotate into the active conformation (Bhattacharya et al., 2008). Given hOPN4 variation at these sites attenuates or abolishes melanopsin-driven activity, it is likely these conserved GPCR motifs have similar functional roles in melanopsin.

A third damaging *hOPN4* variant, Y146C, occurs at a conserved opsin site in TM3. In vertebrate opsins, this is the location of the glutamic acid counterion. In invertebrate opsins, this site is occupied by a conserved tyrosine, with the glutamic acid counterion displaced to EL2. As described in Chapter 1, melanopsin possesses invertebrate-like residues at these two positions, yet *hOPN4* Y146C, at the vertebrate counterion location, causes total loss of function. This site cannot be the melanopsin counterion

because tyrosine is not negatively charged, but may still interact with the Schiff-base, disrupting retinal binding when mutated to abolish responses to light.

Identification of naturally occurring *hOPN4* variants at key functional residues suggests there are humans with non-functional or severely impaired melanopsin activity. Screening individuals with circadian disruption or abnormal non-image forming responses to light could also determine if any of these rare deleterious variants are linked to disease, shedding light on the behavioural role of melanopsin in humans. Interestingly, neither *hOPN4* P10L nor T394I, previously associated with abnormal NIF vision in humans (Higuchi et al., 2013; Lee et al., 2013; 2014; Roecklein et al., 2009;2013), showed robust disruption of intracellular signalling *in vitro*. This suggests they are not critical for melanopsin function, consistent with their location in variable domains. However, they may still cause subtle phenotypes not captured by the calcium imaging screen, discussed in more detail in Chapter 5.

The *in vitro* screen also revealed the most useful properties for identifying critical residues. The most damaging variants occurred at conserved opsin sites in the chromophore binding pocket. Invariant residues in this region are most likely adaptive for opsin function and directly interact with the retinal chromophore, disrupting function even when replaced by amino acids with similar biochemical properties. In contrast, substitutions that substantially alter hydrophobicity or charge, have no observable effect if they occur in more variable domains, such as R186H and G266E which are in the intracellular loops. Prioritising conserved sites in the ligand binding domain could be a good strategy for identifying key functional residues in melanopsin, necessary for future attempts to engineer melanopsin as an optogenetic tool.

Notably, none of the critical residues identified appear specific to melanopsin. This is partly because the activity of GPCRs is highly conserved and they share many key domains. In addition, the simple secondary messenger system used to screen *hOPN4* variants *in vitro*, may have only detected the most damaging mutations at sites fundamental to protein activity.

Constructing intensity-response curves, examining adaptation to repeated light exposure or comparing responses to different wavelengths could help identify the residues responsible for more melanopsin-specific response properties, such as long temporal integration or bistability. However, this more complex testing requires time and resources. Fluorescent calcium imaging combined with bioinformatics provides a high-throughput method for selecting variants worthy of more-in depth characterisation, using techniques such as the targeted-viral approach described in Chapter 5.

In conclusion, several naturally occurring *hOPN4* genetic variants were identified that possess significant functional consequences. These variants are rare and do not appear to affect protein localisation, suggesting they are critical determinants of function, not structure. All *hOPN4* variants that caused abnormal intracellular signalling were highly conserved between different species and were located in conserved opsin and GPCR sequence motifs. These properties suggest that melanopsin shares a number of key functional residues with these protein families and will be useful in future attempts to explore melanopsin structure-function.

Chapter 5

Functional assessment of *hOPN4* SNPs, P10L and T394I, using targeted AAV delivery *in vivo*

Chapter 4 characterised a number of naturally-occurring human melanopsin (*hOPN4*) variants *in vitro*. Of these, only P10L and T394I have been reported to be associated with abnormal NIF behaviour in humans. In this chapter, the functional properties of these two SNPs are assessed *in vivo*, using a novel targeted viral technique.

5.1 Introduction

A number of studies have suggested an association between *hOPN4* SNPs P10L and T394I, and abnormal NIF behaviours, such as pupil light responses (PLR) and sleep-wake timing (Higuchi et al., 2013; Lee et al., 2013; 2014; Roecklein et al., 2009;2013), described in detail in Section 1.4.4, SNPs. These studies imply both SNPs disrupt melanopsin activity, but do not provide evidence of causality. It is important to note all SNP studies were conducted in individuals with normal vision, which makes it difficult to isolate the contribution of the melanopsin variant to the abnormal phenotype,

Moreover, based on alignment of melanopsin with other opsins, the position of P10L and T394I does not correspond to any sites known to cause gain- or loss-of-function when mutated. Both P10L and T394I are located in regions of the protein that are poorly conserved between different species, the N and C terminus respectively (Hankins et al., 2008; Borges et al., 2012), suggesting variation at these locations is well tolerated. T394I does occur in a cluster of conserved hydroxyl-bearing sites, potentially phosphorylated for arrestin binding and opsin deactivation (Blasic et al., 2014; Mure et al., 2016). However, mutation of these sites individually does not affect the kinetics of calcium responses in transiently transfected HEK293T cells (Blasic et al., 2014).

As described in Section 4.3.2., both *hOPN4* P10L and T394I expressed in HEK293 cells demonstrate intracellular calcium influx comparable to *hOPN4* wildtype (WT) in response to light, indicating these SNPs function normally *in vitro*. Therefore, there is limited evidence from bioinformatics and *in vitro* studies to support the hypothesis that P10L and T394I affect melanopsin function *in vivo*.

To determine the functional consequences of these specific variants, an animal model of *hOPN4* SNPs was developed that would allow measurement of melanopsin-associated behaviour *in vivo*. A *hOPN4* WT control or experimental *hOPN4* mutant were introduced specifically to ipRGCs of melanopsin knockout mice (*Opn4*^{-/-}) using a conditionally targeted adeno-associated virus (AAV). This model enabled the study of *hOPN4* SNPs in their native ipRGC environment. The same animals can also be tested before and after AAV introduction, to establish whether the *hOPN4* SNPs are capable of restoring normal wildtype behaviour to *Opn4*^{-/-} mice, as well as separating the contribution of rods and cones from that of melanopsin.

This study had two aims. First, to investigate if targeted delivery of *hOPN4* AAV could successfully restore a wildtype (*Opn4*^{+/+}) phenotype to *Opn4*^{-/-} animals. Second, to explore phenotype differences between *hOPN4* WT control and *hOPN4* SNPs, P10L and T394I, and determine if there was any evidence these mutations affect the structure and function of the melanopsin protein.

This study first confirmed *hOPN4* AAV could be successfully delivered to ipRGCs and restore an *Opn4*^{+/+} pupil phenotype to *Opn4*^{-/-} mice. A wide range of NIF responses to light, including PLR and circadian photoentrainment, were then characterised in *Opn4*^{-/-} mice treated with *hOPN4* WT, P10L or T394I AAV to determine if both SNPs were able to fully restore an *Opn4*^{+/+} behavioural phenotype. Finally the response characteristics of ipRGCs expressing *hOPN4* SNPs or *hOPN4* WT during pharmacological blockade of rod-cone signals were examined to identify any impact of SNPs on *hOPN4* function not observed during assays of whole-animal physiology.

5.2 Methods

5.2.1 Experimental Design

Experiments were divided into 3 separate studies - 1) Technique validation, 2) Viral titre optimisation and 3) *in vivo* comparison of human SNPs.

To address the first aim, the *hOPN4* AAV technique was validated by comparing mice with and without endogenous melanopsin (*Opn4*^{+/+} and *Opn4*^{-/-} respectively) with *Opn4*^{-/-} *Cre*^{+/+} animals treated with targeted *hOPN4* WT AAV. A detailed description of the *Opn4*^{-/-} *Cre*^{+/+} mouse is provided in Section 5.2.2. Next, the relationship between virus titre and melanopsin phenotype restoration was examined to optimise AAV-delivery for comparing *hOPN4* SNPs with *hOPN4* WT control. Finally, an extensive study was conducted comparing control *Opn4*^{-/-} *Cre*^{+/+} animals treated with *hOPN4* WT AAV with *Opn4*^{-/-} *Cre*^{+/+} animals treated with *hOPN4* P10L or T394I AAV. This third study addressed the second aim of whether there were observable differences between the two SNPs and wildtype *hOPN4*. All experiments in this third study were double-blind, with animals randomly allocated to different virus groups. Injections, data collection and analysis were completed before the virus group was unmasked. The cohorts used in each of these studies are described in **Table 5.1**.

Table 5.1. Summary of the cohorts used in the 3 studies.

Cohort	Animals Used	Methods	Testing Times
<i>Technique Validation</i>	6 <i>Opn4</i> ^{+/-} 6 <i>Opn4</i> ^{-/-} 6 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> WT AAV (10 ¹² virus particles/ml)	Pupillometry Immunohistochemistry RT-qPCR	<i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} tested 1 month after bilateral intravitreal injection. <i>Opn4</i> ^{+/-} and <i>Opn4</i> ^{-/-} were not injected
<i>Titre Optimisation</i>	4 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> WT AAV (10 ¹¹ virus particles/ml) 5 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> WT AAV (10 ¹⁰ virus particles/ml)	Pupillometry Tissue collected for RT-qPCR and Immunohistochemistry	All tested 1 month after injection
<i>Comparison of hOPN4 SNPs with WT control</i>	8 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> WT AAV 6 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> P10L AAV 6 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> T394I AAV 8 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> WT AAV 6 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> P10L AAV 8 <i>Opn4</i> ^{-/-} <i>Cre</i> ^{+/-} with <i>hOPN4</i> T394I AAV	Pupillometry Multi-electrode array RT-qPCR Passive-infra red activity monitoring Multi-electrode array Tissue collected for Immunohistochemistry	All tested 1 month after injection. All viruses 10 ¹² virus particles/ml Subset of animals also tested before injection (WT n = 4; P10L n = 3; T394I n = 4) All tested 1 month after injection. All viruses 10 ¹² virus particles/ml Subset of animals also tested before injection (WT n = 5; P10L n = 5; T394I n = 4)

5.2.2 Animals

Six *Opn4*^{+/-} *tau-LacZ*^{-/-} and 6 *Opn4*^{-/-} *tau-LacZ*^{+/-} mice on a mixed C57BL/6 and 129/SvJ background were used as controls for the pilot experiment, genotyped using the method described in Section 2.2.5.4. Fifty-nine *Opn4*^{-/-} *Cre*^{+/-} *EYFP*^{+/-} mice on a mixed C57BL/6 and 129/SvJ background were used for the AAV experiments, genotyped using the method described in Section 2.2.5.5. These mice were generated by breeding *Opn4* *Cre* mice with mice containing a lox.STOP.lox EYFP sequence inserted within the Rosa26 locus (Srinivas et al., 2001). EYFP is expressed in all ipRGCs. In the *Opn4*^{-/-} *Cre*^{+/-} mice, Cre-recombinase replaces the *Opn4* open reading frame so Cre expression is driven by the melanopsin promotor exclusively in ipRGCs. This means *Opn4*^{-/-} *Cre*^{+/-} animals have no endogenous melanopsin expression (Ecker et al., 2010; Hughes et al., 2013).

Immunocytochemistry of retina from these animals show no sign of retinal degeneration, suggesting rod and cone function is intact. Unless stated otherwise, all mice were kept under 12:12 light dark cycle with food and water provided *ad libitum*. At the end of all experiments, animals were culled by cervical dislocation (approved Schedule 1 method). Details of ethical approval can be found in Section 2.2.8.1.

5.2.3 Virus Production

Three viruses were produced: AAV2-DIO-*hOPN4* WT, AAV2-DIO-*hOPN4* P10L and AAV2-DIO-*hOPN4* T394I. An AAV2/2 capsid with four tyrosine to phenylalanine mutations, Quad Y-F, (Mowat et al., 2014) was used for more efficient virus transduction (de Silva et al., 2015). A *hOPN4* transgene plasmid was constructed by replacing the inverted ChR2-mCherry sequence in pEF1α-AAV-DIO-ChR2-mCherry (Cardin et al., 2010) with an inverted *hOPN4* sequence (NM033282.3) fused to a 1D4 tag, using restriction enzyme cloning (performed by Dr. Doron Hickey). Further description of the pEF1α-AAV-DIO vector is provided in section 2.1.3.3. Point mutations were introduced by site directed mutagenesis with QuikChange II XL kit (Stratagene) as described in Section 2.2.4 using P10L and T394I mutagenesis primers described in Section 4.2.2, **Table 4.2.** Presence of mutation was confirmed using Sanger sequencing (Source Bioscience) as described in section 2.2.2., using the following primers: EF1α F, 5' – ATTTGCCCTTTTGGAGTTTGG, *hOPN4* midF, 5' – TGGGCACAGTGATCTTGCTGGT, and *hOPN4* midR, 5' – AGGCATAGAACTCGCAGCCTGT.

A double-floxed inverted (DIO) AAV approach was used to ensure *hOPN4* expression only occurs in presence of Cre recombinase (**Figure 5.1**). The inverted *hOPN4* transgene is flanked by two pairs of Lox sites (LoxP and Lox2272) so that in the presence of Cre, the it is inverted into the sense orientation and expression is driven by a ubiquitous EF1α (elongation factor 1α) promoter (Qin et al., 2010).

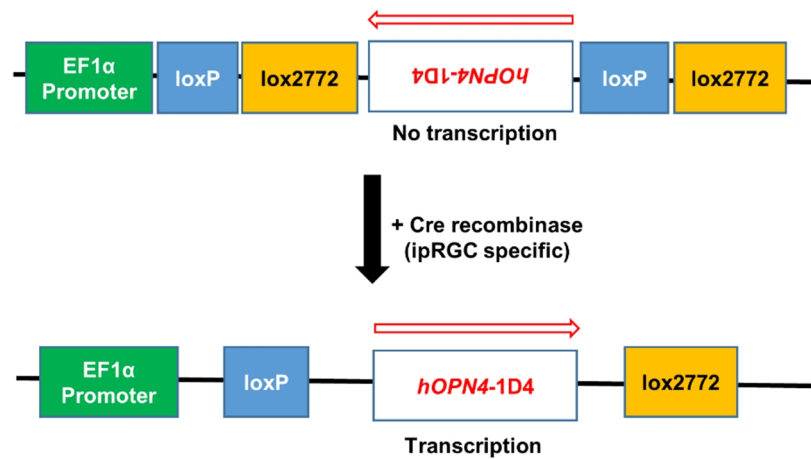


Figure 5.1. Double-floxed inverted orientation AAV (DIO-AAV) for cell-type specific expression. Sequence for human melanopsin gene with 1D4 tag (*hOPN4-1D4*) is inverted and surrounded by two pairs of Lox sites (LoxP and Lox2772). With *hOPN4* sequence in antisense direction, the EF1 α promoter cannot drive transcription. In presence of Cre-recombinase, the *hOPN4* sequence is flipped to the sense orientation and transcription occurs. Cre-recombinase expression is restricted to ipRGCs in *Opn4*^{-/-} *Cre*^{+/+} mice so the DIO-AAV approach ensures *hOPN4* is only expressed in ipRGCs.

Each virus was produced by co-transfection of HEK293T cells grown in a HYPERflask (Corning) with three plasmids. Transfection of each HYPERflask of cells required 118.3 μ g pEF1a-DIO-*hOPN4-1D4*-WPRES (containing ITRs, WPRES and transgene), 125.6 μ g pAAV2-Quad-Y-F (containing *rep* and *cap* genes) and 256.7 μ g pAd Δ F6 (containing helper genes from adenovirus genome). 2 HYPERflasks of HEK293T cells were required for each virus (Corning). Cells were transfected using polyethylenimine (PEI) transfection reagent (de Silva et al., 2015). First sufficient amounts of the 3 plasmids to transfect 2 HYPERflasks were mixed with NaCl (150mM) to 20ml total volume in a 50ml Falcon tube. PEI was then prepared by mixing 2.25ml of PEI (Sigma) with 17.75ml of NaCl (150mM, Sigma), enough for 2 HYPERflasks. 20ml of the PEI-NaCl mix was then added dropwise to the Plasmid-NaCl mix and left to incubate at room temperature for 20mins. The PEI-Plasmid-NaCl mix was then split into two 20ml aliquots. Each aliquot was then added to 450ml of transfection media (DMEM with 4,500mg/L glucose, with 2% FBS, 1% L-glutamine and 1% Pen/Strep) and mixed gently.

Using 20ml of transfection media, the tube containing PEI-Plasmid complex was rinsed and added to the larger volume of transfection media plus PEI-plasmid, and the total volume of 490ml transfection medium with PEI-Plasmid-NaCl was used to replace the culture media of one of the HYPERflasks. This process was then repeated to transfect the second HYPERflask.

Cells were harvested 72hrs later by removing 200ml of media from HYPERflask and shaking vigorously. The cell suspension from one HYPERflask was transferred to a 500ml flask and then centrifuged for 10 mins at 1000rpm at 20°C. The media was removed until approximately 30ml of media was left in the flask. The cells were resuspended and transferred to a 50ml Falcon tube and centrifuged for 10 minutes at 1000rpm at 20°C. The media was then removed and each cell pellet was lysed in 15ml of Lysis buffer (0.15M NaCl, Sigma, and 50mM Tris-HCl, pH8.5, VWR) and 500µl EDTA-free protease-inhibitor cocktail (Roche) in PBS. Cell lysate then underwent four freeze-thaw cycles at -80°C for 1 hour and 37°C for 15mins. The cell lysate was then stored at -80°C until ready for purification.

After 30mins at 37°C with 50U/ml of benzonase (Merck Millipore) added to remove cell DNA contamination, the lysate was centrifuged at 3700 x *g* for 20mins at room temperature.

The AAV was purified by iodixanol gradient ultracentrifugation (de Silva et al., 2015). The different iodixanol (OptiPrep density gradient medium, Sigma-Aldrich) fractions were prepared as follows (sufficient for two gradients necessary to purify a 15ml lysate sample).

15% fraction is 4ml Iodixanol, 3.2ml 5M NaCl, 3.2ml 5x PBS-MK and 5.6ml H₂O.

25% fraction is 5ml Iodixanol, 20µl Phenol Red, 2.4ml 5x PBS-MK and 4.6ml H₂O.

40% fraction is 6.7ml Iodixanol, 2ml 5x PBS-MK and 1.3ml H₂O.

60% fraction is 10ml Iodixanol and 20µl Phenol Red.

PBS-MK is PBS with 5mM magnesium chloride (Sigma-Aldrich) and 12.5mM potassium chloride (Sigma-Aldrich). Phenol Red solution was obtained from Sigma-Aldrich.

The iodixanol gradient was prepared by layering the fractions as follows: 7.2ml of 15% fraction was first pipetted into the ultracentrifuge tube. 4.8ml of the 25% fraction was then pipetted into a separate 15ml Falcon tube. 2.4ml of this 25% fraction aliquot was then drawn up with a Pasteur pipette and, holding the volume at the tip of the pipette, gently moved through the 15% iodixanol fraction until the tip touched the bottom of the ultracentrifuge tube. The 25% fraction was then gently expelled below the 15% fraction, without releasing any air and disturbing the iodixanol. Once empty, the pipette was carefully withdrawn. The remaining volume of the 25% fraction was then pipetted and the process repeated to place it beneath the 15% fraction. Once the 25% fraction was layered in place, the process was repeated to place 4ml of 40% fraction beneath the 25% fraction. The process was again repeated to place 4ml of 60% fraction beneath the 40% fraction. The cell lysate was then added drop-wise to the top of the iodixanol gradient. The ultracentrifuge tube was filled completely and sealed with a centrifuge cap, then centrifuged at 59000rpm for 1.5 hours at 20°C. The cap was then removed and using an 18G needle attached to a 5ml syringe, the ultracentrifuge tube was pierced at the interface of the 60% and 40% phases with bevel positioned upwards. The 40% phase was then collected without collecting any debris from the 25% phase. The collected fraction was then stored at 4°C overnight until ready for concentration.

Virus was concentrated using an Amicon Ultra 100K filter. 5ml of PBS was added to the filter and centrifuged at 3000 x *g* for 15mins, then flow-through was discarded. 5ml PBS was added to the collected iodixanol fraction and mixed by pipetting. The total volume was then added to the filter and centrifuged at 3000 x *g*, until volume was reduced to 500µl. Flow-through was discarded. 15ml PBS was then added to the filter and centrifuged at 3000 x *g*, until the volume was reduced to 500µl, then flow-through was discarded. The previous step was repeated twice more, and on final PBS wash the

volume in the filter was reduced to ~250µl, which contained the purified AAV. The solution was then pipetted over the filter 40 times, then 50µl and 20µl aliquots were prepared and stored at -80°C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to confirm purity of the three viral capsid proteins (V1, VP2 and VP3). 5µl of Laemmli buffer 5x (National Diagnostics) was added to 5µl of concentrated AAV and 5µl of ddH₂O and mixed by pipetting. A negative control of 15µl ddH₂O plus 5µl Laemmli buffer was also prepared.

Both mixtures were then heated at 100°C for 10mins, then vortexed, spun down and placed on ice until ready for use. The full volume of both the AAV and negative control mixture was then added to wells of a 10% SDS gel submerged in running buffer solution (1x Tris/Glycine/SDS) in a gel tank. 5µl of a protein ladder (Geneflow, prestained ladder S6-0024) was also loaded into the SDS gel. The gel was run at 100V for 2 hours.

The gel was then washed three times in ddH₂O, each for 5 mins before being placed in fresh water. The gel was then transferred to EZBlue dye (Sigma) for 30min-2hours until bands were clearly visible on the gel. The gel was then washed in ddH₂O three times, each for 10mins before being placed in fresh water, to remove background staining.

Bands should only be visible where concentrated AAV was loaded into gel and not in the negative control sample. In a concentrated AAV of high purity, only three bands should be visible corresponding to the three capsid proteins, V1 (87 kDa), VP2 (73 kDa) and VP3 (62 kDa). Any additional bands could indicate the AAV was contaminated during production or purification. It is important to note the SDS-PAGE cannot differentiate between empty and fully packaged virus particles.

The viral titre is the number of genome copies of AAV per 1ml of virus and is determined using standard-curve qPCR. The AAV must first be treated with DNase to remove any unpackaged plasmid which may confound qPCR results. The following reaction conditions were used: 1µl of concentrated virus, 1µl of DNase buffer, 1µl of DNase and 7µl of ddH₂O. This reaction was heated at 37°C for 10mins, then 95°C for 10mins.

This high temperature denatures the capsid proteins allowing access to the viral transgene for amplification. The denatured sample was then diluted 1:10 in ddH₂O. A serial dilution of the pEF1α-AAV-DIO-*hOPN4*-1D4 plasmid was then set up to produce positive control samples at 500pg/µl, 50pg/µl, 5pg/µ and 0.5pg/µl. These were used to create a standard curve. Primers were used to amplify part of the *hOPN4* transgene, Forward 5'–GTTGCTGACATCCTGCTCCT, and Reverse 5'–TCCCGGATGGCCCTGAAGAT.

The following reaction conditions were used for qPCR: 5µl iTaq Universal SYBR Green Supermix (Bio-rad), 1µl Forward Primer (2µM), 1µl Reverse Primer (2µM), 2µl ddH₂O and 1µl diluted DNase-treated virus sample or 1µl positive control plasmid sample. Each reaction was performed in triplicate. Thermal cycling conditions were: 95°C for 3mins, then 40 cycles of 95°C for 10s and 60°C for 30s. A melt curve was produced at 70°C.

A standard curve was generated by converting the mass (g) of the positive control samples to amplicon copies, calculated using <http://cels.uri.edu/gsc/cndna.html>. The equation used is:

$$\text{Amplicon Copies} = \frac{\text{mass (g)}}{\text{molecular weight of amplicon (g per mol)}} \times \text{Avogadro's constant}$$

Equation 5.1

Where Avogadro's constant = 6.02×10^{23} per mole.

The number of amplicon copies for each of the positive control samples of varying concentration were then log transformed and plotted against the *Ct* value (described in

more detail in Section 2.2.6.3) to produce a standard curve. The number of genome copies in the diluted DNase-treated virus sample could then be calculated using this standard curve. The viral titre (number of AAV genome copies per 1ml of virus) could then be calculated by accounting for dilution of the AAV sample.

5.2.4 Intravitreal injections

Animals were anaesthetised by intraperitoneal injection of xylazine (10µg/g body weight) and ketamine (80µg/g body weight). Pupils were dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride eye drops (Bausch & Lomb). A 6mm coverslip was positioned on gel lubricant (Viscotears, Novartis) applied to the cornea, to allow visualisation of injection under a surgical microscope (M620 F20, Leica, Wetzlar, Germany). 1.5µl of virus ($2.2 \times 10^{10-12}$ virus particles/ml) was injected into the vitreous through the sclera using a Hamilton syringe with a 10mm 34-gauge needle (65N, Hamilton AG). The eye was stabilised during injection by holding the superior or inferior rectus muscle with notched forceps. All animals received bilateral intravitreal injections of the same virus to each eye. Both needle and syringe were flushed 5-10 times with sterile PBS between injections. Anaesthesia was reversed by intraperitoneal injection of atipamezole (10µg/g body weight). During recovery, 0.5% proxymetacaine hydrochloride (local anaesthetic) and 0.5% chloramphenicol (broad spectrum antibiotic, both Bausch & Lomb) was applied to injected eyes. All animals began testing 1 month after injection. Of the 59 animals injected, 2 experienced adverse effects from injection (loss of pupil constriction identified during testing). These animals were excluded from analysis.

5.2.5 Pupillometry

Pupillometry set up and data analysis were as described in Section 2.2.8.2. For all animals, the exposed eye was treated with a 1% tropicamide solution (Bausch & Lomb). All recordings started with 2s of dark, followed by 10s of light exposure and then up to 40s of dark. This allowed examination of dark-adapted pupil size, pupil constriction and the recovery from constriction of the contralateral eye, respectively.

Images were analysed using ImageJ (<http://rsbweb.nih.gov/ij>). Two components of pupil response were examined: minimum constriction and post-illumination pupil response (PIPR). Minimum pupil constriction was analysed as described in Section 2.2.8.2.

PIPR was defined using semi-automated analysis (**Figure 5.2**). Pupil images were manually thresholded using default method, described in Ridler & Calvard (1978). Pupil area was then defined by calculating area of all objects larger than 100 pixels² with circularity greater than 0.4 (scale is 0-1, flat line to perfect circle). This tended to limit results to the largest object in each image – the pupil. Any artefacts were removed from analysis. Pupil area from each frame was then normalised to the maximum area found during the first 2s dark (maximum dark-adapted pupil size) and plotted over time. This provided the kinetics of the pupil response. The normalised pupil area at 0, 5, 10, 15, 20, 25 and 30s after lights off was fitted to a linear trendline using least squares minimisation. The slope of this trendline, rate of recovery, was used as a measure of PIPR. Larger slope values meant faster recovery and more dilation, whereas smaller slope values meant slow recovery and sustained constriction.

Each animal was tested multiple times for each light condition to minimise any variation due to handling or stress. All sessions were analysed and the most complete recording was used for each animal. This was defined as the recording with the greatest constriction, longest recovery and fewest artefacts caused by movement or stress, identifiable as sudden changes in pupil area.

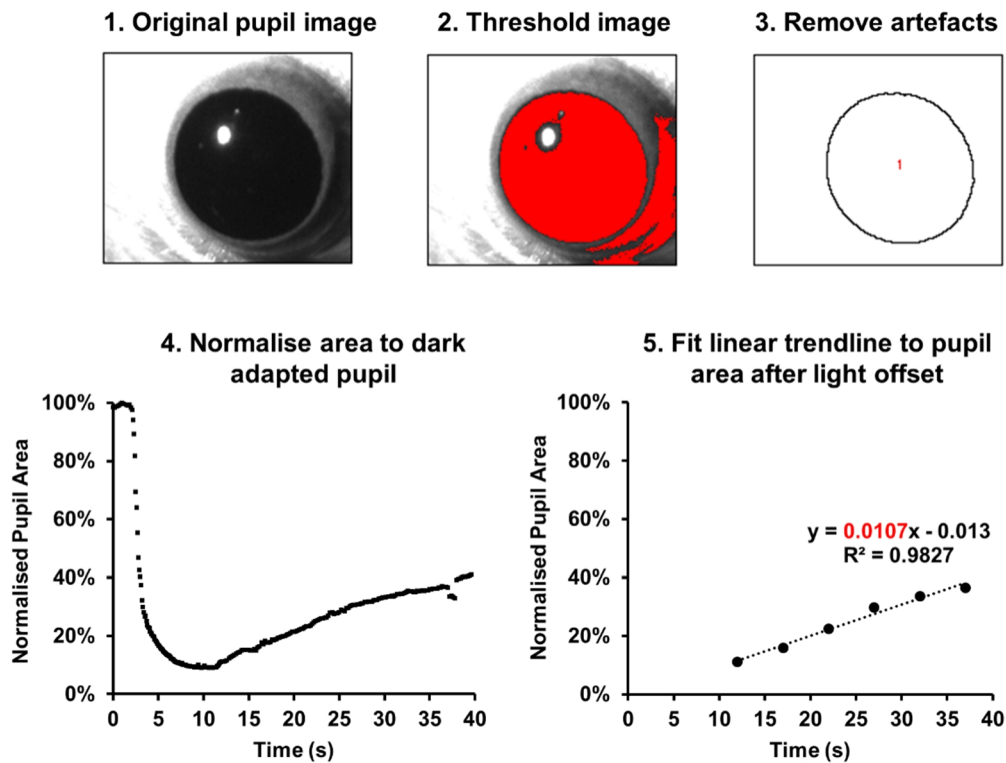


Figure 5.2. Pupillometry analysis for post-illumination pupil response (PIPR). Images were thresholded using ImageJ software to define pupil area (regions below threshold are shown in red). Small non-circular artefacts were removed to leave the pupil as sole object in each frame. The area of each object per frame was then normalised to the area of the first frame. A linear trendline (dotted line) was fitted to normalised pupil area at 0, 5, 10, 15, 20, 25 seconds after light offset. The slope of this trendline (highlighted in red) was used to quantify the rate of recovery as a measure of post-illumination pupil response (PIPR)

5.2.6 Passive infra-red (PIR) circadian screen

Animals were housed individually in large cages (44cm x 26cm x 12cm) with a transparent acrylic block placed under the food hopper to keep animal in PIR detection range at all times. Six cages were arranged in each light-tight chamber with light provided by white LEDs of variable intensity. A pyroelectric (passive) infra-red detector (PIR) was positioned above the centre of each cage to detect motion. PIRs detect infra-red radiation emitted by the surroundings, particularly heat from living organisms.

As shown in **Figure 5.3**, each PIR consists of two sensors and acts as a motion detector by recording the differences in infra-red radiation detected by each sensor. These two sensors are set at an arbitrary floating value and cancel each other out. When one sensor detects more or less infra-red than the other, caused by a warm body entering or leaving the detection range of each sensor, the floating values no longer cancel out and motion is detected. By using a multi-faceted Fresnel lens, the range of each sensor can be increased without losing precision. By calibrating the height of each PIR to the cage, motion detection is restricted to the cage beneath the sensor. Further details of this method can be found in Brown et al. (in preparation). Raw data was collected from each PIR every 100ms for 10s and then averaged to give a percentage time active every 10s. This provides a measure of activity every 10s for the duration of recording, including small movements, such as grooming or head turns, and larger movements, such as running.

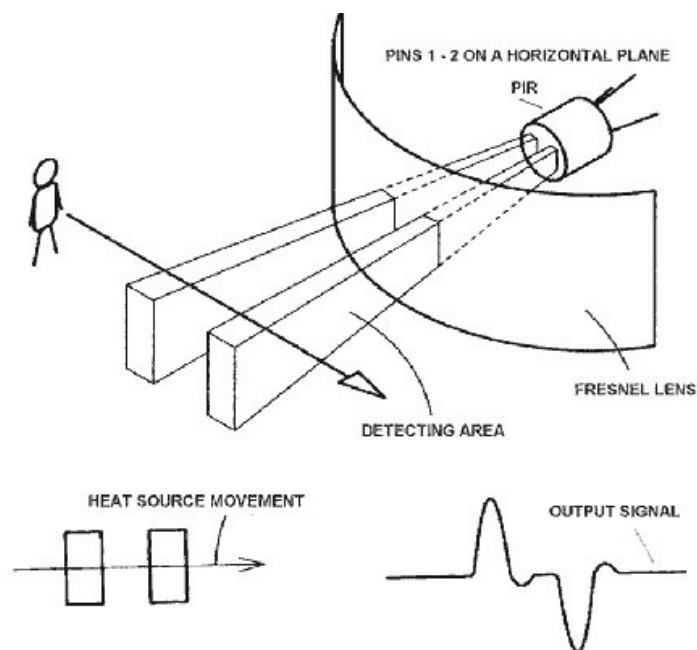


Figure 5.3. Passive infra-red detection for motion detection. Each PIR consists of two pins or sensors which can detect infrared radiation, in the form of heat emitted by living animals. As the heat source moves into and out of the detection range of sensor, this creates an output signal used to measure motion. A Fresnel lens expands the detection range of each sensor. Proximity PIR diagram by Adafruit Learning System (retrieved from learn.adafruit.com/assets/511) used under Creative Commons Attribution-ShareAlike 3.0 license (creativecommons.org/licenses/by-sa/3.0/).

For animals screened using PIRs before injection, the following light schedules were used (**Figure 5.4A**). Activity was first recorded under a 12:12 light:dark (LD, zeitgeber time, ZT0 = 0600) cycle under 350-400 lux ($108\text{-}124\mu\text{W}/\text{cm}^2$ or 14.5-14.6 log quanta) light for 7 days to measure entrainment and activity fragmentation. Animals were then placed in constant darkness (DD) for 13 days to record freerunning period. Animals were returned to 12:12 LD cycle (ZT0 = 0000) for a further 14 days. An earlier light onset was used to prevent a jet lag effect caused by animals re-entraining to the previous LD cycle after free-running. Animals were then placed in constant darkness (DD) for 11 days. On the first day of DD a 15min 100 lux ($32\mu\text{W}/\text{cm}^2$ or 13.94 log quanta) white light pulse was given at ZT15 to induce a phase shift.

The activity screen for animals 1 month after injection differed slightly in the duration and zeitgeber times of each light condition (**Figure 5.4B**). The initial 12:12 LD cycle was for 9 days (ZT0 = 0700), followed by 10 days of DD and 14 days of 12:12 LD cycle (ZT0 = 0200). Animals were returned to DD for 14 days with a 100lux 15min white light pulse on first day of DD at ZT15, as previously. Animals were then placed in 12:12 LD for 7 days before being released into 100lux constant light (LL) for 14 days to monitor period lengthening. LL was not recorded in animals before injection, in case the constant light damaged the retina and confounded the effects of injection.

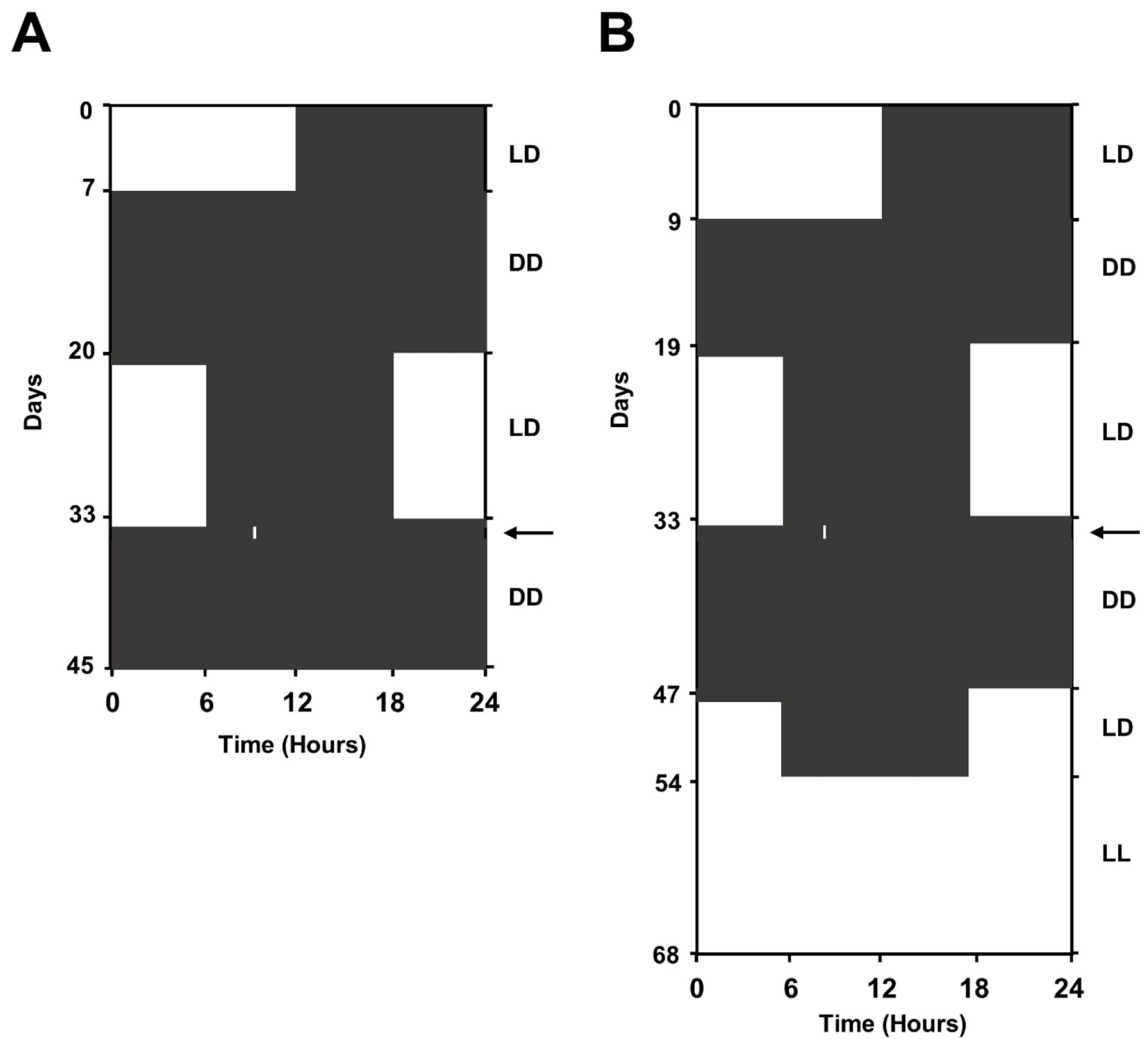


Figure 5.4. Light schedules for PIR circadian screen. (A) Circadian screen before injection. Animals were placed in 12:12 white light: dark cycle (LD, 350-400lux), followed by constant darkness (DD), then back to LD. They were then returned to DD and on the first day of DD (shown by arrow), given a 15min white light pulse (100lux) at ZT15. **(B)** Circadian screen after injection. The same light schedule was used as before, with an additional week in LD, followed by 14 days in constant light (LL) added at end of the screen. Black and white shading corresponds to dark and light, respectively.

Data were processed using custom python scripts that resampled raw PIR data into 1min bins. **Table 5.2** describes the circadian parameters examined using Clocklab (Actimetrics) for Matlab (Mathworks).

Table 5.2. Circadian parameters used to assess activity during PIR screen.

Parameter	Unit	Definition	Reason for investigation
<i>Phase Angle of Entrainment</i>	Hours	Average time difference between onset of activity and offset of light	Rhythm consistency and entrainment to external zeitgeber
<i>Activity Bouts</i>	Bouts	Defined using Clocklab default criteria (max gap. 18min, threshold 5%)	Diurnal rhythm fragmentation
<i>Activity Bout Duration</i>	Minutes	Average duration of activity bouts over given time period	Diurnal rhythm fragmentation
<i>Phase Shift magnitude</i>	Hours	Time difference between onset of activity on day of light pulse and regression line fit to activity onsets 3-10 days after light pulse. Transients from first 2 days after light pulse were excluded (Daan & Pittendrigh, 1976)	Attenuated in <i>Opn4^{-/-}</i> mice (Panda et al, 2002; Ruby et al, 2002)
<i>Total Activity</i>	Activity Counts	Total number of activity counts over given time period	Elevated in <i>Opn4^{-/-}</i> mice (Muindi et al, 2013)
<i>Tau in LD</i>	Hours	Circadian period (tau) length derived from activity onsets in 12:12 LD	Entrainment to zeitgeber
<i>Tau in DD</i>	Hours	Freerunning period (tau) length derived from activity onsets in DD	Internal circadian period
<i>Tau in LL</i>	Hours	Period (tau) length derived from activity onsets in LL	Attenuated in <i>Opn4^{-/-}</i> mice (Panda et al, 2002; Ruby et al, 2002)

Two non-parametric circadian parameters (Van Someren et al., 1999), intraday variability and interday stability, were examined using custom python scripts.

Intraday variability is a non-parametric measure of frequency and size of changes in activity throughout 24hrs. It was calculated using the following equation:

$$IV = \frac{n \sum_{i=2}^n (x_i - x_{i-1})^2}{(n - 1) \sum_{i=1}^n (x_i - \bar{x})^2}$$

Equation 5.2

Where n = total number of data, i = number of data points per day (24 for hourly bins), x_i are individual data points, $\bar{x}$ is mean of all data.

Interday stability is a measure of similarity of activity pattern across different days (Van Someren et al., 1999). It was calculated as:

$$IS = \frac{n \sum_{h=1}^p (\bar{x}_h - \bar{x})^2}{p \sum_{i=1}^n (x_i - \bar{x})^2}$$

Equation 5.3

Where n = total number of data, p = number of data points per day (24 for hourly bins), x_h are hourly means, $\bar{x}$ is mean of all data and x_i are individual data points.

5.2.7 Multi-electrode array recordings

Multi-electrode array (MEA) data collection and analysis were performed as described in Section 2.2.9 with some minor changes. Following isolation, retinæ were perfused for 15-30mins in Ames media, then switched to Ames media containing a pharmacological cocktail to block signals from rods and cones for the duration of recording. Between 100-200ml of Ames media with rod cone blockers was recirculated for the duration of recording. This rod/cone blockade prevented bipolar cell responses using 100µm l(+)-2-amino-4-phosphonobutyrate (l-AP4, a group III metabotropic glutamate receptor agonist), 40µm 6,7-dinitroquinoxaline-2,3-dione (DNQX, AMPA/kainate receptor antagonist), and 30µm d-2-amino-5-phosphonovalerate (d-AP5, NMDA receptor antagonist). Retinæ were perfused in Ames with rod/cone blockade for 30mins prior to recording of light responses.

Light stimuli, filters and light measurement were as described in Section 2.2.9. Prior to all recordings, retinæ were stimulated with 480nm (10^{14} photons/cm²/s) light for 10s, then dark-adapted for 15mins to produce an equal dark adaptation baseline. Light stimulus protocols are shown in **Figure 5.5**. 480nm IRC recordings were conducted as described in Section 2.2.9. Briefly, ipRGC responses to 10s 480nm light stimuli were measured over a 5-log range of intensities, from lowest (10^{11} photons/cm²/s) to highest light intensity (10^{15} photons/cm²/s), with interstimulus interval increasing with intensity (from 2 to 15mins).

Where possible, the response to repeated light exposures was also recorded. For these repeated stimulus recordings, retinæ were stimulated with 10s 480nm $\pm$ 10nm light (10^{15} photons/cm²/s) every 50s for 10 stimulus presentations. Similarly, if possible the response to longer duration light pulses was recorded. For these recordings, after the 30s baseline, retinæ were exposed to 1min of 480nm light at 10^{12} , 10^{13} and 10^{14} photons/cm²/s and then recorded for up to 2mins. Ten to fifteen minutes recovery time was used between each recording.

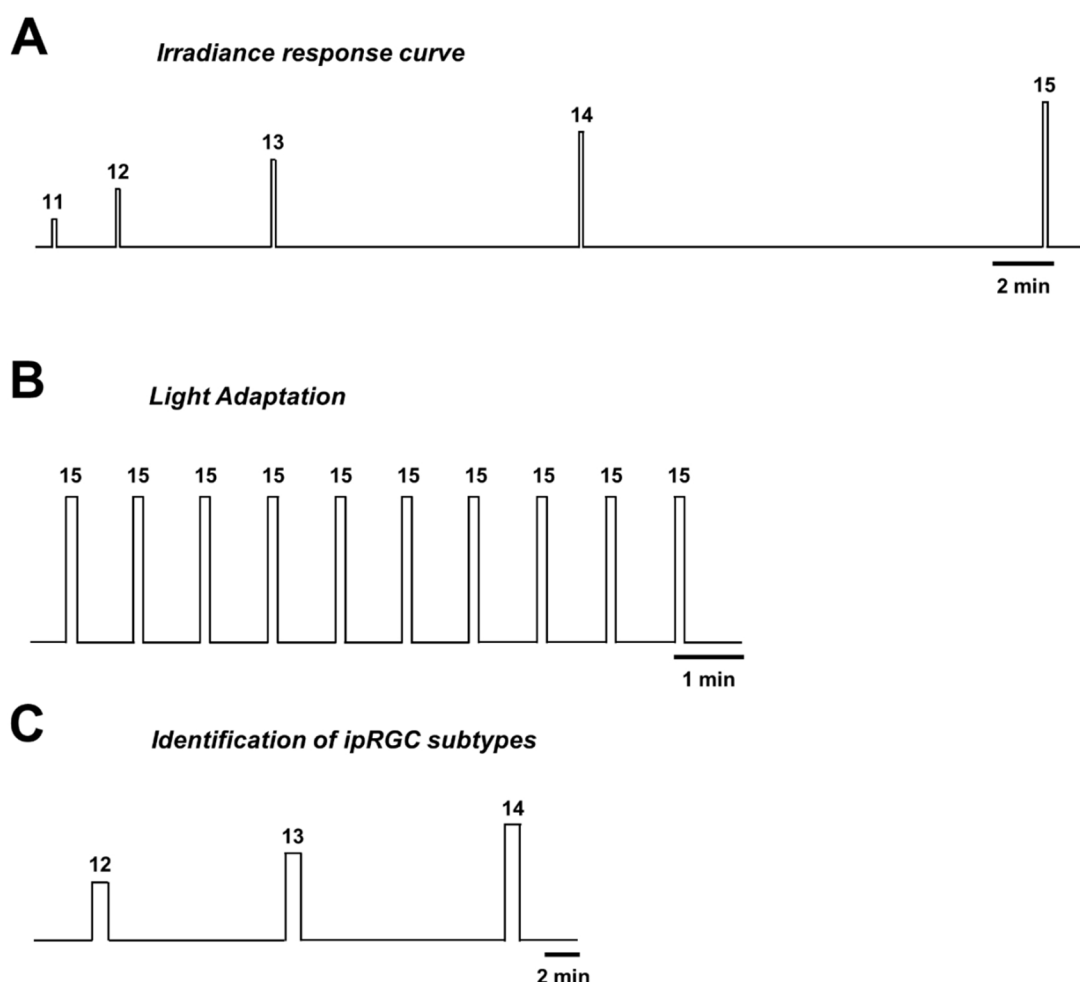


Figure 5.5. Light stimulus protocols for MEA experiments. (A) All recordings were exposure to light stimuli to generate irradiance response curves. Light pulses were 10s duration. Where possible, retinæ were also given (B) a light adaptation protocol, with repeated 10s light pulses every 50s, and (C) longer 1min light pulses for identification of ipRGC subtypes. The flat line indicates the interstimulus interval before and after each light stimulus. The vertical bar indicates the duration and timing of the light stimuli. The number over each bar is the stimulus intensity in log quanta, with height of the bar scaled to represent intensity in log quanta. All stimuli were 480nm (10nm half bandwidth).

Spike data was extracted using MC rack software (Multi Channel Systems). Data was filtered using a 200Hz high pass filter and spikes were sorted using amplitude thresholding of 3 standard deviations from baseline. Any spike larger than this was included. Spikes were then sorted into 1s bins (spikes/s).

5.2.8 Immunohistochemistry

Animals were culled and enucleated. Eyes were pierced with a 28-gauge needle, placed in 4% paraformaldehyde in PBS and stored at 4°C for up to 24hrs. Eyes were then transferred to 30% (w/v) sucrose in PBS and stored at 4°C. Retinae were dissected from cryoprotected eyes and permeabilised in PBS with 1% Triton-X (Sigma) in PBS for 1hr at room temperature. Retinae were blocked in PBS with 10% donkey serum (Sigma) in PBS with 1% Triton X for 1hr, then incubated in primary antibody for 72hrs at 4°C. Primary antibodies were diluted in 2.5% donkey serum in 1% Triton-X in PBS. Retinae were simultaneously incubated with chicken polyclonal anti-EYFP (GFP-1020, AVES Labs, 1:1000) and rabbit polyclonal anti-human melanopsin (Santa Cruz, 1:250). After 5 washes in PBS with 1% Triton-X, retinae were transferred to secondary antibody diluted in 2.5% donkey serum in 1% Triton X in PBS for 16hrs at 4°C. Retinae were double labelled with donkey anti-chicken Alexa-488 and donkey anti-rabbit Alexa-568 secondary antibodies (both Life Technologies, 1:200). Retinae were then washed 5 times in 1% Triton-X in PBS, with DAPI added (1:5000) in last wash step for 10mins at RT. Retinae were mounted in Prolong Gold Anti-Fade reagent (Life Technologies).

Fluorescent images were acquired using an inverted LSM710 laser scanning confocal microscope (Zeiss) with Zen2010 acquisition software (Zeiss), excitation at 405nm, 488nm and 561nm and emission at 440-480nm, 505-550nm and 600-700nm for DAPI, green and red fluorescence, respectively. Brightness and contrast enhancements carried out using ImageJ software (<http://rsbweb.nih.gov/ij>).

5.2.9 RT-qPCR

Animals were culled, enucleated, retina immediately dissected and stored in RNAlater (Ambion). RNA was extracted using RNeasy mini kit (Qiagen) as described in Section 2.2.6.1. RNA was quantified using spectrophotometer (Nanodrop) and cDNA was generated using qScript cDNA synthesis kit (Quanta Biosciences), as described in Section 2.2.6.2. The cDNA was used in RT-qPCR with Quantifast SYBR green reagents (Qiagen) as described in section 2.2.6.3., using an StepOne Plus qPCR machine (Applied Biosystems) to quantify expression levels of human-specific or common human-mouse *Opn4* (Primer sequences shown in **Table 5.3**).

Table 5.3. *Opn4* and housekeeping gene primers used for RT-qPCR

Primer Target	Sequence (5' – 3')
<i>Arbp F</i>	CGACCTGGAGTCCAACTAC
<i>Arbp R</i>	ATCTGCTGCATCTGCTTG
<i>Gapdh F</i>	TGCACCACCAACTGCTTAG
<i>Gapdh R</i>	GATGCAGGGATGATGTTC
<i>B2m F</i>	GCTATCCAGAAAACCCCTCAA
<i>B2m R</i>	CATGTCTCGATCCCAGTAGACGGT
<i>Psmb2 F</i>	AAATGCGGAATGGATATGAAT
<i>Psmb2 R</i>	GAAGACAGTCAGCCAGGTT
<i>Opn4 F (Mouse-human)</i>	TATGCCCTGGCCTGGAGTCT
<i>Opn4 R (Mouse-human)</i>	TAGTCCCAGGAGCAGGATGT
<i>hOPN4 F (Human Specific)</i>	GTTGCTGACATCCTGCTCCT
<i>hOPN4 R (Human Specific)</i>	TCCCGGATGGCCCTGAAGAT

Levels of *Opn4* were normalised to three housekeeping genes: *Arbp* (Acidic ribosomal phosphoprotein P0), *Gapdh* (Glyceraldehyde-3-Phosphate Dehydrogenase) and *B2m* (Beta-2-Microglobulin) as described in Section 2.2.6.3. Levels of *hOPN4* were normalised to four housekeeping genes; *Arbp*, *Gapdh*, *B2m* and *Psmb2* (Proteasome subunit, beta type, 2). Housekeeping gene primer sequences are shown in **Table 5.3**.

5.2.10 Statistical Analysis

Statistical tests were conducted using SPSS 22.0 (IBM). Unless stated otherwise, all comparisons between more than two genotypes or viruses were tested using One-way between-subjects analysis of variance (ANOVA) with virus or genotype as the independent variable. Unless otherwise stated, all comparisons before and after injection for three virus groups were tested using two-way mixed ANOVA with virus type as a between-subjects factor (3 levels – *hOPN4* WT, P10L and T394I AAV) and injection as a within-subjects factor (2 levels – before and after injection). For One-way or Two-way ANOVA comparisons between *hOPN4* AAV groups, a post-hoc Dunnett's test was carried out to explore significant main effects of virus, using *hOPN4* WT as the control group for comparisons. For all other ANOVAs, main effects were explored using a post-hoc Tukey's HSD test. Any significant interactions were explored using multiple Student's t-tests (if appropriate) with a Sidak alpha correction for multiple comparisons (new alpha reported in parenthesis). A significance threshold of $p \leq 0.05$ was used. In all figures * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Error bars show standard error of the mean.

5.3 Results

5.3.1 Technique Validation

5.3.1.1 ipRGC-specific AAV delivery of *hOPN4* in *Opn4^{-/-} Cre^{+/+}* mice

The chosen technique of melanopsin delivery was validated before examining the effects of *hOPN4* SNPs, P10L and T394I. Using targeted AAV to deliver *hOPN4* specifically to ipRGCs of *Opn4^{-/-}* animals would only be considered a good model if it met certain criteria. First, the virus should be successfully transduced and *hOPN4* protein translated in the mouse retina. Second, *hOPN4* expression must be restricted to the ipRGCs. Third, the *hOPN4* protein must be functional and restore a wildtype phenotype to *Opn4^{-/-} Cre^{+/+}* animals. A series of experiments that demonstrate AAV-delivery of *hOPN4* meets these criteria for technique validation are described below.

First, AAV-delivered *hOPN4* in *Opn4^{-/-} Cre^{+/+}* retina was compared with levels of endogenous melanopsin from *Opn4^{+/+}* retinas using RT- qPCR with primers that amplified both human and mouse melanopsin. As shown in **Figure 5.6A**, following injection, *hOPN4* mRNA was expressed in the retina of *Opn4^{-/-} Cre^{+/+}* mice, confirming the *hOPN4* AAV successfully transduced the mouse retina. A Student's t-test demonstrated *hOPN4* mRNA expression had a significant 20-fold increase in expression compared to levels of endogenous mouse melanopsin, $t(6) = 5.18$, $p = 0.002$. This increased expression is most likely due to the ubiquitous EF1 α promotor that drives the *hOPN4* AAV transcription without feedback from the cell.

Successful virus transduction was further substantiated by immunostaining of flatmount retina from AAV-treated *Opn4^{-/-} Cre^{+/+}* mice. Human melanopsin labelling was visible in treated retinas (**Figure 5.6B-C**). Morphology and structure of retina from the injected eyes appeared normal. Injected *Opn4^{-/-} Cre^{+/+}* mice also possess an EYFP reporter that exclusively labels ipRGC cells. When double labelled, *hOPN4* staining was only observed in EYFP-labelled cells, confirming that *hOPN4* expression was restricted to

ipRGCs. hOPN4 labelling was observed in the plasma membrane and processes of ipRGCs (**Figure 5.6B**), suggesting the hOPN4 protein is being successfully folded and trafficked to the plasma membrane by the mouse ipRGC cell machinery. The distribution of hOPN4 expression appeared to be widespread throughout the retina, indicating good viral coverage during injection. A slight gradient in transduction efficiency was evident in some retina, with fewer cells being transduced further from the injection site, but overall, high levels of viral transduction were observed with the many EYFP-labelled ipRGCs co-labelled with hOPN4 (**Figure 5.6C**).

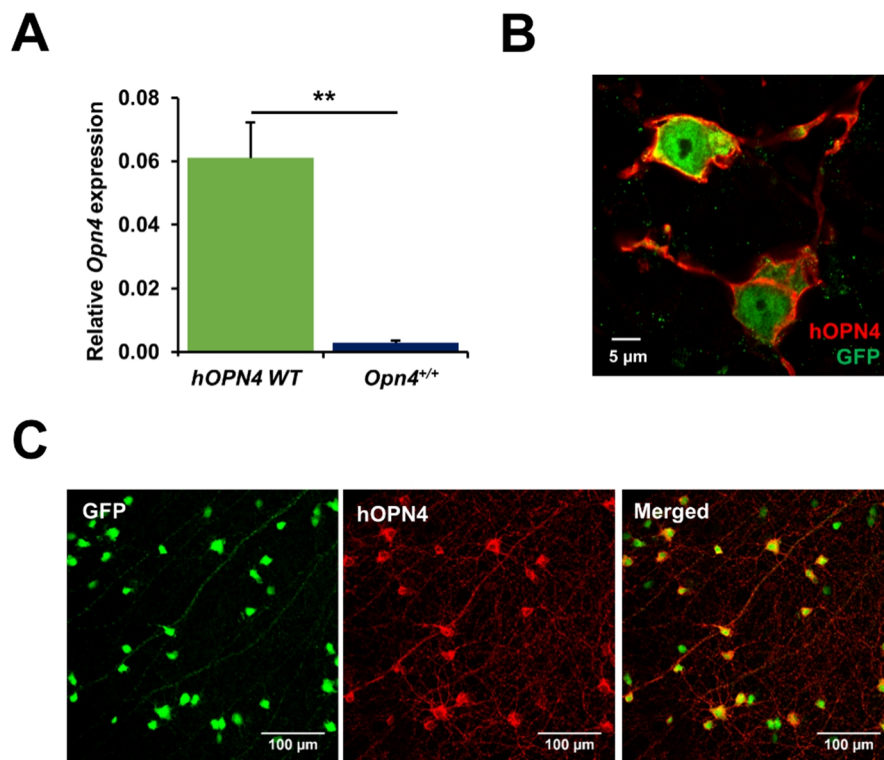


Figure 5.6. ipRGC-specific AAV delivery of *hOPN4* in *Opn4*^{-/-} *Cre*^{+/+} mice (A) Relative mRNA expression of melanopsin in *Opn4*^{+/+} animals and *Opn4*^{-/-} *Cre*^{+/+} treated with *hOPN4* AAV (*hOPN4* WT), assessed using primers that bind to identical sequence in mouse and Human *Opn4*. Normalised to 3 housekeeping genes (*Arbp*, *Gapdh* and *B2m*), n = 5-7 for each group. Error bars = SEM. ** p < 0.01, Student's t-test (B-C) Immunostaining shows colocalisation of AAV-delivered hOPN4 (red) in ipRGCs labelled with genetic eYFP reporter (green) in *Opn4*^{-/-} *Cre*^{+/+} retina. ICC and image acquisition by Dr. Steven Hughes.

RT-qPCR and immunohistochemistry demonstrate that AAV-delivery of *hOPN4* meets the first two criteria for technique validation - the virus is successfully transduced and *hOPN4* protein is synthesised exclusively in ipRGCs of *Opn4*^{-/-} *Cre*^{+/+} mice.

5.3.1.2 Restoration of wildtype pupil phenotype to *Opn4*^{-/-} *Cre*^{+/+} mice using ipRGC-specific *hOPN4* AAV delivery

AAV-delivered *hOPN4* met the first two criteria for technique validation. To test the third, whether the *hOPN4* protein was functional and capable of restoring a wildtype behavioural phenotype to *Opn4*^{-/-} *Cre*^{+/+} mice, the pupillary light responses (PLR) of AAV-treated animals were examined. PLR is a commonly used assay of melanopsin function. Previous reports have demonstrated *Opn4*^{-/-} mice have attenuated pupil constriction during bright, but not dim, light exposure compared to *Opn4*^{+/+} animals (Lucas et al., 2003). In macaques, Gamlin et al., (2007) demonstrated that the sustained constriction after light offset, known as the post-illumination pupil response (PIPR), is unaffected by pharmacological blockade of rod and cone signals. This suggests PIPR is primarily driven by melanopsin, although the study did not block melanopsin function to determine whether the reverse was true – that PIPR can also be driven by rods and cones.

Both minimum pupil size and PIPR were examined to assess the function of AAV-delivered *hOPN4* in *Opn4*^{-/-} *Cre*^{+/+} mice. *Opn4*^{-/-} mice were used as a negative control to assess improvement in melanopsin function. *Opn4*^{+/+} mice were used as a positive control group to determine whether PLR was restored to normal physiological levels. As shown in **Figure 5.7A-B**, *Opn4*^{-/-} *Cre*^{+/+} animals treated with *hOPN4* WT AAV exhibited rapid pupil constriction to ~5% of dark-adapted pupil size in response to a bright 10s light stimulus (480nm, 4.6x10¹⁴ photons/cm²/s) and maintained a sustained pupil constriction after light offset for up to 30s. This is comparable to the PLR seen in *Opn4*^{+/+} mice. In comparison, *Opn4*^{-/-} showed attenuated constriction to ~10% dark-adapted pupil size, and exhibited a faster recovery of pupil size after lights offset.

Minimum pupil size differed significantly between the three genotypes (**Figure 5.7C**), $F(2,15) = 5.258$, $p = 0.019$ (One-way between subjects ANOVA). A post-hoc Tukey's HSD test showed *Opn4*^{+/+} had significantly greater constriction than *Opn4*^{-/-} ($p = 0.030$), replicating previous findings. The same test revealed *Opn4*^{-/-} *Cre*^{+/+} constriction after treatment with *hOPN4* WT AAV was also significantly greater than in *Opn4*^{-/-} ($p = 0.037$), but not significantly different from *Opn4*^{+/+}. This suggests AAV-delivered *hOPN4* successfully restored a full constriction phenotype to *Opn4*^{-/-} *Cre*^{+/+} mice, comparable to that seen in *Opn4*^{+/+} mice.

PIPR (**Figure 5.7D**), measured as rate of pupil dilation after light offset (lower values indicate sustained constriction, higher values indicate faster recovery), was also significantly different between genotypes, $F(2,13) = 6.493$, $p = 0.01$ (One-way between subjects ANOVA). A post hoc Tukeys HSD test found, as anticipated, that *Opn4*^{+/+} had significantly slower recovery in pupil size compared to *Opn4*^{-/-} ($p = 0.012$). It also revealed *hOPN4* WT showed significantly more sustained PIPR compared *Opn4*^{-/-} ($p = 0.030$), but was no different from *Opn4*^{+/+}. This demonstrates AAV-delivered *hOPN4* is also capable of driving sustained pupil constriction after lights off, and that the restored PIPR is similar in magnitude to that seen in *Opn4*^{+/+} mice.

The restoration of full pupil constriction and PIPR to *Opn4*^{-/-} *Cre*^{+/+} mice indicates the AAV-delivered *hOPN4* meets the third criteria for technique validation - the *hOPN4* protein is functional and capable of restoring melanopsin-driven behaviours. Collectively, the RT-qPCR, immunohistochemistry and pupillometry results validate the use of AAV-delivered *hOPN4* for modelling *hOPN4* SNP function.

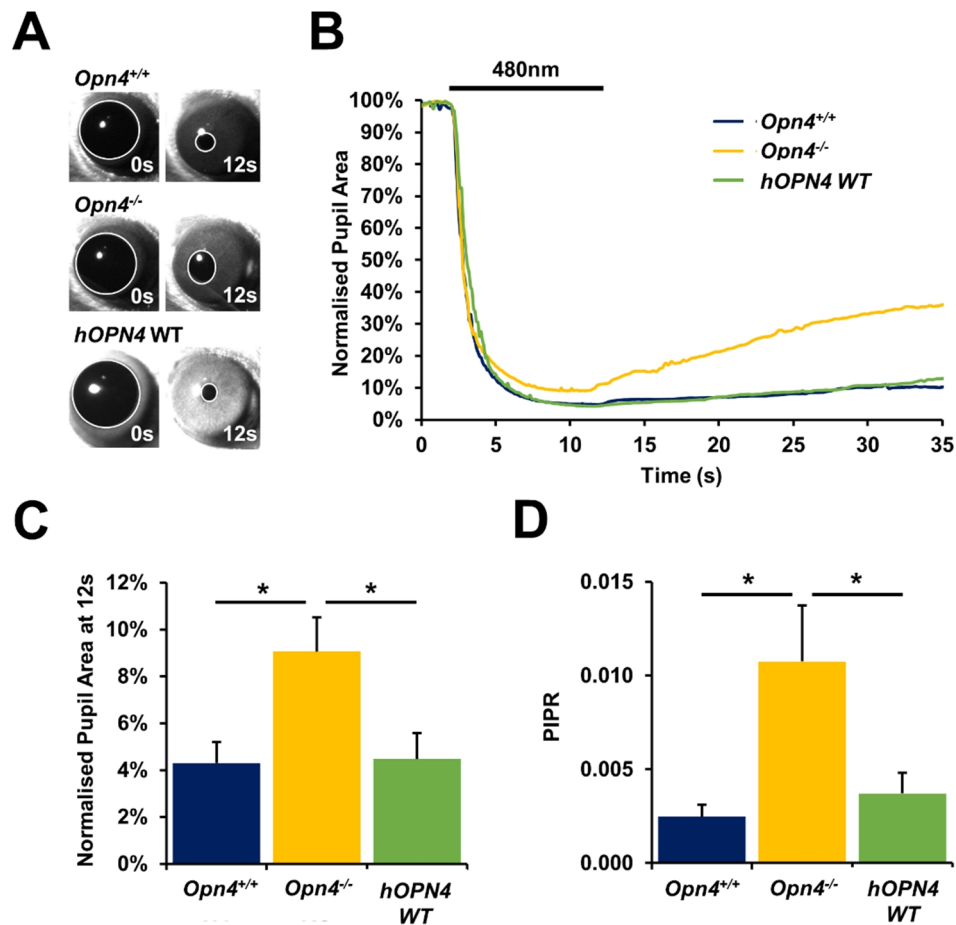


Figure 5.7. Pupil light response is restored in *Opn4*^{-/-} *Cre*^{+/+} animals with ipRGC-specific *hOPN4* WT expression. (A) Representative images of pupil size before (0s) and at end of light pulse (12s) (B) Kinetics of pupil light response to a 10s light pulse (480nm±10nm, 4.6x10¹⁴ photons/cm²/s, shown as black bar above traces) from *Opn4*^{-/-}, *Opn4*^{+/+} and *Opn4*^{-/-} *Cre*^{+/+} treated with *hOPN4* WT AAV (*hOPN4* WT) animals. Traces are averages of n = 6 animals in each group. (C) Normalised minimum pupil area at end of light exposure (12s). (D) Post-illumination pupil response (PIPR), calculated as rate of recovery in pupil area after light offset. Error bars = SEM. * p < 0.05, Tukey's HSD post-hoc test

5.3.2 Titre Optimisation

5.3.2.1 Number of virus particles injected determines restoration of pupil phenotype

The technique validation experiments demonstrated *hOPN4* AAV can restore pupil light responses to normal physiological levels. The viral titre used to validate the technique was 2.2×10^{12} virus particles/ml. Based on the ICC, this titre resulted in good transduction efficiency with viral coverage across the retina. However, supraphysiological levels of human melanopsin mRNA were also found, suggesting the current AAV protocol may lead to excess hOPN4 protein synthesis. It is unclear from the technical validation experiments whether these elevated levels of *hOPN4* mRNA are necessary for phenotype restoration. Ideally, the amount of virus introduced would result in melanopsin expression at physiological levels, while still reliably restoring behavioural phenotype. This study attempted to establish the relationship, if any, between virus concentration (viral titre) and phenotype restoration.

To do this, a small number of *Opn4^{-/-} Cre^{+/+}* animals were injected with *hOPN4* WT AAV diluted either 10 times (Medium titre - 2×10^{11} virus particles/ml) or 100 times (Low Titre - 2×10^{10} virus particles/ml) in PBS. The PLR of these mice to 480nm light was examined and compared to the *hOPN4* WT results from the technique validation experiments (High Titre - 2×10^{12} virus particles/ml), to determine whether the PLR restoration was dependent on viral titre.

The kinetics of the high and medium titre group were comparable, with constriction to around 7% followed by sustained constriction after lights off (**Figure 5.8A-B**). However, the low titre condition appeared to have attenuated constriction, approx. 13%, and a more pronounced recovery after lights offset. A significant main effect of titre on maximum constriction was found, $F(2,14) = 13.33$, $p = 0.001$ (One-way Between Subjects ANOVA, **Figure 5.8C**). A Tukey's HSD post-hoc test revealed pupil constriction was significantly attenuated in the low titre group compared to the high titre group ($p = 0.001$), but that there was no difference between low and medium, or high and medium groups, suggesting that viral titre can affect the magnitude of pupil constriction restoration.

While examining whether this titre-response relationship was also true for PIPR, it was found the variance in the three groups was found to be significantly different, ($p < 0.001$, Levene's test). As can be seen in **Figure 5.8D**, although most animals demonstrated good constriction and PIPR in the high and medium titre condition, there seemed to be more variation in the low titre group. In particular, two animals injected with low titre did not appear to have restored PLR (**Figure 5.8B**), suggesting low titre AAV did not reliably restore pupil constriction and PIPR.

Immunostaining of *Opn4^{-/-} Cre^{+/+}* retina treated with different titres revealed a dose-dependent effect of titre on transduction efficiency (**Figure 5.9**). At low titres, very few EYFP-labelled ipRGCs expressed hOPN4 WT, suggesting low transduction rates. Despite this, nearly full constriction was still observed in some Low Titre animals, consistent with previous findings that small numbers of residual ipRGCs can sustain PLR (Güler et al., 2008). This suggests injection of higher titre virus is required for full viral coverage and high transduction efficiency.

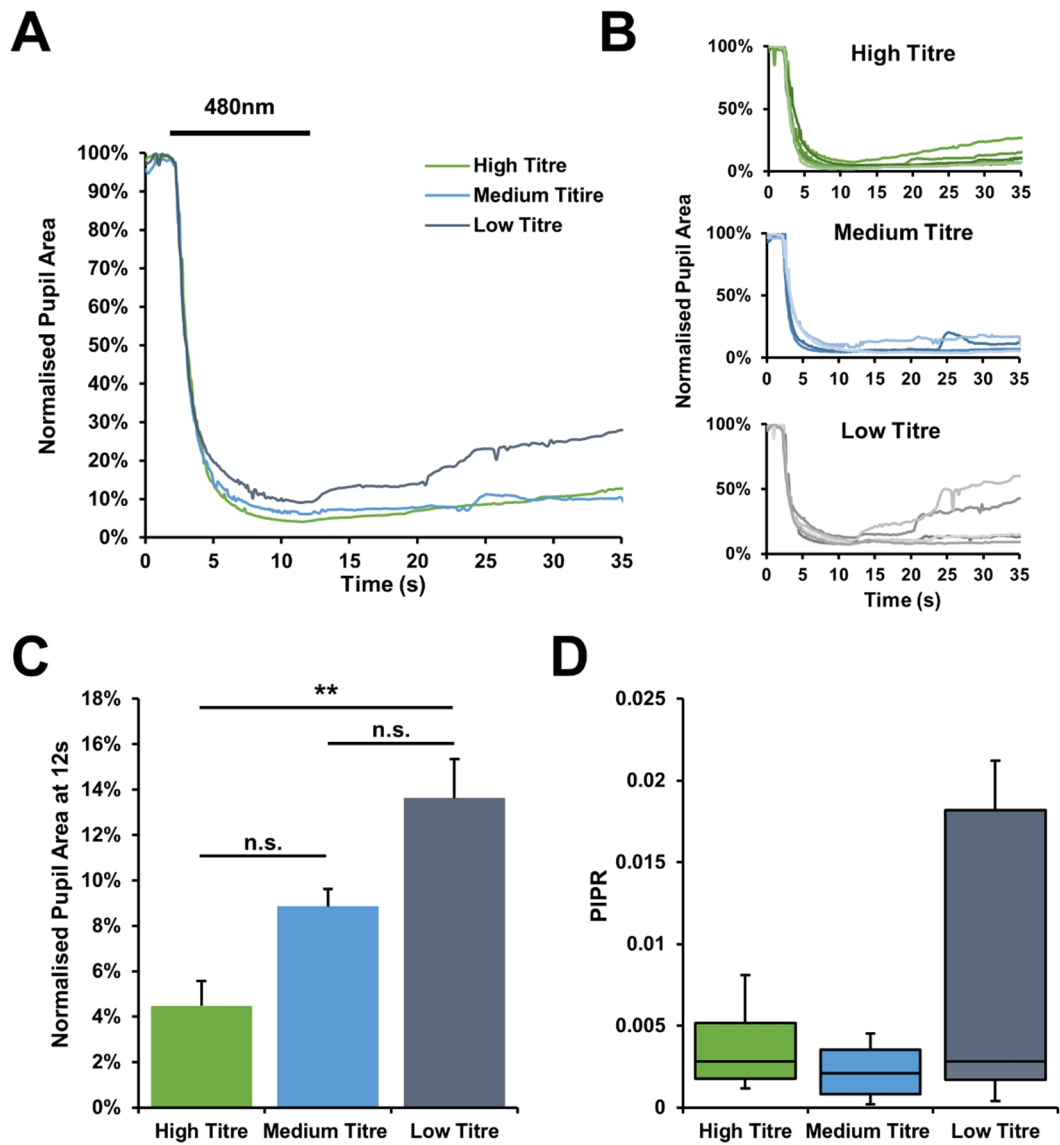


Figure 5.8. PLR restoration is dependent on virus titre (A) Kinetics of average pupil light response to 10s 480nm light pulse (4.8×10^{14} photons/cm²/s, shown as black bar) from *Opn4*^{-/-} *Cre*^{+/+} mice 1 month after injection of 1.5ul *hOPN4* WT AAV at high (2×10^{12} virus particles/ml), medium (2×10^{11} virus particles/ml) or low titre (2×10^{10} virus particles/ml) **(B)** Individual traces from animals injected with high, medium or low titre virus **(C)** Normalised Pupil Area at 12s to 480nm light **(D)** Boxplots of PIPR (rate of recovery). n = 4-6 animals for each group. Error bars = SEM. n.s. = non-significant and * p < 0.05, Tukey's HSD post-hoc test.

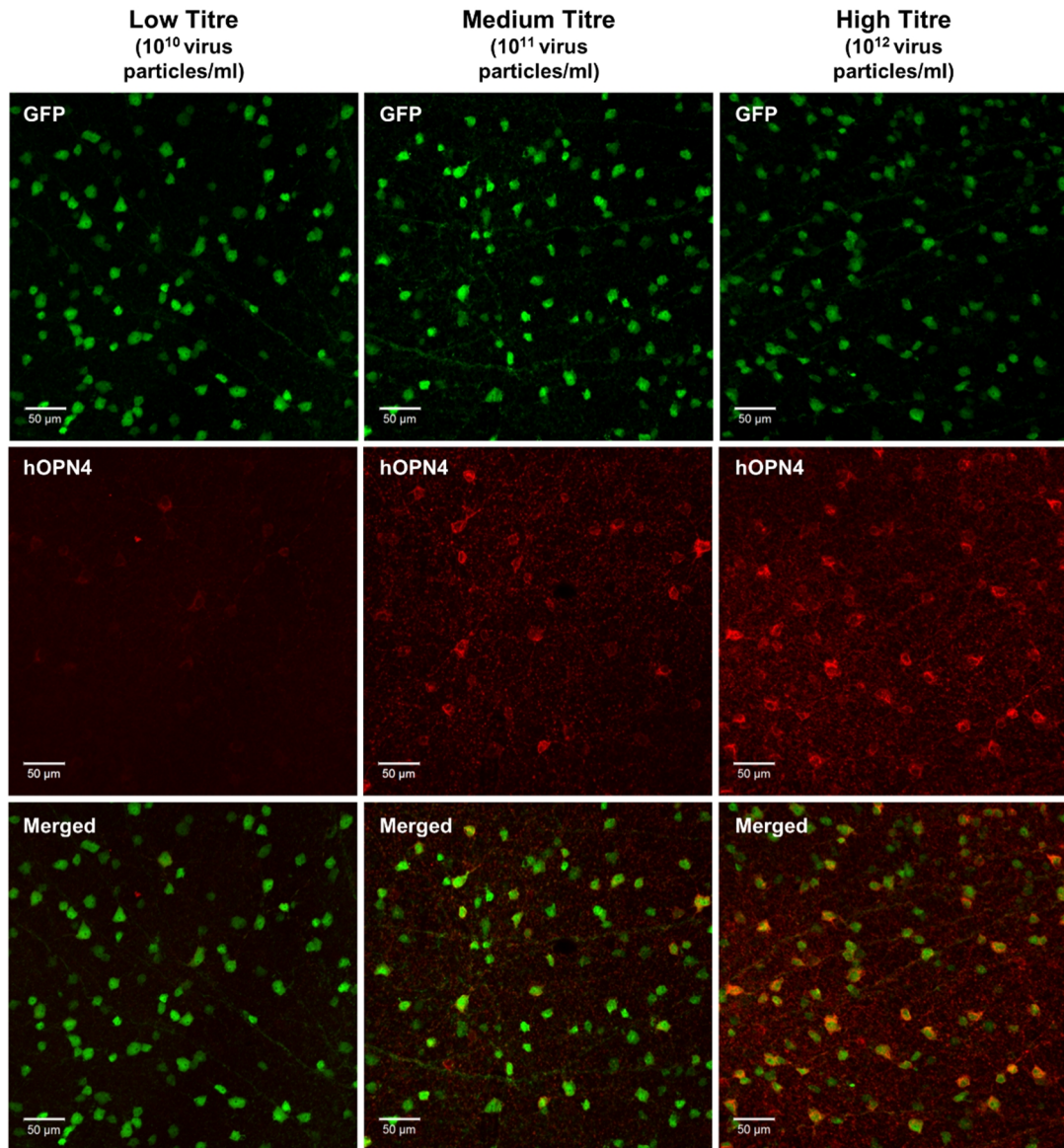


Figure 5.9. Effect of titre on transduction efficiency. Immunostaining of *Opn4*^{-/-} *Cre*^{+/+} retina treated with *hOPN4* WT AAV at high (2×10^{12} virus particles/ml), medium (2×10^{11} virus particles/ml) and low (2×10^{10} virus particles/ml) titre shows fewer EYFP-labelled ipRGCs (green) express AAV-delivered *hOPN4* WT as titre injected decreases. Immunostaining and images acquired by Dr. Steven Hughes.

Given the significant effect of titre on PLR and transduction efficiency, the high viral titre (2×10^{12} virus particles/ml) was used for future experiments comparing *hOPN4* P10L and T394I with *hOPN4* WT control.

5.3.3 Effect of *hOPN4* SNPs, P10L and T394I, on OPN4 function

With a valid technique to model *hOPN4* *in vivo*, the main study focused on investigating the impact of *hOPN4* SNPs, P10L and T394I on OPN4-driven behaviour. Since P10L is associated with behaviours that are difficult to model in mice, such as SAD, the ability of this SNP to cause an observable deficit in a range of well-characterised melanopsin-associated behaviours was assessed. In comparison, three studies specifically associated *hOPN4* T394I with abnormal PLR. Pupillometry was therefore an obvious starting point for testing the *hOPN4* T394I AAV model, although see **Appendix 3** for an explanation of why this study did not attempt to reproduce the chromatic pupillometry result from Roecklein et al. (2013).

In this study, all *Opn4*^{-/-} *Cre*^{+/+} mice injected with *hOPN4* P10L AAV or *hOPN4* T394I AAV were compared with a *hOPN4* WT AAV control group. These comparisons were intended to identify any deficits in melanopsin function due to the introduction of SNPs, while controlling for confounding factors, such as effect of injection or anaesthesia. A subset of animals from each group were also investigated to see how their behaviour changed before and after injection. This helped confirm restoration of melanopsin-dependent behaviours, as well as whether phenotype restoration was similar between the three viruses.

The aim of this study was to determine whether *hOPN4* SNPs, P10L and T394I affect the function of the human melanopsin protein and produce an observable attenuation in melanopsin-driven behaviour.

5.3.3.1 AAV-delivered *hOPN4* P10L and T394I are able to restore PLR to *Opn4*^{-/-} *Cre*^{+/+} mice comparable to *hOPN4* WT AAV

First, the pupil light responses (PLR) of the subset of *Opn4*^{-/-} *Cre*^{+/+} mice tested before and after injection with *hOPN4* P10L, T394I or WT AAV were examined. Both maximum constriction and PIPR are attenuated to bright blue light in *Opn4*^{-/-} mice (Lucas et al., 2003), so improvements in both these components of PLR should be observed if the AAV-delivered *hOPN4* SNPs are functional.

All three viruses led to an improvement in pupil constriction and restoration of PIPR, demonstrated by sustained constriction for up to 30s after a bright 10s 480nm light (**Figure 5.10A-B**). Minimum pupil size was consistently smaller after injection compared to before injection for all three viruses (**Figure 5.10C**), $F(1,7) = 33.82$, $p = 0.001$ (Two-way mixed ANOVA). There was no main effect of virus, $F(2,7) = 0.63$, $p = 0.560$, or interaction of virus by injection on minimum pupil size, $F(2,7) = 1.95$, $p = 0.212$, suggesting the improvement in constriction after injection was comparable for *hOPN4* SNPs and WT control.

Post-illumination pupil constriction was more sustained after injection, resulting in slower rates of recovery and smaller slope values (**Figure 5.10D**). This was confirmed by a significant main effect of injection on PIPR, $F(1,7) = 23.82$, $p = 0.002$ (Two-way mixed ANOVA). Again, there was no main effect of virus, $F(2,7) = 2.02$, $p = 0.203$, or interaction of virus by injection on PIPR, $F(2,7) = 2.06$, $p = 0.198$, implying restoration of PIPR was similar between the three viruses.

Opn4^{-/-} *Cre*^{+/+} mice demonstrated significant improvement in maximum constriction and PIPR after injection. The lack of significant interactions of virus by injection indicates that pupil phenotype restoration was similar for *hOPN4* WT control and *hOPN4* SNPs, P10L and T394I. This suggests that all 3 *hOPN4* AAVs successfully transduce and form functional hOPN4 protein and that the presence of *hOPN4* SNPs does not appear to impact the restoration of a wildtype melanopsin pupil phenotype.

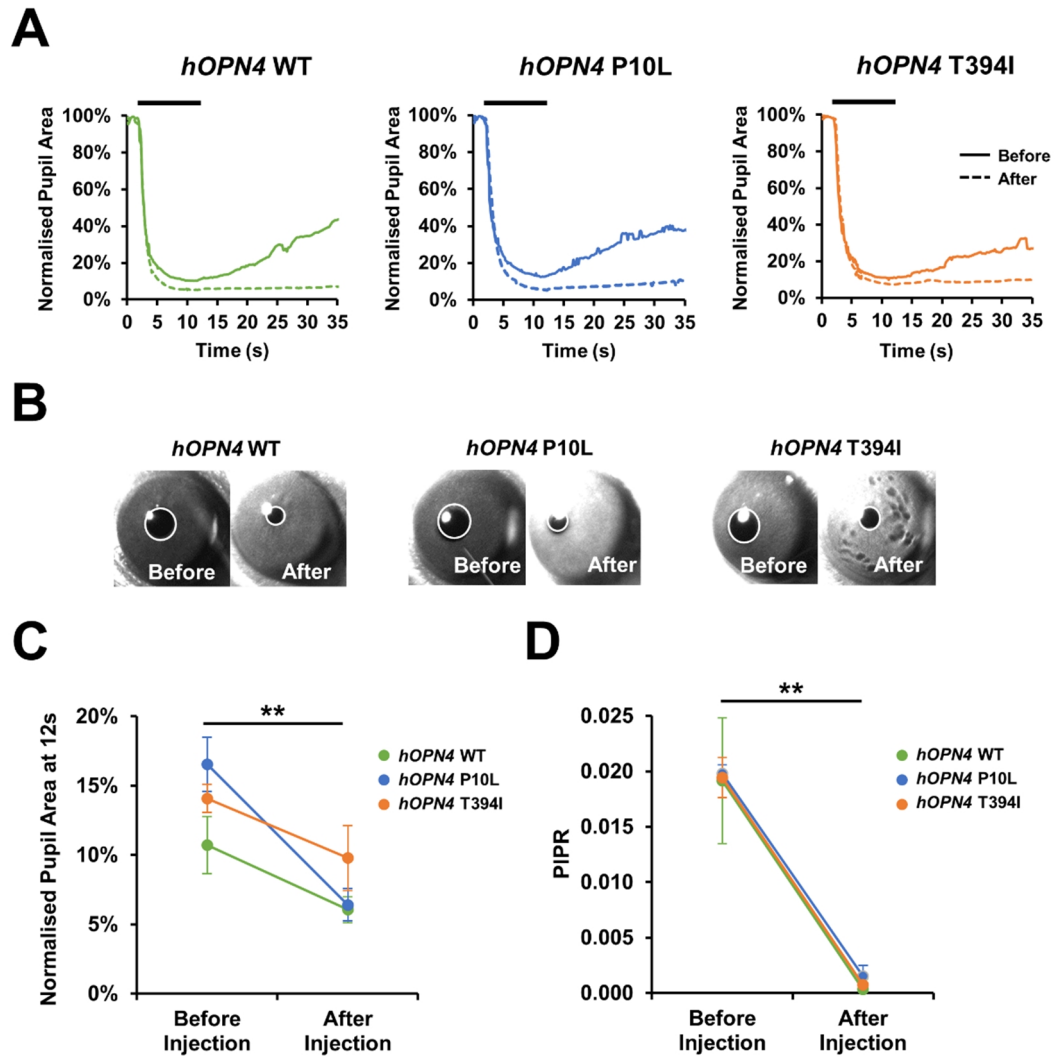


Figure 5.10. *hOPN4* P10L and *hOPN4* T394I are able to restore PLR to *Opn4* Cre animals comparable to *hOPN4* WT AAV (A) Kinetics of average pupil light response to 10s 480nm light pulse (4.8×10^{14} photons/cm²/s, shown as black bar) from *Opn4*^{-/-} *Cre*^{+/-} mice before and 1 month after injection of *hOPN4* WT, P10L or T394I AAV. (B) Representative images of pupil constriction at end of 10s 480nm light before and after injection. (C) Normalised Pupil Area at 12s (end of 10s 480nm light pulse) before and after injection. (D) PIPR to 480nm light before and after injection. $n = 3-4$ animals for each group. Error bars = SEM. ** $p < 0.01$, Main effect of injection, Two-way mixed ANOVA.

5.3.3.2 PLR does not differ between *hOPN4* P10L, T394I and WT control

Opn4^{-/-} Cre^{+/-} mice injected with *hOPN4* P10L and T394I AAV had very similar pupil constriction kinetics to control animals injected with *hOPN4* WT AAV when exposed to bright 480nm light (**Figure 5.11A-B**). There was no difference in minimum constriction, $F(2,15) = 1.69$, $p = 0.218$ or PIPR, $F(2, 15) = 0.29$, $p = 0.754$ (One-way between subjects ANOVA) between the three *hOPN4* AAV conditions.

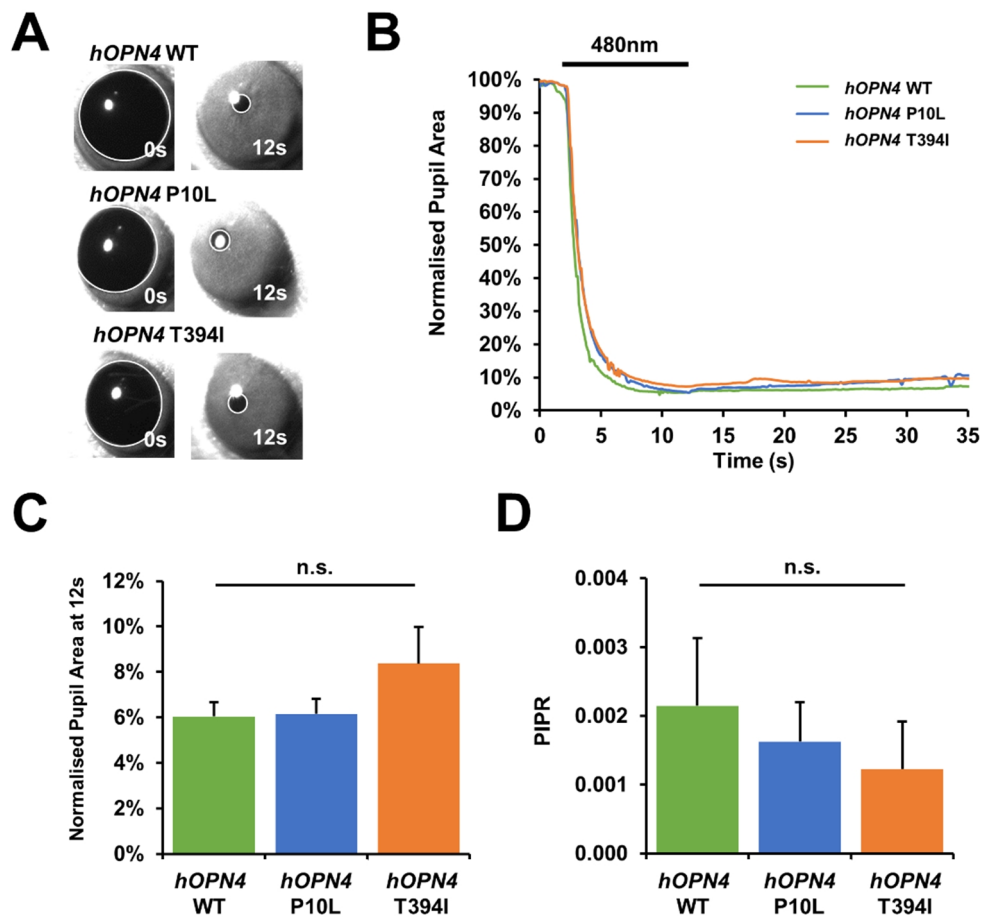


Figure 5.11. PLR for *hOPN4* P10L and T394I AAV is comparable with *hOPN4* WT AAV (A) Representative images of pupil constriction at 0 (dark-adapted) and 12s (end of light exposure) (B) Kinetics of average pupil light response to 10s 480nm light pulse (4.8×10^{14} photons/cm²/s, shown as black bar) from *Opn4^{-/-} Cre^{+/-}* mice 1 month after injection of *hOPN4* WT, P10L or T394I AAV. (C) Normalised Pupil Area at 12s (end of 10s 480nm light pulse) to 480nm light (D) PIPR to 480nm light. $n = 5-8$ animals for each group. Error bars = SEM. n.s = not significant, main effect of virus, One-way ANOVA.

Opn4^{-/-} Cre^{+/+} mice injected with *hOPN4* P10L or T394I AAV appear to have normal pupil light responses. This suggests that introduction of these SNPs does not disrupt protein function, or at least not sufficiently to cause a significant pupil phenotype as suggested by Higuchi et al. (2013) and Lee et al. (2013).

5.3.3.3 Entrainment to light-dark cycle and freerunning in constant dark are unaffected by AAV-delivered *hOPN4*

Next, circadian behaviour of AAV-treated *Opn4^{-/-} Cre^{+/+}* mice was investigated. It has previously been suggested these behaviours are disturbed in individuals with P10L and T394I (Roecklein et al., 2012; Lee et al., 2014). To test this association, a circadian screen using passive infra-red (PIR) detection was conducted to monitor the locomotor activity of *Opn4^{-/-} Cre^{+/+}* mice injected with *hOPN4* WT, P10L and T394I AAV.

As with the pupillometry experiments, a small number of animals were compared from each group before and after injection (n=4-5 for each virus). This was to confirm that *hOPN4* AAV could successfully restore a wildtype phenotype to a range of NIF photic behaviour beyond pupil light response. Comparisons before and after injection also ensured behaviour not associated with melanopsin remained constant throughout testing.

Figure 5.12 shows representative actograms from the first half of the circadian screen, where animals were kept in a 12:12 light dark (LD) cycle (350lux white light) for up to 7 days before being released into constant dark (DD) for up to 10 days. LD assessed entrainment and activity levels under light dark cycle, whereas DD was used to measure the period (τ) of the core circadian clock (Albrecht & Foster, 2002; Jud et al., 2005; Hughes et al., 2015). Actograms of the same mice before and 1 month after injection confirmed that both pre and post-injection *Opn4^{-/-} Cre^{+/+}* mice could synchronise their activity with the LD cycle. These mice were genuinely entrained, rather than masking to light, as activity onset began from the same time on the first day of DD as in LD. There did not appear to be any change in freerunning period in DD before and after injection.

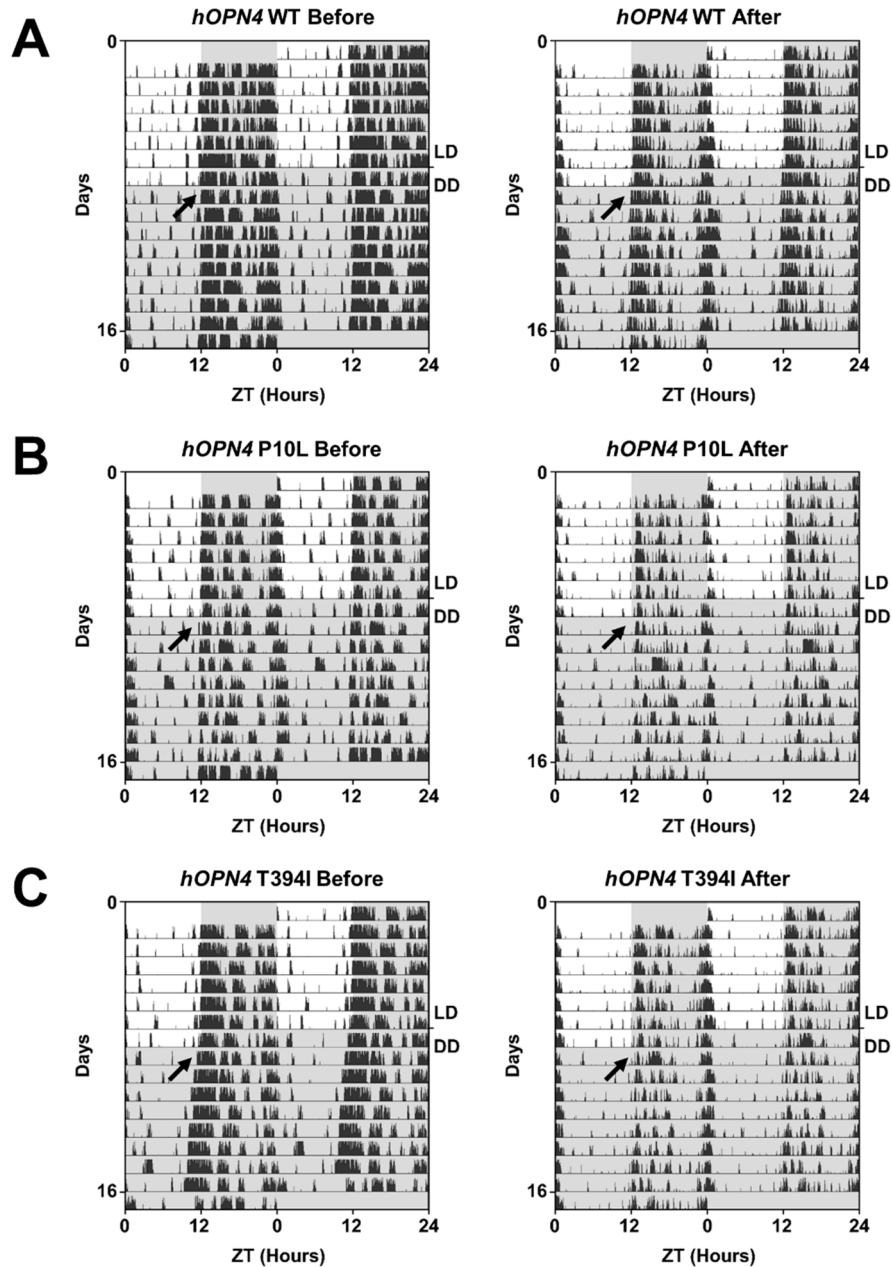


Figure 5.12. Entrainment to 12:12 LD cycle and free-running under constant dark are comparable before and after injection. Representative actograms show activity recorded by PIR detection from three *Opn4*^{-/-} *Cre*^{+/+} mice before (left) and after (right) bilateral intraocular injection of **(A)** *hOPN4* WT, **(B)** *hOPN4* P10L and **(C)** *hOPN4* T394I AAV. Animals were in 12:12 LD cycle under white light (approx. 350lux) for 7 days, then released into constant dark (DD) for 10 days. Actograms are double plotted on a 24hr timescale. Grey shading indicates when animals were in darkness. Activity data displayed in 1min bins. Black arrows show onset of activity on first day of DD.

The actograms before injection highlight the degree of individual variation observed between animals. Although these three animals all had the same genetic background, their activity patterns are quite different, particularly during the dark phase. This emphasises the importance of using within-subjects controls, testing the same animals before and after injection, to determine if there has been any behavioural change due to AAV-delivered *hOPN4*.

5.3.3.4 Timing of activity-rest rhythm relative to zeitgeber is altered by restoration of OPN4 light input.

When average activity patterns in 12:12 LD were compared before and after injection, several differences in timing of activity relative to the light dark cycle were identified (**Figure 5.13**). In particular, delays in activity onset and time to peak activity were observed after AAV injection, possibly indicating a change in photoentrainment of activity rhythms.

All animals exhibited a phase advance of activity onset relative to light offset, but this anticipatory activity was decreased from 30-40 minutes before injection to around 5-10mins after injection (**Figure 5.13D**). Phase angle of entrainment - the difference in timing of the external zeitgeber (12:12 light dark cycle) and entrained activity rhythm – was significantly reduced after injection, $F(1,11) = 40.83$, $p < 0.001$ (Two-way mixed ANOVA). There was no main effect of virus, $F(2,11) = 0.59$, $p = 0.57$, or interaction of virus with injection, $F(2,11) = 0.78$, $p = 0.48$. This implies the delay in activity onset was similar after injection of *hOPN4* WT, P10L and T394I AAV.

In order to determine if activity timing was altered throughout the active phase, time to peak activity was examined (**Figure 5.13E**). Activity consistently peaked soon after dark onset before decreasing, and the timing of this activity peak was significantly delayed after injection, $F(1, 11) = 29.68$, $p < 0.001$ (Two-way mixed ANOVA). Again, no main effect of virus was found, $F(2,11) = 0.78$, $p = 0.48$, or interaction of virus with injection,

$F(2,11) = 1.13$, $p = 0.357$. This suggests *hOPN4* AAV delivery leads to a shift in entrainment of locomotor activity rhythms relative to the 12:12 light dark cycle.

Core clock function, measured as period (τ) in DD, was unaffected by injection, $F(1,11) = 0.08$, $p = 0.782$ (Two-way mixed ANOVA) or virus, $F(2,11) = 0.91$, $p = 0.430$ (**Figure 5.13F**). No interaction of virus with injection was found, $F(2,11) = 0.61$, $p = 0.563$. AAV-delivered *hOPN4* did not significantly alter the endogenous circadian rhythm, consistent with previous findings that τ in DD is unchanged in *Opn4*^{-/-} mice (Ruby et al., 2002, Panda et al., 2002).

Although phase angle of entrainment has not been examined in the literature on *Opn4*^{-/-} mice, Lupi et al. (2008) described increased variation in activity onset in *Opn4*^{-/-} mice. If the decreases in phase angle of entrainment and delayed peak activity in *Opn4*^{-/-} *Cre*^{+/+} mice after injection are due to additional light input provided by AAV-delivered *hOPN4*, this would provide further evidence for a role of melanopsin in setting phase of entrainment. The additional OPN4 light input could be leading to greater synchronisation of activity rhythms to the zeitgeber.

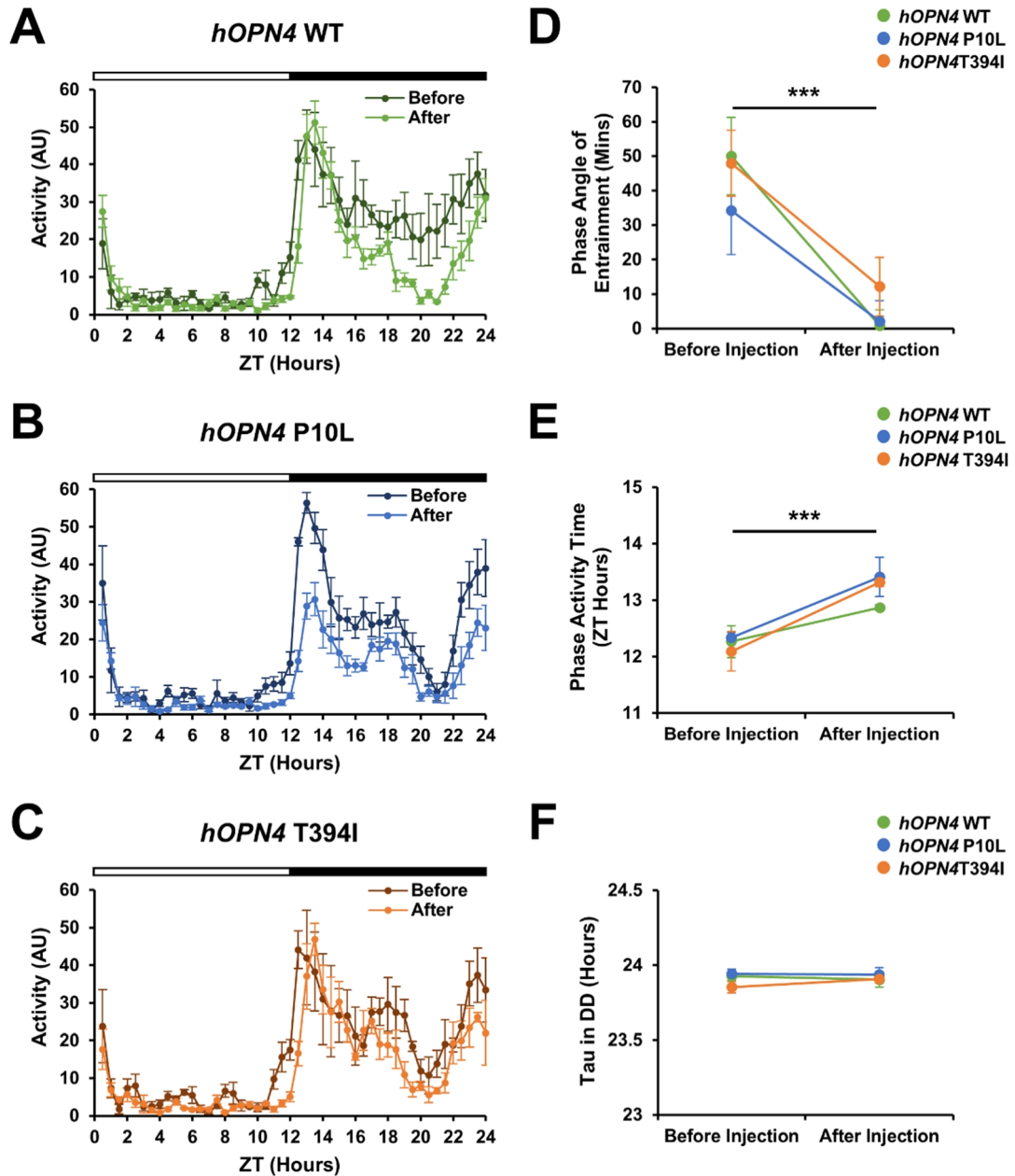


Figure 5.13. *hOPN4* AAV delays phase angle of entrainment and peak activity timing. Activity data derived from PIR recordings from 7 days of activity in 12:12 350lux LD cycle. Average activity profiles of *Opn4*^{-/-} *Cre*^{+/+} before and after bilateral intraocular injection of (A) *hOPN4* WT, (B) P10L and (C) T394I AAV. White and black bar shows timing of light and dark. Data in 30min bins (D) Phase angle of entrainment (average time difference between activity onset and light offset) (E) Time of Peak Activity (ZT Hours). (F) Free-running period (Tau) in constant dark (DD) derived from activity onsets during 10 days in DD. n = 4-5 for each group. Error bars = SEM. *** p < 0.001, Main effect of injection, Two-way mixed ANOVA.

5.3.3.5 Activity patterns before and after restoration of OPN4 light input

Phase of circadian entrainment appeared to be altered by delivery of *hOPN4* AAV to ipRGCs, without changing the core circadian rhythm. Next, distribution of activity was examined to see if this was also affected by the introduction of *hOPN4* AAV (**Figure 5.14**). Fragmentation of activity rhythms was not anticipated, since this had not been described in *Opn4*^{-/-} mice, but was tested for consistency throughout the study.

Both the actograms and activity profiles indicated *Opn4*^{-/-} *Cre*^{+/+} mice were less active after injection. This was confirmed by a significant main effect of injection on daily activity in LD, which was reduced after injection, $F(1,11) = 26.53$, $p < 0.001$ (Two-way mixed ANOVA, **Figure 5.14A**). There was no main effect of virus, $F(2,11) = 0.36$, $p = 0.707$, or interaction of virus with injection, $F(2,11) = 0.52$, $p = 0.610$, suggesting all three viruses led to a similar suppression of activity after injection. *Opn4*^{-/-} animals show increased activity levels compared to *Opn4*^{+/+} under 12:12 LD (Muindi et al., 2013), suggesting the attenuated activity of *hOPN4* AAV-injected *Opn4*^{-/-} *Cre*^{+/+} mice is due to restoration of OPN4 light input.

In order to identify the source of this attenuated activity, bout analysis was performed on activity in LD using Clocklab (Actimetrics). Counterintuitively, the number of activity bouts increased slightly after injection (**Figure 5.14B**), $F(1,11) = 13.05$, $p = 0.004$ (Two-way mixed ANOVA), with no main effect of virus, $F(2,11) = 0.37$, $p = 0.700$, or interaction of virus with injection, $F(2,11) = 2.01$, $p = 0.18$. However, average bout duration was significantly decreased after injection, $F(1,11) = 46.58$, $p < 0.001$ (Two-way mixed ANOVA) with no main effect of virus, $F(2,11) = 0.11$, $p = 0.90$, or interaction of virus with injection, $F(2,11) = 2.01$, $p = 0.091$ (**Figure 5.14C**). This suggests daily activity decreased because individual activity bouts were significantly shorter. After injection, activity was broken up into shorter more frequent activity bouts, indicating a more fragmented activity rhythm.

To confirm activity was more fragmented, intraday variability was examined, a non-parametric measure of the frequency and magnitude of activity changes from hour-to-hour (Van Someren et al., 1999). No main effect of injection was found, $F(1,11) = 1.21$, $p = 0.295$, virus, $F(2,11) = 1.31$, $p = 0.308$, or interaction of injection with virus on intraday variability, $F(2,11) = 0.06$, $p = 0.944$ (Two-way mixed ANOVA, **Figure 5.14D**). This suggests distribution of activity bouts throughout the day did not change substantially after injection.

A second non-parametric parameter, interday stability, measured how tightly coupled the activity rhythm is to the zeitgeber, by calculating how similar the activity patterns are across multiple days (Van Someren et al., 1999). No main effect of injection was found, $F(1,11) =$, $p = 0.491$, no main effect of virus, $F(2,11) = 0.76$, $p = 0.575$, and no interaction of injection with virus, $F(2,11) = 1.95$, $p = 0.191$ (Two-way mixed ANOVA, **Figure 5.14E**). Day-to-day variation in LD was similar before and after injection, as expected since animals were entrained to the LD cycle both pre- and post-injection. This does not appear to be affected by changes in timing of activity relative to zeitgeber.

Despite changes to activity bouts, activity-rest patterns appear to be relatively stable throughout the experiment. The increased number and decreased duration of activity bouts after injection are not necessarily due to restoration of OPN4 light input. They could reflect aging, which disrupts rhythm and increases fragmentation (Banks et al., 2015). Animals were 13 weeks older at the start of the second circadian screen, although were on average only 23 weeks old, before the age Banks and colleagues noticed significant effects of age on rhythm fragmentation. Alternatively, these could be long-term effects of anaesthesia, although this also seems unlikely as animals were allowed to recover for 1 month after injection before testing resumed. Non-parametric measures suggests the stability and fragmentation of activity was comparable before and after injection, as anticipated.

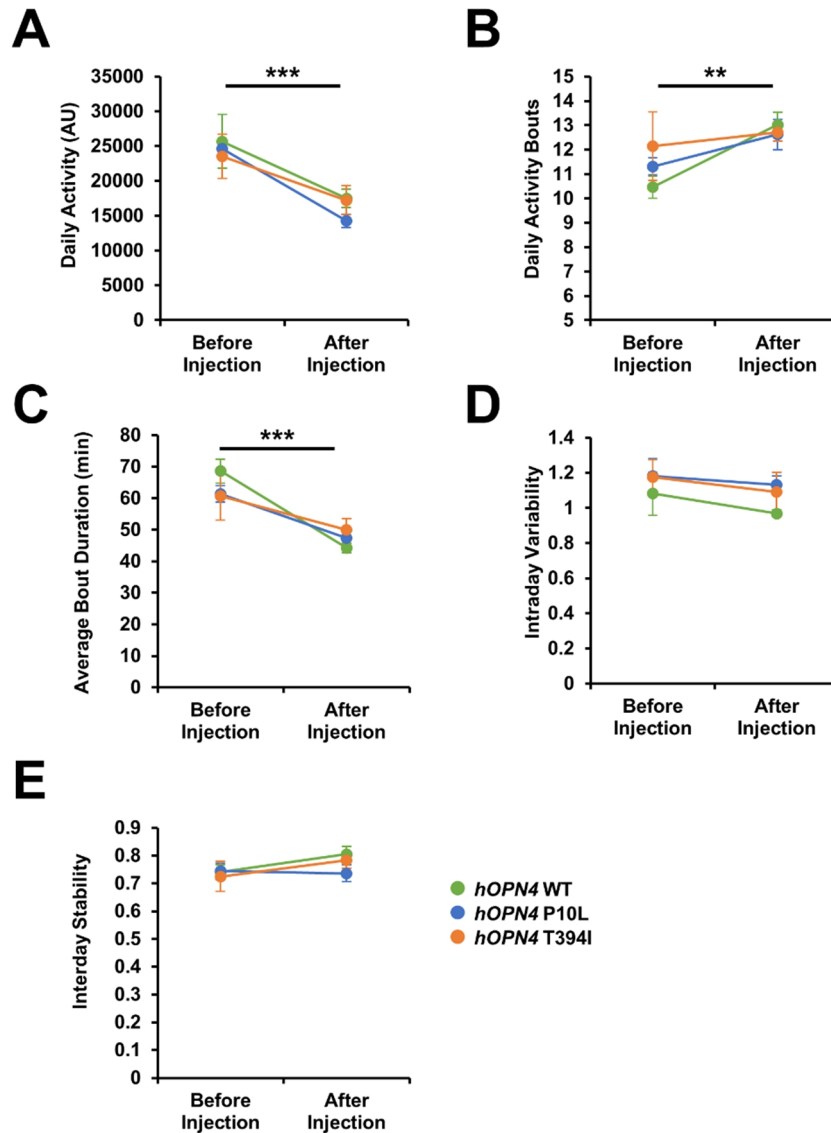


Figure 5.14. Daily activity is attenuated and activity rhythms are more fragmented after injection. Activity data derived from PIR recordings from 7 days of activity in 12:12 350lux white light dark (LD) cycle from *Opn4^{-/-} Cre^{+/+}* mice before and after bilateral intraocular injection of *hOPN4* WT, P10L and T394I AAV. **(A)** Total activity in 24hours **(B)** Number of activity bouts in 24hours. **(C)** Duration of daily activity bouts. Nonparametric measures of activity rhythms **(D)** Intraday variability and **(E)** Intraday Stability. *n* = 4-5 for each group. Error bars = SEM. ** *p* < 0.01 and *** *p* < 0.001, Main effect of injection, Two-way mixed ANOVA.

5.3.3.6 Increased phase shifting after restoration of OPN4 light input

In the first half of the circadian screen, several changes in circadian behaviour were identified after injection which have not previously been tested in *Opn4*^{-/-} mice, such as attenuated phase angle of entrainment. This highlights an advantage of the *hOpn4*-AAV method. By comparing *Opn4*^{-/-} *Cre*^{+/+} before and after restoration of OPN4 light input, novel roles for melanopsin can be identified. However, these changes are hard to define as OPN4-related, since they may also be confounded with age at time of testing.

The data examined in the first half of the circadian screen did not specifically test whether *hOPN4* AAV successfully restores melanopsin-driven circadian behaviour. To do this, phase shifting was examined, which is consistently attenuated in *Opn4*^{-/-} mice (Panda et al., 2002; Ruby et al., 2002). The second half of the circadian screen included a nocturnal light pulse at ZT15 to elicit a phase delay. A Type II Aschoff protocol was used, where the light pulse is given in the first day of constant dark after a week of entrainment to LD (Jud et al., 2005). The intensity and duration of light pulse, 15mins of 100lux white light, were selected to replicate previous studies of phase shifting in *Opn4*^{-/-} and maximise observable changes in phase shift before and after AAV-delivery of *hOPN4* (Panda et al., 2002; Ruby et al., 2002).

Although phase delays were elicited both pre and post-injection (**Figure 5.15**), the magnitude of the phase shift was significantly increased after injection, $F(1, 11) = 7.77$, $p = 0.018$. There was no main effect of virus, $F(2,11) = 0.26$, $p = 0.77$, or interaction of virus and injection, $F(2,11) = 0.86$, $p = 0.451$, suggesting phase shift magnitude was similar for *hOPN4* WT and the two *hOPN4* SNPs. Importantly, this implies the *hOPN4*-AAV model is able to replicate a normal *Opn4* WT phenotype in more complex melanopsin-associated behaviours, such as phase shifting, in addition to restoring PLR. This further validates the chosen technique for the investigation of abnormal circadian phenotypes due to introduction of *hOPN4* SNPs.

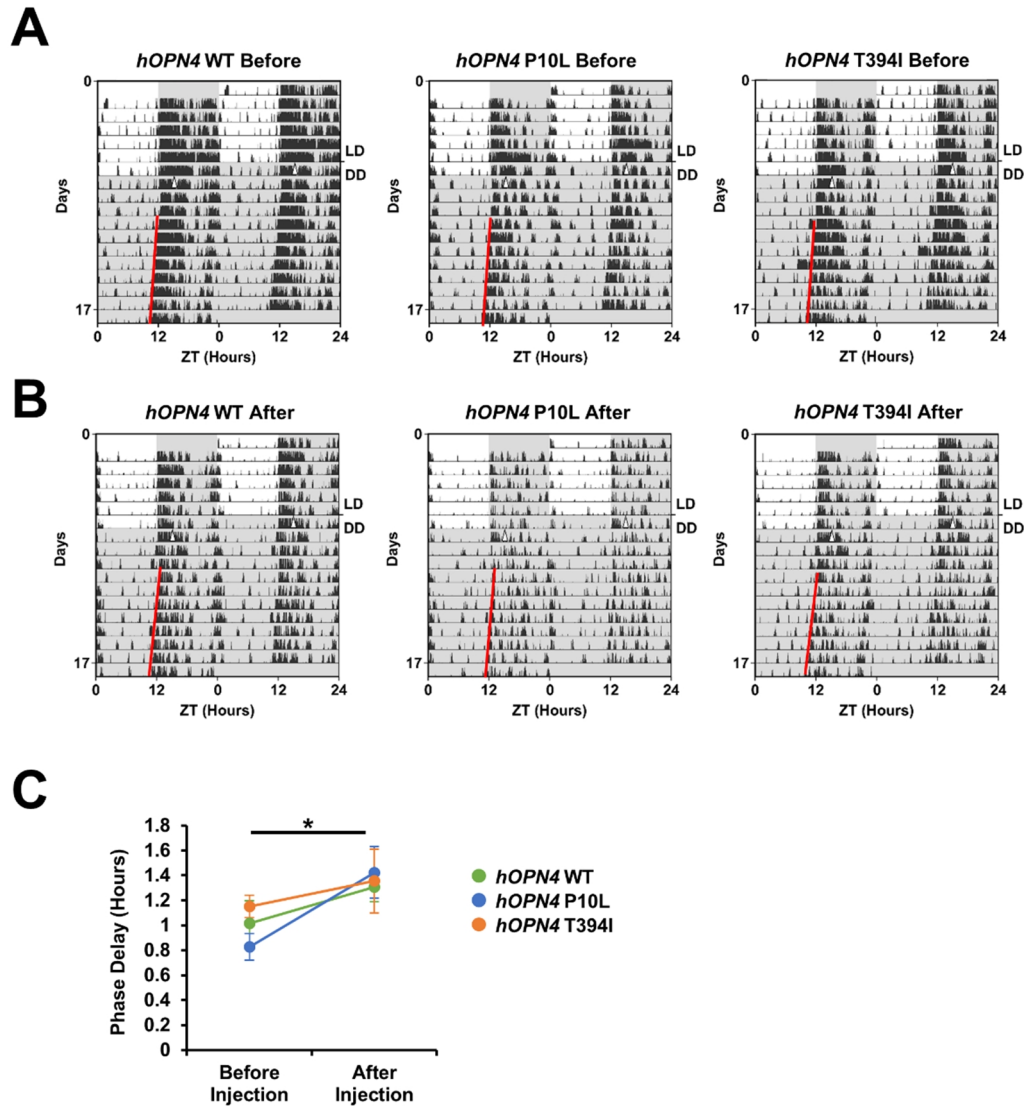


Figure 5.15. AAV-delivered *hOPN4* enhances phase shifting to nocturnal light pulse. Representative actograms showing activity recorded by PIR detection from the same three *Opn4*^{-/-} *Cre*^{+/+} mice before (**A**) and after (**B**) bilateral intraocular injection of *hOPN4* WT (left), P10L (middle) and T394I (right) AAV. Animals were in 12:12 LD cycle under white light (approx. 350lux) for 6 days, then released into constant dark (DD) for up to 10days. On the first day of DD animals were given a 15min 100lux white light pulse at ZT15 (shown as white triangle). Actograms are double plotted on a 24hr timescale. Grey shading indicates darkness. Regression line (red) was used to calculate phase shift from onset of activity on first day of DD. Activity data displayed in 1 min bins. (**C**) Average phase delay before and after injection. n = 4-5 for each group. Error bars = SEM. * p < 0.05, Main effect of injection, Two-way mixed ANOVA.

5.3.3.7 Period lengthening in constant light after restoration of OPN4 light input

Another highly replicated phenotype of *Opn4*^{-/-} mice is attenuated period lengthening in constant light, LL (Ruby et al., 2002). In order to avoid potential damage to the retina that might have confounded the results after *hOPN4*-AAV delivery, constant light was not included in the circadian screen conducted prior to injection. Instead, animals were placed in 100lux constant light at the end of the circadian screen after injection only (**Figure 5.16**). Therefore, comparisons before and after injection were not possible for individual mice that would provide further evidence that *hOPN4*-AAV can restore a range of previously described melanopsin-driven behaviours. Nevertheless, the interaction of virus type with the relationship between tau in LD and LL could still be assessed. For example, attenuated period lengthening in LL after injection of a *hOPN4* SNP compared to *hOPN4* WT control would indicate abnormal melanopsin function.

A two-way mixed ANOVA was conducted with light condition as the within subjects variable (2 levels – LL and 12:12 LD cycle) and virus as the between subjects variable (3 levels – *hOPN4* WT, P10L and T394I) to assess effect of light condition and virus on period (tau). Tau in LL was significantly longer than in LD as anticipated, $F(1,13) = 50.79$, $p < 0.001$. As shown in **Figure 5.16D**, there was no significant main effect of virus $F(2,13) = 0.13$, $p = 0.88$ and, more notably, no significant interaction of lighting condition with virus, $F(2,13) = 0.29$, $p = 0.814$, which suggests the increase in tau seen in LL compared to LD was equivalent for all three virus conditions. It appears the introduction of *hOPN4* SNPs did not affect restoration of melanopsin-associated period lengthening in LL.

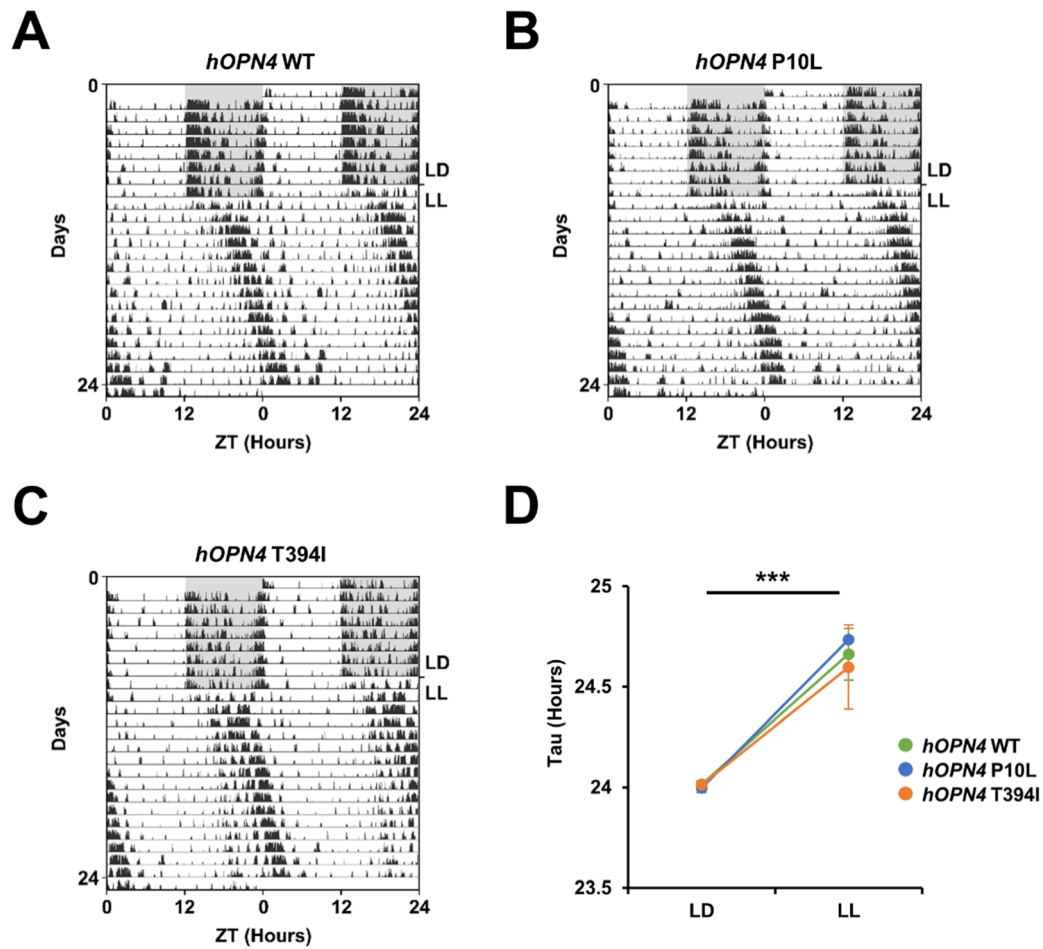


Figure 5.16. Period lengthening in constant light is similar for *hOPN4* WT, P10L and T394I. Representative actograms showing activity recorded by PIR detection from three *Opn4^{-/-} Cre^{+/+}* mice after bilateral intraocular injection of (A) *hOPN4* WT (B) P10L and (C) T394I AAV. Animals were in 12:12 LD cycle under white light (approx. 350lux) for 8 days, then released into constant white light at 100lux (LL) for 16 days. Actograms are double plotted on a 24hr timescale. Grey shading indicates when animals were in darkness. Activity data displayed in 1 min bins. Regression line (red) fit to activity onset used to calculate period. (D) Comparison of Tau in 12:12 LD and LL after injection. $n = 6-8$ for each group. Error bars = SEM, *** $p < 0.001$, Main effect of injection, Two-way mixed ANOVA.

5.3.3.8 Circadian behaviours are comparable for *hOPN4* WT, P10L and T394I

Comparisons before and after injection were useful for several reasons. First, it demonstrated the *hOPN4* AAV technique could successfully restore circadian behaviour known to be attenuated in *Opn4*^{-/-} mice, such as phase shifting. Second, it revealed some potentially novel melanopsin-driven behaviours, such as the effects on phase angle of entrainment. However, whenever each circadian parameter was examined using a Two-way mixed ANOVA with virus as a between-subjects factor (3 levels – *hOPN4* WT, P10L and T394I) and injection as a within-subjects factor (2 levels – before and after injection), no interaction of virus with injection was found. Neither *hOPN4* P10L nor T394I demonstrated greater or attenuated improvement after injection compared to *hOPN4* WT, suggesting there may not be any difference in circadian behaviour between the three groups, or that any difference was relatively small and below the ability to discriminate in this study.

To examine the effects of *hOPN4* SNPs on circadian behaviour, all animals were compared after injection using a One-way between-subjects ANOVA. Any significant main effects of virus were then explored further using a Dunnett's post-hoc test with *hOPN4* WT as the control group. As shown in **Table 5.4**, each of the circadian and activity parameters described previously were tested. No significant main effect of virus was found for any parameter, except duration of daily activity bouts. Post-hoc tests revealed this was because activity bouts in *hOPN4* T394I were significantly longer than in *hOPN4* WT ($p = 0.018$). No significant difference was found in bout duration between *hOPN4* WT and P10L ($p = 0.623$). However, as other measures of rhythm fragmentation, such as bout number and total activity, did not differ between *hOPN4* WT and T394I, it is more likely this due to variation between animals, which as previously noted can be quite high, than an effect of T394I on *hOPN4* function.

Table 5.4. Descriptive statistics for circadian activity parameters and results of One-way Between subjects ANOVA. Statistics for LD derived from 7 consecutive days under 12:12 LD cycle with 350lux white light. Statistics for DD and LL derived from 10 consecutive days of constant conditions (dark or 100lux white light, respectively). Sample sizes are same for all parameters, except Tau in LL (sample size given in parenthesis). Significant results ($p < 0.05$) are highlighted in bold. Significant main effects of virus were further examined using a Dunnett's post-hoc test with *hOPN4* WT as control group. SEM = standard error of mean, H = hours, M = minutes, AU = arbitrary units.

Parameter	<i>hOPN4</i> WT (n = 8) Mean (SEM)	<i>hOPN4</i> P10L (n = 6) Mean (SEM)	<i>hOPN4</i> T394I (n = 8) Mean (SEM)	One-way Between Subjects ANOVA Main Effect (Dunnett's Post-Hoc Test)
<i>Tau</i> in LD (H)	24.02 (0.02)	24.00 (0.02)	24.01 (0.02)	$F(2,21) = 0.21, p = 0.810$
<i>Tau</i> in DD (H)	23.93 (0.04)	23.91 (0.05)	23.94 (0.02)	$F(2,21) = 0.14, p = 0.876$
<i>Tau</i> in LL (H)	24.66 (0.13) (n = 7)	24.74 (0.08) (n = 4)	24.60 (0.21) (n = 5)	$F(2,15) = 0.17, p = 0.849$
Daily Activity in LD (AU)	16947 (1188)	13157 (1362)	18756 (2044)	$F(2,21) = 2.81, p = 0.085$
Number of Activity Bouts in LD	12.79 (0.40)	12.07 (0.75)	11.52 (0.53)	$F(2,21) = 1.49, p = 0.251$
Duration of Activity Bout in LD (M)	45.46 (1.31)	48.46 (2.40)	54.98 (3.16)	$F(2,21) = 4.31, p = 0.029$ (WT vs T394I, $p = 0.018$) (WT vs P10L, $p = 0.623$)
Phase Angle of Entrainment in LD (H)	3.62 (5.05)	2.78 (3.05)	0.77 (6.02)	$F(2,21) = 0.09, p = 0.916$
Time to Peak Activity (H)	12.85 (0.04)	13.41 (0.28)	13.13 (0.13)	$F(2,21) = 3.04, p = 0.072$
Interday Stability in LD (AU)	0.80 (0.02)	0.75 (0.03)	0.81 (0.02)	$F(2,21) = 1.45, p = 0.265$
Intraday Variability in LD (AU)	1.03 (0.04)	1.06 (0.08)	0.96 (0.09)	$F(2,21) = 0.49, p = 0.622$
Phase Delay (H)	1.24 (0.09)	1.36 (0.18)	1.24 (0.20)	$F(2,21) = 0.17, p = 0.847$

Overall, no effect of *hOPN4* T394I and P10L was detected on entrainment of activity-rest rhythms, as suggested by Lee et al. (2014) and Roecklein et al. (2012). This supports the pupillometry data to suggest that introduction of these SNPs does not affect protein function sufficiently to cause an observable change in OPN4-driven behaviour.

5.3.3.9 Increases in immobility-defined sleep after injection are light-phase dependent

The identification of novel changes in activity after injection, particularly those related to total activity and phase angle of entrainment, led to the question of whether sleep was also affected in AAV-treated *Opn4^{-/-} Cre^{+/+}* animals. The relationship between melanopsin and light-induced sleep has previously been demonstrated in *Opn4^{-/-}* animals, which exhibit attenuated sleep induction during nocturnal light pulses (Altimus et al., 2008; Lupi et al., 2008; Tsai et al., 2009; Muindi et al., 2013). The light pulse used for phase shifting in this study was not long enough to determine if animals differed in latency to sleep induction before and after injection. However, Tsai et al. (2009) also described attenuated sleep in *Opn4^{-/-}* animals during light phase of a 12:12 LD cycle. Both *hOPN4* P10L and T394I have been associated with abnormal sleep timing (Lee et al., 2014; Roecklein et al., 2014), suggesting distribution of sleep in 12:12 LD was worth investigating in the *hOPN4* AAV model.

Using PIR data, sleep was defined as a period of immobility of minimum 40s (Fisher et al., 2012; Pack et al., 2007). This measure of sleep has a strong correlation with both video-tracking and EEG sleep determination (Brown et al., in preparation). The average immobility-defined sleep profiles of *Opn4^{-/-} Cre^{+/+}* animals in 12:12 LD after injection of all three *hOPN4* AAVs showed a delay in sleep offset compared to before injection (**Figure 5.17A-C**), most likely related to delay of activity onset seen after injection in all three virus conditions.

Total daily sleep was significantly increased after injection, $F(1,11) = 16.98$, $p = 0.002$, without a main effect of virus, $F(2,11) = 0.30$, $p = 0.746$, or interaction of virus with injection, $F(2,11) = 0.57$, $p = 0.584$ (Two-way mixed ANOVA, **Figure 5.17D**). When the distribution of sleep was examined throughout the light dark cycle, it was found this was due to significantly increased sleep in the dark phase, $F(1,11) = 15.58$, $p = 0.002$, with no significant main effect of virus, $F(2,11) = 0.79$, $p = 0.478$ or interaction, $F(2,11) = 1.51$, $p = 0.264$ (Two-way mixed ANOVA, **Figure 5.17E**). Sleep during light phase was unaffected by injection of *hOPN4* AAV, $F(1,11) = 1.72$. No main effect of virus was found, $F(2,11) = 0.51$, $p = 0.612$, or interaction of injection by virus, $F(2,11) = 1.13$, $p = 0.359$ on light phase sleep (Two-way mixed ANOVA, **Figure 5.17F**).

There was an overall increase in daily sleep in all three *hOPN4* AAV groups after injection. In all three groups, this was due to increased dark phase sleep, as light phase sleep remained unchanged. This explains why *Opn4^{-/-} Cre^{+/+}* mice were less active post-injection – they were sleeping more during their active phase. However, it also contradicts previous reports of attenuated sleep in light phase (Tsai et al., 2008) or no difference in sleep during 12:12 LD in *Opn4^{-/-}* mice (Lupi et al., 2008; Altimus et al., 2009). As with the change in activity bout distribution after injection, it is unlikely these effects are due to age or long-term effects of anaesthesia as mice were still relatively young and given ample recovery time before post-injection testing. Therefore, these effects on sleep bout distribution are probably due to restoration of OPN4-light input.

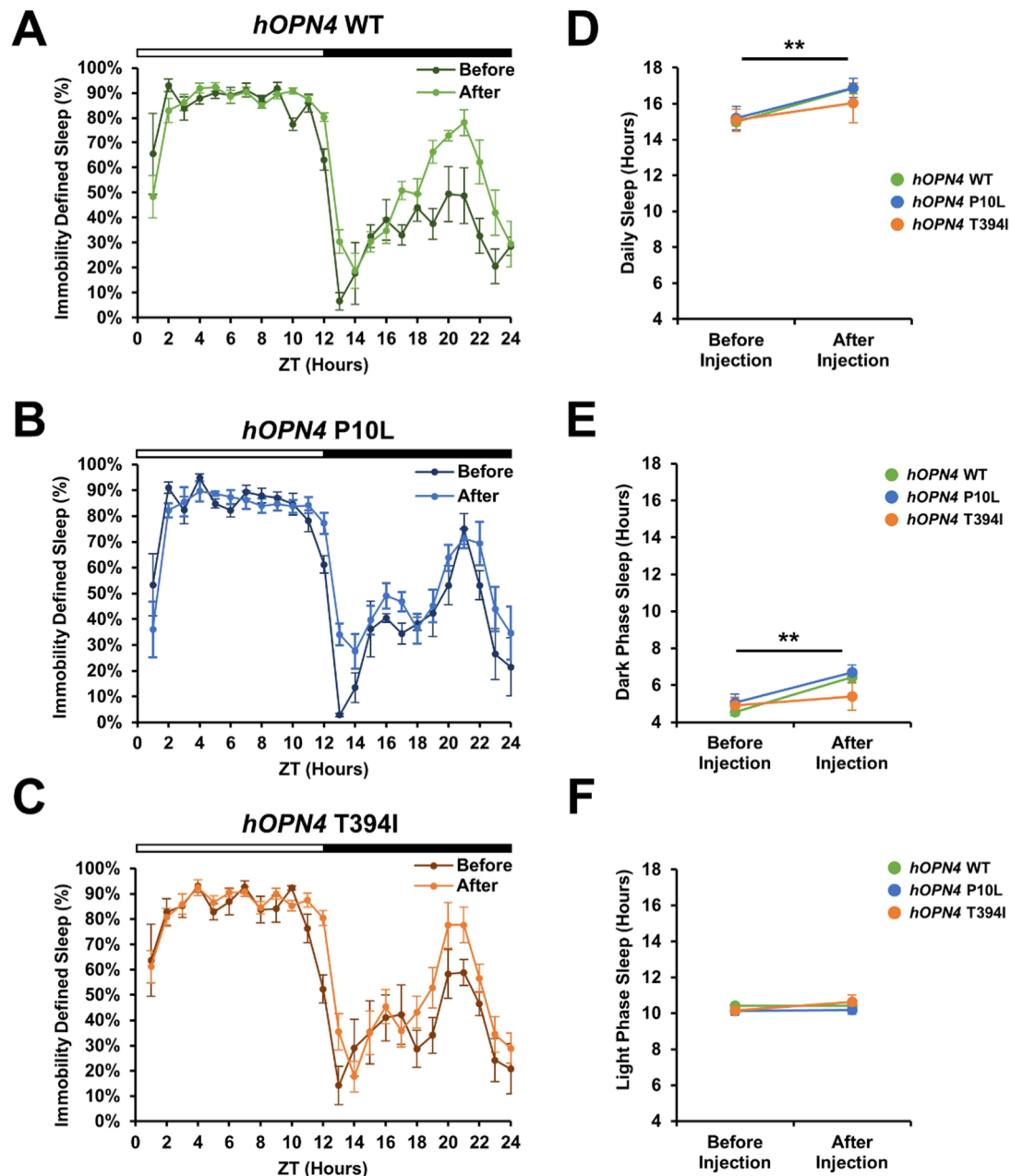


Figure 5.17. Immobility-defined sleep is increased after injection during dark phase of 12:12 LD cycle. Sleep data derived from PIR recordings from 5 days in 12:12 350lux white light-dark cycle. Sleep defined as minimum 40s immobility. Average sleep profiles in 1hr bins for *Opn4*^{-/-} *Cre*^{+/+} mice before and after bilateral intraocular injection of (A) *hOPN4* WT, (B) P10L and (C) T394I AAV. Data in 1hr bins. White and black bar shows timing of light and dark. (D) Average sleep in 24hrs (E) Sleep during Light Phase (ZT0-12) (F) Sleep during Dark Phase (ZT12 – 24). n = 4-5 for each group. Error bars = SEM. ** p < 0.01, Main effect of injection, Two-way mixed ANOVA.

5.3.3.10 Sleep bout distribution throughout light-dark cycle

Changes to sleep rhythm were investigated, using the total number and average duration of sleep bouts as a measure of sleep fragmentation (**Figure 5.18**). Both these measures were highly variable between individual mice but, as shown in **Figure 5.18A**, a small but significant increase in number of daily sleep bouts after injection was observed, $F(1,11) = 7.00$, $p = 0.023$, with no main effect of virus, $F(2,11) = 0.37$, $p = 0.697$, or interaction of virus and injection, $F(2,11) = 2.15$, $p = 0.163$ (Two-way mixed ANOVA). There was no significant change in average daily bout duration after injection, $F(1,11) = 1.02$, $p = 0.334$, and no main effect of virus, $F(2,11) = 0.37$, $p = 0.697$, or interaction, $F(2,11) = 0.88$, $p = 0.442$ (Two-way mixed ANOVA, **Figure 5.18B**). This increase in daily sleep bouts, without any change in average bout duration, suggests that *Opn4^{-/-} Cre^{+/+}* mice were able to maintain sleep normally both before and after injection.

Since the increase in sleep after injection was restricted to the dark phase, the distribution of sleep bouts throughout the LD cycle was considered, to determine whether increase in sleep bouts was also confined to the dark phase. First, it was confirmed there was no main effect of injection on total sleep bouts during the light phase, $F(2,11) = 3.34$, $p = 0.095$, and no main effect of virus, $F(2,11) = 0.52$, $p = 0.607$, or interaction of virus with injection, $F(2,11) = 0.407$, $p = 0.675$ (Two-way mixed ANOVA, **Figure 5.18C**).

Total sleep bouts during the dark phase did increase significantly after injection, $F(1,11) = 10.98$, $p = 0.007$ (Two-way mixed ANOVA). However, a significant interaction of injection by virus type was also found, $F(2,11) = 5.43$, $p = 0.023$, suggesting the increase after injection was not equal for each virus condition (**Figure 5.18D**). No main effect of virus was found, $F(2,11) = 0.306$, $p = 0.742$. Three post-hoc Sidak-corrected ($\alpha = 0.017$) paired t-tests were conducted, comparing dark phase sleep bouts in

each AAV group before and after injection to identify the source of the significant virus by injection interaction.

Both *hOPN4* WT, $t(4) = -1.201$, $p = 0.345$, and *hOPN4* T394I, $t(3) = -0.43$, $p = 0.697$, showed no significant difference before and after injection. *hOPN4* P10L showed a greater increase after injection, $t(4) = -3.70$, $p = 0.021$, although this was not significant with the Sidak-corrected alpha. However, it does imply the significant main effect of injection on dark-phase sleep bouts is driven primarily by the increase seen after injection of *hOPN4* P10L.

There was no significant main effect of injection, $F(1,11) = 1.62$, $p = 0.23$, virus, $F(2,11) = 0.48$, $p = 0.633$, or interaction, $F(2,11) = 0.01$, $p = 0.99$ (Two-way mixed ANOVA) on light-phase sleep bout duration (**Figure 5.18E**). There was also no significant main effect of injection, $F(1,11) = 0.35$, $p = 0.57$, virus, $F(2,11) = 0.20$, $p = 0.825$, or interaction, $F(2,11) = 2.80$, $p = 0.104$ (Two-way mixed ANOVA), on dark-phase bout duration (**Figure 5.18F**). This suggests maintenance of sleep under both light conditions was unaffected by injection of *hOPN4* AAV.

The average duration of sleep bouts in the dark and light phase did not change with injection, suggesting there was no increase in sleep fragmentation, characterised by shorter sleep bouts. The increase in dark phase bouts after injection was primarily driven by an increase in dark phase bouts in *Opn4^{-/-} Cre^{+/+}* mice injected with *hOPN4* P10L. Since *hOPN4* WT showed no difference in dark phase bouts before and after injection, and differences in this parameter has not been described in *Opn4^{-/-}*, it seems unlikely the increased bouts after injection of *hOPN4* P10L are melanopsin-related as these would also be observed after introduction of the melanopsin positive control.

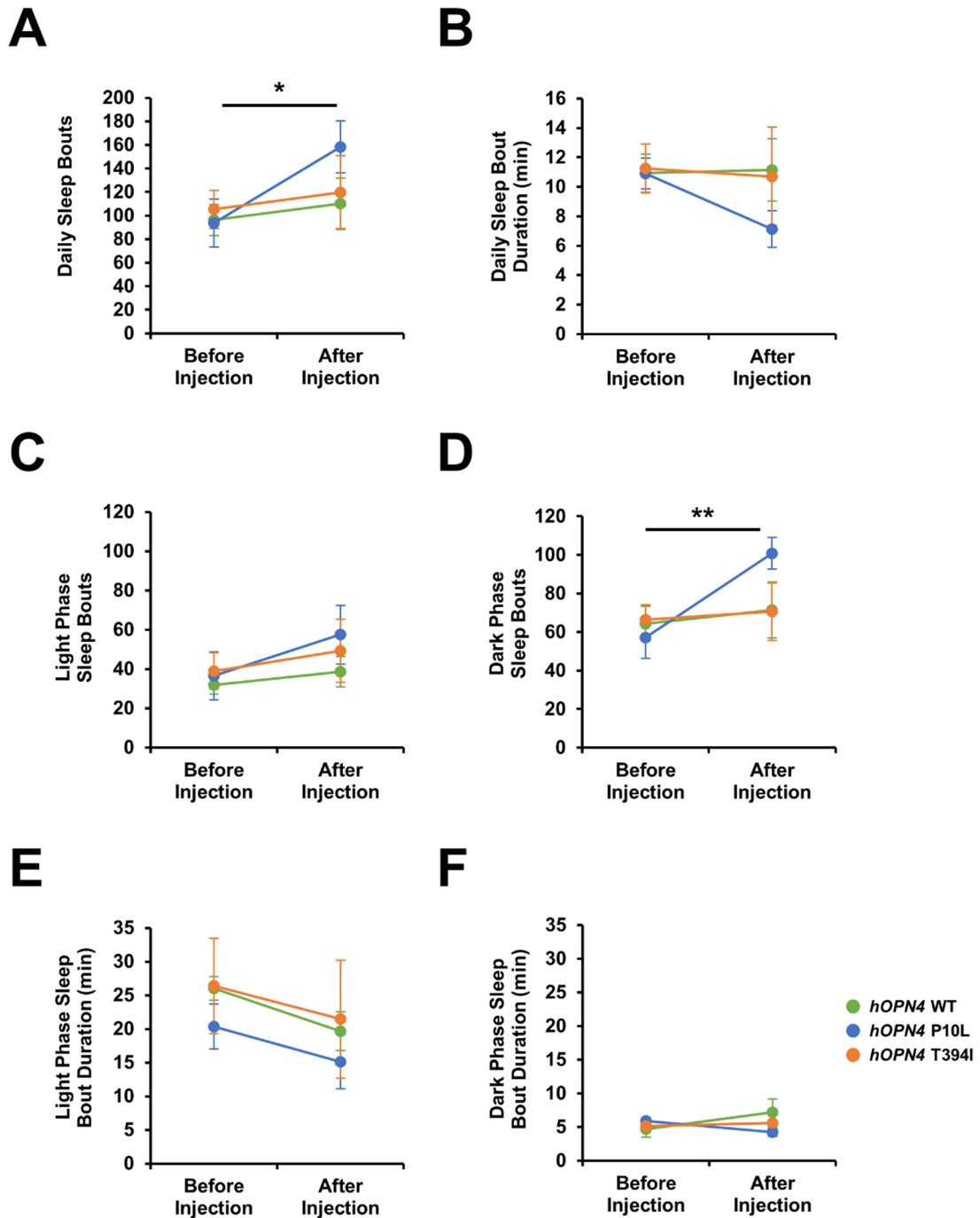


Figure 5.18. Sleep bout frequency, but not duration, is altered after injection. Sleep data derived from PIR recordings of 5 days in 12:12 350lux white light-dark cycle from *Opn4^{-/-} Cre^{+/+}* mice before and after bilateral intraocular injection of *hOPN4* WT, P10L and T394I AAV. Sleep bout defined as minimum 40s immobility until next recorded movement (**A**) Average number of sleep bouts in 24hrs. (**B**) Average duration of sleep bouts in 24hrs. (**C**) Average number of sleep bouts during Light Phase (ZT0-12) (**D**) Average duration of sleep bouts in Light Phase (ZT0-12). (**E**) Average number of sleep bouts during Dark Phase (ZT12-24) (**F**) Average duration of sleep bouts in Light Phase (ZT12-24). n = 4-5 for each group. Error bars = SEM. * p < 0.05 and ** p < 0.01 Main effect of injection, Two-way mixed ANOVA.

5.3.3.11 Comparison of sleep parameters for *hOPN4* WT, P10L and T394I

As with the activity data, the two-way mixed ANOVAs used to analyse the effect of virus and injection on different sleep parameters did not reveal a significant main effect of virus. However, unlike the activity and pupillometry data, a significant interaction of virus with injection on one sleep parameter was found, dark phase sleep bouts, indicating the different virus conditions may not equally restore this aspect of melanopsin function after injection.

To confirm this, the full cohort of each *hOPN4* AAV group was compared, using a between subjects One-way ANOVA, for each of the sleep parameters investigated above. The results shown in **Table 5.5**, demonstrate the three viruses did not differ significantly for any parameters, except daily sleep bouts and dark phase sleep bouts. Post-hoc Dunnett's test revealed the significant main effect of virus these parameters was due to the greater number of bouts in *hOPN4* P10L compared to *hOPN4* WT. There was no difference in daily sleep bouts or dark phase sleep bouts between *hOPN4* T394I and *hOPN4* WT control.

Animals injected with *hOPN4* P10L demonstrated an increased number of sleep bouts, with no change in sleep bout duration. This increase was restricted to the dark phase. Although previous studies have identified disparities between *Opn4*^{-/-} and *Opn4*^{+/+} mice during the dark phase only (Tsai et al., 2008; Muindi et al., 2013), these have been exclusively related to nocturnal light-pulse induced sleep. There have been no reports of altered sleep during dark phase in *Opn4*^{-/-} mice entrained to a 12:12 LD cycle. Therefore, the increased frequency of dark phase sleep bouts is unlikely due to introduction of P10L affecting melanopsin protein function. Especially as the total sleep and total activity of *Opn4*^{-/-} *Cre*^{+/-} mice injected with *hOPN4* P10L was indiscernible from animals treated with *hOPN4* WT, this increase in sleep bouts appears relatively minor and is likely an artefact of variation in the injection procedure.

Collectively, the sleep and activity data from the PIR circadian screen do not identify any differences in known OPN4-related behaviours between *Opn4^{-/-} Cre^{+/+}* mice injected with *hOPN4* WT control and *hOPN4* SNPs, P10L and T394I. This suggests the presence of these mutations does not affect *hOPN4* function sufficiently to disrupt NIF behaviour as described by Lee et al. (2013) and Roecklein et al. (2012).

Table 5.5. Descriptive statistics for immobility-defined sleep and results of One-way Between subjects ANOVA. Sleep data derived from 5 consecutive days under 12:12 LD cycle with 350lux white light. Significant results ($p < 0.05$) are highlighted in bold. Significant main effects of virus were further examined using a Dunnett's post-hoc test with *hOPN4* WT as control group (p values given in parenthesis). SEM = standard error of mean, H = hours, M = minutes.

Parameter	<i>hOPN4</i> WT (n = 8) Mean (SEM)	<i>hOPN4</i> P10L (n = 6) Mean (SEM)	<i>hOPN4</i> T394I (n = 8) Mean (SEM)	One-way Between Subjects ANOVA Main Effect (Dunnett's Post-Hoc Test)
Total Daily Sleep (H)	16.96 (0.54)	17.38 (0.14)	16.54 (0.42)	F(2,21) = 0.62, $p = 0.547$
Total Sleep in Light Phase (H)	10.49 (0.13)	10.33 (0.12)	10.75 (0.34)	F(2,21) = 1.08, $p = 0.360$
Total Sleep in Dark Phase (H)	6.47 (0.63)	7.05 (0.20)	5.79 (0.57)	F(2,21) = 2.68, $p = 0.095$
Total Daily Sleep Bouts	110.33 (19.89)	167.63 (12.38)	104.08 (25.48)	F(2,21) = 4.15, $p = 0.032$ (WT vs P10L, $p = 0.048$) (WT vs T394I, $p = 0.944$)
Total Sleep Bouts in Light Phase	37.75 (6.66)	58.33 (4.55)	35.73 (10.64)	F(2,21) = 1.85, $p = 0.184$
Total Sleep Bouts in Dark Phase	72.58 (13.69)	109.30 (8.14)	68.35 (14.93)	F(2,21) = 5.49, $p = 0.013$ (WT vs P10L, $p = 0.023$) (WT vs T394I, $p = 0.920$)
Average Daily Sleep Bout Duration (M)	10.67 (1.61)	6.88 (1.59)	11.44 (3.65)	F(2,21) = 2.66, $p = 0.096$
Average Sleep Bout Duration in Light Phase (M)	19.64 (5.10)	14.68 (3.25)	29.29 (11.68)	F(2,21) = 2.26, $p = 0.131$
Average Sleep Bout Duration in Dark Phase (M)	6.63 (0.67)	4.12 (0.82)	5.78 (1.07)	F(2,21) = 1.62, $p = 0.224$

5.3.3.12 AAV-restored intrinsic ipRGC responses can be recorded using multi-electrode array (MEA)

ipRGCs have intrinsic responses mediated directly by melanopsin phototransduction, and extrinsic responses that are synaptically-driven by rod and cone signals, via bipolar and amacrine cells (Wong et al., 2007). In an *Opn4*^{-/-} animal, only extrinsic responses can be detected in ipRGCs, but in the *hOPN4* AAV treated *Opn4*^{-/-} *Cre*^{+/+} animals, intrinsic ipRGC responses should also be observed, provided melanopsin phototransduction has been successfully restored. To confirm this, multi-electrode array (MEA) recordings of AAV-treated retinæ were conducted.

MEAs record extracellular field potentials, generated by action potentials (Meister et al., 1994). By recording from the ganglion cell side of AAV-treated retina, the light-induced action potentials generated by ipRGCs are detected. Pharmacology was used to block synaptic input from bipolar and amacrine cells, preventing signals from rods and cones reaching the ipRGCs and isolating intrinsic melanopsin-driven responses. Under these conditions, light-induced responses from retina of *Opn4*^{-/-} *Cre*^{+/+} mice treated with *hOPN4* WT, P10L and T394I AAV were recorded (**Figure 5.19**). Since these responses were recorded during blockade of synaptic input, they must be intrinsic, meaning the AAV-delivered *hOPN4* has restored melanopsin phototransduction to ipRGCs. In addition, the observed ipRGC responses had long onset latency, continuous activity throughout light exposure and prolonged post-stimulus firing. These are all characteristic of intrinsic ipRGC activity recorded from *Opn4*^{+/+} retina using MEA (Tu et al., 2005; Weng et al., 2013).

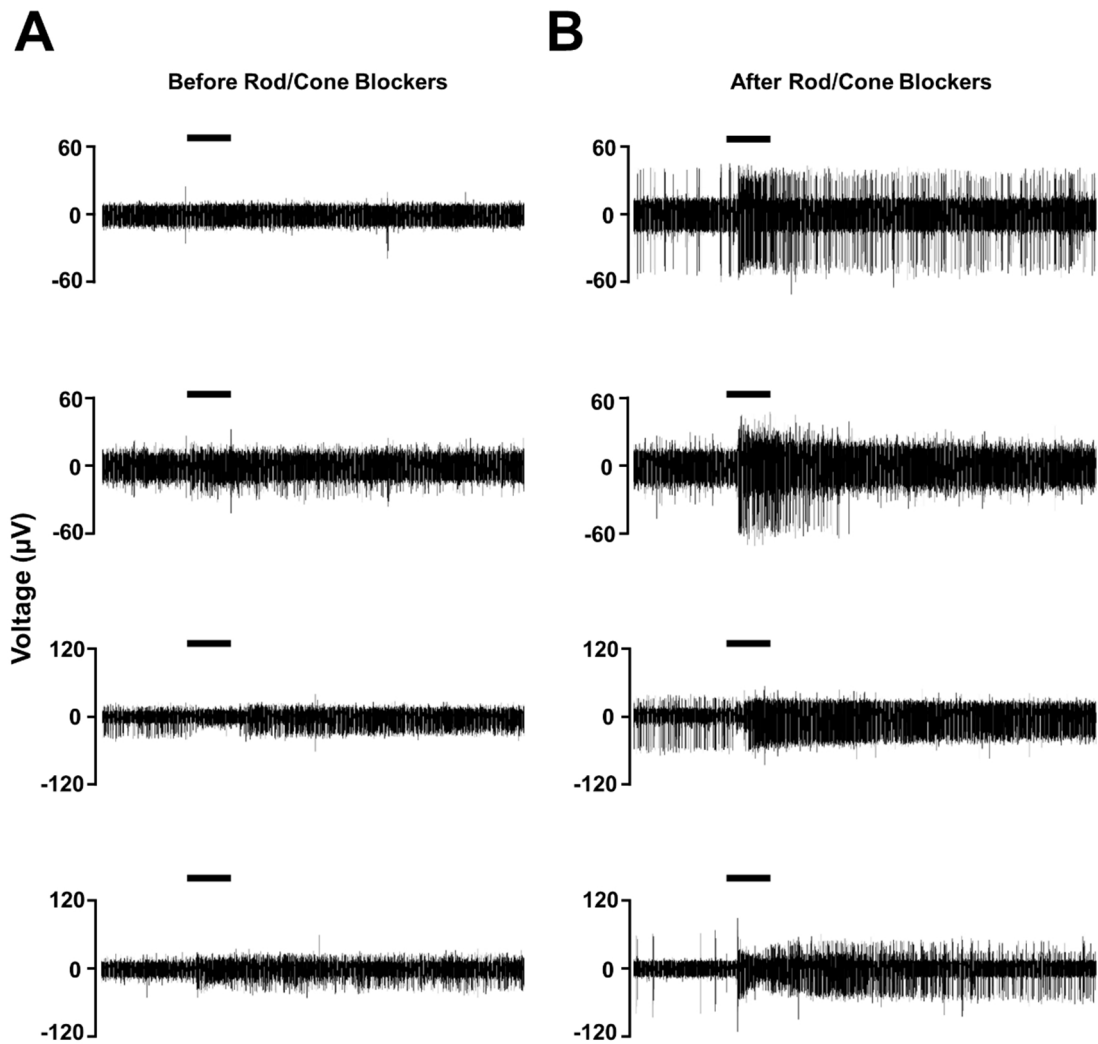


Figure 5.19. AAV-restored intrinsic ipRGC responses can be observed after pharmacological blockade of rod/cone signals. Example ipRGC responses to 10s 480nm light pulse (1.1×10^{14} photons/cm²/s, shown as black bar above trace) during MEA recording of a *hOPN4* WT AAV-treated *Opn4*^{-/-} *Cre*^{+/+} retina. Vertical panels show same electrodes before and after pharmacological blockade. **(A)** Response during perfusion in Ames media only **(B)** Response during perfusion in Ames media with pharmacological blockade of rod/cone signals (100μM I-AP4, 40μM DNQX, 30μM d-AP5).

There did not appear to be any consistent relationship between the activity of ipRGCs before and after synaptic blockade. For some, the synaptic input appeared suppressive or inhibitory, preventing responses being observed until rod/cone signalling was blocked. For others, ipRGC responses to light could be observed regardless of synaptic input. All subsequent MEA recordings were conducted under rod/cone blocking

conditions, as this allowed examination of melanopsin-driven responses in isolation and comparison of the effects of *hOPN4* SNPs with *hOPN4* WT controls on ipRGC activity.

5.3.3.13 Higher number of ipRGC responses observed for retina treated with *hOPN4* WT AAV compared to *hOPN4* P10L or T394I AAV

Intrinsic ipRGC responses could be recorded from *hOPN4* AAV-treated retina. This would only be possible if melanopsin phototransduction had been restored. However, as **Table 5.6** demonstrates, ipRGC responses were only observed on a small proportion of retina examined with the MEA, approximately 1 in every 5. It should be emphasised this relatively low success rate does not mean the *hOPN4* AAV failed to transduce ipRGCs in retina where no light-induced activity was detected using MEA. The presence of *hOPN4* mRNA and protein in injected retina, as well as restoration of *Opn4*^{+/+} behavioural phenotypes, all suggest that the *hOPN4* AAVs reliably deliver functional melanopsin to ipRGCs of *Opn4*^{-/-} *Cre*^{+/+} animals.

Instead the low recording rate may reflect methodological difficulties. Variation in the viral coverage between injections may have contributed to this, as the 60 MEA electrodes only cover a small portion of the retina and without a fluorescent marker, the retina could not be positioned to ensure the electrodes were in contact with a successfully transduced area. The immunohistochemistry of AAV-treated retina suggests viral coverage was widespread throughout the retina, but it is possible recordings were accidentally made from regions of low viral transduction.

Furthermore, the low frequency of successful recordings was exacerbated by recording from non-degenerate retina. Detecting ipRGC responses from *Opn4*^{+/+} non-degenerate retinas is also difficult, with similar rates of successful recordings, whereas ipRGCs can be consistently recorded in animals with retinal degeneration, suggesting this is not simply a viral coverage issue. Although total number of retinas used is often not reported, MEA recordings of adult non-degenerate retina typically have low ipRGC

numbers (Zhao et al., 2014), suggesting obtaining successful ipRGC recordings from non-degenerate adult retinas is a significant challenge for other research groups as well.

This is likely because the physical proximity of the ipRGCs to electrodes is insufficient in non-degenerate retinas due to the presence of the inner limiting membrane (ILM). The ILM is the basement membrane separating the retina from the vitreous humour. Sufficient contact of tissue with electrodes throughout the recording is required for a high signal to noise ratio necessary to detect ipRGC responses (Reinhard et al., 2014). Although still relatively thin, previous attempts to record from ipRGCs in non-degenerate retina often remove the ILM by enzymatic digestion (Schmidt & Kofuji, 2011b). During dissection of the retina, the ILM can also be partially removed but is often left intact to avoid tearing the tissue. Enzymatic digestion of retina prior to MEA recording was attempted but did not increase the frequency of successful recordings.

In order to improve contact with electrodes, the MEA chamber was coated with polyethylenimine and laminin to increase adherence to the electrodes and used glass-coated metal harps were used to press tissue against electrodes, but this may have been insufficient. It appears the ILM breaks down in animals with retinal degeneration (Kolstad et al., 2010), which may explain why ipRGCs can be more reliably recorded in degenerate retina. Future MEA recordings of *hOPN4* AAV-treated retina should probably be carried out in a retinally degenerate *Opn4^{-/-} Cre^{+/+}* line to improve number of retina with ipRGC responses.

The proportion of retina with detectable ipRGC responses was similar between the three virus conditions. However, retina treated with *hOPN4* WT AAV generally had more ipRGC responses per retina than either *hOPN4* P10L or T394I AAV. Notably the lowest number of ipRGCs responses recorded for *hOPN4* WT (range 14 - 38) was greater than the highest number recorded for *hOPN4* P10L (range 2 – 10 per retina) and just below the maximum recorded for *hOPN4* T394I (range 3 – 17 per retina).

Table 5.6. Retina expressing AAV-delivered *hOPN4* P10L and T394I show fewer ipRGC responses compared to *hOPN4* WT control during multi-electrode array recording (MEA). Electrodes were classified as ipRGCs if they had light-induced activity and irradiance-dependent spike firing. Light-induced activity was defined as an increase in 10s rolling average greater than 10 spikes/s from baseline after exposure to a 10s light pulse (480nm, 1.1×10^{14} photons/cm²/s). Intensity-dependent spike firing was identified as irradiance response curves (IRCs) fit to maximum firing rate data with $R^2 < 0.6$. SEM = standard error of mean.

Parameter	<i>hOPN4</i> WT	<i>hOPN4</i> P10L	<i>hOPN4</i> T394I
<i>Total retina tested</i>	12	12	14
<i>Average days from injection to MEA recording (SEM)</i>	111.2 (5.0)	116.7 (2.2)	113.5 (5.0)
<i>Retina with ipRGC responses</i>	3	2	2
<i>Total ipRGC responses</i>	83	12	20
<i>Average ipRGC responses per retina (SEM)</i>	27.3 (6.9)	6.0 (4.0)	10.0 (7.0)

With only small numbers of retina available for each group, the number of ipRGCs per retina is not significantly different between groups, $F(2,6) = 3.17$, $p = 0.150$ (One-way between subjects ANOVA), but the trend is worth noting. It could indicate a greater density of transduced ipRGCs with *hOPN4* WT AAV, suggesting it had more efficient viral transduction. However, ICC of retina treated with *hOPN4* WT AAV has not revealed any noticeable difference in transduction density compared to the *hOPN4* SNP AAVs (**Figure 5.20**).

The small number of retina with successful recording of ipRGC responses demonstrates the advantages and limitations of the MEA technique. Any ipRGC responses recorded during synaptic blockade must be from AAV-restored *hOPN4* expression and are therefore a direct measure of *hOPN4* function. However, the relatively low ipRGC recording rate and small MEA recording area also makes it difficult to identify the degree of intrinsic ipRGC restoration across the retina.

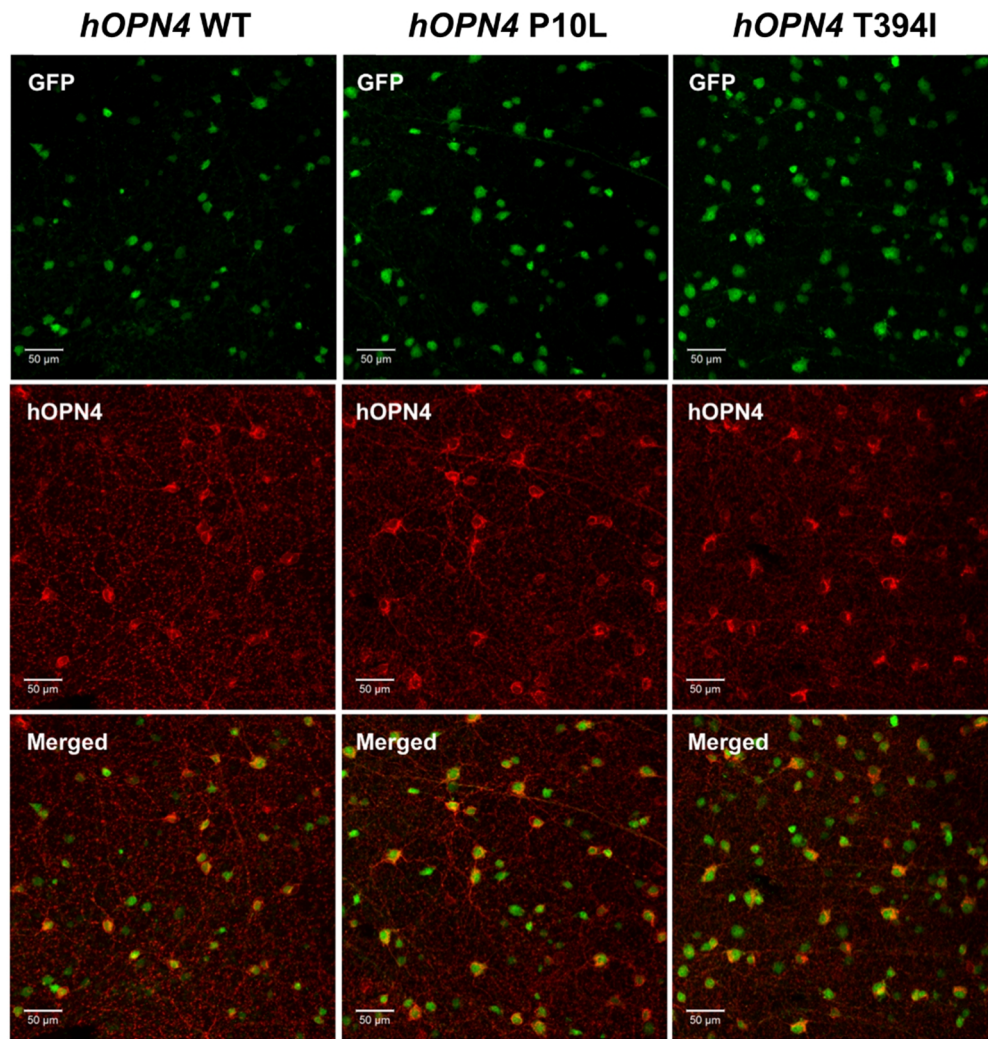


Figure 5.20. Transduction efficiency is comparable between *hOPN4* SNP and WT AAV. Immunostaining of *Opn4*^{-/-} *Cre*^{+/+} retina treated with *hOPN4* WT, P10L or T394I AAV at high titre (2×10^{12} virus particles/ml). AAV-delivered *hOPN4* (red) co-localises with eYFP-labelled ipRGCs (green). Immunostaining performed and images acquired by Dr. Steven Hughes.

5.3.3.14 Properties of ipRGC responses from *hOPN4* AAV treated retina

Where possible, the ipRGC responses to 10s 480nm light pulses of increasing intensity from 1.1×10^{11} – 10^{15} photons/cm²/s were recorded, with recovery time proportional to previous light pulse. Endogenous *11-cis* retinal in the retina was sufficient to allow responses and retinæ were not supplemented with additional chromophore. Since the amplitude of spikes varies with distance from the electrodes, spike firing rate (spikes per second) was used to characterise ipRGC responses.

As demonstrated in **Figure 5.21**, spike firing was rarely observed in the lowest intensity tested but rapidly increased after light onset at higher intensities. Stimulus intensity appeared to affect the maximum firing rate and the kinetics of the ipRGC response, with slower and more prolonged responses observed at lower intensities. These are similar to the ipRGC responses recorded from *Opn4*^{+/+} retina (Tu et al., 2005; Wong et al., 2007) suggesting *hOPN4* AAV restores normal intrinsic ipRGC behaviour. The kinetics of ipRGC spike firing were quantified by examining the baseline firing rate, time to peak firing rate and time to half-maximum firing rate.

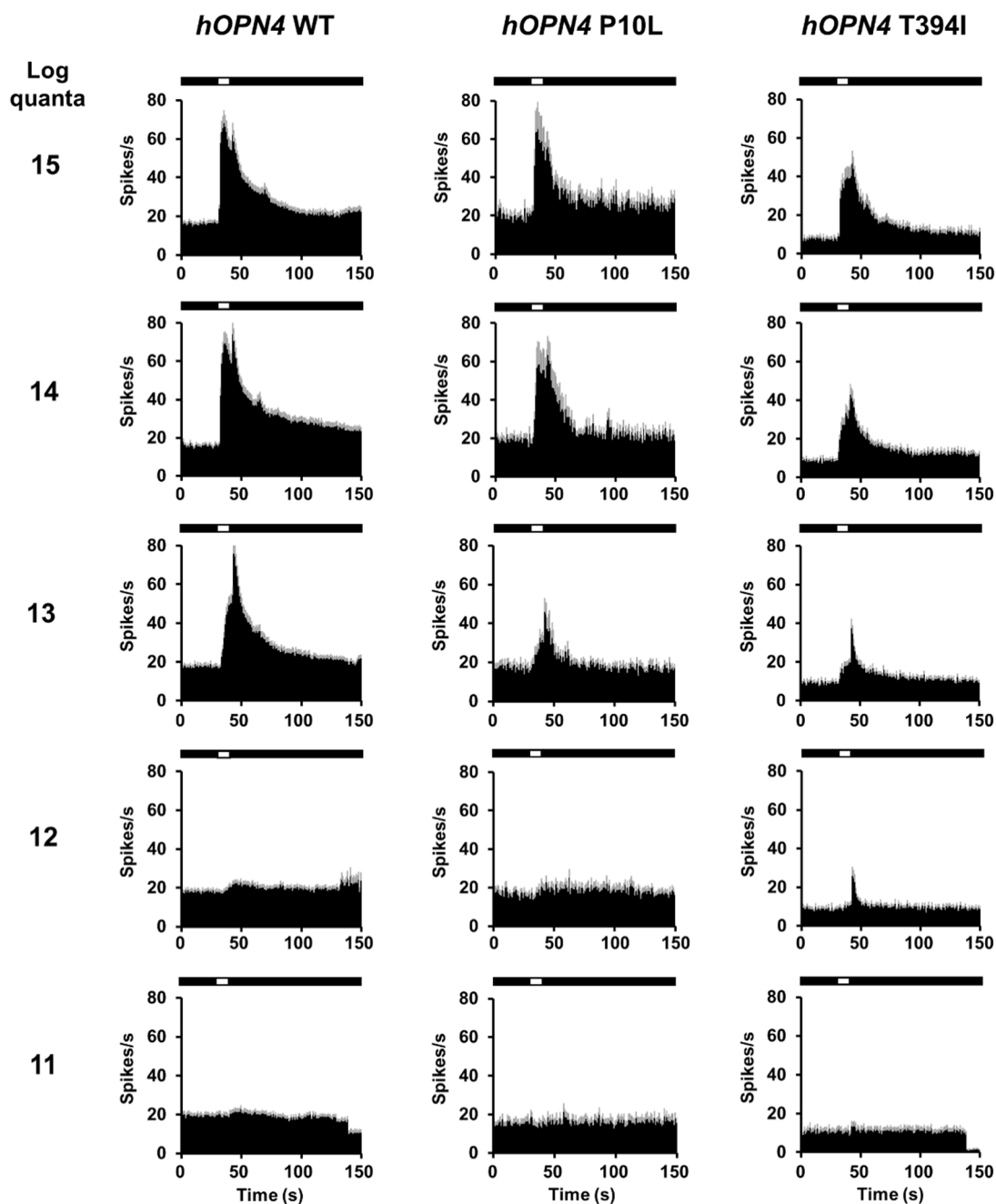


Figure 5.21. ipRGCs spike firing to a single 10s light pulse across a 5-log range of intensities. Average ipRGC spike firing during MEA recording of *Opn4^{-/-} Cre^{+/+}* retina treated with *hOPN4* WT (left), P10L (middle) and T394I (right) AAV. Retinae were exposed to a 10s 480nm light pulse (shown as white section of black bar above trace) after 30s in darkness. Light intensity was increased from 11 to 15log quanta, with increasing inter-stimulus intervals to prevent response saturation. N values: WT = 82, P10L = 12, T394I = 20. Error bars = SEM.

A Two-way mixed ANOVA with intensity as within-subjects factor (4 levels – 12, 13, 14 and 15log quanta, 11log quanta was excluded due to absence of any ipRGC response) and virus as a between-subjects factor (3 levels – *hOPN4* WT, P10L and T394I AAV) was used to explore spike firing characteristics (**Figure 5.22**). Since the assumption of sphericity was violated, a Greenhouse-Geisser correction was used to adjust degrees of freedom. Baseline firing did not vary with intensity, $F(2.6, 290.9) = 0.12$, $p = 0.935$ (**Figure 5.22B**), as expected since this occurred prior to any light exposure.

This is reassuring, as it demonstrates the baseline did not drift over the duration of recording and appropriate interstimulus intervals were selected to allow recovery from light-induced response. There was no significant interaction of virus x intensity, $F(5.2, 290.9) = 1.89$, $p = 0.093$, but a significant main effect of virus was found, $F(2, 111) = 5.39$, $p = 0.006$. A Dunnett's test revealed this was due to a significantly lower baseline firing rate in *hOPN4* T394I compared to *hOPN4* WT control ($p = 0.004$). No difference was found between *hOPN4* WT and P10L ($p = 0.964$). It is not clear how baseline firing rate could be affected by *hOPN4* T394I expression, since this should be independent of melanopsin phototransduction.

Using time to peak firing rate as a measure of response latency, a two-way mixed ANOVA, with Greenhouse Geisser correction as above, found a main effect of intensity on time to peak, $F(1.6, 179.0) = 46.05$, $p < 0.001$, caused by responses peaking significantly slower at low intensities (**Figure 5.22C**). No main effect of virus was found, $F(2, 111) = 1.21$, $p = 0.301$, but did find a significant interaction, $F(3.2, 179.0) = 4.05$, $p = 0.007$.

Eight Student's t-tests, were used to explore the significant interaction and compare *hOPN4* WT with P10L or T394I at each intensity. With Sidak correction for multiple comparisons ($\alpha = 0.0064$), *hOPN4* T394I had significantly faster to peak at low intensity light (10^{12} photons/cm²/s) than *hOPN4* WT control, $t(48.0) = 3.34$, $p = 0.002$. However, *hOPN4* T394I did not differ from *hOPN4* WT at any other intensity and no differences were found between *hOPN4* P10L and WT control, suggesting response onset was broadly comparable between both *hOPN4* SNPs and *hOPN4* WT.

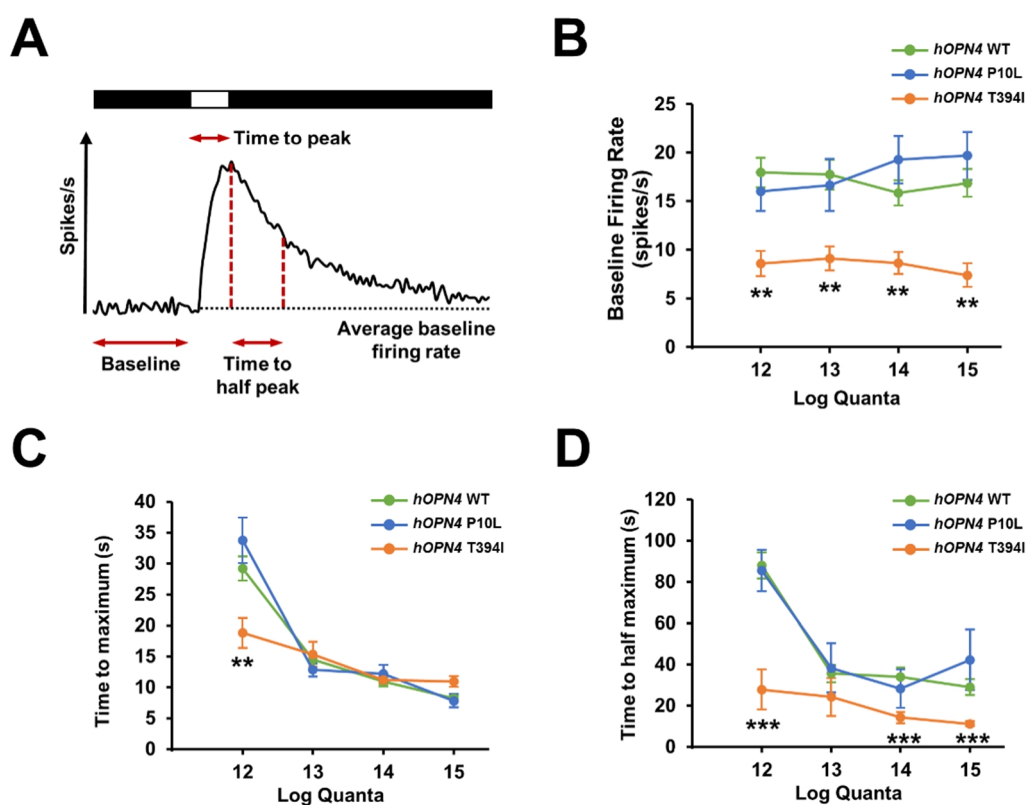


Figure 5.22. Differences in kinetics of ipRGC spike firing to 10s 480nm light pulse between *hOPN4* WT control and *hOPN4* T394I, but not *hOPN4* P10L (A) Definition of response parameters. Baseline firing rate was calculated as average spikes/s during first 30s of recording. Time to peak defined as time from light onset time to maximum firing rate. Time to half peak defined as time from peak firing rate to half-maximal firing rate. Light stimulus shown as white bar above trace **(B-D)** Average ipRGC responses recorded from *Opn4*^{-/-} *Cre*^{+/+} retina treated with *hOPN4* WT (n = 83), P10L (n = 12) and T394I (n = 20) AAV **(B)** Baseline firing rate **(C)** Time to maximum firing rate **(D)** Time to half-maximum firing rate. Error bars = SEM. ** $p < 0.01$ and *** $p < 0.001$ Sidak-corrected t-tests.

Finally time to half-maximum firing rate was used as a measure of response decay (**Figure 5.22D**). ipRGC responses tend to be highly sustained, so it was interesting to see if this was also true for spike firing rates of AAV-treated ipRGCs. Again a significant main effect of intensity was found, $F(2.3, 252.7) = 18.348$, $p < 0.001$, driven by slower response decay after low intensity light exposure compared to high intensity light. A main effect of virus was also found, $F(2,111) = 7.715$, $p = 0.001$, but a significant interaction, $F(4.6, 252.7) = 3.34$, $p = 0.008$, suggested this was probably driven by faster response decay in T394I that was not consistent across different intensities. The significant interaction was explored with 8 Student's t-tests (Sidak corrected alpha = 0.0064). Post-hoc tests revealed *hOPN4* WT did not significantly differ from *hOPN4* P10L, but *hOPN4* T394I had significantly faster response decay than *hOPN4* WT control at all intensities ($p < 0.001$) except 10^{13} photons/cm²/s.

This is intriguing as *hOPN4* T394I appears to have faster response offset to a wide range of light intensities but only relatively small changes to response onset, which are specific to low intensity light. This suggests there may be specific functional difference between *hOPN4* WT and T394I affecting response offset kinetics. It also suggests there is not a non-specific virus-related difference between *hOPN4* WT and T394I AAV. For example, if viral load was lower in *hOPN4* T394I, deficits may be expected across a range of ipRGC spike firing properties. Instead T394I appears to have relatively normal response onset kinetics but significant attenuation of response offset. This is the first indication that introduction of T394I may affect function of hOPN4 protein.

5.3.3.15 ipRGC sensitivity is significantly reduced in *hOPN4* T394I AAV-treated retina

A major advantage of MEA recording during synaptic blockade is that it allows study of melanopsin function in isolation, without confounding rod and cone signalling that occur during behavioural testing. In particular, sensitivity can be examined. Determining sensitivity is not feasible *in vivo*, where rod-cone input creates a small dynamic range between completely normal function and complete loss of Opn4 function. It is important to investigate sensitivity, as it may determine if *hOPN4* P10L or T394I cause the melanopsin protein to function inefficiently. High intensity light was used for behavioural experiments, as studies of *hOPN4* P10L and T394I in humans suggested that any effects of the SNPs were only observable at high intensities (Higuchi et al., 2013; Lee et al., 2014), but it would be prudent to establish if these intensities were actually saturating.

Sensitivity was measured by fitting a sigmoidal intensity-response curve (IRC) to the normalised maximum firing rate across a 5-log range of intensities (**Figure 5.23**) using non-linear regression by least-squares minimisation. A two-parameter model was used where slope and EC50 were allowed to vary during this process to ensure the best IRC fit to the data. More detail on non-linear curve fitting and IRC curves is provided in Section 3.2.10. To briefly recap, EC50 is the intensity producing half-maximum response derived from this IRC, which can be used as measure of sensitivity. Slope determines the steepness of the curve and the dynamic range of response.

A higher EC50 value meant lower sensitivity, as more light was required to reach half-maximum response (**Figure 5.23B**). A One-way between subjects ANOVA revealed there was a significant main effect of virus on EC50, $F(2,113) = 5.527$, $p = 0.005$. A Dunnett's post-hoc test revealed this was due to a significant decrease in sensitivity for *hOPN4* T394I (EC50 = 13.1log quanta) compared to *hOPN4* WT (EC50 = 12.7log

quanta), $p = 0.002$. There was no difference in sensitivity between *hOPN4* P10L ($EC_{50} = 12.8$) and WT, $p = 0.736$.

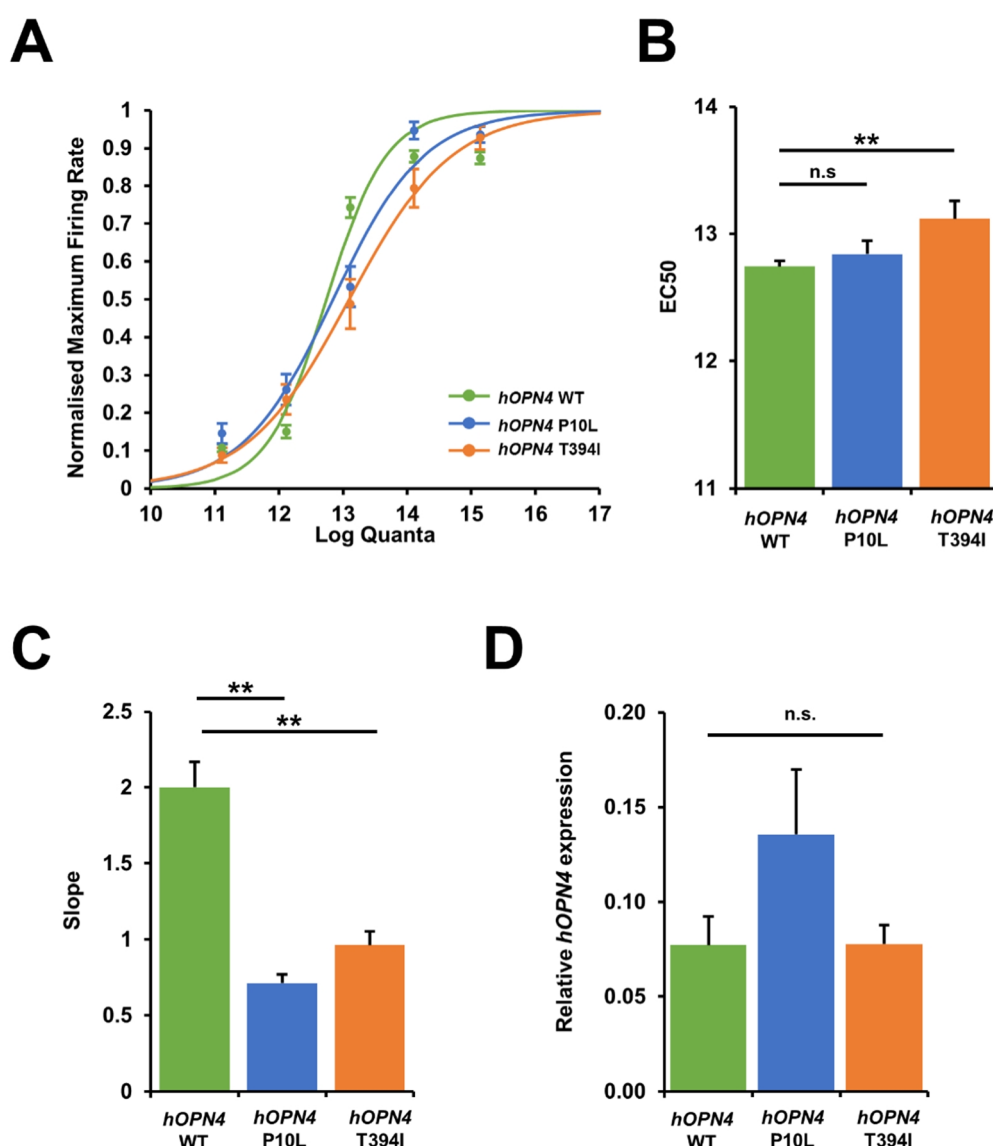


Figure 5.23. ipRGC sensitivity is significantly reduced in AAV-delivered *hOPN4* T394I compared to *hOPN4* WT control. (A) Irradiance response curves (IRC) fit to normalised maximum firing rate in response to 10s light pulse (480nm, 10^{11} - 10^{15} photons/cm²/s). ipRGC data from MEA recordings of *Opn4*^{-/-} *Cre*^{+/+} retina injected with *hOPN4* WT ($n = 82$), P10L ($n = 12$) and T394I ($n = 20$) AAV. IRCs were fit to data using non-linear regression, allowing both hill slope and EC_{50} to vary. (B) EC_{50} - the intensity which produced half-maximal response, derived from the IRC (C) Slope of best fit IRC. (D) Relative mRNA expression of *hOPN4* in *Opn4*^{-/-} *Cre*^{+/+} retina injected with *hOPN4* WT, P10L and T394I AAV. Normalised to 4 housekeeping genes (*Arbp*, *Gapdh*, *Psmb2* and *B2m*). $n = 6$ -7 retina for each group. Error bars = SEM, ** $p < 0.01$, Dunnett's Post-Hoc Test

A significant difference in slope was identified, $F(2,113) = 8.608$, $p < 0.001$ (**Figure 5.23C**), which Dunnett's post-hoc tests revealed was due to significantly steeper slopes in *hOPN4* WT compared to both *hOPN4* P10L ($p = 0.004$) and T394I ($p = 0.004$), suggesting *hOPN4* WT had a narrower dynamic range than either *hOPN4* SNP.

Sensitivity can also be affected by expression level. If less protein is available to drive a response, more light is required for normal function and responses are less sensitive. Using RT-qPCR with *hOPN4* specific primers, no significant difference was found in expression of *hOPN4* mRNA in the retina of *Opn4^{-/-} Cre^{+/+}* animals injected with *hOPN4* WT, P10L or T394I AAV, $F(2,18) = 2.295$, $p = 0.133$ (One-way Between Subjects ANOVA, **Figure 5.23D**). This implies transduction efficiency and viral load were similar between different virus groups. It also suggests that the attenuated sensitivity observed in *Opn4^{-/-} Cre^{+/+}* mice injected with *hOPN4* T394I was not due to differences in expression level. Coupled with the attenuated response offset observed in the same ipRGCs, the MEA data suggest introduction of *hOPN4* T394I affects protein function to cause abnormal intrinsic ipRGC responses to light.

5.3.3.16 Consistent ipRGC sensitivity across different AAV-treated retina

Particularly for the *hOPN4* T394I and *hOPN4* P10L AAV injected retina, the majority of ipRGC responses were derived from a single retina. Pooling ipRGCs from different retina may have accidentally weighted results towards a single retina – which might make the result more susceptible to variation from injection quality and could account for the decrease in sensitivity. If sensitivity was consistent across retina, this would suggest it was indicative of protein function, rather than a transduction or injection related confound.

Figure 5.24A-C shows the IRCs derived from the average ipRGC responses of each retina. There is very little variation in EC50 between different retina treated with *hOPN4* P10L or T394I (**Figure 5.24D**) even though the individual data points used to fit the IRC show greater variation. When the EC50 values calculated for each retinae were compared using a One-way ANOVA, it was found sensitivity was significantly different between retina treated with *hOPN4* WT AAV, $F(2,81) = 7.08$, $p = 0.001$. A Post-hoc Tukey's HSD test revealed this difference was between retina 2 and 3 ($p = 0.001$) no other comparison was significant (1 vs 2, $p = 0.547$; 1 vs 3, $p = 0.172$). Independent samples t-tests showed there was no significant difference in EC50 for P10L, $t(10) = 0.235$, $p = 0.819$, or T394I, $t(18) = 0.415$, $p = 0.683$.

Sensitivity of *hOPN4* T394I ipRGCs was consistently low, as demonstrated by higher EC50 values, for both retina from which ipRGC responses were recorded. Sensitivity was slightly more variable between retinae treated with *hOPN4* WT AAV, with one WT retina demonstrating significantly higher sensitivity compared to one other retina. Given the relatively high numbers of ipRGC responses recorded in *hOPN4* WT treated retinas, it seems unlikely that the average ipRGC sensitivity of *hOPN4* WT has been unfairly biased by variation in one retina.

This suggests the decreased sensitivity observed for *hOPN4* T394I was not variation from *hOPN4* AAV technique. It also suggests that a low number of ipRGCs per retina does not necessarily reflect poor injection quality, as the ipRGC sensitivity appears consistent regardless of number of ipRGC responses per retina.

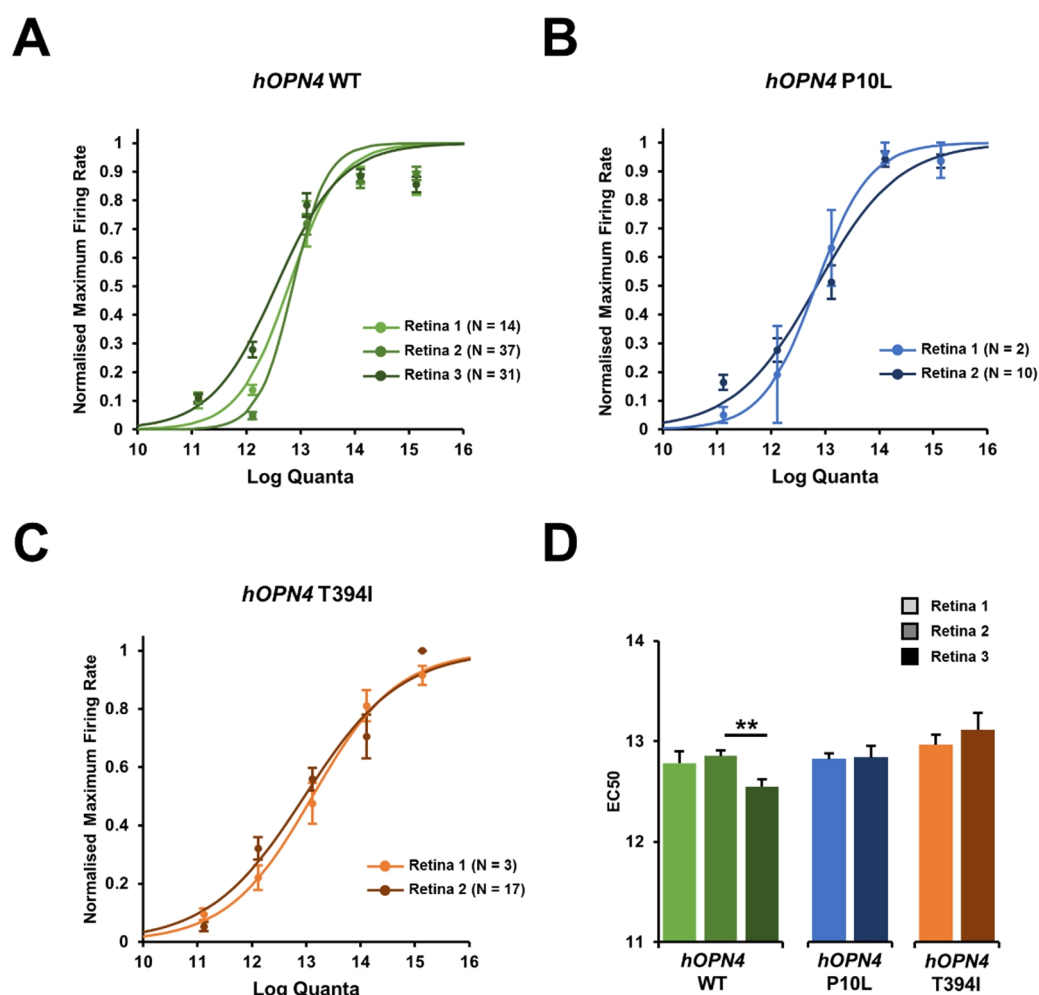


Figure 5.24. Sensitivity is consistent between retina treated with same AAV, despite different frequency of ipRGC responses (A-C) Irradiance response curves (IRC) fit to normalised maximum firing rate in response to 10s light pulse (480nm, 10^{11} - 10^{15} photons/cm²/s). IRC fit to data from individual *Opn4*^{-/-} *Cre*^{+/+} retina treated with (A) *hOPN4* WT, (B) P10L and (C) T394I AAV. Individual retina data calculated from average of all ipRGC responses for a given retina (n values for ipRGC of each retina given in parenthesis). (D) Comparison of EC50 calculated for individual retina for each *hOPN4* AAV. Error bars = SEM. ** p < 0.01, Tukey's HSD Post-Hoc test.

5.3.3.17 Light adaptation of *hOPN4* AAV-treated retina

Another important response property of ipRGCs is light adaptation. This is the ability to adjust sensitivity of a response depending on previous light exposure, in order to remain responsive to a range of light intensities greater than the dynamic range of the response. Wong et al. (2005) used a paired flash experiment to demonstrate that *Opn4*^{+/+} ipRGCs show desensitisation to repeated light exposure, indicating light adaptation, and that light adaptation is able to persist throughout short periods of darkness between light pulses. A repeated light pulse paradigm was used to examine light adaptation of *hOPN4*-AAV retina. Retina with robust ipRGC responses, were exposed to ten high intensity light pulses (480nm, 10^{15} photons/cm²/s), 10s duration, every 50s (**Figure 5.25A-C**).

The responses to the first and second light pulses were compared to determine if there was any change in sensitivity after light exposure, demonstrated as a decrease in response amplitude. Response amplitude is measured as the difference between baseline firing rate before light and maximum spike firing rate after light onset. Therefore it was necessary to establish whether baseline firing rate changed with additional light pulses, before response amplitude could be examined.

Using a Two-way mixed ANOVA, with light pulse number as a within-subjects factor (2 levels – first and second light pulse) and virus as a between subjects factor (3 levels – *hOPN4* WT, P10L and T394I), the average baseline firing rate in the 10s before each light onset was assessed (**Figure 5.25D**). Firing rate significantly increased for the second light pulse, $F(1,107) = 13.69$, $p < 0.001$, suggesting the ipRGC response to the first light pulse was not complete by the start of the second light pulse.

A significant main effect of virus was also found, $F(2,107) = 4.268$, $p = 0.016$, which a Dunnett's post-hoc test revealed was due to significantly higher firing rate in *hOPN4* T394I compared to *hOPN4* WT control ($p = 0.009$). This is opposite direction of the baseline firing difference previously found between *hOPN4* T394I and *hOPN4* WT (**Figure 5.22B**). No difference was found between *hOPN4* P10L and WT control. No interaction of virus by light pulse was found, $F(2,107) = 1.46$, $p = 0.236$, suggesting all three *hOPN4* AAV groups were still responding to first light pulse by start of the second light pulse.

Having established that firing rate did not return to baseline before second light pulse, response amplitude was examined (**Figure 5.25E**). Using the same Two-way mixed ANOVA described above, response size was significantly smaller to the second light pulse, $F(1,107) = 39.62$, $p < 0.001$, indicating that all three *hOPN4* AAV retinæ were desensitised by the first light pulse, suggesting they were able to adapt to the light. No main effect of virus was found, $F(2,107) = 1.89$, $p = 0.156$, and no virus by light pulse interaction, $F(2,107) = 1.23$, $p = 0.296$. This suggest that adaptation was comparable between the three *hOPN4* AAV groups. To confirm this, responses to the second light pulse normalised to the first light pulse (**Figure 5.25F**) were compared using a One-way between subjects ANOVA, and no significant difference was found between the three groups, $F(2,109) = 0.24$, $p = 0.790$.

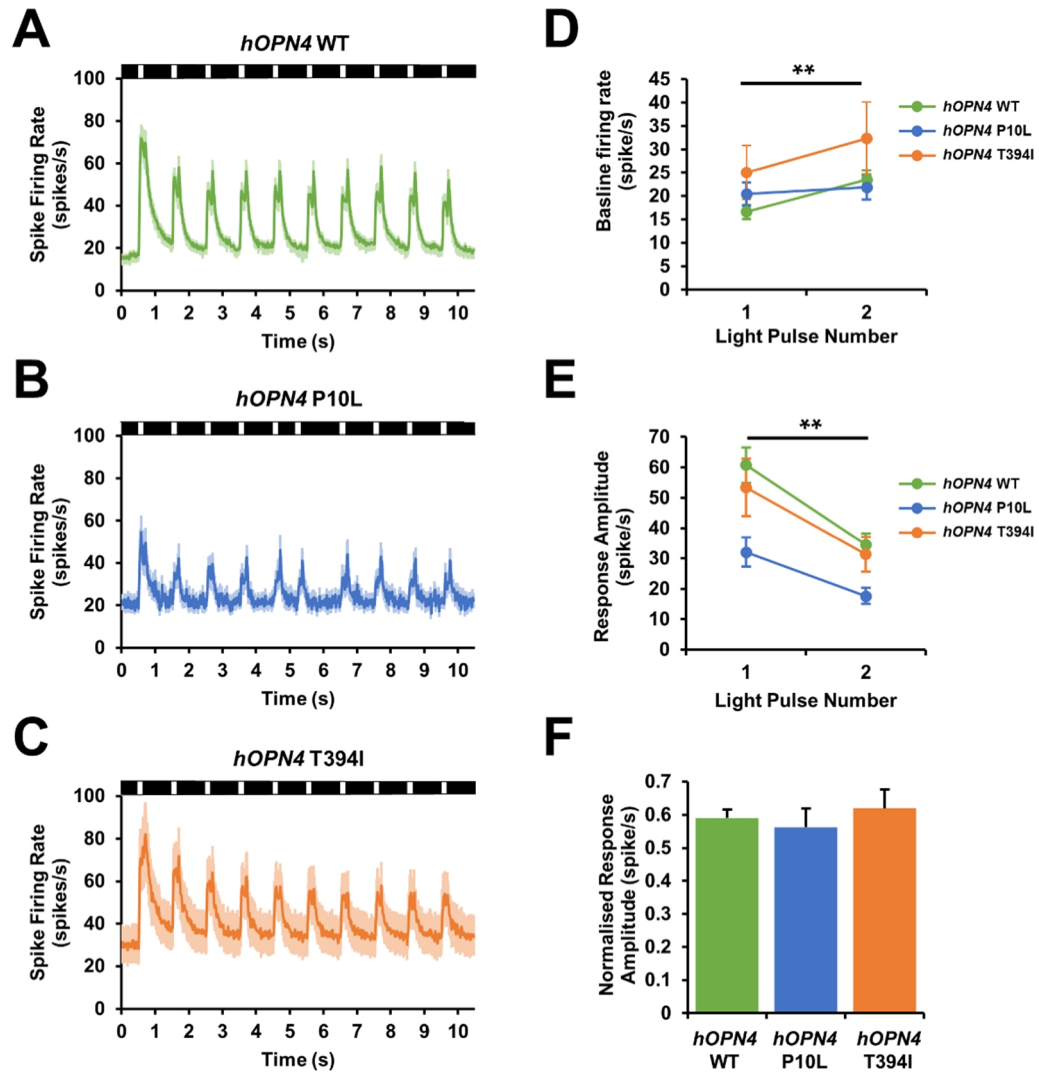


Figure 5.25. *hOPN4* WT, P10L and AAV treated retina show light adaptation to repeated light pulses (A-C) ipRGC responses to 10x 10s 480nm light pulses (10^{15} photons/cm²/s) from retina treated with *hOPN4* WT, P10L and T394I AAV. Retinae were exposed to light every 50s (6th light pulse for *hOPN4* P10L was given 20s early). **(D)** Baseline firing rate, defined as average firing rate in 10s before light onset, for first and second light pulse. **(E)** Response amplitude defined as maximum increase in firing rate from baseline after light onset for first and second light pulse. **(F)** Response amplitude normalised to first light pulse. *hOPN4* WT (n = 82 from 3 retina), P10L (n = 12 from 1 retina), T394I (n = 16 from 2 retina). Error bars = SEM. ** p < 0.01, Main effect of light pulse number, Two-way mixed ANOVA.

It was important to investigate whether the changes in response amplitude and firing rate before light pulse continued with additional light pulses or if they plateaued after a certain number of light pulses. This was to confirm the decrease in response size represented a genuine decrease in sensitivity and not response compression, where bright light limits ability to encode further light intensity increases (Wong et al., 2005), for example by reducing the amount of retinal chromophore available for phototransduction. If response size continued to decrease with additional light pulses, this might suggest that the ipRGC responses were compressed, whereas if response size reached a plateau, this would imply the ipRGCs were genuinely adapting to the light.

The baseline firing rate of *hOPN4* WT, P10L and T394I AAV appeared to be relatively consistent with additional light pulses. To confirm this, the gradient of a linear trendline fit to baseline firing data was assessed (**Figure 5.26A**), using 3 One-sample t-tests to determine if the gradient was significantly different from zero (flat line indicates no trend in baseline firing over time). A Sidak correction for multiple comparisons was used ($\alpha = 0.017$). Only *hOPN4* T394I demonstrated a significant negative trend over time, $t(15) = -3.00$, $p = 0.009$. Neither *hOPN4* WT, $t(81) = 0.92$, $p = 0.359$ or P10L, $t(11) = 0.13$, $p = 0.902$, had gradients significantly different from zero. This is consistent with the previous finding that T394I had faster response decay compared to WT (**Figure 5.22D**), making responses from T394I-treated ipRGCs more likely to be complete by next light pulse.

The baseline firing gradient was also compared between the three *hOPN4* AAV-treatment groups (**Figure 5.26B**), using a One-way between subject's ANOVA. No main effect of virus was found, $F(2,109) = 0.614$, $p = 0.543$, suggesting that although *hOPN4* T394I has a small but significant trend to decrease baseline firing rate with additional light pulses, this was not significantly different from *hOPN4* WT control.

Repeating the linear trendline approach for normalised response size (**Figure 5.26C**), only *hOPN4* WT, $t(81) = -3.13$, $p = 0.002$ had significant negative gradient for response size. The gradient of *hOPN4* P10L, $t(11) = -0.67$, $p = 0.519$, and T394I, $t(15) = -2.43$, $p = 0.028$, did not significantly differ from zero. When the three *hOPN4* AAV groups were compared with a One-way between subjects ANOVA (**Figure 5.26D**), a significant main effect of virus was found, $F(2,109) = 3.73$, $p = 0.284$, but post-hoc Dunnett's test revealed that neither *hOPN4* P10L ($p = 0.838$) or *hOPN4* T394I ($p = 0.292$) was significantly different from *hOPN4* WT control, suggesting the main effect was driven by a difference between *hOPN4* T394I and P10L.

To summarise, baseline firing rate increased and response size decreased significantly for second light pulse compared to the first light pulse. For any subsequent light pulses, baseline firing rate and response size remained relatively constant. This suggests that the observed decrease in sensitivity is due to light adaptation rather than other physiological mechanisms, such as photobleaching.

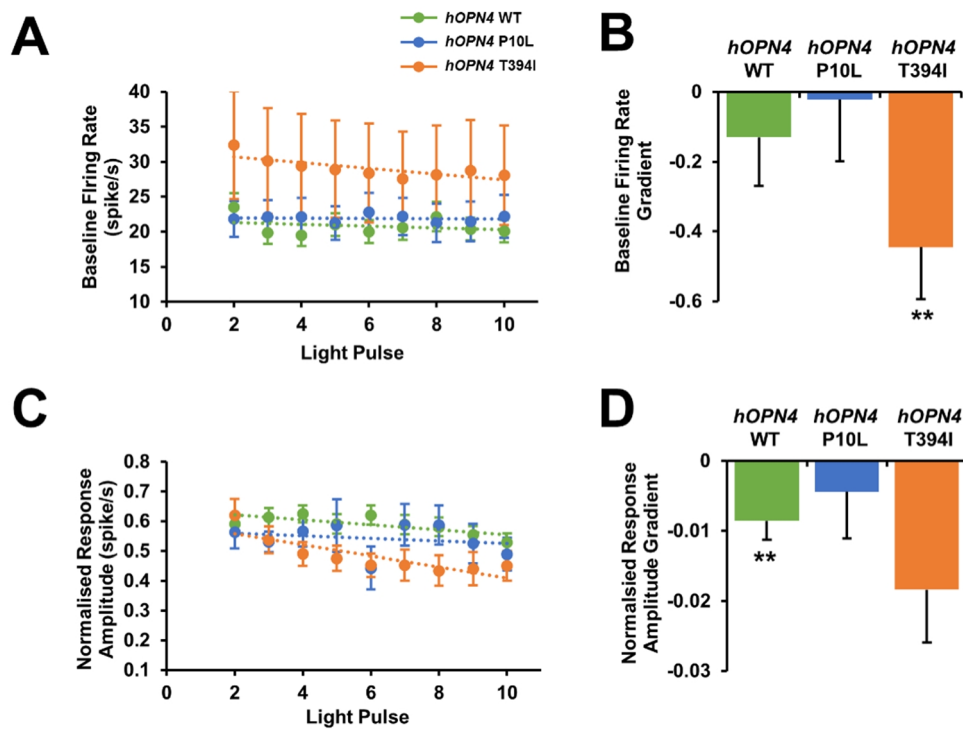


Figure 5.26. Light adaptation to repeated light pulses is not consistent between *hOPN4* AAV WT, P10L and T394I. 480nm light pulses (10^{15} photons/cm²/s) were given every 50s, 10s duration. **(A)** Baseline firing rate, defined as average firing rate in 10s before light onset, for 2nd to 10th light pulse. Dotted line shows average linear trendline. **(B)** Average gradient of linear trendline fit to individual ipRGC baseline firing data for light pulse 2-10. **(C)** Response amplitude normalised to first light pulse, defined as maximum increase in firing rate from baseline, for 2nd to 10th light pulse. Dotted line shows average linear trendline. **(D)** Average gradient of linear trendline fit to individual ipRGC response amplitude for light pulse 2-10. *hOPN4* WT (n = 82 from 3 retina), P10L (n = 12 from 1 retina), T394I (n = 16 from 2 retina). Error bars = SEM, ** p < 0.01, One-sample t-test compared to 0.

5.3.3.18 Comparison of ipRGC spike firing in *Opn4*^{-/-} *Cre*^{+/+} retina treated with *hOPN4* WT AAV and *Opn4*^{+/+} retina

Having compared the ipRGC responses of *hOPN4* AAV treated retina, MEA data from 4 *Opn4*^{+/+} retina with ipRGC responses were examined. This data was collected by Dr. Steven Hughes, under the same conditions as the *hOPN4* AAV MEA recordings. By comparing ipRGC responses of *Opn4*^{+/+} retina with *hOPN4* WT AAV-treated *Opn4*^{-/-} *Cre*^{+/+} retina, the virally-restored ipRGC activity could be compared with physiological normal ipRGC activity. This would contribute to the assessment of *hOPN4* AAV treated *Opn4*^{-/-} *Cre*^{+/+} mice as a model for screening melanopsin mutations.

As shown in **Figure 5.27A-D**, sensitivity of *hOPN4* WT ipRGCs (EC₅₀ – 12.7log quanta) was significantly higher than *Opn4*^{+/+} ipRGCs (EC₅₀ – 13.1log quanta), $t(153.0) = -4.37$, $p < 0.001$ (Independent samples student's t-test, equal variance not assumed). The IRC fits for *hOPN4* WT and *Opn4*^{+/+} did not differ by slope, $t(154.5) = 1.83$, $p = 0.069$, or R^2 , $t(150.0) = 1.31$, $p = 0.192$. The increased sensitivity observed in AAV-treated retina is likely associated with the higher expression level of *hOPN4* protein compared to the naturally occurring levels found in mice, as previously demonstrated using qPCR (**Figure 5.8A**). It is worth noting that *hOPN4* AAV-treated mice are more sensitive, since this means they will saturate at lower light intensities than previously described for *Opn4*^{+/+}. This may help explain why some behaviour looks different between the AAV-treated mice and the literature on *Opn4*^{+/+} mice

The spike firing properties of *Opn4*^{+/+} retinæ were compared to *hOPN4* WT AAV-treated retina across a range of intensities, using a two-way mixed ANOVA, with genotype as a between subjects factor (2 levels – *Opn4*^{+/+} and *hOPN4* WT AAV) and intensity as a within subjects factor (4 levels – 12, 13 14 and 15log quanta), using a Greenhouse-Geisser correction to adjust for unequal variance, to examine baseline firing rate, time to peak firing rate and time to half-maximum firing rate.

First, baseline firing was examined (**Figure 5.27E**). Significant main effects of genotype, $F(1,158) = 13.55$, $p < 0.001$, and intensity, $F(2.8, 443.4) = 3.94$, $p = 0.004$, were found, as well as a significant interaction, $F(2.8, 443.4) = 6.603$, $p < 0.001$, which indicated the differences in intensity and genotype were not consistent across each factor. The significant interaction was explored with 4 Students t-tests, with Sidak correction for multiple comparisons ($\alpha = 0.013$). *Opn4*^{+/+} had significantly higher baseline firing rate than *hOPN4* WT AAV at 15, 14 and 12 log quanta (all $p < 0.001$) but not 13 log quanta ($p = 0.103$) due to a decrease in *Opn4*^{+/+} baseline firing at this intensity. This also suggests that baseline firing was relatively consistent across intensities as expected for both AAV-treated and *Opn4*^{+/+} ipRGCs. It is not clear why baseline firing would be different in AAV-treated retina since baseline firing should be independent of OPN4 phototransduction.

Latency to peak firing rate was examined (**Figure 5.27G**) using the same two-way ANOVA as above. A significant main effect of intensity was found, $F(1.7, 269.9) = 110.17$, $p < 0.001$, with shorter latency to peak firing at higher intensities, as anticipated (Walch et al., 2015), but no main effect of virus, $F(1,158) = 2.81$, $p = 0.095$, or intensity by genotype interaction, $F(1.7, 269.9) = 1.098$, $p = 0.327$. This implies that despite differences in sensitivity, both *Opn4*^{+/+} and *hOPN4* WT AAV ipRGCs were able to respond to light stimuli at similar speeds across a range of light intensities. This suggests the *hOPN4*-AAV treated ipRGCs are able to replicate the response onset kinetics of *Opn4*^{+/+} ipRGCs and are a good model for normal ipRGC behaviour.

Using time to half-maximum as a measure of response decay (**Figure 5.27H**), significant main effects of intensity, $F(2.1, 336.8) = 66.52$, $p < 0.001$, and genotype, $F(1,158) = 9.52$, $p = 0.002$, were found, which indicated both groups had slower time to half-maximum firing rate at lower intensities and that *Opn4*^{+/+} had a slower time to half peak than *hOPN4* WT AAV. Although there was no interaction of intensity by genotype, $F(2.1, 336.8) = 2.22$, $p = 0.085$, 4 Student's t-tests with Sidak correction for multiple comparisons ($\alpha = 0.013$), demonstrated that time to half-maximum was only different between *Opn4*^{+/+} and *hOPN4* WT AAV at the three highest intensities, 13-15log quanta ($p < 0.01$). This could indicate *Opn4*^{+/+} ipRGCs sustain high firing rates for longer than *hOPN4* WT AAV ipRGCs. However, the AAV-treated ipRGCs still possess the same intensity-dependent trend as *Opn4*^{+/+} ipRGCs, suggesting they are also a good model of response offset kinetics.

Finally, although this study has focused on average ipRGC responses to compare *Opn4*^{+/+} and *hOPN4* WT AAV, it is worth noting different ipRGC subtypes exhibit heterogeneous spike firing properties in adult *Opn4*^{+/+} retina (Reifler et al., 2015; Tu et al., 2006; Walch et al., 2015). This diversity of ipRGC responses in *Opn4*^{-/-} *Cre*^{+/+} retina treated with *hOPN4* WT AAV is discussed further in **Appendix 4**.

To summarise, *hOPN4* WT AAV-restored ipRGCs have broadly similar response characteristics to *Opn4*^{+/+} ipRGCs. They maintain constant baseline firing rate throughout recording, as well as demonstrating intensity-dependent response onset and offset kinetics comparable with *Opn4*^{+/+} ipRGCs. The virally restored ipRGCs do show modest differences in sensitivity and latency to half-maximum firing rate compared to ipRGC responses driven by native mouse melanopsin. These differences could be artefacts of high expression level of AAV-restored *hOPN4* or because different species are being compared— native mouse and AAV-delivered human. Overall *hOPN4* WT AAV appears to be a good model of native ipRGC activity, with the potential to mimic *Opn4*^{+/+} spike firing even closer if expression level could be more controlled.

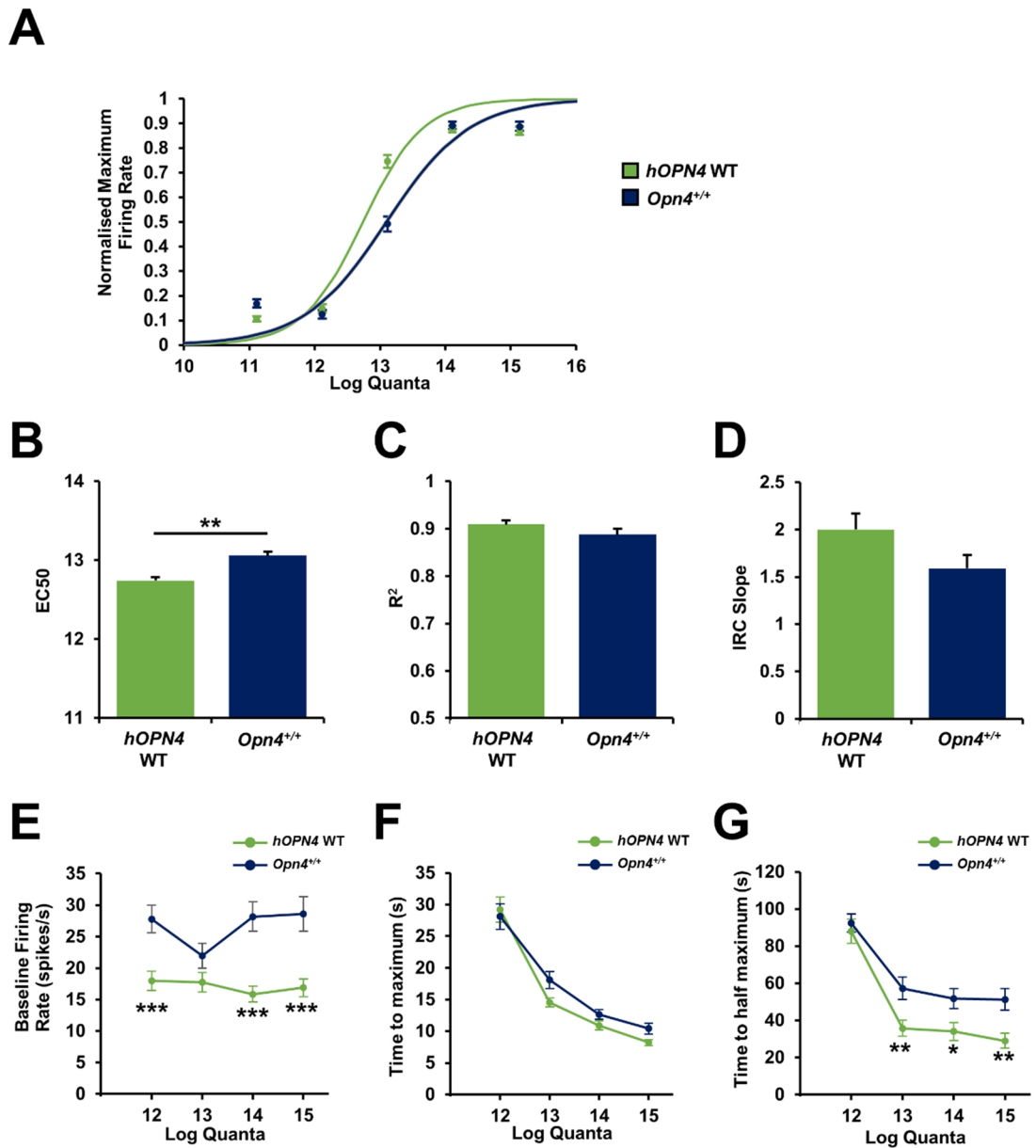


Figure 5.27. Comparison of ipRGC responses from retina of *Opn4*^{-/-} *Cre*^{+/+} treated with *hOPN4* WT AAV and *Opn4*^{+/+} retina. (A) Irradiance response curves (IRCs) fit to data from all responsive electrodes pooled from retina of *Opn4*^{-/-} *Cre*^{+/+} treated with *hOPN4* WT AAV (n = 82) and *Opn4*^{+/+} retina without injection (n = 78). Average (B) EC50, (C) R² and (D) Slope of best fitting IRC for pooled responsive electrodes. Average (E) Baseline firing rate, (F) Time to maximum firing rate and (G) Time to half-maximum firing rate for pooled responsive electrodes. Error bars = SEM. * p < 0.05, ** p < 0.01 and *** p < 0.001, Sidak-Corrected t-tests.

5.4 Discussion

This study used ipRGC-specific delivery of *hOPN4* AAV to *Opn4^{-/-} Cre^{+/+}* mice to model the effects of *hOPN4* SNPs on a range of melanopsin-associated behaviours. Since this was a novel approach, the *hOPN4*-AAV technique was first validated to determine whether it was able to replicate an *Opn4^{+/+}* phenotype to *Opn4^{-/-} Cre^{+/+}* mice. As demonstrated by immunohistochemistry and RT-qPCR, *hOPN4* AAV is successfully transduced and *hOPN4* protein synthesised in the mouse retina (**Figure 5.6**). As shown in **Figure 5.7**, AAV-delivered *hOPN4* was functional and capable of driving a melanopsin phototransduction cascade, leading to restoration of an *Opn4^{+/+}* behavioural phenotype and intrinsic ipRGC spike firing in *Opn4^{-/-} Cre^{+/+}* mice. These characteristics, such as maximum constriction and PIPR, appear to be restored to normal physiological levels, based on comparison with *Opn4^{+/+}* mice.

Furthermore, *hOPN4* AAV was able to restore a wildtype phenotype to a wide range of melanopsin-associated behaviours. In addition to PLR, increases in phase shifting and total activity level were observed after injection to levels, behaviours known to be attenuated in *Opn4^{-/-}* mice (Hattar et al., 2003; Panda et al., 2002; Ruby et al., 2002). These NIF behaviours were restored to *Opn4^{+/+}* levels. This indicates *hOPN4* is expressed at sufficient levels in a variety of ipRGCs subtypes to restore melanopsin light input to the different brain areas that drive NIF responses, such as OPN for pupillometry and SCN for circadian entrainment, as discussed in Chapter 1.

Overall, ipRGC-specific expression of *hOPN4* in *Opn4^{-/-} Cre^{+/+}* mice appears to be a good model for normal native melanopsin function, suggesting the technique is valid for modelling the effects of *hOPN4* SNPs. If *hOPN4* WT expression in mouse retina is fully functional, amino acid substitutions which disrupt *hOPN4* protein function should cause observable changes to melanopsin-dependent behaviour when expressed in mouse retina using targeted-AAV.

There are some limitations using this targeted-AAV model for screening *hOPN4* function. First, these mice have genetically ablated melanopsin and therefore have never had melanopsin expression. The role of *OPN4* during development is still unclear. Recent papers have suggested *Opn4*^{-/-} mice have disrupted retinal blood vessel development (Rao et al., 2013), which viral restoration would be unable to rectify. If there are additional roles for melanopsin in development that cannot be sustained by rod-cone light input, this could limit the ability to *hOPN4* AAV to restore a normal behavioural phenotype.

Second, the *hOPN4*-AAV model relies on viral restoration, which can only approximate normal physiology. Without an *Opn4* promotor, normal regulation of protein is absent, as are rhythms in protein expression. Using a ubiquitous EF1α promotor and high viral concentration leads to overexpression of *hOPN4* in the retina, based on comparison with *Opn4*^{+/+} mice by RT-qPCR. As EF1α drives high expression in all transduced ipRGCs, some of the diversity of expression level between subtypes (Schmidt et al., 2011) is lost in the *hOPN4* AAV model. These limitations highlight the importance of using *Opn4*^{-/-} *Cre*^{+/+} mice treated with *hOPN4* WT AAV as a control group, rather than *Opn4*^{+/+} mice, as this allows examination of the effect of SNPs on protein function in an over-expressing model, while controlling for confounds related to the *hOPN4* AAV technique.

The second aim of the study was to assess the impact of *hOPN4* SNPs, P10L and T394I on *hOPN4* protein function. Several studies have described associations between individuals with *hOPN4* SNPs, P10L and T394I, and a variety of NIF behaviours, including PLR and sleep-wake timing (Higuchi et al., 2013; Lee et al., 2013, 2014; Roecklein et al., 2009, 2012, 2013). Since the abnormal behaviours in these individuals are partially driven by melanopsin, the implication is the presence of these *hOPN4* SNPs disrupts the function of the *hOPN4* protein. Using the *hOPN4* AAV model, the melanopsin-driven responses of *hOPN4* WT could be directly compared with *hOPN4*

P10L and T394I, to determine if these SNPs cause the behavioural disturbances described above.

Both *hOPN4* P10L and T394I AAV appeared to restore a normal wildtype phenotype to *Opn4^{-/-} Cre^{+/+}* mice, comparable to *hOPN4* WT AAV. The behavioural disruption associated with P10L and T394I in humans was not observed in this study, either by directly replicating the study, such as PLR under bright blue light (Lee et al., 2013), or using analogous tests to assess similar behaviour in mice, such as the PIR circadian screen.

Indeed, no differences were identified between *hOPN4* SNPs and WT control in any known melanopsin-related behaviour. Where behavioural differences were identified between *hOPN4* WT and SNPs, such as longer activity bouts in T394I and more frequent dark phase sleep bouts in P10L, it was difficult to determine whether these were genuine effects of variation, since these behaviours are not necessarily mediated by melanopsin, and are potentially false positives. For both *hOPN4* P10L and T394I, the results of behavioural suggest neither SNP impacts grossly upon NIF responses to light.

Sensitivity, response kinetics and adaptation to repeated light pulses were all comparable between ipRGCs expressing *hOPN4* P10L and WT, confirming this SNP does not affect melanopsin function. Combined with the lack of a behavioural phenotype in *Opn4^{-/-} Cre^{+/+}* mice treated with *hOPN4* P10L AAV, this raises the possibility that the results in the original human SNP studies examining P10L were false positives. Given the genetic heterogeneity and difficulty controlling for confounding factors in a complex multi-factorial disease, such as SAD, this is not unlikely.

Alternatively, the association of P10L with SAD and abnormal seasonality by chronotype interactions could be due to genetic factors in linkage disequilibrium with P10L. Another SNP may cause the phenotype, but segregation of this SNP with P10L could lead P10L to appear with higher frequency in individuals with the phenotype. Since there was no

specific melanopsin-associated phenotype seen in individuals with P10L, unlike the deficits in PLR seen in people with T394I, it is perhaps not surprising that introduction of this SNP has no impact on melanopsin function.

In comparison, *hOPN4* T394I demonstrated abnormal ipRGC responses, suggesting lack of behavioural phenotype was due to rod-cone input or the high expression levels of AAV-delivered *hOPN4* compensating for partial loss of melanopsin function. ipRGCs from *hOPN4* T394I AAV treated retina had faster response decay and attenuated sensitivity compared to *hOPN4* WT AAV treated ipRGCs. These responses do not appear to be related to general loss of function, since T394I ipRGCs have normal response onset kinetics and light adaptation. This would imply a specific change in *hOPN4* protein function after introduction of T394I.

It is difficult to determine how T394I affects OPN4 protein function. *hOPN4* T394I is functional and correctly trafficked to the plasma membrane of ipRGCs, so it is unlikely the protein structure has been misfolded (**Figure 5.20**). The equivalent position of T394 has been proposed as one of several phosphorylation sites in the C-terminus of mouse melanopsin necessary for arrestin binding and deactivation of mouse melanopsin (Blasic et al., 2014; Cameron & Robinson, 2014; Mure et al., 2009; 2016), which might explain the abnormal response decay kinetics observed during MEA recordings of T394I. However, mutagenesis studies suggest substitution of individual phosphorylation sites is insufficient to disrupt melanopsin activity (Blasic et al., 2014; Mure et al., 2009b). Therefore, it is unclear whether loss of single phosphorylation site after introduction of T394I could produce the phenotype described in the literature and shown by ipRGCs treated with *hOPN4* T394I AAV.

Alternatively, T394I could be affecting the stability of the protein as it transitions between active and inactive states during photoisomerisation and deactivation. Emanuel & Do (2015) suggest the equilibrium between these different states is necessary for the unique sustained signalling and temporal integration exhibited by melanopsin. If T394I

interfered with this, it could alter melanopsin sensitivity and response kinetics. However, the position of T394I in the C-terminus makes this improbable, as opsin activation appears to be predominantly controlled by residues in the transmembrane domain interacting with the retinal chromophore (Palczewski et al., 2000).

The viral titre experiments offer a third possible explanation. It appears reducing the viral load of *hOPN4* significantly affects the restoration of PLR, indicating there may be a minimum number of functional ipRGCs or *Opn4* expression level required to sustain normal function. Studies of mice with targeted ablation of ipRGCs using diphtheria toxin show even a small number of surviving ipRGCs is sufficient to sustain PLR (Güler et al., 2008; Hatori et al., 2008). This suggests restoration of phenotype may depend on *Opn4* expression level. If so, attenuated expression of *Opn4* might account for the association between *hOPN4* SNPs and abnormal NIF behaviours, rather than disruption to the structure-function relationship of OPN4. However, this wouldn't necessarily explain the attenuated sensitivity in T394I, as *hOPN4* T394I appears to be expressed at similar levels to *hOPN4* WT AAV in *Opn4*^{-/-} *Cre*^{+/+} retina (see **Figure 5.20** and **4.23D**).

Whatever the cause, the results of this study raise some interesting questions about the capacity for minor loss of *Opn4* function to cause the behavioural change described in Human OPN4 SNP studies. The non-degenerate *Opn4*^{-/-} *Cre*^{+/+} mouse were deliberately selected as a better model of individuals described in Human OPN4 SNP studies. Despite abnormal ipRGC responses after introduction of *hOPN4* T394I AAV, no changes in behavioural phenotype were observed. This suggests only severely deleterious *Opn4* mutations produce measurable change in NIF responses to light if compensatory light input from rods and cones via ipRGCs is available.

If the *hOPN4* AAV technique were used in the future to model *hOPN4* SNPs, it would be wise to assess SNPs in a retinally-degenerate *Opn4*^{-/-} *Cre*^{+/+} mouse to determine if SNPs alter melanopsin function isolated from rod-cone input, as well as screening the same

SNP in a non-degenerate *Opn4*^{-/-} *Cre*^{+/+} mouse to determine if this loss of function translates into a noticeable phenotype in presence of rods and cones.

In conclusion, ipRGC-specific delivery of *hOPN4* AAV was used to model the effects of *hOPN4* SNPs, P10L and T394I on melanopsin protein function. The *hOPN4* AAV model was validated to confirm it was able to restore a wide range of melanopsin-dependent behaviours to *Opn4*^{-/-} *Cre*^{+/+} mice. The behavioural phenotypes associated with either *hOPN4* T394I or P10L were not observed, contradicting the literature on these SNPs in humans. However, small but significant changes to the sensitivity and response kinetics of ipRGCs treated with *hOPN4* T394I were identified, suggesting presence of this SNP disrupts protein function by some mechanism. Whether this is due to abnormal structure-function relationships, protein stability or expression levels is unclear. This study also highlights the importance of investigating *hOPN4* SNPs in isolation, as well as in the intact retina with rod-cone input, to generate a realistic representation of the impact they may have on behaviour.

Chapter 6

Discussion

6.1 Summary of Results

The aim of this thesis was to characterise key functional residues in the photopigment melanopsin. Using a range of techniques, the impact of induced and naturally-occurring non-synonymous substitutions on melanopsin function was examined, and several *Opn4* mutants with abnormal phenotypes were identified, as shown in **Figure 6.1**.

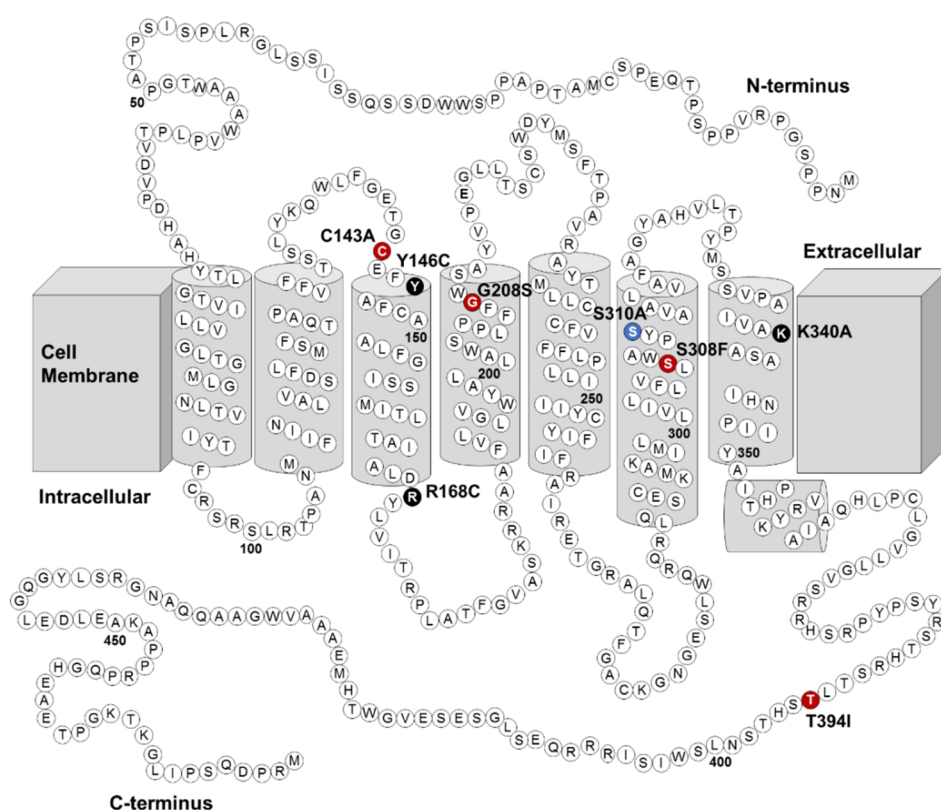


Figure 6.1. Key functional residues in melanopsin. Sites highlighted in black are those identified in this study which are critical for melanopsin function and mutation of these residues causes complete loss of function. Sites highlighted in red are those identified in this study necessary for normal melanopsin function. Substitution of amino acids at these sites disrupts melanopsin activity, attenuating responses to light or changing response kinetics. *Opn4* S310A (highlighted in blue) is involved in melanopsin spectral sensitivity.

First, a novel missense mutation was identified, *Opn4* S310A, which occurs at a conserved opsin spectral tuning site. This mutation leads to loss of sensitivity specifically at long wavelengths, suggesting it causes a blue-shift in melanopsin spectral sensitivity and acts as a spectral tuning site. This is the first exploration of melanopsin spectral tuning sites and the first *in vivo* characterisation of a melanopsin point mutation in the transmembrane domain.

Four naturally-occurring human melanopsin mutations were then identified, which cause robust loss-of-function *in vitro*. Introduction of Y146C and R168C to *hOPN4* abolishes responses to light, while G208S and S308F lead to attenuated response amplitude and delayed response onset, consistent with a loss of sensitivity. All four occur in critical domains for opsin and GPCR activation. R168C and S308F are located in the conserved DRY and xWxPY motifs respectively, which mediate networks of hydrogen bonds necessary for opsin conformation change (Palczewski et al., 2000; Angel et al., 2009). Y146C and G208S are also found in conserved sequences, which interact with the Schiff-base linkage between retinal chromophore and opsin protein (Sakmar et al., 1989; Terakita et al., 2004). The phenotype of these four variants suggest these conserved domains have homologous critical functional roles in melanopsin and there are potentially people with non-functional melanopsin, which could be associated with disease.

Finally, two *hOPN4* SNPs, P10L and T394I, were characterised *in vivo*. The reported behavioural phenotypes, including abnormal sleep-wake timing and attenuated PLR, associated with these SNPs in humans (Higuchi et al., 2013; Lee et al., 2013; 2014; Roecklein et al., 2012; 2013) were not replicated using conditional expression of AAV-delivered *hOPN4* SNPs to *Opn4*^{-/-} mouse ipRGCs. Instead, a range of *Opn4*-driven behaviours were comparable between *hOPN4* SNPs and the WT positive control. However closer examination of ipRGC responses in *hOPN4* T394I AAV-treated retina

revealed attenuated sensitivity and faster offset kinetics, suggesting this site may be functionally significant for melanopsin activity.

In comparison, introduction of P10L had no effect on ipRGC responses, indicating this site is not critical for melanopsin function. This study represents the first attempt to establish a causal role for melanopsin SNPs in abnormal NIF behaviour *in vivo*.

Several types of key functional sites in melanopsin were identified. Some, such as R168C and S308F, occur at highly conserved GPCR motifs. These sites are necessary in all GPCRs and it was anticipated their substitution would severely disrupt melanopsin function. Others, such as Y146 and K340 necessary for formation of stable Schiff base linkage, were located at sites critical for opsin function. These opsin-specific sites were equally expected to alter melanopsin activity when mutated. Finally, there were sites with functional roles specific to melanopsin. These are potentially the most interesting, as they may improve understanding of some of the more unusual functional properties in OPN4. For example, mutation of T394 causes loss of sensitivity and alters OPN4 offset kinetics, but has no homology with key functional sites in other opsins, suggesting its role is specific to melanopsin. Similarly, although S310A is located at a known opsin spectral tuning site, mutating this site produces a spectral tuning shift which does not match other opsins. This indicates spectral tuning in melanopsin may be distinct from other opsins, discussed in more detail below.

6.2 Thesis Outcomes

At the start of this thesis, five objectives were outlined for accomplishing the aim of identifying key functional residues in melanopsin. The remainder of this chapter discusses how each these objectives were addressed and the implications of these findings.

6.2.1 Strategies for identification of critical functional residues

It is not practical or necessary to explore every amino acid in the melanopsin protein sequence. Given that some sites in the OPN4 protein are highly variable between species, it suggests that many sites are not critical for melanopsin function and will tolerate substitutions. The aim of this study was to identify sites that produced a measurable change in melanopsin phenotype when mutated, as this would demonstrate that the amino acid at this specific position was critical for normal melanopsin function.

Distinguishing key residues from sites that tolerate substitutions requires a balance of simple high-throughput assays of melanopsin function, such as the *in vitro* calcium imaging screen described in Chapter 4, with more complex and time-consuming in-depth characterisation, such as the MEA, PLR and circadian tests of AAV-delivered *hOPN4* described in Chapter 5.

High-throughput *in vitro* screening can be useful for detecting mutants which cause severe disruption of OPN4 function, such as Y146C or R168C, while more complex assays can characterise mutants with more subtle phenotypes that might appear normal in relatively simple *in vitro* screens. For example, *hOPN4* T394I exhibited normal intracellular calcium influx in response to light when expressed in HEK293T cells, but attenuated sensitivity and faster response onset during MEA recordings of ipRGCs transduced with *hOPN4* T394I AAV. This suggests that certain phenotypes may only be

observed when expressed in a native environment using extensive testing not practical for high-throughput screening.

Where more-detailed testing requires recovery of an animal model, a significant investment of time and resources is made that the mutation occurs at a critical functional residue in the melanopsin protein. Therefore, a number of strategies were developed that helped identify key sites worthy of further investigation. The most useful approach was bioinformatics, focusing on melanopsin residues conserved between different species. Almost all of the critical functional residues characterised in this thesis were highly conserved, or occurred at a conserved difference between melanopsin genes in the case of *Opn4* S310A (**Figure 3.6**). Similarly, mutations explored at highly variable sites, such as *hOPN4* W283C, had no observable impact on melanopsin activity. An invariant site suggests substitutions are poorly tolerated at this position and that the amino acid is adaptive for protein structure or function. Therefore, it makes sense to focus on conserved residues for future investigations.

Structural and sequence homology with other opsins or GPCRs was invaluable for identifying potential critical sites. With mutations in conserved opsin or GPCR motifs, there is the added benefit that the functional role of the site may have already been explored in a different protein, which can allow targeted testing of the mutant. For example, S310A occurs at a known opsin spectral tuning site. This provided a strong hypothesis on how S310A may affect melanopsin function, causing a blue shift in λ_{max} , and allowed *in vivo* assays to focus on testing spectral sensitivity. However, relying on this approach means only known critical GPCR or opsin residues will be explored. This can be useful, for example when comparing the functional role of a known critical site between opsins with different functional properties, but can also mean that more melanopsin-specific critical sites are neglected.

The location of the melanopsin mutation can also inform whether it is worthy of further investigation. For example, mutation of individual residues in the chromophore binding pocket are more likely to disrupt function than single mutations in other regions. Mutation of these transmembrane sites can alter interactions with the retinal chromophore or interfere with hydrogen-bond networks to change protein stability, affecting how melanopsin responds to light and its sensitivity or response timing.

In comparison, it appears individual residues in the extracellular and intracellular domain are less important. Protein and enzyme interactions with melanopsin seem to rely on multiple residues, where the substitution of one can be compensated for by others, as in the cluster of phosphorylation sites in the melanopsin C-terminus. Mutation of individual phosphorylated-residues in this domain had little effect on melanopsin deactivation kinetics, but simultaneous replacement of all or some of the residues caused delayed offset of response (Blasic et al., 2014; Mure et al., 2016), suggesting β -arrestin requires multiple phosphorylation sites to bind and deactivate melanopsin signalling.

Finally, these results suggests caution is required when using disease-association for identification of critical melanopsin residues, especially where other strategies suggest mutation of this site will be inconsequential. The limited evidence for an association between *hOPN4* SNP P10L and SAD (Roeklein et al., 2009; 2012) led this study to examine the effects of this mutation both *in vitro* and *in vivo*, even though protein alignments demonstrated this site was poorly conserved between species, and that mice had leucine (the minor allele) at the same position (see **Table 4.5**). With a non-specific phenotype anticipated and no suggested mechanism for mutation consequence, a number of melanopsin-driven behaviours were screened *in vivo* and ultimately did not identify any effect of this SNP. In this instance, the negative result was still valuable, discussed in more detail below, but does demonstrate the need for a strong hypothesis when investigating disease-associated residues as candidates for key functional sites.

To summarise, structural and sequence homology, particularly highly conserved residues or those in known opsin critical domains, was the most useful strategy for identifying potential functional residues for further investigation.

6.2.2 Identification of novel mutations in the mouse *Opn4* gene

The majority of mutagenesis studies of melanopsin structure-function relationships have explored post-translational modification sites, such as glycosylation in the N-terminus (Fahrenkrug et al., 2009) or phosphorylation in the C-terminus (Blasic et al., 2012a; 2012b; 2014). The study of these sites has contributed to the understanding of how melanopsin interacts with enzymes and other proteins to produce normal melanopsin-driven activity. In this thesis, the focus was on novel mutations in the transmembrane region that could alter spectral sensitivity, bistability or tertiary structure, properties which have not yet been investigated in melanopsin using mutagenesis. Novel mutations in this domain could assist in characterising structure-function relationships within the melanopsin protein.

To achieve this, a genotype-driven screen was conducted to identify chemically induced mutations in the mouse *Opn4* gene. The location and type of mutation were not site-directed, as ENU introduces nucleotide substitutions randomly throughout the genome. Instead a specific region was targeted, exon 6, which was most likely to cause interesting phenotypes if mutated. ENU mutants were also evaluated *in vitro* and using bioinformatics to determine if they were worthy of further investigation. Had the mutations identified during the ENU archive screen of exon 6 been silent or predicted to possess normal function, genotype-driven screening would have been continued for a different *Opn4* exon. The big advantage of ENU mutagenesis is that animal models can be recovered for *in vivo* testing, which was another important objective of this thesis.

As a result of the ENU archive screen described in Chapter 3, a novel melanopsin mutant, *Opn4* S310A was identified and a putative melanopsin spectral tuning site was characterised. Although the spectral sensitivity of melanopsin, specifically the $\lambda_{\max}$, has been extensively examined using a wide variety of techniques (see Chapter **Table 1.3**), the residues which cause 480nm $\lambda_{\max}$ have not yet been identified, perhaps because these were assumed to be equivalent to spectral tuning sites in other opsins.

The studies of *Opn4* S310A suggests that although opsin spectral tuning sites have similar roles in melanopsin, the magnitude of spectral tuning shifts caused by mutation of these sites is not comparable with other opsins. S310A produces a much smaller shift than anticipated, likely due to interaction of residues in the chromophore binding pocket. These interactions limit the ability of individual mutations to substantially blue shift melanopsin, which may require multiple concurrent mutations (Lin et al., 1998). Future examination of spectral tuning sites in melanopsin could compare the ability of single and concurrent *Opn4* mutations at known opsin spectral tuning sites (outlined in **Appendix 1**) to shift spectral sensitivity to longer or shorter wavelengths.

The residues in the melanopsin chromophore binding pocket are potentially unique among vertebrate visual pigments. How these sites interact with the retinal chromophore to mediate unusual functional properties of melanopsin, particularly its apparent ability to endogenously regenerate chromophore after exposure to different wavelengths of light, is currently unknown. *Opn4* S310A is the first description of a melanopsin mutation in the chromophore binding domain, apart from the retinal attachment site (Kumbalasiri et al., 2007). Homology with vertebrate rhodopsin may prove less useful in identification of critical sites in this region, as there are clear functional differences in how these opsins interact with the retinal chromophore. Instead, attempts to identify critical residues in this region should focus on amino acids conserved between species and examine sequence homology with residues in the chromophore binding pocket of invertebrate bistable opsins, such as squid or *Drosophila* rhodopsin.

The ability to engineer different spectral sensitivity mutants has a number of potential applications. Some are pragmatic, for example shifting the peak spectral sensitivity of melanopsin away from the excitation range of fluorescent calcium indicators to separate light necessary for imaging from light stimulus for melanopsin phototransduction, allowing measurement of baseline calcium before light exposure. Spectrally-shifted melanopsins could also be used *in vivo* to distinguish between opsins in the mammalian retina. By using metamers, perceptually identical lights with different wavelength composition (Brown et al., 2013), and combining spectrally-shifted melanopsin with other mutants, such as the red-cone opsin knock-in mouse (Lall et al., 2010; Smallwood et al., 2003), which shifts cone spectral sensitivity to longer wavelengths, the interactions of rods, cones and melanopsin to produce NIF responses to light could be explored in the intact retina. Melanopsin will also be a much more effective optogenetic tool if its response properties can be manipulated to suit the proposed application, which will only be possible with continued exploration of the spectral tuning sites in melanopsin and how these differ from other opsins.

6.2.3 Characterisation of naturally-occurring *hOPN4* missense variants

In addition to the identification and characterisation of novel melanopsin mutations, such as S310A, naturally-occurring missense mutations in the human *OPN4* gene were also characterised in this thesis. There is both common and rare genetic variation in the human *OPN4* gene, demonstrated by whole-genome and exome sequencing projects, such as the 1000 genomes project (1000 Genomes Project Consortium, 2015), collated in public databases. It is important to recognise which melanopsin variants occur at key residues that might affect NIF behaviour when mutated and whether this disruption could lead to disease.

In Chapter 4, a number of non-synonymous *hOPN4* variants were identified which caused complete or partial loss-of-function. This suggests that there are potentially individuals with severely disrupted melanopsin signalling, which could affect their NIF responses to light. Whether this will be associated with disease is unclear, given that individuals homozygous for these mutations will likely have normal rod-cone function, which can partially compensate for loss of OPN4-driven light input.

Although there are many missense variants, the majority are very rare. For example, in the 1000 Genomes project S308F has a minor allele frequency of 0.0006 because out of 5005 individual genotypes, only 3 examples of the minor allele were recorded (all heterozygous). For many of the *hOPN4* variants screened in Chapter 4, no examples were reported of individuals homozygous for substitution. This is surprising given the limited effect these variants can have on behaviour. Complete loss of melanopsin function will only cause attenuation of NIF behaviour if rods and cones are present, yet it appears deleterious variants have not been tolerated during human evolution. The scarcity of missense variants suggests normal melanopsin function is adaptive and any sequence variation in the melanopsin gene which disrupts this is poorly tolerated.

The ability of melanopsin variants to affect NIF responses to light in the intact retina was the reason the two previously reported SNPs were explored *in vivo*. As described above, there was limited bioinformatics evidence for how these SNPs, particularly P10L, might affect melanopsin function leading to the proposed phenotype. Targeted expression of both *hOPN4* P10L and T394I AAV in mouse ipRGCs restored a wildtype phenotype and whole animal behaviour comparable to *hOPN4* WT AAV, contradicting previously suggested associations of these SNPs with abnormal NIF behaviour. Given that significant attenuation in sensitivity of T394I ipRGC responses was later found, this suggests rods and cones, possibly combined with high transgene-expression levels, can compensate for partial loss of melanopsin function to produce normal NIF behaviour.

This raises important questions about whether melanopsin variants are associated with seasonal mood disorders in individuals with normal outer retinal function. Reporting this association encourages development of commercial products, such as high-intensity blue light therapy or genetic testing targeted at treatment and diagnosis of these SNPs. These are only effective if variants are shown to impact melanopsin-driven activity sufficiently that the disturbance in NIF behaviour cannot be compensated for by light input from other opsins. The results described in this thesis suggest this is not the case. This does not mean melanopsin variants cannot cause sleep or circadian rhythm disruption, but that the two previously reported SNPs, *hOPN4* P10L and T394I, do not appear to be critical sites for normal NIF responses *in vivo*.

6.2.4 Characterisation of *Opn4* mutants *in vivo*

One of the main objectives of this thesis was to characterise the impact of melanopsin point mutations *in vivo*, which has only recently started to be investigated (Mure et al., 2016). As demonstrated by the siRNA studies of long and short isoform knockdown (Jagannath et al., 2015), structural changes in melanopsin can have a substantial impact on whole-animal behaviour. It was unclear whether this was also true for subtle sequence changes, such as mutations at key functional sites.

Two approaches were adopted for investigating point mutations *in vivo*: chemical-induced mutations (*Opn4* S310A) and targeted expression of virally-delivered mutant melanopsin (*hOPN4* P10L and T394I). The advantages and limitations of both techniques, as well as *in vivo* mutagenesis testing in general, are discussed here. The AAV technique is more flexible and comparatively fast, from virus production to injection and behavioural testing in 3-4 months. However, this approach is less physiological, with transgene expression driven by a constitutive promotor at high levels in all ipRGCs, potentially removing some of the functional specialisation of ipRGC subtypes. This approach is also more susceptible to experimental error, with variation in transduction efficiency due to injection and viral titre (see **Figure 5.8 – 4.9**).

In comparison, ENU melanopsin mutations are heritable and expression occurs at physiological levels regulated by the endogenous melanopsin promotor. This approach also allows the study of mutants during development or in young animals, which is not possible using the AAV technique. However, the ENU approach is also more time and resource consuming, as it requires backcrossing animals for several generations to remove off-target effects (other ENU-induced mutations in the genome), which could confound results. This approach is also less flexible, as only mutants identified during the screen can be recovered and as discussed previously, there is no control over the location of this type of mutation.

One alternative method for more targeted mutagenesis *in vivo*, not explored in this thesis, is the use of site-specific nucleases, such as CRISPR or TALENS (Gaj et al., 2013). This approach allows targeted mutagenesis of the genome, so the position and type of mutation can be chosen as in the AAV approach, but the impact of the mutation can also be studied during development and expression is controlled by the endogenous promoter, as in ENU mutants. However, as with the ENU approach, there are also potential off-target effects (Zhang et al., 2015), which need to be removed by backcrossing, which is time-consuming and costly. This approach would be suitable for investigating critical functional sites with strong hypothesised effect on development, or which required expression at physiological levels.

Regardless of approach, melanopsin mutations are worth investigating *in vivo* for several reasons. First, the protein is expressed in its native environment, so folding and trafficking occurs with endogenous chaperones. These appear to be particularly important for melanopsin, as demonstrated by the difficulties purifying melanopsin from heterologous expression discussed in Chapter 1.

Second, it allows characterisation of intrinsic ipRGC responses and impact on whole-animal behaviour. With all the components of the endogenous melanopsin phototransduction cascade, from ion channels to enzymes, more subtle effects of mutations may be detected in ipRGCs, such as the attenuated sensitivity found in ipRGCs expressing *hOPN4* T394I despite no change in calcium responses being observed in transfected cells *in vitro*.

During completion of this thesis, Mure et al. (2016) published a study using targeted AAV-delivery of mouse melanopsin in ipRGCs of retinally degenerate *Opn4^{-/-} Cre^{+/+}* mice. Similar to the results described in Chapter 5, restoration of OPN4-driven light input resulted in a wildtype behavioural phenotype, with injected animals showing normal phase shifting and PLR. This study also examined the effect of mutating phosphorylation sites and demonstrated a cluster of conserved serine and threonine residues in the C terminus that are necessary for normal deactivation of melanopsin signalling *in vivo*. Their findings support the validity of targeted-AAVs for investigating melanopsin structure-function relationships, as well as the technique validation experiments described in Chapter 5.

6.2.5 Development of techniques for screening of *Opn4* mutants *in vivo*

The final objective was the development of a high-throughput method for screening *Opn4* mutants *in vivo*. As described above, *in vivo* testing can be necessary for in-depth characterisation of key functional sites.

Chapter 5 validated the targeted AAV technique, confirming *hOPN4* expression exclusively in ipRGCs and that intravitreal injections reliably resulted in good coverage across the retina. The behavioural experiments demonstrate AAV-delivered *hOPN4* can restore wildtype phenotype to a large number of NIF responses to light, known to be attenuated in *Opn4^{-/-}* mice, suggesting it is suitable as an *in vivo* model of melanopsin function.

The targeted AAV technique is highly adaptable, with mutations easily introduced to wildtype *hOPN4* AAV plasmid to produce different viruses. As all viruses are injected into *Opn4^{-/-} Cre^{+/+}*, this approach allows the effects of different mutations to be investigated *in vivo* without maintaining different colonies of animals, or recovering new mutant lines.

The *Opn4^{-/-} Cre^{+/+}* line is currently on a C57Bl/6 background and possesses normal outer retinal function. This is ideal for characterising the effects of melanopsin mutations in presence of rod-cone input to ipRGCs, such as modelling the effects of *hOPN4* variants on NIF behaviour in individuals with normal vision. Maintaining a retinally degenerate *Opn4^{-/-} Cre^{+/+}* line may also be useful for isolating melanopsin function, specifically to explore key functional residues, as well as improving the consistency of MEA recordings. This could enable mutation effect on ipRGC responses to be reliably characterised, for example continuing the S310A work to explore other spectral tuning sites and their combined effect on melanopsin $\lambda_{\max}$ by generating multiple MEA action spectra.

There is also the potential to adapt this technique beyond exploring human melanopsin variants, for example expression of only one mouse *Opn4* isoform in all ipRGCs to differentiate between the function of the two isoforms in different ipRGC subtypes. The melanopsin transgene could also be replaced with genetically encoded calcium indicators, allowing measurement of OPN4-driven calcium responses in ipRGCs of heterozygous *Opn4^{+/-} Cre^{+/-}* animals.

The issue of high transgene expression level driven by a constitutive promoter could potentially be regulated by replacing the Ef1 α promoter with a tetracycline-inducible promoter. If there is a dose-dependent effect of tetracycline on transgene expression, it could help limit transgene expression in the retina to physiological levels. It could also reduce time between testing before and after transgene expression, avoiding the confounding effects of age.

To summarise, ipRGC specific AAV delivery is a highly adaptable technique, which allows the characterisation of multiple melanopsin mutants *in vivo* without the time or expense of recovering multiple animal lines. This approach will be invaluable for future exploration of key functional melanopsin residues.

6.3 Conclusions

A number of critical functional residues in the melanopsin protein were characterised. A novel chemical-induced melanopsin mutation was identified at a putative spectral tuning site and several naturally-occurring *hOPN4* variants located in conserved opsin and GPCR domains were found abolish or attenuate melanopsin activity. The functional role of these sites in opsin hydrogen-bond networks and chromophore binding is likely shared with melanopsin. Finally, two *hOPN4* SNPs, P10L and T394I, were examined using conditional AAV expression in ipRGCs. Presence of either SNP had no effect on NIF behaviour, although minor changes were found in intrinsic responses of ipRGCs expressing T394I, suggesting this site may have a role in melanopsin function. These SNPs do not appear critical for NIF behaviour, suggesting *hOPN4* variants will have limited effect in individuals with normal vision. The methods developed in this thesis for identification and *in vivo* characterisation of critical functional residues in melanopsin are highly applicable for future investigations of structure-function relationships in the melanopsin protein.

Appendices

Appendix 1 - Opsin spectral tuning sites in melanopsin.

Table i. Amino acid at equivalent position of known opsin spectral tuning sites in zebrafish OPN4M2 and OPN4X1 and mouse OPN4. Amino acid substitutions that cause spectral tuning shifts are shown for Bovine Rhodopsin (BovRho) or Human LWS (HumLWS) and MWS (HumMWS) cone visual pigment. 3 sites rule = LWS/MWS cone visual pigment spectral tuning sites. Direction and magnitude of $\lambda_{\max}$ shift shown from wildtype $\lambda_{\max}$. Predicted direction of OPN4 mutations at each site is shown by colour (blue = short wavelength shift, red = long wavelength shift).

Residue	Position	Zebrafish OPN4X1	Zebrafish OPN4M2	Mouse OPN4	Opsin Mutation	$\lambda_{\max}$ Shift	Possible OPN4 Mutations
83	TM2	D	D	D	D83N _{BovRho}	-8nm ^a -5nm ^b	D83N
90	TM2	Q	Q	Q	G90S _{BovRho}	-13nm ^c -11nm ^d	Q90S
117	TM3	G	G	G	A117G _{BovRho}	-4nm ^d	G117A
118	TM3	A	A	A	T118A _{BovRho}	-16nm ^c	A118T
122	TM2	I	I	I	E122D _{BovRho}	-23nm ^c	I122D
164	TM4	S	S	A	3 sites rule A164S _{BovRho} S164A _{HumLWS} A164S _{HumMWS}	+2nm ^e -5nm ^f +2nm ^g	S164A
181	EL2	E	E	E	H181Y _{HumLWS}	-28nm ^h	E181Y
261	TM6	F	F	F	3 sites rule Y261F _{HumLWS} F261Y _{BovRho} F261Y _{HumMWS}	-7nm ^f +10nm ^e +6nm ^g	F261Y
265	TM6	W	W	W	W265Y _{BovRho}	-15nm ^d	W265Y
269	TM6	A	S	S	3 sites rule A269T _{HumMWS} T269A _{HumLWS} A269S _{BovRho}	+10nm ^g -14nm ^f +14nm ^e	S269A
292	TM7	A	A	A	A292S _{BovRho} A292S _{BovRho} A292S _{BovRho}	-9.5nm ^c -9nm ^d -10nm ^b	A292S
295	TM7	A	A	A	A295S _{BovRho}	-5nm ^d	A295S

^a Nathans et al (1990)

^b Fasick & Robinson (1998)

^c Janz & Farrens (2001)

^d Lin et al (1998)

^e Chan et al (1992)

^f Merbs & Nathans (1993)

^g Asenjo et al (1994)

^h Sun, Macke & Nathans (1997)

Appendix 2 - Human *OPN4* missense variants in dbSNP

Table ii. 96 *hOPN4* missense variants in dbSNP. MAF = Minor Allele Frequency (eg: 0.04 means minor allele observed in 4% of genotyped individuals). 1K Genomes = 1000 Genomes Project (1000 Genomes Project Consortium, 2015).

Mutation	ID	Conservation between Species	Location in Protein	Validation	MAF	SIFT	POLY PHEN-2
S5L	rs143330369	Conserved in Mammals	NT	1K Genomes, Multiple Submissions	0.001	0.04	0.002
P7Q	rs148229125	Not conserved	NT	Multiple Submissions		0.06	0
V9A	rs558565190	Not conserved	NT	1K Genomes, Multiple Submissions	0.0002	0.42	0.153
P10L	rs2675703	Not conserved	NT	1K Genomes, Multiple Submissions	0.078	1	0
A21S	rs552813212	Not conserved	NT	1K Genomes, Multiple Submissions	0.0002	0.13	0.089
R41Q	rs771237976	Not conserved	NT	Multiple Submissions		1	0
R41P	rs771237976	Not conserved	NT	Multiple Submissions		0.1	0.002
P43A	rs375718325	Not conserved	NT	Multiple Submissions		0.26	0.039
T52I	rs375209241	Conserved in Opn4M	NT	Multiple Submissions		0.12	0.055
A55V	rs137859210	Conserved in Mammals	NT	Multiple Submissions		0.26	0.488
A56T	rs192643153	Conserved in Mammals	NT	1K Genomes, Multiple Submissions	0.001	0.17	0.024
T62M	rs185200496	Conserved in Opn4M	NT	1K Genomes, Multiple Submissions	0.001	0.04	0.406
H68P	rs753064364	Conserved in Opn4M	NT	Multiple Submissions		0	0.939
T83M	rs202029105	Conserved in Opn4M, T/I in Opn4X	TM1	1K Genomes, Multiple Submissions	0.0002	0.02	0.588
T90M	rs201532005	Conserved in Opn4M	TM1	1K Genomes, Multiple Submissions	0.0002	0.08	0.105
F95L	rs573102269	Conserved in all species	TM1	1K Genomes, Multiple Submissions	0.0002	0	0.999
F95Y	rs760967151	Conserved in all species	TM1	Multiple Submissions		0	1
R102W	rs543587644	Conserved in most species (Q in chicken)	IL1	Multiple Submissions		0.02	0.344
R102Q	rs375300822	Conserved in most species (Q in chicken)	IL1	1K Genomes, Multiple Submissions	0.0002	0.09	0.507
L112I	rs751704144	Conserved in all species	TM2	Multiple Submissions		0	0.999
V126L	rs201523315	Conserved in Opn4M	TM2	1K Genomes, Multiple Submissions	0.0002	0.07	0.612
E144K	rs371561135	Conserved in Opn4M	TM3	1K Genomes, Multiple Submissions	0.0002	0.25	0.081
Y146C	rs200099863	Conserved in all species	TM3	1K Genomes, Multiple Submissions	0.0002	0	0.992
Y146F	rs200099863	Conserved in all species	TM3	1K Genomes, Multiple Submissions	0.0002	0	0.983
L161R	rs763437689	Conserved in all species	TM3	Multiple Submissions		0	0.999
T162M	rs140087560	Conserved at T/M in Opn4M, L/M in Opn4X	TM3	Multiple Submissions		0.51	0.217
R168C	rs143641898	Conserved in all species	TM3	Multiple Submissions	0.0005	0.01	0.929
P175L	rs370421133	Conserved in all species	IL2	Multiple Submissions		0	0.999
A182V	rs139556219	Not conserved	IL2	Multiple Submissions		1	0.006

Table ii Continued).

Mutation	ID	Conservation between Species	Location in Protein	Validation	MAF	SIFT	POLY PHEN-2
S183P	rs151123640	Conserved in most species (T in amphioxus)	IL2	1K Genomes, Multiple Submissions	0.0002	0	0.998
S183A	rs151123640	Conserved in most species (T in amphioxus)	IL2	1K Genomes, Multiple Submissions	0.0002	0	0.987
R186H	rs141089672	Conserved in most species (K in Chicken/Zebrafish M1)	IL2	Multiple Submissions	0.0005	0.01	0.934
A187E	rs200719705	Not conserved	IL2	Multiple Submissions		0	0.971
A187V	rs200719705	Not conserved	IL2	Multiple Submissions		0.17	0.408
V194I	rs368062538	Not conserved	TM4	1K Genomes, Multiple Submissions	0.0002	0.11	0.072
V194L	rs368062538	Not conserved	TM4	1K Genomes, Multiple Submissions	0.0002	0.07	0.123
V194F	rs368062538	Not conserved	TM4	1K Genomes, Multiple Submissions	0.0002	0	0.931
S202N	rs771769205	Conserved in most species (A in amphioxus)	TM4	Multiple Submissions		0	1
G208S	rs549998450	Conserved in all species	TM4	1K Genomes, Multiple Submissions	0.0002	0	0.999
A211T	rs367851084	Conserved in Opn4M, S in Opn4X	EL2	Multiple Submissions		0	0.966
A211P	rs367851084	Conserved in Opn4M, S in Opn4X	EL2	Multiple Submissions		0	0.995
V213M	rs202171086	Conserved in most species (I in Lizard and Zebrafish Opn4X)	EL2	1K Genomes, Multiple Submissions	0.0002	0	0.997
G216R	rs201432667	Conserved in all species	EL2	1K Genomes, Multiple Submissions	0.0002	0	1
T229K	rs115439401	Conserved in Opn4M, S/T in Opn4X	EL2	1K Genomes, Multiple Submissions	0.0002	0.04	0.993
T229M	rs115439401	Conserved in Opn4M, S/T in Opn4X	EL2	1K Genomes, Multiple Submissions	0.0002	0	0.997
V232M	rs143017128	Conserved in Opn4M, N in Opn4X	EL2	1K Genomes, Multiple Submissions	0.0002	0.06	0.989
V232A	rs543378130	Conserved in Opn4M, N in Opn4X	EL2	1K Genomes, Multiple Submissions	0.0002	0.23	0.327
R233H	rs376729483	Conserved in most species (K in Zebrafish Opn4X)	TM5	Multiple Submissions		0.04	1
R233L	rs376729483	Conserved in most species (K in Zebrafish Opn4X)	TM5	Multiple Submissions		0	1
A234V	rs199722703	Conserved in Opn4M, S in Opn4X	TM5	1K Genomes, Multiple Submissions	0.0002	0	0.911
V243M	rs148282551	Conserved in most species (M in amphioxus)	TM5	Multiple Submissions		0.04	1
V243L	rs148282551	Conserved in most species (M in amphioxus)	TM5	Multiple Submissions		0	1
V243G	rs369240840	Conserved in most species (M in amphioxus)	TM5	Multiple Submissions		0.03	1

Table ii (Continued).

Mutation	ID	Conservation between Species	Location in Protein	Validation	MAF	SIFT	POLY PHEN-2
F244L	rs372288717	Conserved in all species	TM5	Multiple Submissions		0.03	0.734
G266E	rs576858032	Conserved in mammals, S/G/N in non-mammals	IL3	1K Genomes, Multiple Submissions	0.0002	0.07	0.845
G266A	rs576858032	Conserved in mammals, S/G/N in non-mammals	IL3	1K Genomes, Multiple Submissions	0.0002	0.02	0.304
R267W	rs201905519	Not conserved	IL3	1K Genomes, Multiple Submissions	0.0002	0	0.901
W283C	rs145634412	Not conserved	IL3	1K Genomes, Multiple Submissions	0.001	0.16	0.295
R285Q	rs137979675	Not conserved	IL3	Multiple Submissions		0.33	0.028
R287W	rs142548175	Not conserved	IL3	Multiple Submissions		0	0.954
R287Q	rs377404718	Not conserved	IL3	1K Genomes, Multiple Submissions	0.0002	0.06	0.192
R287L	rs377404718	Not conserved	IL3	1K Genomes, Multiple Submissions	0.0002	0.01	0.128
E291K	rs375395379	Conserved in all species	IL3/TM6	Multiple Submissions		0.02	0.624
F305L	rs371493155	Conserved in mammals, Y/F in non-mammals	TM6	Multiple Submissions		0.03	0.595
S308F	rs559392371	Conserved in all species	TM6	1K Genomes, Multiple Submissions	0.001	0	0.972
A319G	rs555049946	Conserved in most species (G in cow and amphioxus)	TM6	1K Genomes, Multiple Submissions	0.0002	0.09	0.12
A324T	rs574631617	Conserved as S/A in most species	EL3	1K Genomes, Multiple Submissions	0.0002	0.01	0.541
V326I	rs144213090	Conserved as V/I in Opn4M, not conserved in Opn4X	EL3	1K Genomes, Multiple Submissions	0.001	0.55	0.021
S333L	rs370996195	Conserved in most species (T in Xenopus, Zebrafish Opn4X)	TM7	1K Genomes, Multiple Submissions	0.001	0.03	0.871
H345Q	rs184720512	Conserved in Opn4M, Y in Opn4X	TM7	1K Genomes, Multiple Submissions	0.0002	0	0.976
I352T	rs142432317	Conserved in all species	TM7	Multiple Submissions		0	0.911
Y357C	rs372004343	Conserved in most species (F in amphioxus)	H8	Multiple Submissions		0	0.999
L365V	rs34191521	Conserved in mammals, not in non-mammals	H8	1K Genomes, Multiple Submissions	0.039	1	0.083
L368V	rs61735658	Conserved in all species	CT	Multiple Submissions		0	0.936
R376H	rs35130918	Not conserved	CT	1K Genomes, Multiple Submissions	0.002	0.14	0.004

Table ii (Continued).

Mutation	ID	Conservation between Species	Location in Protein	Validation	MAF	SIFT	POLY PHEN-2
R377W	rs147596527	Not conserved	CT	Multiple Submissions		0	0.586
R377Q	rs142050572	Not conserved	CT	1K Genomes, Multiple Submissions	0.0002	0.96	0.002
R380C	rs535877312	Not conserved	CT	1K Genomes, Multiple Submissions	0.0002	0.18	0.677
R380H	rs150092638	Not conserved	CT	Multiple Submissions		0.68	0.003
S384N	rs751062041	Not conserved	CT	Multiple Submissions		0.04	0.821
R390H	rs575739858	Conserved in most species (Q in Zebrafish X2 and S in Amphioxus)	CT	1K Genomes, Multiple Submissions	0.0002	0.08	0.019
T392R	rs776209626	Conserved as S/T in most species	CT	Multiple Submissions		0.02	0.188
T392M	rs776209626	Conserved as S/T in most species	CT	Multiple Submissions		0.01	0.953
T394I	rs1079610	Conserved as S/T in most species	CT	1K Genomes, Multiple Submissions	0.247	0.28	0.003
R406W	rs3740337	Not conserved	CT	1K Genomes, Multiple Submissions	0.23	0.04	0.776
R407T	rs781478047	Not conserved	CT	Multiple Submissions		0.12	0.71
R408G	rs199878852	Conserved in mammals, not conserved in non- mammals	CT	1K Genomes, Multiple Submissions	0.0002	0.04	0.237
R408C	rs199878852	Conserved in mammals, not conserved in non- mammals	CT	1K Genomes, Multiple Submissions	0.0002	0	0.985
A426T	rs143623676	Not conserved	CT	Multiple Submissions		0.63	0.007
G444D	rs12262894	Not conserved	CT	1K Genomes, Multiple Submissions	0.013	0.52	0.004
H459Q	rs141877399	Not conserved	CT	Multiple Submissions		0.39	0
E460K	rs368037081	Not conserved	CT	Multiple Submissions		0.15	0.001
E460Q	rs368037081	Not conserved	CT	Multiple Submissions		0.18	0.025
E462K	rs571654939	Not conserved	CT	1K Genomes, Multiple Submissions	0.0002	0.53	0.004
T467S	rs369208074	Not conserved	CT	Multiple Submissions		0.07	0.003
P476L	rs35891072	Not conserved	CT	1K Genomes, Multiple Submissions	0.0002	0.29	0.002

Appendix 3 - Validity of net PIPR to assess OPN4 function

Several studies have specifically associated *hOPN4* SNP, T394I, with abnormal PLR (Higuchi et al., 2013; Lee et al., 2013; Roecklein et al., 2013). Comparable pupillometry assays are required to test the *hOPN4* T394I AAV model to see if it replicated the pupil phenotypes found in humans. Two human studies of *hOPN4* T394I measured minimum constriction (Higuchi et al., 2013; Lee et al., 2013), while a third used chromatic pupillometry to study the effects of *hOPN4* SNPs. Since this last approach has not been studied in mice, it was important to determine if it is a valid assay of OPN4 function.

As mentioned previously, PIPR is the sustained pupil constriction seen after light offset. Chromatic pupillometry is the comparison of PIPR at short and long wavelengths, typically 480nm and 610nm. The logic of this approach is that PIPR will be greatest after blue light, at the wavelength of peak melanopsin sensitivity, but attenuated at longer wavelengths where melanopsin is less sensitive. The comparison of PIPR after blue and red light is referred to as “net PIPR” and is used as an assay of inner retinal function (Kankipati et al., 2010; 2011).

Unlike other aspects of PLR, such as maximum constriction (Lucas et al., 2003), net PIPR has not previously been compared in *Opn4*^{-/-} and *Opn4*^{+/-} mice. Although Gamlin et al. (2007) demonstrated PIPR was unaffected by pharmacological blockade of rod/cone input to ipRGCs, without the corresponding experiment blocking OPN4 activity and demonstrating loss of PIPR and decreased net PIPR, it is simplistic to assume this approach can isolate ipRGC function.

For example, although *Opn4*^{-/-} tend to show faster recovery after lights off compared to *Opn4*^{+/+} mice, sustained constriction post-illumination can occasionally be observed in *Opn4*^{-/-} mice (Hattar et al., 2003). The presence of sustained constriction in some *Opn4*^{-/-} suggests rods and cones are capable of driving PIPR. If this is the case, then *Opn4*^{-/-} may also show normal net PIPR, because the spectral sensitivity of rod and cone photoreceptors also decreases at longer wavelengths. Therefore, chromatic pupillometry and net PIPR may fail to isolate intrinsic ipRGC function. If this is the case, net PIPR would not be an appropriate assay to test *hOPN4* SNPs, P10L and T394I in non-degenerate *Opn4*^{-/-} *Cre*^{+/+} animals.

To assess whether chromatic pupillometry is a valid assay of intrinsic ipRGC function, PIPR was compared to 480nm and 600nm light in *Opn4*^{+/+} and *Opn4*^{-/-} animals. Both *Opn4*^{+/+} and *Opn4*^{-/-} mice have faster recovery and reduced PIPR to long wavelength light compared to short wavelength light (**Figure S1A-B**). PIPR was quantified using two methods. The first measured the post-illumination rate of recovery in pupil size, which provided an inverse measure of PIPR (**Figure S1C**). Smaller slope values indicated slower recovery and increased PIPR, while larger slope values indicated faster recovery and decreased PIPR. Net rate of recovery was calculated as the difference between red and blue slopes. As shown in **Figure S1D**, the net rate of recovery was not significantly different between *Opn4*^{+/+} and *Opn4*^{-/-} mice, $t(8) = -0.29$, $p = 0.77$ (Student's t-test).

The second PIPR method measured the difference between average pupil area 10-15s after lights off and baseline (dark adapted) pupil area (**Figure S1E**). This was expressed as a percentage of baseline pupil area. This method is the same as those used in human net PIPR studies (Kankipati et al., 2010). Net PIPR was calculated as the difference between red and blue PIPR. Again, no significant difference in net PIPR was found between *Opn4*^{+/+} and *Opn4*^{-/-} mice (**Figure S1F**), $t(8) = 0.80$, $p = 0.45$ (Student's t-test).

Both net PIPR and rate of recovery were comparable for *Opn4^{+/+}* and *Opn4^{-/-}* mice suggesting that chromatic pupillometry is not a reliable assay of intrinsic ipRGC function. The average traces (**Figure S1B**) indicate there may have been a trend for net PIPR to decrease in *Opn4^{-/-}*, and with a larger sample size perhaps this difference would reach significance. However, PLR differences between *Opn4^{+/+}* and *Opn4^{-/-}* were reliably observed at a single wavelength (Chapter 5 **Figure 5.10**) close to melanopsin λ_{max} , even with a small sample size. This suggests that chromatic pupillometry is an inferior method to isolate OPN4 function. Since chromatic pupillometry cannot reliably distinguish between normal and total loss of OPN4 function, analysis of *hOPN4* SNP AAV model focused on maximum constriction and PIPR at shorter wavelengths, which are consistently attenuated in *Opn4^{-/-}* compared to *Opn4^{+/+}* mice.

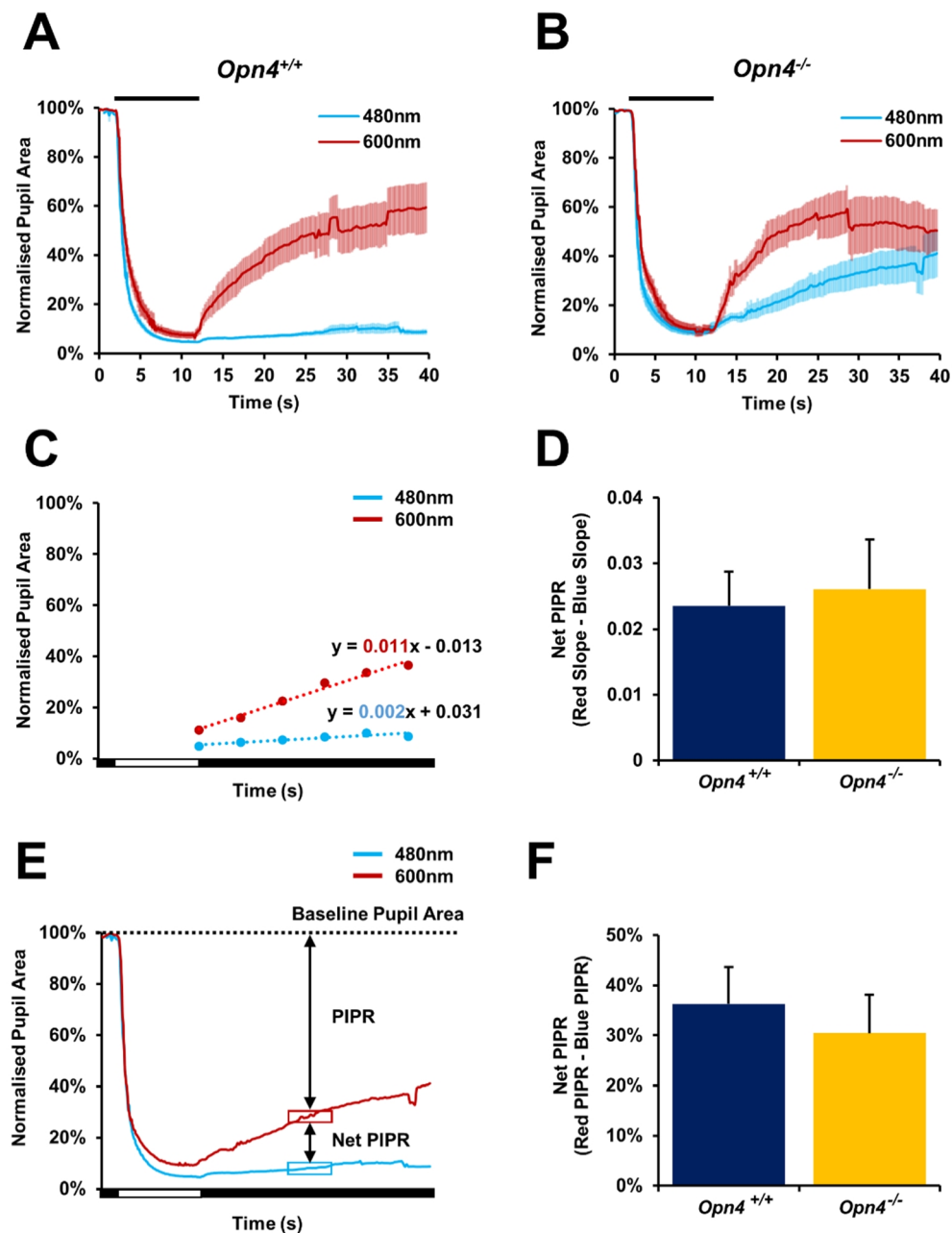


Figure S1. Net rate of recovery and net PIPR is comparable between *Opn4*^{-/-} and *Opn4*^{+/+} mice. Average kinetics of pupil light response to a 10s light pulse (480nm, 4.6×10^{14} photons/cm²/s, 600nm 4.8×10^{14} photons/cm²/s; shown as black bar above traces) from (A) *Opn4*^{+/+} and (B) *Opn4*^{-/-} animals, n = 5-6 for each group, error bars = SEM. (C) Schematic rate of recovery - calculated as slope of linear trendline (dotted line) fit to normalised pupil area after light offset. (D) Average Net PIPR calculated from recovery rate (n = 4-6) (E) Schematic PIPR – difference between baseline pupil (dotted line) and average pupil area 10-15s after light offset (shown as box around traces). (F) Average Net PIPR calculated from average pupil area 10s after light as blue PIPR – red PIPR, (n = 4-6). Black and white bar shows timing of dark and light in E and F.

Appendix 4 - Diversity of ipRGC responses in *hOPN4* WT AAV-treated retina

During MEA recording of AAV-treated retina, spike firing of ipRGC responses was heterogeneous, with diverse response latency and duration recorded by different electrodes from the same retina flatmount (**Figure S2**). Several MEA studies have been able to group ipRGCs into different subtypes based on their spike firing patterns (Tu et al., 2005; Weng et al., 2013; Walch et al., 2015). The ipRGC responses from retina of adult mice can generally be divided into two types: Type II/non-M1 responses and type III/M1 responses. According to Hughes et al. (2016), Type III/M1 responses have very rapid onset of activity, fast time to peak firing, very prolonged post-stimulus discharge and tend to be more sensitive. Type II or non-M1 responses have longer response latency, are slower to reach peak firing, have shorter response duration and are less sensitive compared to type III/M1 ipRGCs.

Since diverse ipRGC responses were observed during MEA recording of *Opn4*^{-/-} *Cre*^{+/+} retina treated with *hOPN4* WT AAV, it was interesting to test whether these could be divided into subtypes based on spike firing properties, as in adult *Opn4*^{+/+} retina and whether the subtypes still showed different sensitivity. As there is no regulation of the *hOPN4* protein synthesis with the *hOPN4* AAV model, theoretically wherever *hOPN4* AAV is expressed, it should all be at similar level. This might remove the variation in expression levels and sensitivity seen between different ipRGC subtypes that occurs in *Opn4*^{+/+} retina (Schmidt et al., 2011).

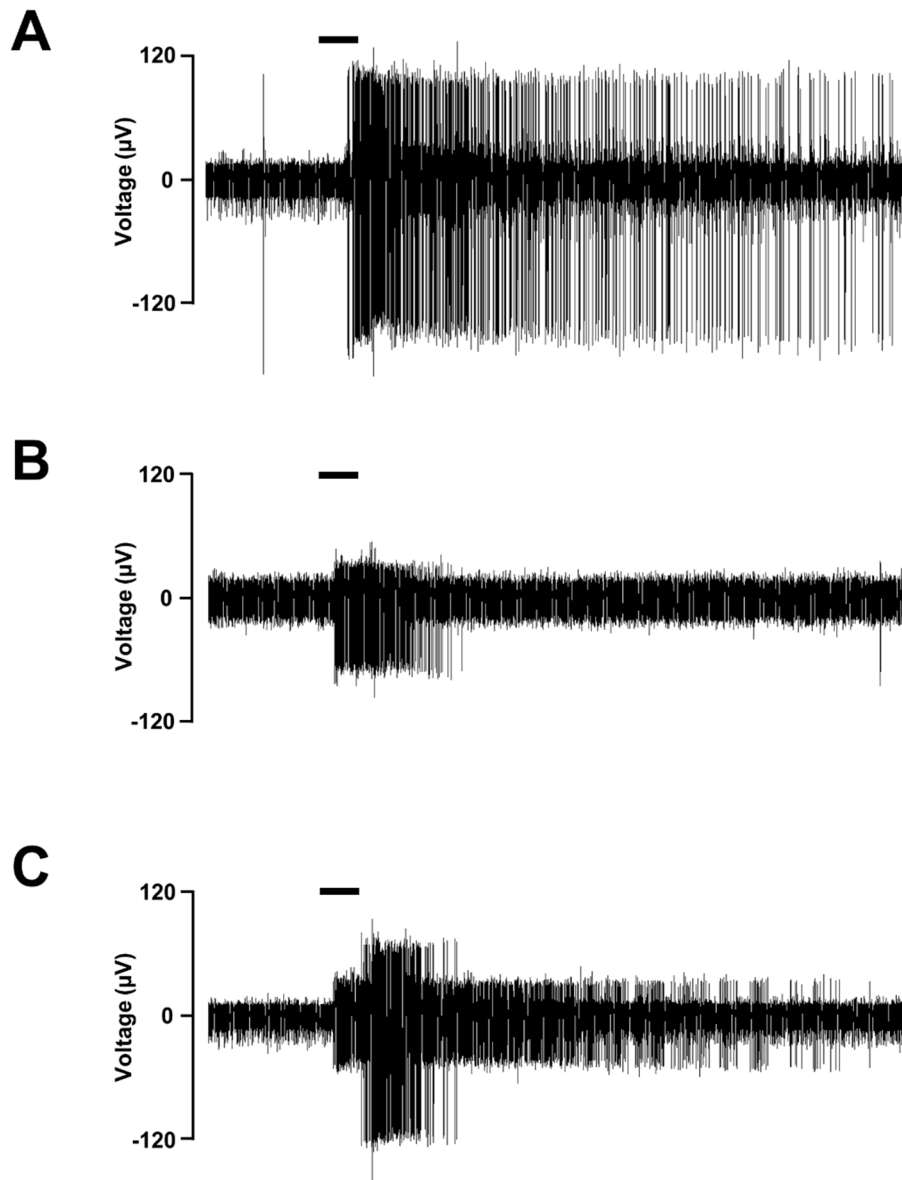


Figure S2. Diverse ipRGC responses from retina of *Opn4^{-/-} Cre^{+/+}* treated with *hOPN4* WT AAV. Traces from individual electrodes during 10s 480nm light pulse (10^{14} photons/cm²/s, shown as black bar above trace) after pharmacological blockade of rod/cone signals. ipRGC responses were heterogeneous with **(A)** some exhibiting prolonged firing after light offset and **(B)** others showing shorter responses. **(C)** Two ipRGC responses recorded on the same electrode. This trace shows a smaller amplitude prolonged response and a larger amplitude delayed short response.

Tu et al. (2005) observed a bimodal distribution in onset latency to 1min sub-saturating light pulses, where one population of ipRGCs responded before 12s (Type III) and another only responded after 12s (Type II). Using this 12s threshold, they found the two ipRGC populations differed in other ways, such as response duration and sensitivity (**Figure S3A**). A similar sub-saturating 1min light pulse (10^{13} photons/cm²/s) was used to try grouping AAV-treated ipRGC responses into type II and type III from two hOPN4 WT AAV-treated retina. The 1min light pulses were completed at the end of the recording and as a result, fewer ipRGC responses were observed and the data was noisier than previously. Using the 12s response latency threshold, two groups were formed with roughly equal numbers in each (>12, n = 21, <12, n = 23). However, the average responses for each group to 1min light pulse (**Figure S3B**) did not match spike firing patterns of types II and III described by Tu et al. (2005).

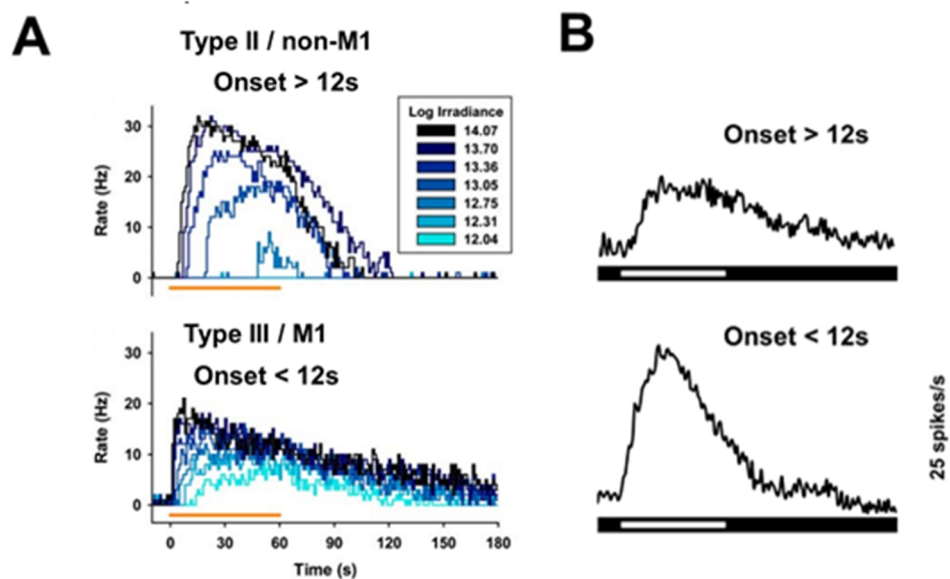


Figure S3. ipRGC subtypes classified by response latency to subsaturating light pulse. ipRGC responses to 1 minute 480nm light pulse ($\sim 10^{13}$ photons/cm²/s, shown as white bar below trace) are grouped into Type II and III subtypes by 12s response onset latency threshold (**A**) Example responses from Type II (top) and Type III (bottom) adapted from Tu et al. (2005) with permission from Elsevier (**B**) Average ipRGC responses from *hOPN4* WT AAV-treated *Opn4*^{-/-} *Cre*^{+/+} retina grouped by response onset relative to 12s threshold.

In particular, responses with short onset latency did not also show long post-stimulus firing or rapid increase in firing rate, as described by Tu et al. (2005). This discrepancy might be caused by difficulty defining response onset because of variable baseline. As the 1min light pulse was the last recording of the session, after completing irradiance response curve and repeated spike firing, the retina may not have been responding optimally. Additionally, the threshold for subtype classification may vary between retina, and so that even *Opn4*^{+/+} responses form less discrete categories than suggested by Tu et al. (2005).

Since ipRGC subtypes are defined by many criteria beyond response onset, including sensitivity, latency to peak and response duration (Reifler et al., 2015; Walch et al., 2015), the distribution of these spike firing properties in *Opn4*^{+/+} and *hOPN4* WT AAV-treated *Opn4*^{-/-} *Cre*^{+/+} retinæ were compared. Bimodal distributions in spike firing properties could indicate subpopulations of ipRGC responses. Latency to peak and half maximum firing rate were examined to a sub-saturating 10s light pulse (480nm, 10¹³ photons/cm²/s) conducted near the start of recording when the retina was responding robustly. Sensitivity was measured as the EC50 value of irradiance response curves described above.

As shown in **Figure S4**, there appeared to be a bimodal distribution in sensitivity in retina treated with *hOPN4* WT AAV, consistent with the description of Type II/non-M1 and Type III/M1 subtypes, which differ in sensitivity. Sensitivity can also be influenced by expression level, which may reflect variation associated with viral transduction. However, if this was the case, a broader normal distribution would be expected rather than the two peaks observed in **Figure S4A**. Both latency to half maximum and peak firing rate appeared to be normally distributed (**Figure S4B-C**). This suggests different ipRGC subtypes may not show distinct response kinetics after transduction with *hOPN4* WT AAV.

The distribution of ipRGC spike firing properties from *Opn4*^{+/+} retina was examined to determine whether native ipRGC responses could also be subdivided into discrete categories, as described by several studies (Reifler et al., 2015; Walch et al., 2015; Zhao et al., 2014). Intriguingly, although a possible bimodal distribution was observed for time to half maximum (**Figure S4B**), both sensitivity and latency to peak appeared to be normally distributed (**Figure S4A,C**). These are parameters frequently used to define subtypes in adult mouse retina, so it is unusual the responses recorded formed a continuum with no clear threshold for defining subpopulations. Previously, Sexton et al. (2015) were also unable to distinguish Type II and Type III cells in adult retina based on ipRGC spike firing properties. This supports these findings, suggesting the reported response characteristics of Type II/non-M1 and Type III/M1 subtypes may represent trends, rather than discrete categories.

Overall, categorising ipRGC responses into subtypes based on spike firing properties can be difficult. As demonstrated by the *Opn4*^{+/+} ipRGC responses, these characteristics are variable, making it difficult to apply thresholds from the literature or identify of new thresholds to define subtypes. It is therefore not too surprising that ipRGC response kinetics of *hOPN4* AAV-treated retina did not form clear subpopulations. However, a binomial distribution of AAV-treated ipRGC sensitivity was identified, which might represent type II and type III subtypes. This implies that despite being driven by a ubiquitous promotor, the *hOPN4* AAV-driven ipRGC responses can at least partly replicate the ipRGC response diversity seen in *Opn4*^{+/+} retina.

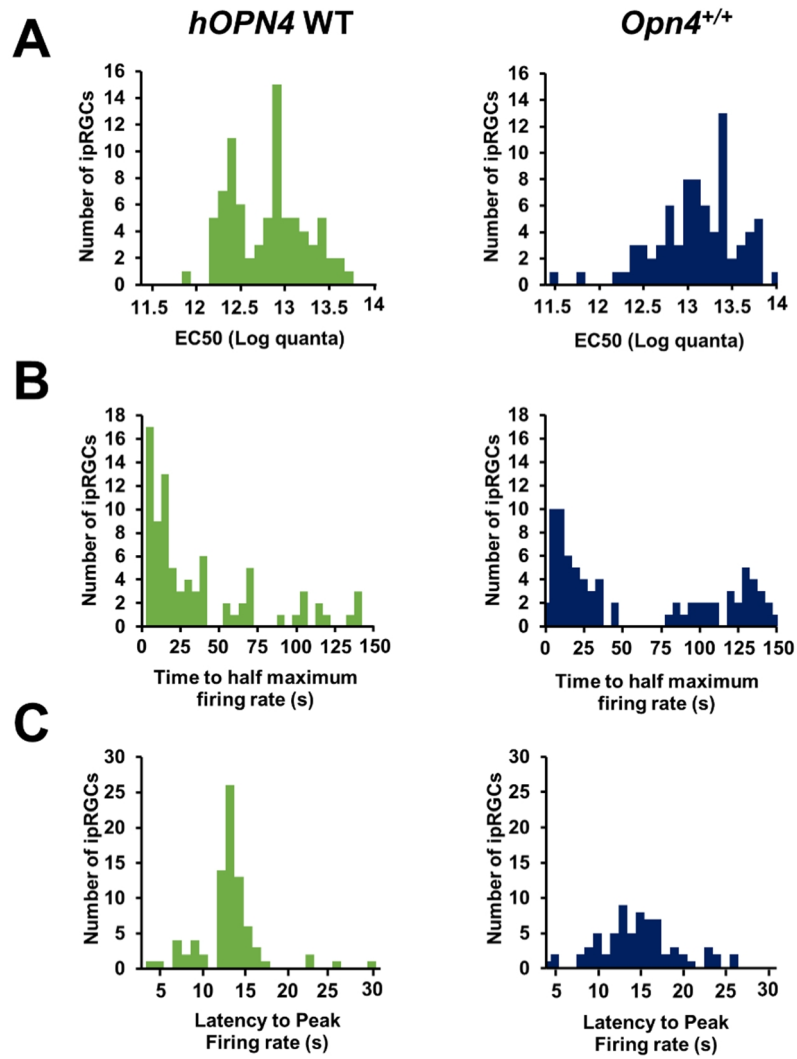


Figure S4. Histograms of ipRGC spike firing properties to identify discrete populations of ipRGC subtypes. ipRGC responses from *Opn4*^{-/-} *Cre*^{+/+} treated with *hOPN4* WT AAV (n = 82, left) and *Opn4*^{+/+} retina (n = 78, right) **(A)** EC50, derived from irradiance response curve fit to ipRGC responses to 10s 480nm light (10^{11} - 10^{15} photons/cm²/s). Bin width = 0.1 log quanta **(B)** Time to half maximum firing rate (5s bin width) and **(C)** Time to peak firing rate (1s bin width) in response to 10s 480nm light pulse (10^{13} photons/cm²/s).

References

- 1000 Genomes Project Consortium. (2015). A global reference for human genetic variation. *Nature*, 526(7571), 68–74.
- 1000 Genomes Project Consortium (2010). A map of human genome variation from population-scale sequencing. *Nature*, 467(7319), 1061–1073.
- Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., Kondrashov, A. S. & Sunyaev, S. R. (2010). A method and server for predicting damaging missense mutations. *Nature Methods*, 7(4), 248–249.
- Ahuja, S., Hornak, V., Yan, E. C. Y., Syrett, N., Goncalves, J. A., Hirshfeld, A., Ziliox, M., Sakmar, T. P., Sheves, M., Reeves, P. J., Smith, S. O. & Eilers, M. (2009). Helix movement is coupled to displacement of the second extracellular loop in rhodopsin activation. *Nature Structural & Molecular Biology*, 16(2), 168–175.
- Albrecht, U. & Foster, R.G. (2002). Placing ocular mutants into a functional context: a chronobiological approach. *Methods*, 28(4), 465–77.
- Altimus, C. M., Güler, A. D., Villa, K. L., McNeill, D. S., Legates, T. A., & Hattar, S. (2008). Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation. *Proceedings of the National Academy of Sciences*, 105(50), 19998–20003.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403–10.
- Angel, T. E., Chance, M. R., & Palczewski, K. (2009). Conserved waters mediate structural and functional activation of family A (rhodopsin-like) G protein-coupled receptors. *Proceedings of the National Academy of Sciences*, 106(21), 8555–8560.
- Asenjo, A. B., Rim, J., & Oprian, D. D. (1994). Molecular determinants of human red/green color discrimination. *Neuron*, 12(5), 1131–1138.
- Azam, M., Khan, M. I., Gal, A., Hussain, A., Shah, S. T., Khan, M. S., Sadeque, A., Bokhari, H., Collin, R. W. J., Orth, U., van Genderen, M., den Hollander, A. I., Cremers, F. P. M. & Qamar, R. (2009). A homozygous p.Glu150Lys mutation in the opsin gene of two Pakistani families with autosomal recessive retinitis pigmentosa. *Molecular Vision*, 15, 2526–34.
- Bailes, H. J., & Lucas, R. J. (2013). Human melanopsin forms a pigment maximally sensitive to blue light ($\lambda_{\text{max}} \approx 479$ nm) supporting activation of G(q/11) and G(i/o) signalling cascades. *Proceedings of the Royal Society London B: Biological Sciences*, 280(1759), 20122987.
- Banks, G., Heise, I., Starbuck, B., Osborne, T., Wisby, L., Potter, P., Jackson, I. J., Foster, R. G., Perison, S. N. & Nolan, P. M. (2015). Genetic background influences age-related decline in visual and nonvisual retinal responses, circadian rhythms, and sleep. *Neurobiology of Aging*, 36(1), 380–93.

- Bareil, C., Hamel, C., Pallarès-Ruiz, N., Arnaud, B., Demaille, J., & Claustres, M. (1999). Molecular analysis of the rhodopsin gene in southern France: identification of the first duplication responsible for retinitis pigmentosa, c.998999ins4. *Ophthalmic Genetics*, 20(3), 173–82.
- Barnard, A. R., Appleford, J. M., Sekaran, S., Chinthapalli, K., Jenkins, A., Seeliger, M., Biel, M., Humphries, P., Douglas, R. H., Wenzel, A., Foster, R. G., Hankins, M. W. & Lucas, R. J. (2004). Residual photosensitivity in mice lacking both rod opsin and cone photoreceptor cyclic nucleotide gated channel 3 alpha subunit. *Visual Neuroscience*, 21(5), 675–83.
- Batten, M. L., Imanishi, Y., Maeda, T., Tu, D. C., Moise, A. R., Bronson, D., Possin, D., Van Gelder, R. N., Baehr, W. & Palczewski, K. (2004). Lecithin-retinol Acyltransferase Is Essential for Accumulation of All-trans-Retinyl Esters in the Eye and in the Liver. *Journal of Biological Chemistry*, 279(11), 10422–10432.
- Baver, S. B., Pickard, G. E., Sollars, P. J., & Pickard, G. E. (2008). Two types of melanopsin retinal ganglion cell differentially innervate the hypothalamic suprachiasmatic nucleus and the olivary pretectal nucleus. *European Journal of Neuroscience*, 27(7), 1763–1770.
- Beiert, T., Bruegmann, T., & Sasse, P. (2014). Optogenetic activation of Gq signalling modulates pacemaker activity of cardiomyocytes. *Cardiovascular Research*, 102(3), 507–16.
- Belenky, M. A., Smeraski, C. A., Provencio, I., Sollars, P. J., & Pickard, G. E. (2003). Melanopsin retinal ganglion cells receive bipolar and amacrine cell synapses. *The Journal of Comparative Neurology*, 460(3), 380–393.
- Bellingham, J., Chaurasia, S. S., Melyan, Z., Liu, C., Cameron, M. A., Tarttelin, E. E., Iuvone, P. M., Hankins, M. W., Tosini, G. & Lucas, R. J. (2006). Evolution of Melanopsin Photoreceptors: Discovery and Characterization of a New Melanopsin in Nonmammalian Vertebrates. *PLoS Biology*, 4(8), e254.
- Berson, D. M., Dunn, F. A. & Takao, M. (2002). Phototransduction by Retinal Ganglion Cells That Set the Circadian Clock. *Science*, 295(5557), 1070–1073.
- Bhattacharya, S., Hall, S. E., & Vaidehi, N. (2008). Agonist-induced conformational changes in bovine rhodopsin: insight into activation of G-protein-coupled receptors. *Journal of Molecular Biology*, 382(2), 539–55.
- Blasic, J. R., Brown, R.L., & Robinson, P. R. (2012a). Light-dependent phosphorylation of the carboxy tail of mouse melanopsin. *Cellular and Molecular Life Sciences*, 69(9), 1551–62.
- Blasic, J. R., Brown, R.L., & Robinson, P. R. (2012b) Phosphorylation of Mouse Melanopsin by Protein Kinase A. *PLoS One*, 7(9), e45387.
- Blasic, J. R., Matos-Cruz, V., Ujla, D., Cameron, E. G., Hattar, S., Halpern, M. E., & Robinson, P. R. (2014). Identification of critical phosphorylation sites on the carboxy tail of melanopsin. *Biochemistry*, 53(16), 2644–9.

- Bockaert, J. L., & Pin, J. P. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. *The EMBO Journal*, 18(7), 1723–1729.
- Borges, R., Johnson, W. E., O'Brien, S. J., Vasconcelos, V., & Antunes, A. (2012). The role of gene duplication and unconstrained selective pressures in the melanopsin gene family evolution and vertebrate circadian rhythm regulation. *PloS One*, 7(12), e52413.
- Bourne, H.R. (1997) How receptors talk to trimeric G proteins. *Current Opinion in Cell Biology*, 9(2), 134-142.
- Bowmaker, J. K. & Dartnell, H. J. (1980) Visual pigments of rods and cones in a human retina. *The Journal of Physiology*, 298(1), 501-511.
- Brainard, G. C., Hanifin, J. P., Greeson, J. M., Byrne, B., Glickman, G., Gerner, E., & Rollag, M. D. (2001). Action spectrum for melatonin regulation in humans: evidence for a novel circadian photoreceptor. *The Journal of Neuroscience*, 21(16), 6405–12.
- Brown L. A., Hasan S., Foster R. G., Peirson S. N. (*in preparation*) COMPASS: Continuous Open Mouse Phenotyping of Activity and Sleep Status.
- Brown, T. M., Gias, C., Hatori, M., Keding, S. R., Semo, M., Coffey, P. J., Gigg, J., Piggins, H. D., Panda, S. & Lucas, R. J. (2010). Melanopsin Contributions to Irradiance Coding in the Thalamo-Cortical Visual System. *PLoS Biology*, 8(12), e1000558.
- Brown, T. M., Tsujimura, S.-I., Allen, A. E., Wynne, J., Bedford, R., Vickery, G., Vugler, A. & Lucas, R. J. (2012). Melanopsin-based brightness discrimination in mice and humans. *Current Biology*, 22(12), 1134–41.
- Brown, T. M., Allen, A. E., al-Enezi, J., Wynne, J., Schlangen, L., Hommes, V., & Lucas, R. J. (2013). The Melanopic sensitivity function accounts for Melanopsin-Driven responses in mice under diverse lighting conditions. *PLoS ONE*, 8(1), e53583.
- Bulka, B., desJardins, M. & Freeland, S. J. (2006) An interactive visualisation tool to explore biophysical properties of amino acids and their contribution to substitution matrices. *BMC Bioinformatics*, 7, 329.
- Bush, W. S. & Moore, J. H (2012). Chapter 11: Genome-Wide Association Studies. *PLoS Computational Biology*, 8(12), e1002822.
- Cai, Z., Tsung, E. F., Marinescu, V. D., Ramoni, M. F., Riva, A., & Kohane, I. S. (2004). Bayesian approach to discovering pathogenic SNPs in conserved protein domains. *Human Mutation*, 24(2), 178–184.
- Cameron, E. G., & Robinson, P. R. (2014). β -Arrestin-dependent deactivation of mouse melanopsin. *PLoS One*, 9(11), e113138.
- Cardin, J. A., Carlén, M., Meletis, K., Knoblich, U., Zhang, F., Deisseroth, K., Tsai, L.-H. & Moore, C. I. (2010). Targeted optogenetic stimulation and recording of neurons in vivo using cell-type-specific expression of Channelrhodopsin-2. *Nature Protocols*, 5(2), 247–254.

- Chalupa, L. M., & Williams, R. W. (2008). *Eye, retina, and visual system of the mouse*. Cambridge, MA: MIT Press.
- Chan, T., Lee, M., & Sakmar, T. P. (1992). Introduction of hydroxyl-bearing amino acids causes bathochromic spectral shifts in rhodopsin. Amino acid substitutions responsible for red-green color pigment spectral tuning. *Journal of Biological Chemistry*, 267(14), 9478–9480.
- Chang, B. S. W., Crandall, K. A., Carulli, J. P., & Hartl, D. L. (1995). Opsin Phylogeny and Evolution: A Model for Blue Shifts in Wavelength Regulation. *Molecular Phylogenetics and Evolution*, 4(1), 31–43.
- Chen, J., Makino, C. L., Peachey, N. S., Baylor, D. A., & Simon, M. I. (1995). Mechanisms of rhodopsin inactivation in vivo as revealed by a COOH-terminal truncation mutant. *Science*, 267(5196), 374–7.
- Chen, S.-K., Badea, T. C., & Hattar, S. (2011). Photoentrainment and pupillary light reflex are mediated by distinct populations of ipRGCs. *Nature*, 476(7358), 92–95.
- Chew, K. S., Schmidt, T. M., Rupp, A. C., Kofuji, P. & Trimarchi, J. M. (2014). Loss of Gq/11 Genes Does Not Abolish Melanopsin Phototransduction. *PLoS One*, 9(5), e98356.
- Coghill, E. L., Hugill, A., Parkinson, N., Davison, C., Glenister, P., Clements, S., Hunter, J., Cox, R. D. & Brown, S. D. M. (2002). A gene-driven approach to the identification of ENU mutants in the mouse. *Nature Genetics*, 30(3), 255-6
- Colley, N. J., Cassill, J. A., Baker, E. K. & Zuker, C. S. (1995). Defective intracellular transport is the molecular basis of rhodopsin-dependent dominant retinal degeneration. *Genetics*, 92, 3070–3074.
- Contín, M. A., Verra, D. M., Salvador, G., Ilincheta, M., Giusto, N. M. & Guido, M. E. (2010). Light Activation of the Phosphoinositide Cycle in Intrinsically Photosensitive Chicken Retinal Ganglion Cells. *Investigative Ophthalmology & Visual Science*, 51(11), 5491-8.
- Daan, S. & Pittendrigh, C.S. (1976) A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents. *Journal of Comparative Physiology*, 106(3), 223-252.
- Dacey, D. M., Liao, H.-W., Peterson, B. B., Robinson, F. R., Smith, V. C., Pokorny, J., Yau, K.-W. & Gamlin, P. D. (2005). Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. *Nature*, 433(7027), 749–54.
- Davies, W. I. L., Zheng, L., Hughes, S., Tamai, T. K., Turton, M., Halford, S., Foster, R. G., Whitmore, D. & Hankins, M. W. (2011). Functional diversity of melanopsins and their global expression in the teleost retina. *Cellular and Molecular Life Sciences*, 68(24), 4115–32.
- Demb, J. B., & Singer, J. H. (2015). Functional Circuitry of the Retina. *Annual Review of Vision Science*, 1(1), 263–289.

- de Silva, S. R., McClements, M. E., Hankins, M. W. & Maclaren R. E. (2015). Adeno-associated viral gene therapy for Retinal Disorders. In Bo, X., & Verhaagen, J. (Eds.). *Gene Delivery and Therapy for Neurological Disorders* (Vol. 98). New York, NY: Springer New York.
- Deupi, X., & Kobilka, B. (2007). Activation of G protein-coupled receptors. *Advances in Protein Chemistry*, 74, 137-166.
- Do, M. T. H., Kang, S. H., Xue, T., Zhong, H., Liao, H.-W., Bergles, D. E., & Yau, K.-W. (2009). Photon capture and signalling by melanopsin retinal ganglion cells. *Nature*, 457(7227), 281-7.
- Do, M. T. H., & Yau, K.-W. (2010). Intrinsically photosensitive retinal ganglion cells. *Physiological Reviews*, 90(4), 1547-81.
- Douglas, R. H. & Jeffery, G. (2014). The spectral transmission of ocular media suggests ultraviolet sensitivity is widespread among mammals. *Proceedings of the Royal Society of London B: Biological Sciences*, 281(1780), 20132995.
- Doyle, S. E., Castrucci, A. M., McCall, M., Provencio, I., & Menaker, M. (2006). Nonvisual light responses in the Rpe65 knockout mouse: rod loss restores sensitivity to the melanopsin system. *Proceedings of the National Academy of Sciences*, 103(27), 10432-7.
- Ebihara, S., & Tsuji, K. (1980). Entrainment of the circadian activity rhythm to the light cycle: Effective light intensity for a Zeitgeber in the retinal degenerate C3H mouse and the normal C57BL mouse. *Physiology & Behavior*, 24(3), 523-527.
- 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. & Hattar, S. (2010). Melanopsin-expressing retinal ganglion-cell photoreceptors: cellular diversity and role in pattern vision. *Neuron*, 67(1), 49-60.
- Emanuel, A. J., & Do, M. T. H. (2015). Melanopsin tristability for sustained and broadband phototransduction. *Neuron*, 85(5), 1043-55.
- Estevez, M. E., Fogerson, P. M., Ilardi, M. C., Borghuis, B. G., Chan, E., Weng, S., Auferkorte, O. N., Demb, J. B. & Berson, D. M. (2012). Form and Function of the M4 Cell, an Intrinsically Photosensitive Retinal Ganglion Cell Type Contributing to Geniculocortical Vision. *Journal of Neuroscience*, 32(39), 13608-13620.
- Fahrenkrug, J., Falktoft, B., Georg, B., Hannibal, J., Kristiansen, S. B., & Klausen, T. K. (2014). Phosphorylation of rat melanopsin at Ser-381 and Ser-398 by light/dark and its importance for intrinsically photosensitive ganglion cells (ipRGCs) cellular Ca²⁺ signaling. *The Journal of Biological Chemistry*, 289(51), 35482-93.
- Fahrenkrug, J., Falktoft, B., Georg, B., & Rask, L. (2009). N-linked deglycosylated melanopsin retains its responsiveness to light. *Biochemistry*, 48(23), 5142-8.
- Fasick, J. I., & Robinson, P. R. (1998). Mechanism of spectral tuning in the dolphin visual pigments. *Biochemistry*, 37(2), 433-438.

- Fisher, S. P., Godinho, S. I. H., Potheary, C. A., Hankins, M. W., Foster, R. G., & Peirson, S. N. (2012). Rapid Assessment of Sleep-Wake Behavior in Mice. *Journal of Biological Rhythms*, 27(1), 48–58.
- Foster, R. G., Provencio, I., Hudson, D., Fiske, S., De Grip, W., & Menaker, M. (1991). Circadian photoreception in the retinally degenerate mouse (rd/rd). *Journal of Comparative Physiology A*, 169(1), 39–50.
- Franke, R. R., Sakmar, T. P., Graham, R. M., & Khorana, H. G. (1992). Structure and function in rhodopsin. Studies of the interaction between the rhodopsin cytoplasmic domain and transducin. *The Journal of Biological Chemistry*, 267(21), 14767–74.
- Freedman, M. S., Lucas, R. J., Soni, B., von Schantz, M., Muñoz, M., David-Gray, Z., & Foster, R. (1999). Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. *Science*, 284(5413), 502–4.
- Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K. P., & Ernst, O. P. (2003). Role of the conserved NPxxY(x) motif in the rhodopsin ground state and during activation. *Proceedings of the National Academy of Sciences*, 100(5), 2290–5.
- Fu, Y., Zhong, H., Wang, M.-H. H., Luo, D.-G., Liao, H.-W., Maeda, H., Hattar, S., Fishman, L. J. & Yau, K.-W. (2005). Intrinsically photosensitive retinal ganglion cells detect light with a vitamin A-based photopigment, melanopsin. *Proceedings of the National Academy of Sciences*, 102(29), 10339–44.
- Fujiki, K., Hotta, Y., Murakami, A., Yoshii, M., Hayakawa, M., Nicolas, M., Takeda, M., Yano, H., Kishishita, H. Yokoyama, T. & Kanai, A. (1995). Heterozygous Asn-15-Ser And Gly-174-Ser Mutations In Rhodopsin Gene Found In Japanese Retinitis-Pigmentosa. *Investigative Ophthalmology & Visual Science* , 36(4), S890.
- Gaj, T., Gersbach, C. A., & Barbas, C. F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology*, 31(7), 397–405.
- Gamlin, P. D. R., McDougal, D. H., Pokorny, J., Smith, V. C., Yau, K.-W., & Dacey, D. M. (2007). Human and macaque pupil responses driven by melanopsin-containing retinal ganglion cells. *Vision Research*, 47(7), 946–54.
- Gooley, J. J., Lu, J., Chou, T. C., Scammell, T. E., & Saper, C. B. (2001). Melanopsin in cells of origin of the retinohypothalamic tract. *Nature Neuroscience*, 4(12), 1165–1165.
- Govardovskii, V. I., Fyhrquist, N., Reuter, T., Kuzmin, D. G., & Donner, K. (2000). In search of the visual pigment template. *Visual Neuroscience*, 17(4), 509–28.
- Graham, D. M., Wong, K. Y., Shapiro, P., Frederick, C., Pattabiraman, K., & Berson, D. M. (2008). Melanopsin ganglion cells use a membrane-associated rhabdomeric phototransduction cascade. *Journal of Neurophysiology*, 99(5), 2522–32.
- Güler, 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., Hankins, M. W., Berson, D. M., Lucas, R. J., Yau, K.-W. & Hattar, S. (2008). Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. *Nature*, 453(7191), 102–5.

- Hankins, M. W., & Lucas, R. J. (2002). The Primary Visual Pathway in Humans Is Regulated According to Long-Term Light Exposure through the Action of a Nonclassical Photopigment. *Current Biology*, 12(3), 191–198.
- Hankins, M. W., Peirson, S. N., & Foster, R. G. (2008). Melanopsin: an exciting photopigment. *Trends in Neurosciences*, 31(1), 27–36.
- Hannibal J., Hindersson P., Knudsen S. M., Georg B., & Fahrenkrug J. (2002). The photopigment melanopsin is exclusively present in pituitary adenylate cyclase-activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. *Journal of Neuroscience*, 22(1), RC191.
- Hargrave, P. A. (2001). Rhodopsin. In *Encyclopedia of Life Sciences*. Chichester, UK: John Wiley & Sons, Ltd.
- Hargrave, P. A., & McDowell, J. H. (1992). Rhodopsin and phototransduction: a model system for G protein-linked receptors. *FASEB Journal*, 6(6), 2323–31.
- Hartwick, A. T. E., Bramley, J. R., Yu, J., Stevens, K. T., Allen, C. N., Baldrige, W. H., Solar, P. J. & Pickard, G. E. (2007). Light-evoked calcium responses of isolated melanopsin-expressing retinal ganglion cells. *The Journal of Neuroscience*, 27(49), 13468–80.
- Hatori, M., Le, H., Vollmers, C., Keding, S. R., Tanaka, N., Schmedt, C., Jegla, T. & Panda, S. (2008). Inducible ablation of melanopsin-expressing retinal ganglion cells reveals their central role in non-image forming visual responses. *PLoS One*, 3(6), e2451.
- Hatori, M., & Panda, S. (2010). The emerging roles of melanopsin in behavioral adaptation to light. *Trends in Molecular Medicine*, 16(10), 435–446.
- Hattar, S., Kumar, M., Park, A., Tong, P., Tung, J., Yau, K.-W., & Berson, D. M. (2006). Central projections of melanopsin-expressing retinal ganglion cells in the mouse. *The Journal of Comparative Neurology*, 497(3), 326–349.
- Hattar, S., Liao, H.-W. W., Takao, M., Berson, D. M. & Yau, K.-W. (2002). Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science*, 295(5557), 1065–70.
- Hattar, S., Lucas, R. J., Mrosovsky, N., Thompson, S., Douglas, R. H., Hankins, M. W., Lem, J., Biel, M., Hofmann, F., Foster, R. G. & Yau, K.-W. (2003). Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature*, 424(6944), 75–81.
- Higuchi, S., Hida, A., Tsujimura, S., Mishima, K., Yasukouchi, A., Lee, S., Kinjyo, Y. & Miyahira, M. (2013). Melanopsin gene polymorphism I394T is associated with pupillary light responses in a dose-dependent manner. *PLoS One*, 8(3), e60310.
- Hu, C., Hill, D. D., & Wong, K. Y. (2013). Intrinsic physiological properties of the five types of mouse ganglion-cell photoreceptors. *Journal of Neurophysiology*, 109(7), 1876–89.

- Hughes, S., Jagannath, A., Hankins, M. W., Foster, R. G., & Peirson, S. N. (2015). Chapter 6 – Photic regulation of clock systems. *Methods in Enzymology*, 552, 125–43.
- Hughes, S., Jagannath, A., Hickey, D., Gatti, S., Wood, M., Peirson, S. N., Foster, R. G. & Hankins, M. W. (2015). Using siRNA to define functional interactions between melanopsin and multiple G Protein partners. *Cellular and Molecular Life Sciences*, 72(1), 165–79.
- Hughes, S., Jagannath, A., Rodgers, J., Hankins, M. W., Peirson, S. N., & Foster, R. G. (2016). Signalling by melanopsin (OPN4) expressing photosensitive retinal ganglion cells. *Eye*, 30(2), 247–254.
- Hughes, S., Watson, T.S., Foster, R.G., Peirson, S.N. and Hankins, M.W. (2013). Nonuniform distribution and spectral tuning of photosensitive retinal ganglion cells of the mouse retina. *Current Biology*, 23(17), 1696-1701.
- Hughes, S., Potheary, C. A., Jagannath, A., Foster, R. G., Hankins, M. W., & Peirson, S. N. (2012a). Profound defects in pupillary responses to light in TRPM-channel null mice: a role for TRPM channels in non-image-forming photoreception. *The European Journal of Neuroscience*, 35(1), 34–43.
- Hughes, S., Welsh, L., Katti, C., González-Menéndez, I., Turton, M., Halford, S., Sekaran, S., Peirson, S. N., Hankins, M. W. & Foster, R. G. (2012b). Differential Expression of Melanopsin Isoforms Opn4L and Opn4S during Postnatal Development of the Mouse Retina. *PLoS One*, 7(4), e34531
- Hunt, D. M., Carvalho, L. S., Cowing, J. A., & Davies, W. L. (2009). Evolution and spectral tuning of visual pigments in birds and mammals. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 364(1531), 2941–55.
- Hwa, J., Reeves, P. J., Klein-Seetharaman, J., Davidson, F., & Khorana, H. G. (1999). Structure and function in rhodopsin: further elucidation of the role of the intradiscal cysteines, Cys-110, -185, and -187, in rhodopsin folding and function. *Proceedings of the National Academy of Sciences*, 96(5), 1932–5.
- Jagannath, A., Hughes, S., Abdelgany, A., Potheary, C. A., Di Pretoro, S., Pires, S. S., Vachtsevanos, A., Pillorz, V., Brown, L. A., Hossbach, M., MacLaren, R. E., Halford, S., Gatti, S., Hankins, M. W., Wood, M. J. A., Foster, R. G. & Peirson, S. N. (2015). Isoforms of Melanopsin Mediate Different Behavioral Responses to Light. *Current Biology*, 25(18), 2430–4.
- Janz, J. M., & Farrens, D. L. (2001). Engineering a functional blue-wavelength-shifted Rhodopsin mutant. *Biochemistry*, 40(24), 7219–7227.
- Janz, J. M., Fay, J. F., & Farrens, D. L. (2003). Stability of dark state rhodopsin is mediated by a conserved ion pair in intradiscal loop E-2. *The Journal of Biological Chemistry*, 278(19), 16982–91.

- Jiang, R., Duan, J., Windemuth, A., Stephens, J. C., Judson, R., & Xu, C. (2003). Genome-wide evaluation of the public SNP databases. *Pharmacogenomics*, 4(6), 779–89.
- Johnson, J., Wu, V., Donovan, M., Majumdar, S., Renteria, R. C., Porco, T., Van Gelder, R. N. & Copenhagen, D. R. (2010). Melanopsin-dependent light avoidance in neonatal mice. *Proceedings of the National Academy of Sciences*, 107(40), 17374–17378.
- Jud, C., Schmutz, I., Hampp, G., Oster, H., & Albrecht, U. (2005). A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. *Biological Procedures Online*, 7, 101–16.
- Kankipati, L., Girkin, C. A., & Gamlin, P. D. (2010). Post-illumination pupil response in subjects without ocular disease. *Investigative Ophthalmology & Visual Science*, 51(5), 2764–9.
- Kankipati, L., Girkin, C. A., & Gamlin, P. D. (2011). The post-illumination pupil response is reduced in glaucoma patients. *Investigative Ophthalmology & Visual Science*, 52(5), 2287–92.
- Karnik, S. S., Sakmar, T. P., Chen, H.-B., & Khorana, H. G. (1988). Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. *Proceedings of the National Academy of Sciences*, 85(22), 8459–8463.
- Katoh, K., Misawa, K., Kuma, K., & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, 30(14), 3059–3066.
- Katritch, V., Cherezov, V., & Stevens, R. C. (2013). Structure-Function of the G Protein–Coupled Receptor Superfamily. *Annual Review of Pharmacology and Toxicology*, 53(1), 531–556.
- Kaushal, S., Ridge, K. D., & Khorana, H. G. (1994). Structure and function in rhodopsin: the role of asparagine-linked glycosylation. *Proceedings of the National Academy of Sciences*, 91(9), 4024–8.
- Kemmer, G., & Keller, S. (2010). Nonlinear least-squares data fitting in Excel spreadsheets. *Nature Protocols*, 5(2), 267–281.
- Kim, J.-M., Hwa, J., Garriga, P., Reeves, P. J., RajBhandary, U. L., & Khorana, H. G. (2005). Light-Driven Activation of β_2 -Adrenergic Receptor Signaling by a Chimeric Rhodopsin Containing the β_2 -Adrenergic Receptor Cytoplasmic Loops. *Biochemistry*, 44(7), 2284–2292.
- Kitts, A., & Sherry, S. (2002). The Single Nucleotide Polymorphism Database (dbSNP) of Nucleotide Sequence Variation. National Center for Biotechnology Information (US).
- Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J.-Y., Sackler, R. S., Haynes, C., Henning, A. K., SanGiovanni, J. P., Mane, S. M., Mayne, S. T., Bracken, M. B., Ferris, F. L., Ott, J., Barnstable, C. & Hoh, J. (2005). Complement factor H polymorphism in age-related macular degeneration. *Science*, 308(5720), 385–9.

- Kobilka, B. K. (2007). G protein coupled receptor structure and activation. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1768(4), 794–807.
- Kobilka, B. K., & Deupi, X. (2007). Conformational complexity of G-protein-coupled receptors. *Trends in Pharmacological Sciences*, 28(8), 397–406.
- Kochendoerfer, G. G., Lin, S. W., Sakmar, T. P., & Mathies, R. A. (1999). How color visual pigments are tuned. *Trends in Biochemical Sciences*, 24(8), 300–305.
- Kock, I., Bulgakova, N. A., Knust, E., Sinning, I., & Panneels, V. (2009). Targeting of Drosophila Rhodopsin Requires Helix 8 but Not the Distal C-Terminus. *PLoS One*, 4(7), e6101.
- Koizumi, A., Tanaka, K. F., & Yamanaka, A. (2013). The manipulation of neural and cellular activities by ectopic expression of melanopsin. *Neuroscience Research*, 75(1), 3–5.
- Kolstad, K. D., Dalkara, D., Guerin, K., Visel, M., Hoffmann, N., Schaffer, D. V., & Flannery, J. G. (2010). Changes in adeno-associated virus-mediated gene delivery in retinal degeneration. *Human Gene Therapy*, 21(5), 571–8.
- Koyanagi, M., Kubokawa, K., Tsukamoto, H., Shichida, Y., & Terakita, A. (2005). Cephalochordate melanopsin: evolutionary linkage between invertebrate visual cells and vertebrate photosensitive retinal ganglion cells. *Current Biology*, 15(11), 1065–9.
- Kühn, H., & Wilden, U. (1987). Deactivation of photoactivated rhodopsin by rhodopsin-kinase and arrestin. *Journal of Receptor Research*, 7(1-4), 283–98.
- Kumar, P., Henikoff, S., & Ng, P. C. (2009). Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nature Protocols*, 4(7), 1073–1081.
- Kumaramanickavel, G., Maw, M., Denton, M. J., John, S., Srikumari, C. R., Orth, U., Oehlmann, R. & Gal, A. (1994). Missense rhodopsin mutation in a family with recessive RP. *Nature Genetics*, 8(1), 10–1.
- Kumbalasiri, T., Rollag, M. D., Isoldi, M. C., Castrucci, A. M. de L., & Provencio, I. (2007). Melanopsin triggers the release of internal calcium stores in response to light. *Photochemistry and Photobiology*, 83(2), 273–80.
- Lall, G. S., Revell, V. L., Momiji, H., Al Enezi, J., Altimus, C. M., Güler, A. D., Aguilar, C., Cameron, M. A., Allender, S., Hankins, M. W. & Lucas, R. J. (2010). Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance. *Neuron*, 66(3), 417–28.
- Lee, S., Hida, A., Tsujimura, S., Morita, T., Mishima, K., & Higuchi, S. (2013). Association between melanopsin gene polymorphism (I394T) and pupillary light reflex is dependent on light wavelength. *Journal of Physiological Anthropology*, 32(16).

- Lee, S.-I., Hida, A., Kitamura, S., Mishima, K., & Higuchi, S. (2014). Association between the melanopsin gene polymorphism OPN4*Ile394Thr and sleep/wake timing in Japanese university students. *Journal of Physiological Anthropology*, 33(9).
- Lin, B., Koizumi, A., Tanaka, N., Panda, S., & Masland, R. H. (2008). Restoration of visual function in retinal degeneration mice by ectopic expression of melanopsin. *Proceedings of the National Academy of Science*, 105(41), 16009–14.
- Lin, S. W., Kochendoerfer, G. G., Carroll, K. S., Wang, D., Mathies, R. A., & Sakmar, T. P. (1998). Mechanisms of spectral tuning in blue cone visual pigments. Visible and raman spectroscopy of blue-shifted rhodopsin mutants. *The Journal of Biological Chemistry*, 273(38), 24583–91.
- Liu, M., Dai, J., Liu, W., Zhao, C., & Yin, Z. Q. (2016). Overexpression of melanopsin in the retina restores visual function in Royal College of Surgeons rats. *Molecular Medicine Reports*, 13(1), 321–6.
- Lucas, R.J., Freedman, M. S., Muñoz, M., Garcia-Fernández, J. M. & Foster, R. G. (1999). Regulation of the Mammalian Pineal by Non-rod, Non-cone, Ocular Photoreceptors. *Science*, 284(5413), 505–507.
- Lucas, R. J., Hattar, S., Takao, M., Berson, D. M., Foster, R. G. & Yau, K. W. (2003). Diminished Pupillary Light Reflex at High Irradiances in Melanopsin-Knockout Mice. *Science*, 299(5604), 245–247.
- Lucas, R. J., Douglas, R. H., & Foster, R. G. (2001). Characterization of an ocular photopigment capable of driving pupillary constriction in mice. *Nature Neuroscience*, 4(6), 621–626.
- Lupi, D., Oster, H., Thompson, S., & Foster, R. G. (2008). The acute light-induction of sleep is mediated by OPN4-based photoreception. *Nature Neuroscience*, 11(9), 1068–73.
- Lupi, D., Sekaran, S., Jones, S. L., Hankins, M. W., & Foster, R. G. (2006). Light-evoked FOS induction within the suprachiasmatic nuclei (SCN) of melanopsin knockout (*Opn4*^{-/-}) mice: A developmental study. *Chronobiology International*, 23(1-2), 167–179.
- Macke, J. P., Davenport, C. M., Jacobson, S. G., Hennessey, J. C., Gonzalez-Fernandez, F., Conway, B. P., Heckenlively, J., Palmer, R., Maumenee, I. H., Sieving, P., Gouras, P., Good, W. & Nathans, J. (1993). Identification of novel rhodopsin mutations responsible for retinitis pigmentosa: implications for the structure and function of rhodopsin. *American Journal of Human Genetics*, 53(1), 80–9.
- Marin, E. P., Krishna, A. G., Zvyaga, T. A., Isele, J., Siebert, F., & Sakmar, T. P. (2000). The amino terminus of the fourth cytoplasmic loop of rhodopsin modulates rhodopsin-transducin interaction. *The Journal of Biological Chemistry*, 275(3), 1930–6.

- Matos-Cruz, V., Blasic, J., Nickle, B., Robinson, P. R., Hattar, S., & Halpern, M. E. (2011). Unexpected diversity and photoperiod dependence of the zebrafish melanopsin system. *PloS One*, 6(9), e25111.
- Matsuyama, T., Yamashita, T., Imamoto, Y., & Shichida, Y. (2012). Photochemical properties of mammalian melanopsin. *Biochemistry*, 51(27), 5454–62.
- Mawad, K., & Van Gelder, R. N. (2008). Absence of Long-Wavelength Photoc Potentiation of Murine Intrinsically Photosensitive Retinal Ganglion Cell Firing In Vitro. *Journal of Biological Rhythms*, 23(5), 387–391.
- McNeill, D. S., Sheely, C. J., Ecker, J. L., Badea, T. C., Morhardt, D., Guido, W. & Hattar, S. (2011). Development of melanopsin-based irradiance detecting circuitry. *Neural Development*, 6(1), 8.
- Meister, M., Pine, J., & Baylor, D. A. (1994). Multi-neuronal signals from the retina: acquisition and analysis. *Journal of Neuroscience Methods*, 51(1), 95–106.
- Melyan, Z., Tattelin, E. E., Bellingham, J., Lucas, R. J., & Hankins, M. W. (2005). Addition of human melanopsin renders mammalian cells photoresponsive. *Nature*, 433(7027), 741–5.
- Mendes, H. F., van der Spuy, J., Chapple, J. P., & Cheetham, M. E. (2005). Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy. *Trends in Molecular Medicine*, 11(4), 177–185.
- Menon, S. T., Han, M., & Sakmar, T. P. (2001). Rhodopsin: structural basis of molecular physiology. *Physiological Reviews*, 81(4), 1659–88.
- Merbs, S. L., & Nathans, J. (1993). Role Of Hydroxyl-Bearing Amino Acids In Differentially Tuning The Absorption Spectra Of The Human Red And Green Cone Pigments. *Photochemistry and Photobiology*, 58(5), 706–710.
- Montell, C. (1999). Visual Transduction In Drosophila. *Annual Review of Cell and Developmental Biology*, 15(1), 231–268.
- Morin, L. P., Blanchard, J. H., & Provencio, I. (2003). Retinal ganglion cell projections to the hamster suprachiasmatic nucleus, intergeniculate leaflet, and visual midbrain: Bifurcation and melanopsin immunoreactivity. *The Journal of Comparative Neurology*, 465(3), 401–416.
- Motulsky, H., & Christopoulos, A. (2003). Fitting Models to Biological Data using Linear and Nonlinear Regression Contents at a Glance. Graphpad Prism. Accessed from <http://www.graphpad.com/manuals/prism4/RegressionBook.pdf>
- Mowat, F. M., Gornik, K. R., Dinculescu, A., Boye, S. L., Hauswirth, W. W., Petersen-Jones, S. M., & Bartoe, J. T. (2014). Tyrosine capsid-mutant AAV vectors for gene delivery to the canine retina from a subretinal or intravitreal approach. *Gene Therapy*, 21(1), 96–105.
- Mrosovsky, N., & Hattar, S. (2003). Impaired masking responses to light in melanopsin-knockout mice. *Chronobiology International*, 20(6), 989–99.

- Muindi, F., Zeitzer, J. M., Colas, D., & Heller, H. C. (2013). The acute effects of light on murine sleep during the dark phase: importance of melanopsin for maintenance of light-induced sleep. *The European Journal of Neuroscience*, 37(11), 1727–36.
- Murakami, M., & Kouyama, T. (2008). Crystal structure of squid rhodopsin. *Nature*, 453(7193), 363–367.
- Mure, L. S., Cornut, P.-L., Rieux, C., Drouyer, E., Denis, P., Gronfier, C., & Cooper, H. M. (2009). Melanopsin Bistability: A Fly's Eye Technology in the Human Retina. *PLoS One*, 4(6), e5991.
- Mure, L. S., Hatori, M., Zhu, Q., Demas, J., Kim, I. M., Nayak, S. K., & Panda, S. (2016). Melanopsin-Encoded Response Properties of Intrinsically Photosensitive Retinal Ganglion Cells. *Neuron*, 90(5), 1016–27.
- Nathans, J. (1990). Determinants of visual pigment absorbance: Role of charged amino acids in the putative transmembrane segments. *Biochemistry*, 29(4), 937–942.
- Neitz, M., Neitz, J., & Jacobs, G. H. (1991). Spectral tuning of pigments underlying red-green color vision. *Science*, 252(5008), 971–4.
- Nelson, R. J., & Zucker, I. (1981). Absence of extraocular photoreception in diurnal and nocturnal rodents exposed to direct sunlight. *Comparative Biochemistry and Physiology Part A: Physiology*, 69(1), 145–148.
- Newman, E., & Reichenbach, A. (1996). The Müller cell: a functional element of the retina. *Trends in Neurosciences*, 19(8), 307–312.
- Newman, L. A., Walker, M. T., Brown, R. L., Cronin, T. W., & Robinson, P. R. (2003). Melanopsin forms a functional short-wavelength photopigment. *Biochemistry*, 42(44), 12734–8.
- Nolan, P. M., Hugill, A., & Cox, R. D. (2002). ENU mutagenesis in the mouse: application to human genetic disease. *Briefings in Functional Genomics & Proteomics*, 1(3), 278–89.
- Nolan, P. M., Peters, J., Strivens, M., Rogers, D., Hagan, J., Spurr, N. et al., (2000). A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nature Genetics*, 25(4), 440–3.
- Noorwez, S. M., Kuksa, V., Imanishi, Y., Zhu, L., Filipek, S., Palczewski, K., & Kaushal, S. (2003). Pharmacological Chaperone-mediated in Vivo Folding and Stabilization of the P23H-opsin Mutant Associated with Autosomal Dominant Retinitis Pigmentosa. *Journal of Biological Chemistry*, 278(16), 14442–14450.
- Okada, T., Ernst, O. P., Palczewski, K. & Hofmann, K. P. (2001). Activation of rhodopsin: new insights from structural and biochemical studies. *Trends in Biochemical Sciences*, 26(5), 318–324.
- Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., & Shichida, Y. (2002). Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography. *Proceedings of the National Academy of Sciences*, 99(9), 5982–7.

- Okada, T., Sugihara, M., Bondar, A.-N., Elstner, M., Entel, P., & Buss, V. (2004). The Retinal Conformation and its Environment in Rhodopsin in Light of a New 2.2Å Crystal Structure. *Journal of Molecular Biology*, 342(2), 571–583.
- Oprrian, D. D., Asenjo, A. B., Lee, N., & Pelletier, S. L. (1991). Design, chemical synthesis, and expression of genes for the three human color vision pigments. *Biochemistry*, 30(48), 11367–72.
- Oprrian, D. D., Molday, R. S., Kaufman, R. J., & Khorana, H. G. (1987). Expression of a synthetic bovine rhodopsin gene in monkey kidney cells. *Proceedings of the National Academy of Sciences*, 84(24), 8874–8.
- Pack, A. I., Galante, R. J., Maislin, G., Cater, J., Metaxas, D., Lu, S., Zhang, L., Smith, R. V., Kay, T., Lian, J., Svenson, K. & Peters, L. L. (2007). Novel method for high-throughput phenotyping of sleep in mice. *Physiological Genomics*, 28(2), 232–8.
- Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Trong, I. L., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M. & Miyano, M. (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science*, 289(5480), 739–45.
- Panda, S., Nayak, S. K., Campo, B., Walker, J. R., Hogenesch, J. B., & Jegla, T. (2005). Illumination of the Melanopsin Signaling Pathway. *Science*, 307(5709), 600–604.
- Panda, S., Sato, T. K., Castrucci, A. M., Rollag, M. D., DeGrip, W. J., Hogenesch, J. B., Provencio, I. & Kay, S. A. (2002). Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. *Science*, 298(5601), 2213–6.
- Park, J. H., Scheerer, P., Hofmann, K. P., Choe, H.-W., & Ernst, O. P. (2008). Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature*, 454(7201), 183–187.
- Partonen, T., & Lönngqvist, J. (1998). Seasonal affective disorder. *Lancet*, 352(9137), 1369–74.
- Peirson, S. N., Halford, S., & Foster, R. G. (2009). The evolution of irradiance detection: melanopsin and the non-visual opsins. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 364, 2849–2865.
- Peirson, S. N., Oster, H., Jones, S. L., Leitges, M., Hankins, M. W., & Foster, R. G. (2007). Microarray analysis and functional genomics identify novel components of melanopsin signaling. *Current Biology*, 17(16), 1363–72.
- Peirson, S. N., Thompson, S., Hankins, M. W., & Foster, R. G. (2005). Mammalian Photoentrainment: Results, Methods, and Approaches. *Methods in Enzymology*, 393, 697–726.
- Peirson, S. N., Butler, J.N. & Foster, R.G. (2003). Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Research*, 31(14), e73-e73.

- Perez-Leighton, C. E., Schmidt, T. M., Abramowitz, J., Birnbaumer, L., & Kofuji, P. (2011). Intrinsic phototransduction persists in melanopsin-expressing ganglion cells lacking diacylglycerol-sensitive TRPC subunits. *The European Journal of Neuroscience*, 33(5), 856–67.
- Pires, S. S., Hughes, S., Turton, M., Melyan, Z., Peirson, S. N., Zheng, L., Kosmaoglou, M., Bellingham, J., Cheetham, M. E., Lucas, R. J., Foster, R. G., Hankins, M. W. & Halford, S. (2009). Differential expression of two distinct functional isoforms of melanopsin (Opn4) in the mammalian retina. *The Journal of Neuroscience*, 29(39), 12332–42.
- Pittler, S. J., & Baehr, W. (1991). Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase beta-subunit gene of the rd mouse. *Proceedings of the National Academy of Sciences*, 88(19), 8322–8326.
- Premont, R. T., & Gainetdinov, R. R. (2007). Physiological Roles of G Protein–Coupled Receptor Kinases and Arrestins. *Annual Review of Physiology*, 69(1), 511–534.
- Provencio, I., & Foster, R. G. (1995). Circadian rhythms in mice can be regulated by photoreceptors with cone-like characteristics. *Brain Research*, 694(1-2), 183–90.
- Provencio, I., Jiang, G., Willem, J., Hayes, W. P., & Rollag, M. D. (1998). Melanopsin: An opsin in melanophores, brain, and eye. *Proceedings of the National Academy of Sciences*, 95(1), 340–345.
- Provencio, I., Rodriguez, I. R., Jiang, G., Hayes, W. P., Moreira, E. F., & Rollag, M. D. (2000). A Novel Human Opsin in the Inner Retina. *The Journal of Neuroscience*, 20(2), 600–605.
- Provencio, I., Rollag, M. D., & Castrucci, A. M. (2002). Anatomy Photoreceptive net in the mammalian retina. *Nature*, 415(6871), 493.
- Pulagam, L. P., & Palczewski, K. (2010). Electrostatic compensation restores trafficking of the autosomal recessive retinitis pigmentosa E150K opsin mutant to the plasma membrane. *The Journal of Biological Chemistry*, 285(38), 29446–56.
- Qin, J. Y., Zhang, L., Clift, K. L., Hular, I., Xiang, A. P., Ren, B.-Z., & Lahn, B. T. (2010). Systematic Comparison of Constitutive Promoters and the Doxycycline-Inducible Promoter. *PLoS ONE*, 5(5), e10611.
- Qiu, X., & Berson, D. M. (2007). Melanopsin Bistability in Ganglion-Cell Photoreceptors. *Investigative Ophthalmology & Visual Science*, 48(13), 612.
- Qiu, X., Kumbalasiri, T., Carlson, S. M., Wong, K. Y., Krishna, V., Provencio, I., & Berson, D. M. (2005). Induction of photosensitivity by heterologous expression of melanopsin. *Nature*, 433(7027), 745–749.
- Quwailid, M. M., Hugill, A., Dear, N., Vizer, L., Wells, S., Horner, E., et al. (2004). A gene-driven ENU-based approach to generating an allelic series in any gene. *Mammalian Genome*, 15(8), 585-91.

- Rakoczy, E. P., Kiel, C., McKeone, R., Stricher, F., & Serrano, L. (2011). Analysis of Disease-Linked Rhodopsin Mutations Based on Structure, Function, and Protein Stability Calculations. *Journal of Molecular Biology*, 405(2), 584–606.
- Raman, D., Osawa, S., & Weiss, E. R. (1999). Binding of Arrestin to Cytoplasmic Loop Mutants of Bovine Rhodopsin. *Biochemistry*, 38(16), 5117–5123.
- Rao, S., Chun, C., Fan, J., Kofron, J. M., Yang, M. B., Hegde, R. S., Ferrara, N., Copenhagen, D. R. & Lang, R. A. (2013). A direct and melanopsin-dependent fetal light response regulates mouse eye development. *Nature*, 494(7436), 243–246.
- Rao, V. R., Cohen, G. B., & Oprian, D. D. (1994). Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature*, 367(6464), 639–642.
- Redmond, T. M., Yu, S., Lee, E., Bok, D., Hamasaki, D., Chen, N., Goletz, P., Ma, J.-X., Crouch, R. K. & Pfeifer, K. (1998). Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nature Genetics*, 20(4), 344–350.
- Reifler, A. N., Chervenak, A. P., Dolikian, M. E., Benenati, B. A., Meyers, B. S., Demertzis, Z. D., Lynch, A. M., Li, B. Y., Wachter, R. D., Abufarha, F. S., Dulka, E. A., Pack, W., Zhao, X. & Wong, K. Y. (2015). The rat retina has five types of ganglion-cell photoreceptors. *Experimental Eye Research*, 130, 17–28.
- Reinhard, K., Tikidji-Hamburyan, A., Seitter, H., Idrees, S., Mutter, M., Benkner, B., & Münch, T. A. (2014). Step-by-step instructions for retina recordings with perforated multi electrode arrays. *PLoS One*, 9(8), e106148.
- Ridler, T.W. & Calvard, S. (1978) Picture thresholding using an iterative selection method, *IEEE Transactions on Systems, Man and Cybernetics*, 8, 630-632.
- Roecklein, K. A., Rohan, K. J., Duncan, W. C., Rollag, M. D., Rosenthal, N. E., Lipsky, R. H., & Provencio, I. (2009). A missense variant (P10L) of the melanopsin (OPN4) gene in seasonal affective disorder. *Journal of Affective Disorders*, 114(1), 279–85.
- Roecklein, K. A., Wong, P. M., Franzen, P. L., Hasler, B. P., Wood-Vasey, W. M., Nimgaonkar, V. L., Miller, M. A., Kepreos, K. M., Ferrell, R. E. & Manuck, S. B. (2012). Melanopsin gene variations interact with season to predict sleep onset and chronotype. *Chronobiology International*, 29(8), 1036–47.
- Roecklein, K., Wong, P., Ernecoff, N., Miller, M., Donofry, S., Kamarck, M., Wood-Vasey, M. & Franzen, P. (2013). The post illumination pupil response is reduced in seasonal affective disorder. *Psychiatry Research*, 210(1), 150–8.
- Ruby, N. F., Brennan, T. J., Xie, X., Cao, V., Franken, P., Heller, H. C., & O'Hara, B. F. (2002). Role of melanopsin in circadian responses to light. *Science*, 298(5601), 2211–3.
- Rutkowski, D. T., & Kaufman, R. J. (2004). A trip to the ER: coping with stress. *Trends in Cell Biology*, 14(1), 20–28.
- Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1989). Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. *Proceedings of the National Academy of Sciences*, 86(21), 8309–8313.

- Saliba, R. S., Munro, P. M. G., Luthert, P. J., & Cheetham, M. E. (2002). The cellular fate of mutant rhodopsin: quality control, degradation and aggresome formation. *Journal of Cell Science*, 115(14), 2907–18.
- Schadel, S. A., Heck, M., Maretzki, D., Filipek, S., Teller, D. C., Palczewski, K., & Hofmann, K. P. (2003). Ligand Channeling within a G-protein-coupled Receptor: The Entry And Exit Of Retinals In Native Opsin. *Journal of Biological Chemistry*, 278(27), 24896–24903.
- Scheerer, P., Park, J. H., Hildebrand, P. W., Kim, Y. J., Krauss, N., Choe, H.-W., Hofmann, P. & Ernst, O. P. (2008). Crystal structure of opsin in its G-protein-interacting conformation. *Nature*, 455(7212), 497–502.
- Schmidt, T. M., Chen, S.-K., & Hattar, S. (2011). Intrinsically photosensitive retinal ganglion cells: many subtypes, diverse functions. *Trends in Neurosciences*, 34(11), 572–580.
- Schmidt, T. M., & Kofuji, P. (2009). Functional and Morphological Differences among Intrinsically Photosensitive Retinal Ganglion Cells. *Journal of Neuroscience*, 29(2), 476–482.
- Schmidt, T. M., & Kofuji, P. (2011a). Structure and function of bistratified intrinsically photosensitive retinal ganglion cells in the mouse. *The Journal of Comparative Neurology*, 519(8), 1492–1504.
- Schmidt, T. M., & Kofuji, P. (2011b). An isolated retinal preparation to record light response from genetically labelled retinal ganglion cells. *Journal of Visualized Experiments*, 47, e2367
- Sekaran, S., Foster, R. G., Lucas, R. J., & Hankins, M. W. (2003). Calcium Imaging Reveals a Network of Intrinsically Light-Sensitive Inner-Retinal Neurons. *Current Biology*, 13(15), 1290–1298.
- Semo, M., Gias, C., Ahmado, A., Sugano, E., Allen, A. E., Lawrence, J. M., Tomita, H., Coffey, P. J. & Vugler, A. A. (2010). Dissecting a role for melanopsin in behavioural light aversion reveals a response independent of conventional photoreception. *PLoS One*, 5(11), e15009.
- Sexton, T. J., Bleckert, A., Turner, M. H., & Van Gelder, R. N. (2015). Type I intrinsically photosensitive retinal ganglion cells of early post-natal development correspond to the M4 subtype. *Neural Development*, 10(1), 17.
- Sexton, T. J. & Van Gelder, R. N. (2015). G-Protein Coupled Receptor Kinase 2 Minimally Regulates Melanopsin Activity in Intrinsically Photosensitive Retinal Ganglion Cells. *PLoS One*, 10(6), e0128690.
- Sexton, T. J., Golczak, M., Palczewski, K., & Van Gelder, R. N. (2012). Melanopsin is highly resistant to light and chemical bleaching in vivo. *The Journal of Biological Chemistry*, 287(25), 20888–97.

- Sheffield, V. C., Fishman, G. A., Beck, J. S., Kimura, A. E. & Stone, E. M. (1991). Identification of novel rhodopsin mutations associated with retinitis pigmentosa by GC-clamped denaturing gradient gel electrophoresis. *American Journal of Human Genetics*, 49(4), 699.
- Shen, G.-Q., Abdullah, K. G., & Wang, Q. K. (2009). The TaqMan Method for SNP Genotyping. *Methods in Molecular Biology*, 578, 293–306.
- Sherry, S. T., Ward, M. H., Kholodov, M., Baker, J., Phan, L., Smigielski, E. M., & Sirotkin, K. (2001). dbSNP: the NCBI database of genetic variation. *Nucleic Acids Research*, 29(1), 308–11.
- Shi, L., & Javitch, J. A. (2004). The second extracellular loop of the dopamine D2 receptor lines the binding-site crevice. *Proceedings of the National Academy of Sciences*, 101(2), 440–5.
- Shichida, Y. & Matsuyama, T. (2009). Evolution of opsins and phototransduction. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 364(1531), 2881–95.
- Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G. W., Kobayashi, T., Stevens, R. C. & Iwata, S. (2011). Structure of the human histamine H1 receptor complex with doxepin. *Nature*, 475(7354), 65–70.
- Shirzad-Wasei, N., van Oostrum, J., Bovee-Geurts, P. H., Wasserman, M., Bosman, G. J., & DeGrip, W. J. (2013). Large scale expression and purification of mouse melanopsin-L in the baculovirus expression system. *Protein Expression and Purification*, 91(2), 134–146.
- Small, K. M., Tanguay, D. A., Nandabalan, K., Zhan, P., Stephens, J. C., & Liggett, S. B. (2003). Gene and protein domain-specific patterns of genetic variability within the g-protein coupled receptor Superfamily. *American Journal of Pharmacogenomics*, 3(1), 65–71.
- Smallwood, P. M., Ölveczky, B. P., Williams, G. L., Jacobs, G. H., Reese, B. E., Meister, M., & Nathans, J. (2003). Genetically engineered mice with an additional class of cone photoreceptors: implications for the evolution of color vision. *Proceedings of the National Academy of Sciences of the United States of America*, 100(20), 11706–11.
- Sollars, P. J., Smeraski, C. A., Kaufman, J. D., Ogilvie, M. D., Provencio, I., & Pickard, G. E. (2003). Melanopsin and non-melanopsin expressing retinal ganglion cells innervate the hypothalamic suprachiasmatic nucleus. *Visual Neuroscience*, 20(6), 601–10.
- Soucy, E., Wang, Y., Nirenberg, S., Nathans, J., & Meister, M. (1998). A Novel Signaling Pathway from Rod Photoreceptors to Ganglion Cells in Mammalian Retina. *Neuron*, 21(3), 481–493.
- Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M., & Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Developmental Biology*, 1, 4.

- Sun, H., Macke, J. P., & Nathans, J. (1997). Mechanisms of spectral tuning in the mouse green cone pigment. *Proceedings of the National Academy of Sciences*, 94(16), 8860–5.
- Sun, X., Ågren, H., & Tu, Y. (2014). Functional Water Molecules in Rhodopsin Activation. *The Journal of Physical Chemistry B*, 118(37), 10863–10873.
- Sung, C. H., Schneider, B. G., Agarwal, N., Papermaster, D. S., & Nathans, J. (1991). Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. *Proceedings of the National Academy of Sciences*, 88(19), 8840–8844.
- Tabor, H. K., Risch, N. J., & Myers, R. M. (2002). Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nature Reviews Genetics*, 3(5), 391–397.
- Tai, A. W., Chuang, J.-Z., Bode, C., Wolfrum, U., & Sung, C.-H. (1999). Rhodopsin's Carboxy-Terminal Cytoplasmic Tail Acts as a Membrane Receptor for Cytoplasmic Dynein by Binding to the Dynein Light Chain Tctex-1. *Cell*, 97(7), 877–887.
- Terakita, A., Koyanagi, M., Tsukamoto, H., Yamashita, T., Miyata, T., & Shichida, Y. (2004). Counterion displacement in the molecular evolution of the rhodopsin family. *Nature Structural & Molecular Biology*, 11(3), 284–289.
- Terakita, A., Tsukamoto, H., Koyanagi, M., Sugahara, M., Yamashita, T., & Shichida, Y. (2008). Expression and comparative characterization of Gq-coupled invertebrate visual pigments and melanopsin. *Journal of Neurochemistry*, 105(3), 883–890.
- Thapan, K., Arendt, J., & Skene, D. J. (2001). An action spectrum for melatonin suppression: evidence for a novel non-rod, non-cone photoreceptor system in humans. *The Journal of Physiology*, 535(1), 261–267.
- Torii, M., Kojima, D., Okano, T., Nakamura, A., Terakita, A., Shichida, Y., Wada, A. & Fukada, Y. (2007). Two isoforms of chicken melanopsins show blue light sensitivity. *FEBS Letters*, 581(27), 5327–5331.
- Tsai, J. W., Hannibal, J., Hagiwara, G., Colas, D., Ruppert, E., Ruby, N. F., Heller, C., Franken, P. & Bourgin, P. (2009). Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in *Opn4(-/-)* mice. *PLoS Biology*, 7(6), e1000125.
- Tsukamoto, H., & Terakita, A. (2010). Diversity and functional properties of bistable pigments. *Photochemical & Photobiological Sciences*, 9(11), 1435–43.
- Tsunematsu, T., Tanaka, K. F., & Yamanaka, A. (2013). Ectopic expression of melanopsin in orexin/hypocretin neurons enables control of wakefulness of mice in vivo by blue light. *Neuroscience Research*, 75(1), 23–28.
- Tu, D. C., Owens, L. A., Anderson, L., Golczak, M., Doyle, S. E., McCall, M., Menaker, M., Palczewski, K. & Van Gelder, R. N. (2006). Inner retinal photoreception independent of the visual retinoid cycle. *Proceedings of the National Academy of Sciences*, 103(27), 10426–10431.

- Tu, D. C., Zhang, D., Demas, J., Slutsky, E. B., Provencio, I., Holy, T. E., & Van Gelder, R. N. (2005). Physiologic diversity and development of intrinsically photosensitive retinal ganglion cells. *Neuron*, 48(6), 987–99.
- Van Someren, E. J., Swaab, D. F., Colenda, C. C., Cohen, W., McCall, W. V., & Rosenquist, P. B. (1999). Bright light therapy: improved sensitivity to its effects on rest-activity rhythms in Alzheimer patients by application of nonparametric methods. *Chronobiology International*, 16(4), 505–18.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3(7), 00341-003411.
- Viney, T. J., Balint, K., Hillier, D., Siebert, S., Boldogkoi, Z., Enquist, L. W., Meister, M., Cepko, C. L. & Roska, B. (2007). Local Retinal Circuits of Melanopsin-Containing Ganglion Cells Identified by Transsynaptic Viral Tracing. *Current Biology*, 17(11), 981–988.
- Walch, O. J., Zhang, L. S., Reifler, A. N., Dolikian, M. E., Forger, D. B., & Wong, K. Y. (2015). Characterizing and modeling the intrinsic light response of rat ganglion-cell photoreceptors. *Journal of Neurophysiology*, 114(5), 2955–66.
- Walker, M. T., Brown, R. L., Cronin, T. W., & Robinson, P. R. (2008). Photochemistry of retinal chromophore in mouse melanopsin. *Proceedings of the National Academy of Sciences*, 105(26), 8861–8865.
- Wang, J.-S., & Kefalov, V. J. (2009). An Alternative Pathway Mediates the Mouse and Human Cone Visual Cycle. *Current Biology* 19(19):1665-9.
- Wang, Y., Macke, J. P., Merbs, S. L., Zack, D. J., Klaunberg, B., Bennett, J., Gearhart, J. & Nathans, J. (1992). A locus control region adjacent to the human red and green visual pigment genes. *Neuron*, 9(3), 429–40.
- Warren, E. J., Allen, C. N., Brown, R. L., & Robinson, D. W. (2003). Intrinsic light responses of retinal ganglion cells projecting to the circadian system. *The European Journal of Neuroscience*, 17(9), 1727–35.
- Warren, E. J., Allen, C. N., Brown, R. L., & Robinson, D. W. (2006). The light-activated signaling pathway in SCN-projecting rat retinal ganglion cells. *The European Journal of Neuroscience*, 23(9), 2477–87.
- Wässle, H. (2004). Parallel processing in the mammalian retina. *Nature Reviews Neuroscience*, 5(10), 747–757.
- Weng, S., Estevez, M. E., & Berson, D. M. (2013). Mouse Ganglion-Cell Photoreceptors Are Driven by the Most Sensitive Rod Pathway and by Both Types of Cones. *PLoS One*, 8(6), e66480.
- Wilden, U., Hall, S. W., & Kühn, H. (1986). Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proceedings of the National Academy of Sciences*, 83(5), 1174–8.

- Wong, K. Y., Dunn, F. A., & Berson, D. M. (2005). Photoreceptor adaptation in intrinsically photosensitive retinal ganglion cells. *Neuron*, 48(6), 1001–10.
- Wong, K. Y., Dunn, F. A., Graham, D. M., & Berson, D. M. (2007). Synaptic influences on rat ganglion-cell photoreceptors. *The Journal of Physiology*, 582(1), 279–96.
- Xue, T., Do, M. T. H., Riccio, A., Jiang, Z., Hsieh, J., Wang, H. C., Merbs, S. L., Welsbie, D. S., Yoshioka, T., Weissgerber, P., Stolz, S., Flockerzi, V., Freichel, M., Simon, M. I., Clapham, D. E. & Yau, K.-W. (2011). Melanopsin signalling in mammalian iris and retina. *Nature*, 479(7371), 67–73.
- Yamashita, T., Terakita, A., & Shichida, Y. (2000). Distinct roles of the second and third cytoplasmic loops of bovine rhodopsin in G protein activation. *The Journal of Biological Chemistry*, 275(44), 34272–9.
- Ye, H., Daoud-El Baba, M., Peng, R.-W., Fussenegger, M. (2011). A synthetic optogenetic transcription device enhances blood-glucose homeostasis in mice. *Science*, 332(6037), 1565–8.
- Yokoyama, S., & Radlwimmer, F. B. (1998). The “five-sites” rule and the evolution of red and green color vision in mammals. *Molecular Biology and Evolution*, 15(5), 560–7.
- Yokoyama, S., & Radlwimmer, F. B. (2001). The molecular genetics and evolution of red and green color vision in vertebrates. *Genetics*, 158(4), 1697–710.
- Yokoyama, S., & Tada, T. (2003). The spectral tuning in the short wavelength-sensitive Type 2 pigments. *Gene*, 306, 91–8.
- Yoshimura, T., & Ebihara, S. (1996). Spectral sensitivity of photoreceptors mediating phase-shifts of circadian rhythms in retinally degenerate CBA/J (rd/rd) and normal CBA/N (+/+) mice. *Journal of Comparative Physiology A*, 178(6), 797–802.
- Zaidi, F. H., Hull, J. T., Peirson, S. N., Wulff, K., Aeschbach, D., Gooley, J. J., Brainard, G. C., Gregory-Evans, K., Rizzo III, J. F., Czeisler, C. A., Foster, R. G., Moseley, M. J. & Lockley, S. W. (2007). Short-wavelength light sensitivity of circadian, pupillary, and visual awareness in humans lacking an outer retina. *Current Biology*, 17(24), 2122–8.
- Zhao, X., Pack, W., Khan, N. W., & Wong, K. Y. (2016). Prolonged Inner Retinal Photoreception Depends on the Visual Retinoid Cycle. *The Journal of Neuroscience*, 36(15), 4209–17.
- Zhao, X., Stafford, B. K., Godin, A. L., King, W. M., & Wong, K. Y. (2014). Photoresponse diversity among the five types of intrinsically photosensitive retinal ganglion cells. *The Journal of Physiology*, 592(7), 1619–36.
- Zhang, X.-H., Tee, L. Y., Wang, X.-G., Huang, Q.-S., & Yang, S.-H. (2015). Off-target effects in CRISPR/Cas9-mediated genome engineering. *Molecular Therapy—Nucleic Acids*, 4(11), e264.
- Zufferey, R., Donello, J. E., Trono, D., & Hope, T. J. (1999). Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors. *Journal of Virology*, 73(4), 2886–2892.