



Investigating the effect of melanopsin gene therapy in the degenerate retina

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Abstract

Retinal degenerations such as retinitis pigmentosa affect 1 in 3000 people causing visual loss and blindness. In these conditions, photoreceptors are lost whereas other retinal cells and central visual pathways remain largely intact. A strategy for restoring vision in patients with end-stage disease would be to introduce a light sensitive protein into remaining inner retinal cells to make them directly light sensitive.

The aim of this thesis was to assess whether the human photopigment melanopsin, normally present in intrinsically photosensitive ganglion cells, could be delivered to other residual cells of the degenerate retina using an adeno-associated viral (AAV) vector, and whether this could restore visual function.

Several AAV serotypes were tested in the *rd1* mouse model of end-stage retinal degeneration. The most effective was found to have a single tyrosine to phenylalanine mutation in the AAV capsid (rAAV2/8 Y733F), and this vector was also able to transduce bipolar cells in human retinal samples cultured *ex-vivo*. Further evaluation of the vector was then performed to identify whether delivery using a ubiquitous or bipolar cell specific promoter gave optimal expression, and that comparing a wild type to a codon optimised sequence was also assessed. Longevity of expression was also assessed with robust levels of melanopsin seen in the degenerate retina fifteen months after a single subretinal injection.

Retina transduced using the optimised vector was tested for functional light responses using *ex-vivo* multi-electrode array recordings and *in vivo* light pulsing, with results from both highly indicative of functional photosensitivity mediated by expressed melanopsin. Furthermore, laser speckle cortical imaging demonstrated a light-dependent change in blood flow in the visual cortex of treated mice.

Finally to assess whether visual function was restored, *in vivo* testing of the pupil light response revealed augmented pupil constriction compared to controls two months after subretinal injection of the vector. This effect was maintained and even greater at thirteen months. Further behavioural testing demonstrated that following vector administration both two and thirteen months previously, mice were able to detect a change in visual environment, whereas control *rd1* mice were not.

These data indicate that the increased photosensitivity derived from expression of human melanopsin in the degenerate retina can generate functionally useful information, which can restore light-driven responses and even enhance performance in a test that relies on image-forming vision in mice with end-stage retinal degeneration. Therefore with further development, this optogenetic strategy has great potential for the treatment of patients with end-stage retinal degeneration.

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Dedication

This thesis is dedicated to my family: my parents, husband and daughter for their ongoing support, love and patience.

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Chapter 1 Introduction

1.1 Overview

Inherited retinal degenerations such as retinitis pigmentosa (RP) affect approximately 1 in 3000 people, leading to significant visual morbidity and blindness. The mechanism of blindness is via loss of photoreceptors, i.e. rods and cones, but the remaining retinal layers and central visual pathways remain largely structurally intact.

A strategy to restore visual function in these patients is to use gene therapy in order to introduce a light sensitive protein to cells of the residual retina and make them photosensitive in the absence of classical photoreceptors. For this purpose, use of a native mammalian protein would be highly advantageous since it would be unlikely to provoke an immune response. In recent years a third mammalian photoreceptor has been discovered: a subset of retinal ganglion cells, the majority of which project to areas of the brain involved in non-image forming functions, are directly light sensitive due to expression of the melanopsin (Opn4) gene. This protein is G-protein coupled allowing signal amplification, and is sensitive to light at physiological intensities.

The research question is to develop melanopsin gene therapy using an adeno-associated viral (AAV) vector: to assess whether human melanopsin can be expressed in residual neurons of the degenerate retina involved in image-forming circuitry and examine whether this can restore visual function in a model of end-stage retinal degeneration.

1.2 The Retina

The retina is a complex, light sensitive tissue, which forms the innermost layer of the eye, figure 1.1, and mediates conversion of light into action potentials that are transmitted to the brain. It consists of ten layers and multiple cell types, which span from the retinal pigment epithelium adjacent to the choroid, to the inner limiting membrane underlying the vitreous, figure 1.2. The structure of the retina in vertebrates is 'inverted' where incident light passes through the majority of these layers before striking photoreceptors and activating phototransduction.

The primate retina is divided into central and peripheral areas, the former also known as the macula which has a higher density of cone photoreceptors, whereas the latter contains predominantly rod photoreceptors. In the centre of the macula lies the fovea, and its centre is known as the foveal pit in which only cones are present and the cell bodies of second and third order neurons are displaced laterally (1).

1.2.1 Retinal pigment epithelium

The retinal pigment epithelium (RPE) is a monolayer of hexagonal epithelial cells containing pigment granules that is located between the choriocapillaris and neurosensory retina and has a crucial role in the visual cycle. Tight junctions between RPE cells form part of the outer blood retinal barrier, which is important for maintaining immune privilege of the eye and also facilitates highly specific transport between the subretinal space and choroidal circulation (2). In this way, the RPE is involved in controlling ion homeostasis in the subretinal space and further influences this environment via the secretion of a number of molecules such as vascular endothelial growth factor (3) and ciliary neurotrophic factor (4).

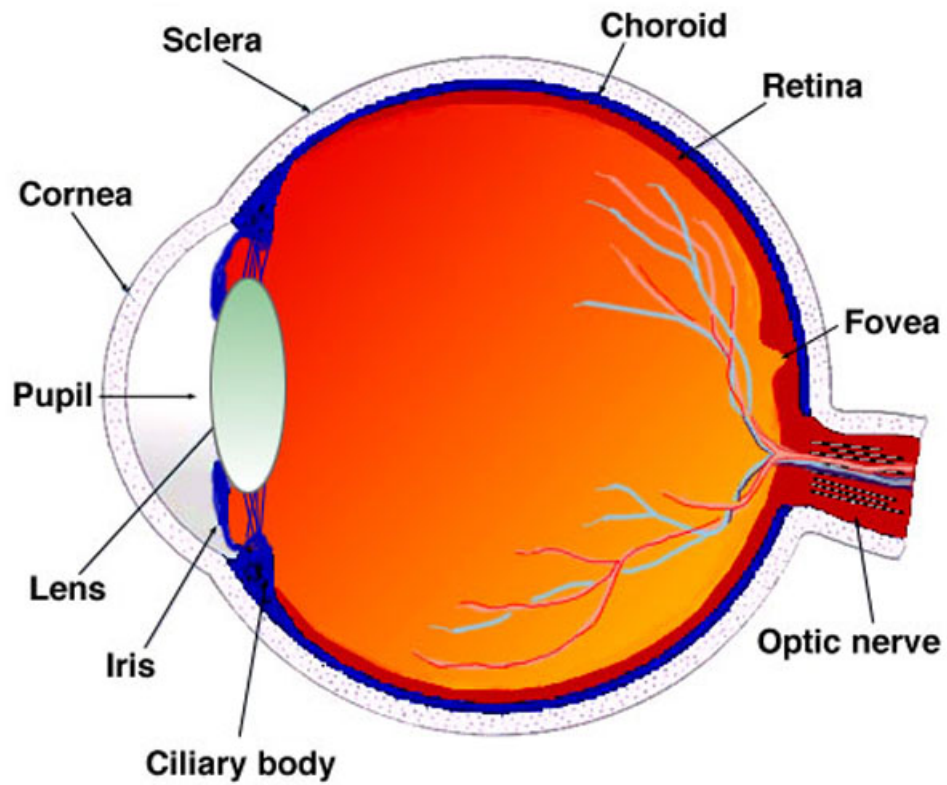


Figure 1.1 The human eye

Diagram of the human eye showing key structures. Adapted from (1).

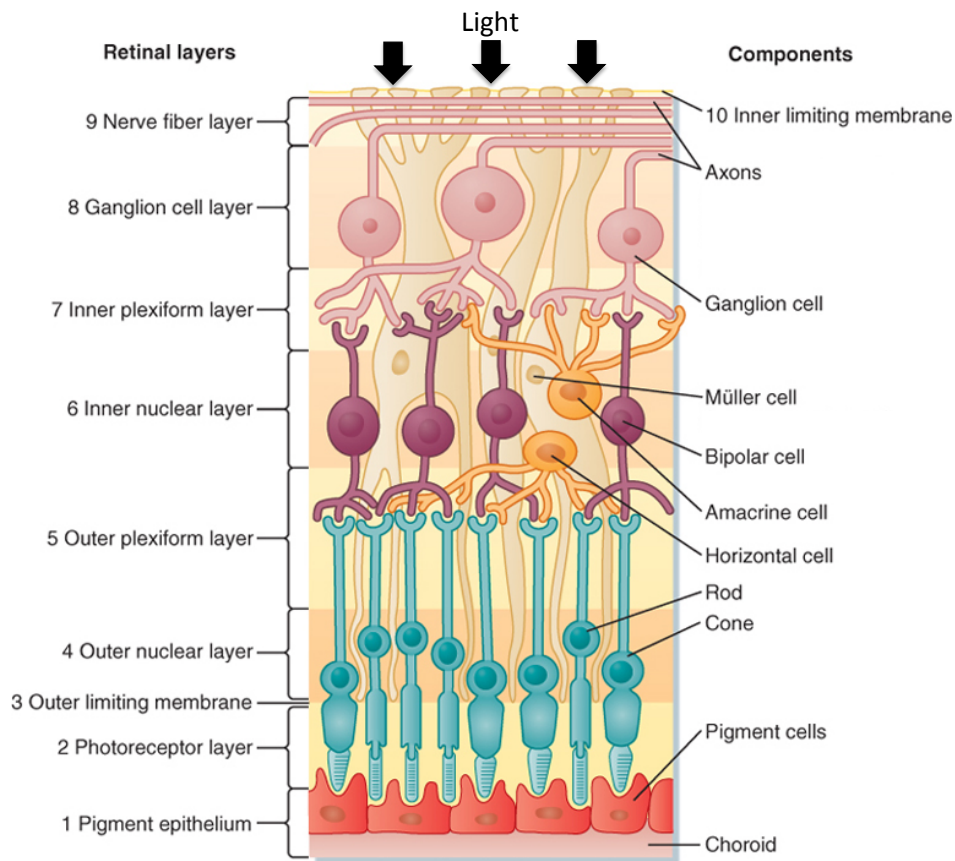


Figure 1.2 Layers of the retina

Diagram illustrating the ten layers and multiple cell types that comprise the human retina. Adapted from (5).

1.2.2 Photoreceptors and phototransduction

Structurally, photoreceptors consist of four distinct regions: the outer segment, inner segment, cell body and synaptic terminal. The outer segment lies in close association with the retinal pigment epithelium and is densely packed with discs containing visual pigment. This is linked via a thin cilium to the inner segment, which contains a large number of mitochondria as well as endoplasmic reticulum and Golgi apparatus to meet the high metabolic needs of the photoreceptor cell. The cell body is located in the outer nuclear layer (ONL): cone cell bodies form a single layer adjacent to the outer limiting membrane of the retina, whereas rod cell bodies populate the remainder of the ONL (6). The synaptic terminals of photoreceptors are located in the outer plexiform layer (OPL) via which the light mediated signal is transmitted to second order neurons in the retina.

1.2.2.1 Phototransduction and the visual cycle

A G protein signalling pathway mediates classical phototransduction. Photoreceptor outer segments are filled with membrane discs, which contain the visual pigment rhodopsin or cone opsin. These proteins have seven transmembrane domains and are covalently bound via a protonated Schiff base to the vitamin A derived chromophore 11-*cis* retinal in the dark (7). Using rod phototransduction as an example, when incident light hits the photoreceptor, photon absorption leads to a change in configuration of retinal from the 11-*cis* isomer to the *all-trans* isomer (8), which activates the rhodopsin molecule (R to R*). This triggers exchange of GDP for GTP on the alpha subunit of the G protein transducin, producing $G\alpha^*$ -GTP, figure 1.3. Two $G\alpha^*$ -GTP molecules then bind and inactivate the inhibitory γ subunits of phosphodiesterase (PDE), resulting in activation of PDE (PDE*) which hydrolyses cyclic GMP (9). This reduction of cGMP causes closure of cGMP-gated cation channels in the disc membrane and hyperpolarisation of the photoreceptor in response to light.

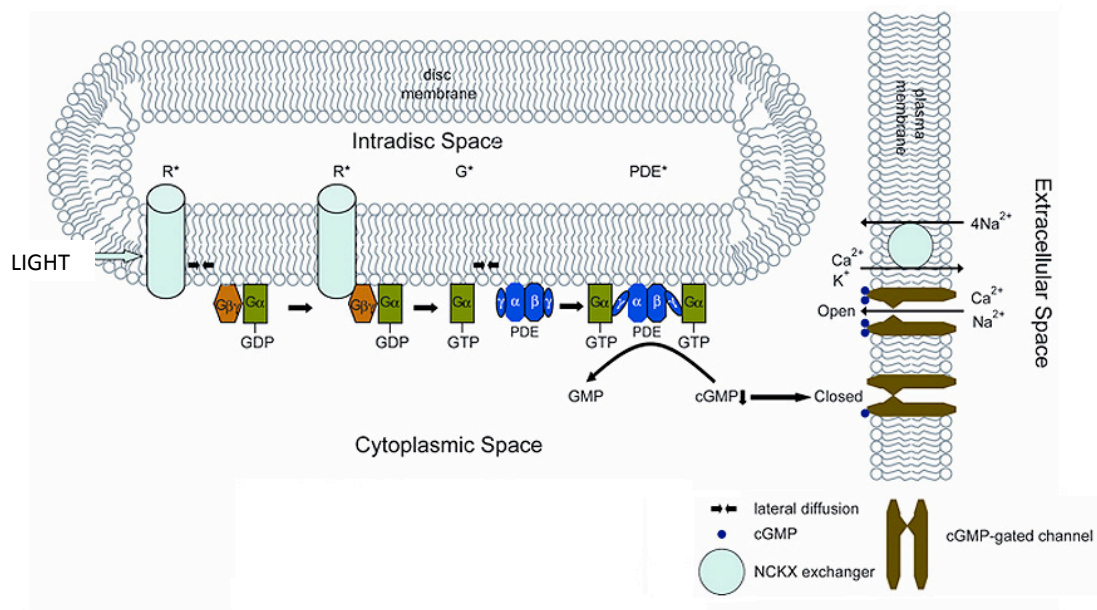


Figure 1.3 Schematic of rod phototransduction

Following absorption of a photon, activated rhodopsin triggers a G protein mediated cascade which results in closure of cyclic GMP gated channels and halts the influx of cations into the cell. This results in cell hyperpolarisation. Adapted from (10).

One of the most important features of this transduction cascade is the potential for signal amplification. Each activated rhodopsin molecule can interact with over 800 transducin molecules, resulting in the closure of over 200 ion channels in the outer segment disc membrane from the absorption of one photon alone (11).

Termination of the phototransduction cascade involves deactivation of each of the elements involved: R^* is phosphorylated by rhodopsin kinase and capped by the arrestin protein, transducin deactivates via hydrolysis of GTP to GDP which inhibits PDE and reduces the rate of cGMP hydrolysis (12). In the classical visual cycle of rod phototransduction, *all-trans* retinal is converted into *all-trans* retinol, which crosses the subretinal space into the RPE and is converted back to 11-*cis* retinal via several enzymatic steps, figure 1.4. In cone phototransduction, all-trans retinol enters the Muller cell where it is converted to 11-*cis* retinol (13). The latter then enters the inner segment of the cone photoreceptor where it is converted back to 11-*cis* retinal.

1.2.3 Bipolar cells

In the mammalian retina there are more than ten types of bipolar cell, the majority of which synapse with cones and one with rods (14). However, since rods form the majority of photoreceptors in the retina, rod bipolar cells are actually the most common cell type. The bipolar cell dendrite penetrates into the rod spherule with metabotropic glutamate receptors (mGluR6) on its surface, to terminate at synaptic ribbons (15-17). One rod bipolar cell contacts 15 to 20 rods in the central retina and as many as 40 to 50 rods in the peripheral retina. Of the cone bipolar cells, seven are diffuse and synapse with multiple cone photoreceptors, and the remaining three types (midget and blue cone specific) synapse in a one-to-one fashion with cones (1). In the mouse retina there are ten types of bipolar cell, nine of which synapse with cones and one with rods (18).

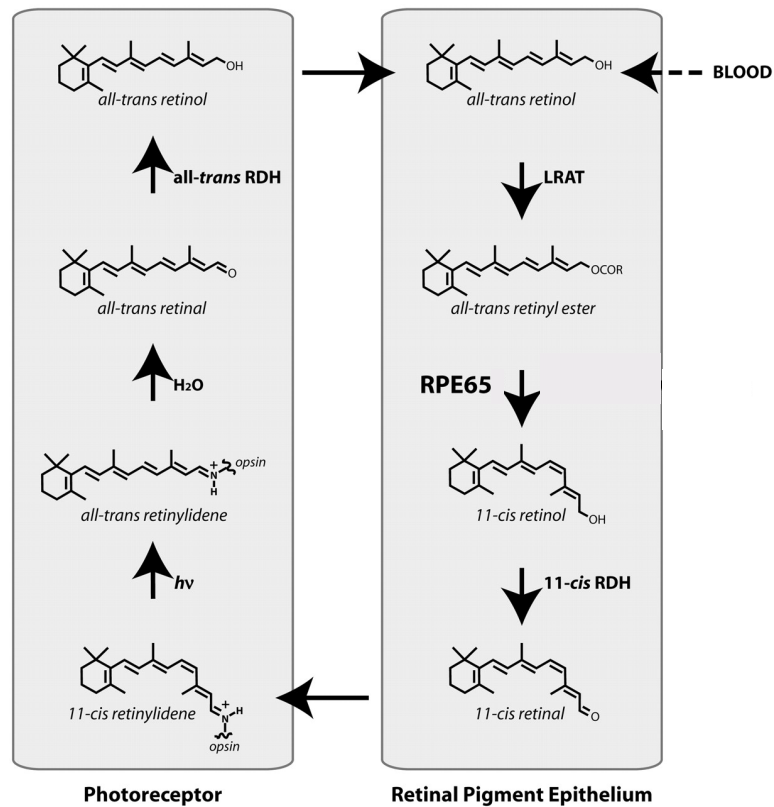


Figure 1.4 Schematic of the retinoid visual cycle

11-cis retinal is regenerated after its conversion to all-trans retinal following light activation. RDH= retinol dehydrogenase, LRAT = lecithin retinol acetyltransferase, RPE65= Retinal pigment epithelium-specific 65 kDa protein. Adapted from (19).

The multiplicity of connections allows for image processing and the creation of ON and OFF pathways, where a light stimulus excites one population of cells and inhibits another as will be discussed below.

1.2.4 Horizontal cells

The cell bodies of horizontal cells are located in the inner nuclear layer (INL) but their dendrites and axon terminals extend within the outer plexiform layer (OPL) to mediate lateral connections between cells. In the primate retina there are three types of horizontal cell: HI, HII and HIII (20). HI cells have a relatively small dendritic tree in the central retina, and a larger dendritic tree in the peripheral retina. The dendrites of these cells synapse with cones as part of the ribbon synapse whereas its long axon extends in the OPL and synapses with rods at its terminal processes. HII cells only synapse with cones, have a more spidery dendritic tree and a short axon the terminal processes of which synapse with short wavelength cones only. HIII cells are similar to HI cells in morphology but are larger, and currently are only known to synapse with long and medium wavelength cones (21). In the murine retina, there is only one class of horizontal cell which is similar to the HI cell (22). Retinal horizontal cells are connected to homologous neighbours via gap junctions, with a variety of gap junctions existing between neurons depending on the arrangement of connexions or hemichannels (23). These serve to extend the receptive field of horizontal cells even further. Synapses from horizontal to bipolar cells have been documented in non-mammalian retinas (24). In the mammalian retina, horizontal cells hyperpolarise in response to light: this is a comparatively slow response and is dependent on light intensity and duration.

1.2.5 Amacrine cells

Amacrine cells are interneurons that integrate and modulate signals at the second synapse in the retina, within the inner plexiform layer (IPL). There are at least 25 types of amacrine cell in the mammalian retina (25). These are categorised according to their morphological characteristics and by which IPL sublamina their dendrites terminate. Cajal subdivided the IPL into 5 layers (26), and further work elucidated two functionally discrete layers: the ON sublamina adjacent to the ganglion cell layer and the OFF sublamina adjacent to the INL (27). The majority of amacrine cells are inhibitory and synapse with both bipolar cells and ganglion cells in the IPL.

1.2.6 Ganglion cells

Ganglion cells are the output cells of the retina and receive synapses from bipolar and amacrine cells. They transmit the visual information they receive via action potentials along their axons to retino-recipient areas of the brain. There are numerous types of ganglion cell which can be classified according to dendritic morphology, the IPL layer in which they make synapses (26), see below, and functional properties (28). In many cell types structural differences also correlate with function: for example large ganglion cells with wide dendritic trees process information regarding motion (magnocellular or M cells), whereas those with fine, narrow dendritic trees are involved in the processing of information regarding fine detail (parvocellular or P cells) (29).

1.2.7 Retinal glial cells

Müller glial cells span the entire thickness of the retina; their connections with photoreceptor inner segments form the outer limiting membrane (OLM), their cell bodies sit within the INL and distal connections of their end feet form the inner limiting membrane (ILM). Müller cells have numerous functions within the retina including removal of neural waste products such as carbon dioxide and ammonia; protecting neurons from excess neurotransmitter exposure, for example via the uptake of glutamate, and have a homeostatic role by regulating the potassium concentration of the extracellular environment (30). Astrocytes are located in the nerve fibre layer of the retina and enter the retina from the optic nerve (31). They envelop ganglion cell axons and blood vessels and contribute to the blood-brain barrier. Microglia are found in all layers of the retina and act as macrophages after retinal injury.

1.2.8 Retinal cell connectivity

The circuitry of the retina is complex with simultaneous convergence, divergence and modulation of the signal transmitted. Following hyperpolarisation of the photoreceptor in response to a light stimulus, a rod bipolar cell depolarises. A single rod bipolar cell synapses with 15 to 30 rods in the ONL, with bipolar cell dendrites and horizontal cell axonal processes in very close proximity in the rod spherule, figure 1.5. Horizontal cells modulate the visual signal from rods to mediate contrast sensitivity: excitation of a single photoreceptor causes feedback inhibition of a ring of surrounding ones. Therefore only the ganglion cell directly above the stimulated rod is excited and those surrounding it are inhibited giving a centre-surround organisation (32).

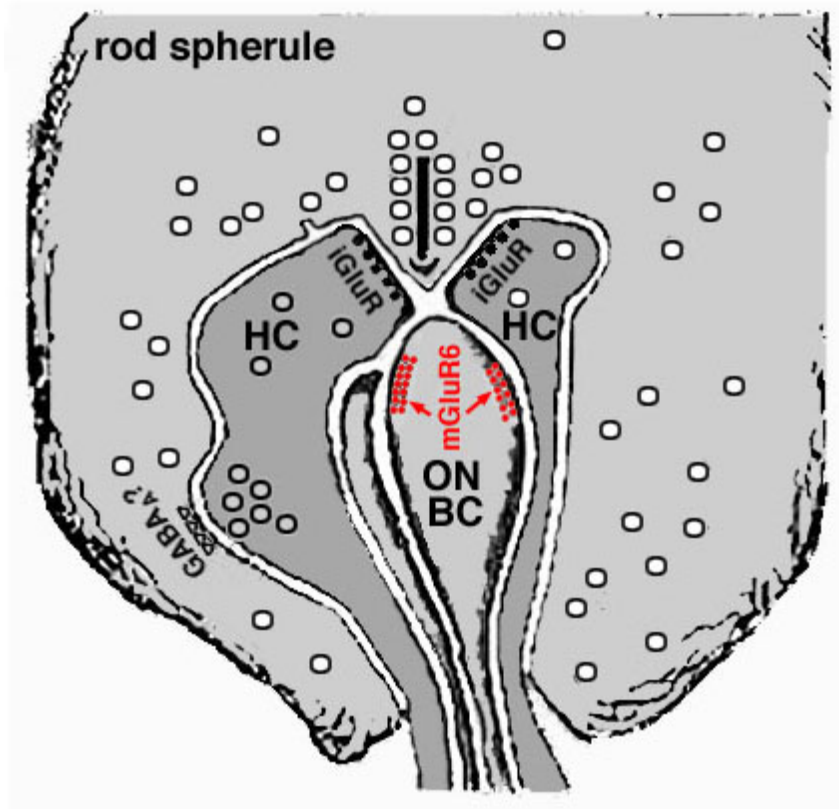


Figure 1.5 Schematic of the ribbon synapse

This illustrates the presence of rod ON bipolar cell (BC) and horizontal cell (HC) dendrites within the invagination of the rod spherule. Metabotropic glutamate receptors (mGluR6) are present on the bipolar cell post-synaptic membrane. Adapted from (33).

Rod bipolar cell axons synapse only with AII and A17 amacrine cells which in turn provide inhibitory input to OFF ganglion cells (34). AII amacrine cells also synapse with cone bipolar cells (32), which provide excitatory input to ON ganglion cells. By this means, visual signals from rods reach both ON and OFF ganglion cells, figure 1.6.

The cone pathway varies from that of the rods beginning at the ONL since there are multiple types of cone bipolar cell. Some cone bipolar cells form ribbon synapses with cone photoreceptors with mGluR6 glutamate receptors on the post-synaptic membrane, and these bipolar cells depolarise in response to a light stimulus. Other cone bipolars make flat or basal synapses with cones and express AMPA/kainate receptors on the post-synaptic membrane; these bipolar cells hyperpolarise in response to a light stimulus (14). ON cone bipolar cell axons terminate in the ON sublamina of the IPL and synapse with ON ganglion cells. Likewise OFF bipolar cell axons terminate in the OFF sublamina of the IPL and synapse with OFF ganglion cells. Therefore two streams of information pass from cones to ganglion cells, one signalling light stimuli that are brighter than background (the ON pathway) and the other stimuli that are darker than background (the OFF pathway), figure 1.6 (14, 35). This is further enhanced by the lateral inhibition of horizontal cells, which mediate the centre-surround pattern as seen in the rod pathway.

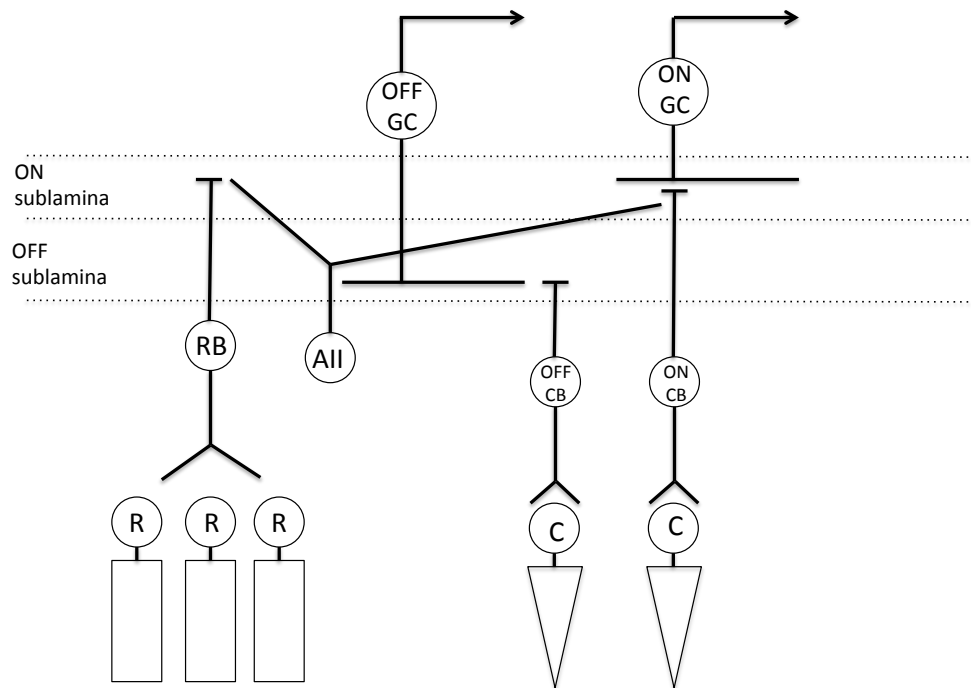


Figure 1.6 Schematic of retinal circuitry

Rod photoreceptors synapse with rod bipolar cells which in turn provide input to All amacrine cells. These provide inhibitory input to OFF ganglion cells, and also synapse with ON cone bipolar cells to activate ON ganglion cells. Cone photoreceptors synapse with bipolar cells which depolarise in response to a light stimulus (ON cone bipolars) which in turn synapse with ON ganglion cells. Other cone bipolar cells hyperpolarise in response to cone hyperpolarisation (OFF cone bipolars) which synapse with OFF ganglion cells, creating two parallel streams of information for visual processing. R= rod, C= cone, RB= rod bipolar cell, CB= cone bipolar cell, AII= All amacrine cell, GC= ganglion cell.

1.2.9 Central visual pathways and visual cortex

Following complex processing of the visual signal in the retina, this information leaves the eye via action potentials transmitted by the optic nerve. In primates, there is decussation of axons arising from the nasal retina at the optic chiasm, so that information regarding one half of the visual field from both eyes is transmitted to the same side of the brain. Following this, the majority project to the lateral geniculate nucleus (LGN). This is a six layered structure in non-human primates, with layers 1 and 2 receiving input from magnocellular ganglion cells and layers 3-6 receiving input from parvocellular ones. Other fibres innervate the superior colliculus (involved in saccadic eye movements), the pretectal nuclei (involved in the pupil light reflex and smooth pursuit eye movements), and the suprachiasmatic and pregeniculate nuclei involved in non-image forming vision (36).

With regards to image forming vision, fibres pass from the LGN via the optic radiation to the primary visual cortex (V1) located at the posterior pole of the occipital lobe in primates. There are six major cortical layers (Brodmann layers) of which layers 4C α receives input from the magnocellular pathway from the LGN, and layers 4C β from the parvocellular pathway, maintaining segregation of these streams of information (37). The spatial or retinotopic arrangement of information from the retina is also preserved in V1. In addition, there is a columnar arrangement of neurons where vertical aligned neurons comprise a functional or anatomical unit (38). From V1, information is disseminated to over thirty further cortical areas for further visual processing (39).

1.3 Melanopsin

In recent years, a third class of retinal photoreceptor has been discovered, separate from rods and cones. This arose from the observation that mice lacking classical photoreceptors are able to maintain a circadian rhythm that is entrained to light, but removal of the eye abolishes this effect (40, 41). The implication that a further photoreceptive cell in the eye must exist, led to the discovery of a population of ganglion cells that are intrinsically photosensitive (ipRGCs) (42, 43).

Previous work had identified a novel opsin in dermal melanophores, brain and the eye of *Xenopus laevis* (44), named melanopsin (or Opn4), which was subsequently demonstrated to be present in the mammalian inner retina, specifically in ganglion and amacrine cells (45). Confirmation that this was the photopigment present in intrinsically photosensitive ganglion cells came from knockout studies where melanopsin knock-out mice showed altered circadian entrainment (46), and pupil responses (47). Furthermore, mice lacking rods, cones and melanopsin showed a loss of all visual and accessory visual functions (48), confirming that melanopsin was the photopigment present in ipRGCs (49, 50).

1.3.1 Melanopsin structure

The human melanopsin gene is mapped to chromosome 10q22 and consists of 10 exons (45). Similar to other opsins, its protein structure involves seven transmembrane domains, with a lysine residue (lysine-294) in the seventh transmembrane domain where chromophore binding can occur. Melanopsin also has a pair of cysteine residues in the second and third extracellular loops between which disulphide bridges form for stabilisation of its tertiary structure consistent with other opsins (44), figure 1.7.

However, in many respects melanopsin is significantly different from other mammalian opsins with only approximately 30% homology with classical visual pigments (44, 51). In fact melanopsin shows greatest homology with invertebrate opsins, such as octopus rhodopsin (39% homology) and phylogenetic analysis indicate a common ancestor for both melanopsin and invertebrate opsins (44). The chromosomal location and gene structure of melanopsin also differs from other mammalian opsins, for example melanopsin contains nine intron insertion sites in comparison to four for rhodopsin and five for red/green cone opsin (45), figure 1.8. Other differences include the presence of tyrosine instead of glutamate at the counterion position for Schiff-base linkage of the opsin protein to chromophore, and an extended intracellular C terminal tail (44).

The murine melanopsin sequence has approximately an 86% homology with its human counterpart (45) with regards to the transmembrane and loop domains but has significantly different cytoplasmic and extracellular tails. In the murine retina there are two isoforms of melanopsin generated by alternate splicing of a single melanopsin gene: a long isoform (Opn4L) and a novel short isoform (Opn4S) which only differ in the length of their C terminal tails (52), the latter being more prevalent.

1.3.2 Melanopsin phototransduction

Melanopsin has a peak sensitivity to light at 480nm, as measured by single cell recordings from ipRGCs (42) and confirmed by the action spectrum of the pupil light reflex in rodless/coneless mice (53). The kinetics of its light response are typically slow and sustained but more transient responses have also been described (54). The melanopsin phototransduction cascade has not been clearly elucidated and work in this area is evolving. As described in section 1.2.2.1, opsins are G protein coupled receptors bound to the chromophore 11-*cis* retinal.

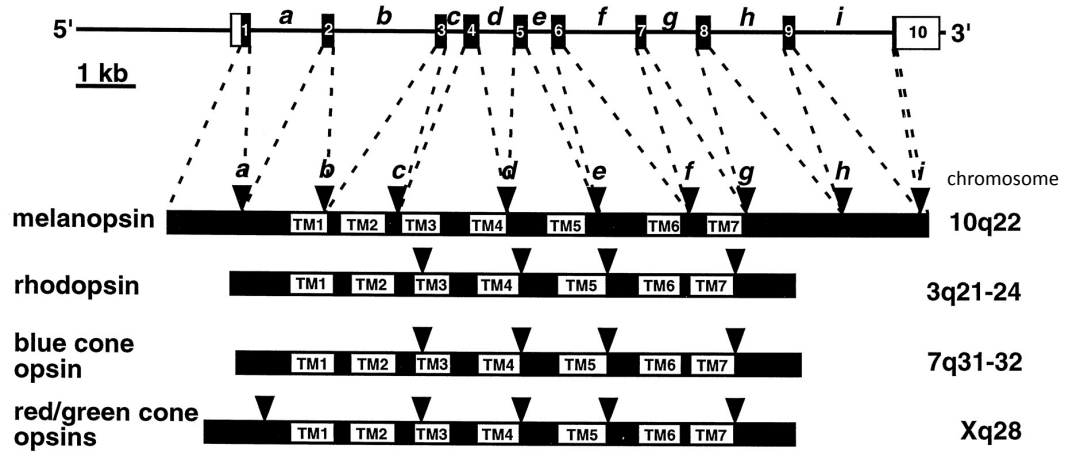


Figure 1.8 Gene structure of human melanopsin

This is shown in compared to rhodopsin, blue cone opsin and red and green cone opsins. TM= transmembrane domain, arrows indicate positions of introns. Adapted from (45).

Light causes photoisomerisation of 11-*cis* retinal to all *trans* retinal, resulting in G protein activation and subsequently that of a downstream signalling cascade. The resemblance of melanopsin to invertebrate opsins indicates that melanopsin may use a similar transduction cascade: activation of a G_q/G_{11} type G protein with resultant activation of phospholipase C which generates inositol phosphate ($\text{Ins}(1,4,5)\text{P}_3$) and diacylglycerol (DAG) that ultimately leads to opening of transient receptor potential channels (TRPCs) resulting in cell depolarisation (51). In support of this hypothesis, blockers of the G_q/G_{11} pathway but not the G_i/G_o pathway abolish light responses when administered to *Xenopus* oocytes induced to express melanopsin (55). Furthermore, 2-aminoethoxydiphenyl-borane (2-APB) which is an inhibitor of TRP ion channels blocks the light responses mediated by ipRGCs *in vitro* and attenuates the pupil light response *in vivo* (56).

Melanopsin appears to function as a bistable pigment in that it is able to regenerate 11-*cis* retinal from all-*trans* retinal using long wavelength light. This is likely to be due to its presence in ipRGCs which are a significant distance away from the RPE which regenerates the chromophore for rods and cones (51).

Intrinsically photosensitive ganglion cells respond at higher light intensities than classical photoreceptors (57), but this is not due to a lack of sensitivity of melanopsin itself. Each photon captured by melanopsin elicits a large and prolonged light response and ipRGCs, like rods, are capable of signalling single photon absorption events to the brain (58). However, the density of melanopsin molecules in the ipRGC cell membrane is approximately 10^4 times lower than in rod or cone opsins, resulting in a significant phototransducing role only in bright light (58), consistent with the role of ipRGCs as irradiance detectors for circadian photoentrainment, and for the pupil light response which is most relevant at high light intensities.

1.3.3 Classes of melanopsin expressing ipRGCs

It has now become evident that there are multiple subclasses of ipRGCs with up to five different cell types classified as M1-M5 based on morphology and localisation of dendrites, figure 1.9. The initially described cells now correspond to the M1 subtype which have dendrites that terminate in the OFF sublamina of the inner plexiform layer (59). M2, M4 and M5 cell dendrites terminate in the ON sublamina (60) and M3 cell dendrites reach both sublaminae (61). Some melanopsin ganglion cell bodies themselves may be located in the inner part of the inner nuclear layer, with one study showing five per cent of melanopsin expressing cells to be displaced here (59). With regards to morphology, M2 cells have larger cell bodies and dendritic arbors than M1 cells, M3 cells are rare and have similar characteristics to M2 cells. M4 cells have the largest somas and dendritic arbors, whereas M5 cells have small cell bodies and dendrites (60).

Along with morphological differences, ipRGCs also show different light-evoked responses. Using calcium imaging, ganglion cell physiological responses were examined in whole-mounts of retinas lacking rods and cones. Three types of light evoked calcium responses were observed: sustained, transient and repetitive suggesting different functional classes of ipRGCs (54). Further work has demonstrated that the various morphological cell types also show different physiological properties. M1 cells have a large photosensitive response and a more depolarised resting membrane potential than M2 cells (61). Intrinsically photoreceptive ganglion cells also receive input from rods and cones via bipolar and amacrine cells. These inputs appear to have more influence on M2 cells, whose light response is influenced by melanopsin, rod and cone signalling (57).

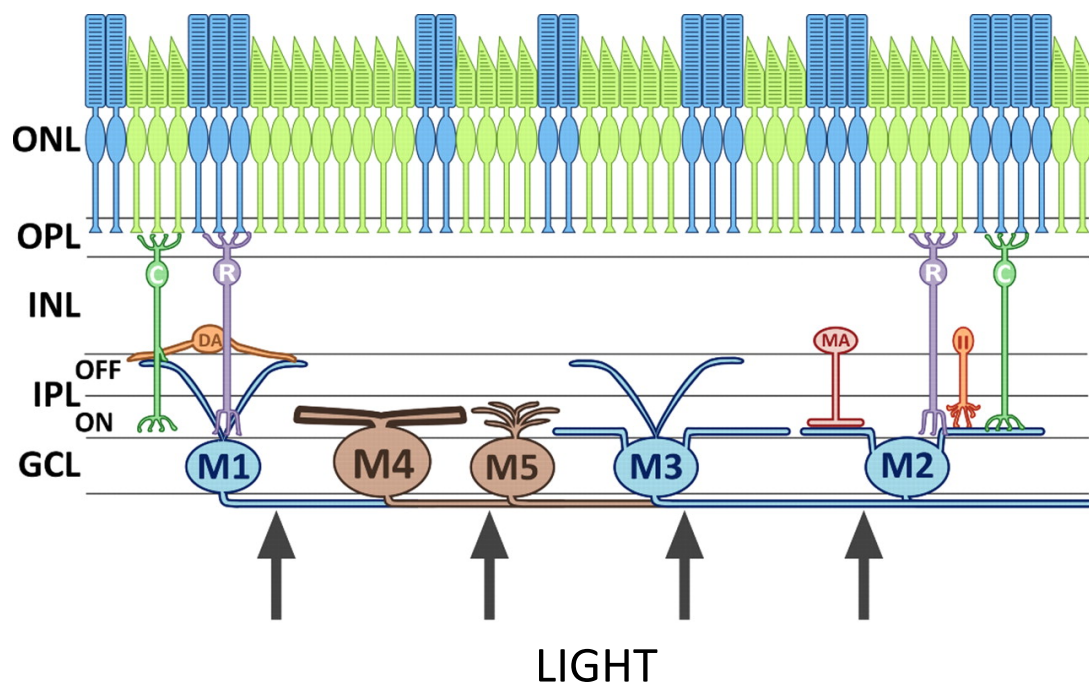


Figure 1.9 Classes of melanopsin expressing intrinsically photosensitive ganglion cells and their connections.

M1 cells dendrites terminate in the OFF sublamina of the inner plexiform layer, whereas dendrites all other cell types terminate in the ON sublamina. DA, MA, and II are populations of amacrine cells. Adapted from (57).

1.3.4 Connectivity of ipRGCs within the retina

There appear to be complex connections of ipRGCs within the retina. In the majority of cases, ganglion cells with dendrites that innervate the outer or OFF sublamina of the inner plexiform layer receive input from OFF bipolar cells, and those with dendrites terminating in the ON sublamina of the IPL receive input from ON bipolar cells. However, despite M1 dendrites terminating in the OFF sublamina and M2 in the ON sublamina of the IPL, both receive predominantly ON inputs. This has been demonstrated to arise since ON bipolar cells passing through the OFF sublamina make *en passant* synapses with M1 ganglion cell dendrites, providing them with input from the ON pathway (62).

M1 cell dendrites have been shown to co-localise with (63) and signal back to dopaminergic amacrine cells in the retina (64), with signalling therefore occurring in the opposite direction to classical ON and OFF pathways. In addition, melanopsin expressing ganglion cells also appear to communicate with other cells in the ganglion cell layer via gap junctions, since application of a gap junction blocker reduces the number of cells showing light evoked calcium responses in the retina by half (54). The function of both these types of connection is still unknown.

1.3.5 Central projections of ipRGCs

Intrinsically photosensitive ganglion cells project to brain regions involved in a variety of non-visual functions, and more recently have been shown to project to some areas involved in the classical visual pathway, figure 1.10. Both M1 and non-M1 melanopsin expressing ganglion cells project to the suprachiasmatic nucleus (SCN) and intergeniculate leaflet (IGL) which facilitate circadian entrainment in response to light (59). M1 cells project to the shell of the olivary pretectal nucleus (OPN) and this is likely to be the predominant drive for the pupillary light reflex (60). Non-M1 cells project to the core of the OPN, the function of this innervation is currently unknown (60).

In the thalamus, a well characterised projection of ipRGCs is to the ventral region of the lateral geniculate nucleus (LGN), a region involved in non-visual light detection (65). Further work characterising projections in the mouse has shown that the more recently documented non-M1 cells project to regions of the superior colliculus (60) and also the dorsal LGN (66), both of which are regions associated with image forming vision and direction of gaze. In the primate retina, a subset of melanopsin expressing cells that also receive rod/cone input have been shown to project to the LGN. These are a rare group of 'giant' cells with an opponent receptive field exhibiting an S-cone mediated OFF response and L/M cone mediated ON response (67).

Melanopsin expressing ganglion cells also project to a number of other hypothalamic targets including the ventrolateral preoptic nucleus (VLPO) which is known to be involved in sleep regulation, and the lateral hypothalamus involved in neuroendocrine function (59). Other targets include the medial amygdala where light and olfactory inputs converge to influence reproductive behaviour (59). The majority of projections are crossed, apart from those to the SCN which are bilateral and symmetrical (59).

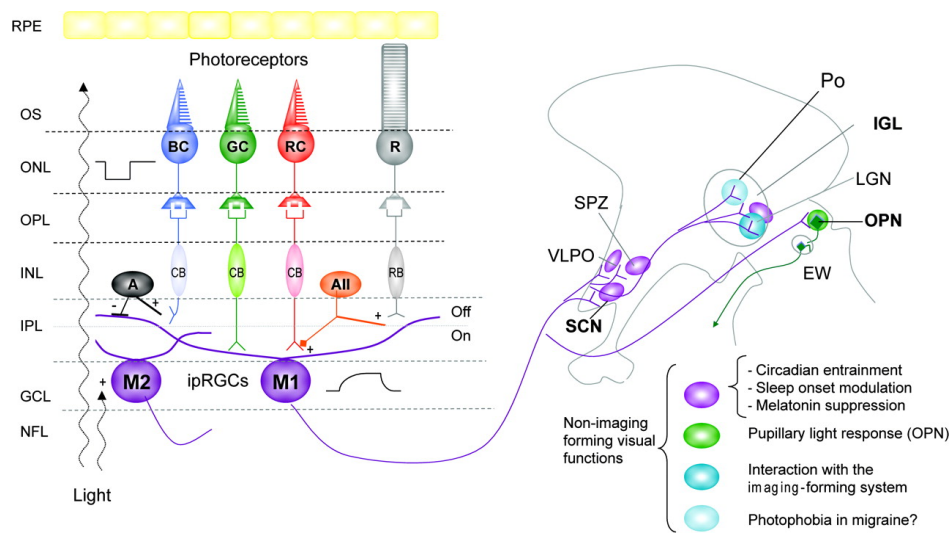


Figure 1.10 Central projections of intrinsically photosensitive ganglion cells.

Connections are shown to the suprachiasmatic nucleus (SCN), ventrolateral preoptic nucleus (VLPO), subparaventricular zone (SPZ), intergeniculate leaflet (IGL), lateral geniculate nucleus (LGN), olivary pretectal nucleus (OPN) and Edinger Westphal nucleus (EW). Superior colliculus projections are not shown. Adapted from (68).

1.3.6 The role of ipRGCs in image forming vision

The majority of work on processes driven by ipRGC light responses has focussed on non-visual functions. However, there is evidence that ipRGCs are involved in the regulation of classical visual pathways. The retinal cone pathway exhibits diurnal variation as can be demonstrated by a significant variation in cone ERG at different times of day. This diurnal variation is a function of both light exposure and circadian phase and is significantly reduced in melanopsin knockout mice (69). Further work has compared ERG and dorsal LGN responses in 'daylight' conditions versus light selectively depleted of melanopsin stimulating wavelengths ("mel-low") in a mouse model where cone wavelengths have been spectrally shifted to long wavelengths. Reversible changes were demonstrated in the photopic ERG between these two conditions, and furthermore changes were also seen in recordings made simultaneously from the dorsal LGN. More dorsal LGN units responded to finer spatial patterns in the daylight condition suggesting that environmental light conditions encoded by melanopsin in ipRGCs act to adjust the feature selectivity of dorsal LGN neurons and optimise the visual circuit according to irradiance (70).

The observation that non-M1 cells project to the dorsal LGN and superior colliculus in the murine thalamus raises the question of whether ipRGC light responses are used for image forming vision. Using a model in which rod transducin and cone cyclic nucleotide gated channel knockouts eliminate rod and cone transduction respectively (*Gnat1*^{-/-} and *Cnga3*^{-/-}) and a water maze test, one study showed that these mice were able to use the visual cue of a sinusoidal grating on a computer screen to identify the presence of a hidden platform. *Gnat1*^{-/-} and *Cnga3*^{-/-} mice required a higher number of trials than wild type mice, however total knockout mice (TKO, with melanopsin knock out superimposed on this background) were unable to complete the task (60). TKO mice did

show an ability to detect differences in luminance (60), which other studies have documented to be due to some residual rod pathway activity (71), and therefore there is a need for this work to be repeated in other mouse models.

1.4 Retinal degenerations

The clinical diagnosis of retinitis pigmentosa (RP) is the most common presentation of inherited retinal degeneration and affects approximately 1 in 4,000 people (72). This diagnosis encompasses a spectrum of genetic conditions with onset ranging from childhood to late adult life. Inheritance may be autosomal dominant, autosomal recessive, X-linked or mitochondrial and over 180 causative genes have so far been identified (<https://sph.uth.tmc.edu/retnet/sum-dis.htm>), figure 1.11. Clinical symptoms feature night blindness followed eventually by central visual loss, consistent with rod photoreceptor loss preceding that of cones. Histologically, this loss of photoreceptors leads to a secondary migration of the RPE into the retina giving a characteristic “bone spicule” appearance. Other retinal degenerations include cone-rod dystrophies (where cone loss precedes that of rods) such as in ABCA4 (ATP-binding cassette, sub-family A, member 4) mutations, or non-progressive retinal dysfunction syndromes such as achromatopsia where there is a loss of cone function without significant degeneration.

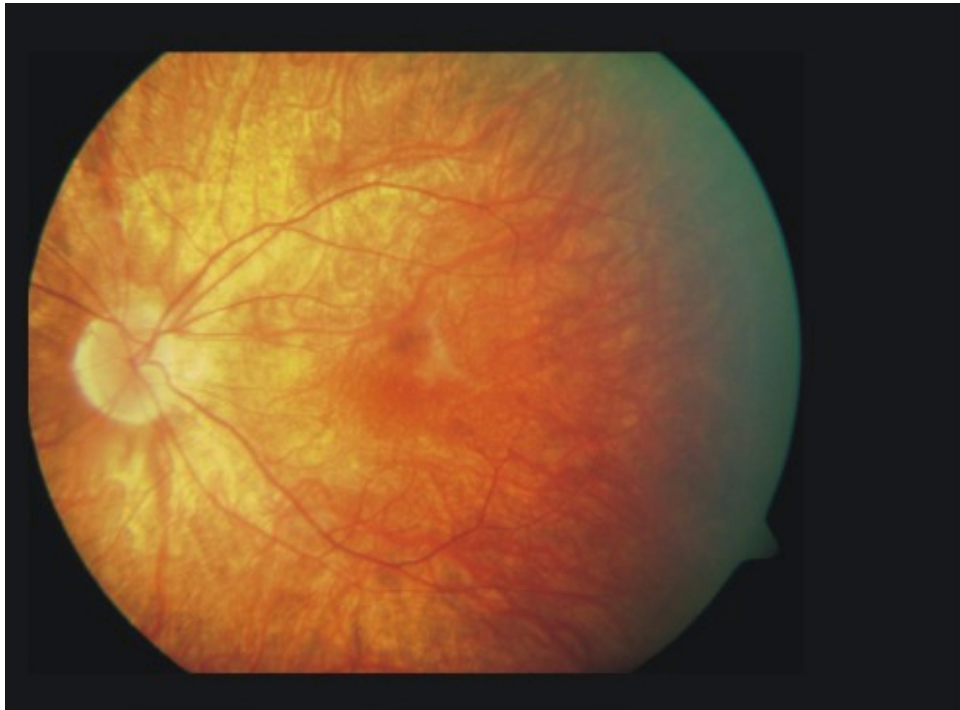


Figure 1.11 Colour fundus photograph of a patient with end-stage retinal degeneration.

This patient has a mutation in the retinitis pigmentosa GTPase regulator (RPGR) gene leading to extensive retinal degeneration.

1.4.1 The *rd1* mouse model

Animal models are used extensively in order to understand retinal degenerations and develop potential therapies and of these, the best characterised are mouse models. Although the mouse retina lacks a fovea with its concentration of cone photoreceptors, its peripheral retina is largely similar to human structure. The *rd1* model is one of the best characterised mouse models of retinal degeneration. These mice have a murine viral insert and a second nonsense mutation in the *Pde6b* gene, which encodes the rod-specific cGMP phosphodiesterase $\beta 6$ subunit (*Pde6b^{rd1}*) (73). Mutations in this gene have also been found in humans with autosomal recessive retinitis pigmentosa (74).

In the *rd1* mouse, retinal degeneration is rapid with a lack of functional cGMP phosphodiesterase leading to a toxic accumulation of cGMP in rod photoreceptors. Only two percent of rods remain at postnatal day 17 (P17) and no rods are detected in the central retina by P36 and in the far periphery by P65 (75). Cone degeneration follows but has a slower time course with half the cone population lost between P17 to P36. A small population of cones exists even at 18 months, with approximately 1.5% of cone nuclei remaining at this time (75). These mice have a phenotype of vessel attenuation and patchy pigmentation on fundal examination (76), and histologically show a thinning followed by complete loss of the outer nuclear layer. The inner retina remains structurally intact and therefore the population of ipRGCs in this model is unaffected maintaining the pupillary light reflex at high light intensities (47) and other non-image forming light responses. Despite the presence of endogenous mouse melanopsin in this model, the almost complete loss of photoreceptor cells makes it a good candidate for investigating therapeutic approaches for end-stage retinal degenerations.

1.4.2 Therapeutic approaches for retinal degenerations

Therapeutic options for patients with retinal degenerations are currently limited, but there are a number of strategies being investigated. For early to moderate disease where there are remaining areas of structurally intact retina and the causative genetic defect is known, gene replacement therapy (77-79) is likely to be the optimal approach for minimising further retinal degeneration. Alternatively, administration of a neuroprotective agent such as ciliary neurotrophic factor (CNTF) (80) or rod-derived cone viability factor (RdCVF) (81) may reduce or arrest loss of photoreceptor cells, however in clinical trials CNTF treatment has not resulted in any improvements in visual acuity in patients with RP (82). For end-stage disease where the retina is largely degenerate, potential options include electronic implants (83, 84), stem cell therapies to reconstruct the outer retina (85) or optogenetic approaches (86-89).

1.5 AAV Gene therapy for retinal degenerations

Gene therapy is a promising treatment for inherited retinal disease since the eye is a site of relative immune privilege, the vector can be comparatively easily administered (90) and outcomes can be monitored both subjectively by the patient and objectively by the investigator. Gene replacement therapy is likely to be the most effective approach but relies on the causative gene being identified and areas of retina being structurally intact. For autosomal recessive forms of RP which are usually caused by missense or null mutations, the defective gene could be directly replaced using administration of a viral vector such as an adeno-associated viral vector (AAV), provided the gene was of an appropriate size for AAV. In autosomal dominant conditions, where there is often a toxic accumulation of abnormal protein, AAV could be used to overexpress a healthy copy of the gene (91) or knockdown the abnormal gene product (92). The greatest

advancements in the treatment of RP have been made with gene replacement approach, as will be discussed in greater detail below.

1.5.1 Adeno-associated viruses

Adeno-associated viral vectors (AAV) have been used extensively to deliver genetic material to a cell for the purpose of gene therapy, and could be delivered to the eye via a subretinal or intravitreal route. AAV are single-stranded DNA parvoviruses that are not associated with pathogenicity in humans. AAV2 is the best characterised serotype and has a 4.7kb genome that consists of two open reading frames encoding rep and cap genes on either side of which lies a palindromic region known as an inverted terminal repeat (ITR).

The ITR is 145 base pairs in length (93) of which the first 125 base pairs have a palindromic sequence and form a hairpin structure, figure 1.12a. The remaining 20 base pairs are known as the D sequence and remain unpaired. The ITR is the origin of AAV replication and acts as the primer for second strand DNA synthesis by DNA polymerase. Two important regions within the ITR are the rep binding elements (RBE) and terminal resolution site (trs) which are used by the viral rep protein to process the intermediates formed after second strand synthesis (94). The ITR is also important for AAV packaging and insertion into the host genome (95). The latter process occurs into a specific site on human chromosome 19 (AAV2) (96), but the majority of wild type AAV DNA exists as circular double-stranded episomes in the host cell (97). This makes AAV more predictable than other viruses such as lentiviruses, which may integrate randomly causing insertional mutagenesis.

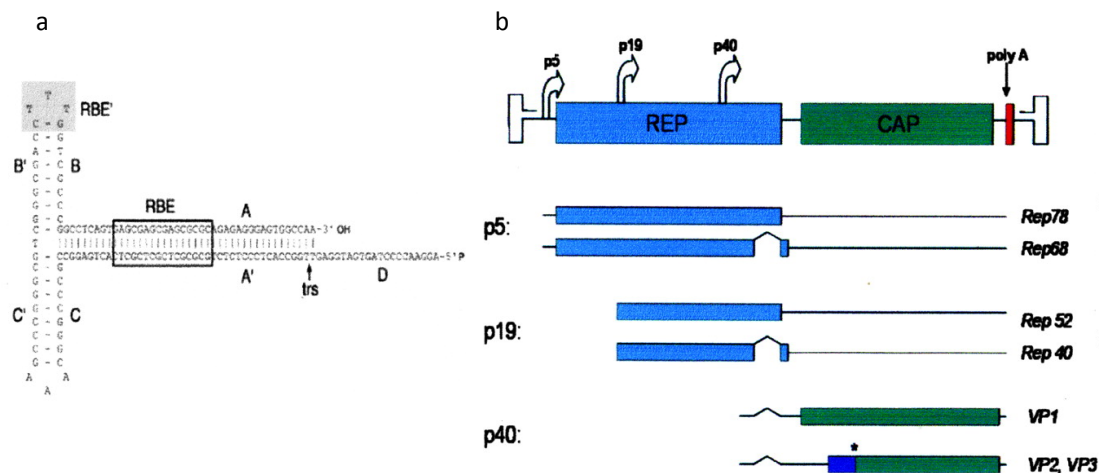


Figure 1.12 Adeno-associated viruses.

The structure of the AAV ITR (a) illustrates its palindromic sequence and secondary structure. (b) Transcription occurs from three promoters (p5, p19 and p40) and the 4 rep proteins created by alternative splicing. An alternative start codon ACG is used for VP2, whereas AUG (indicated by *) is used for translation of VP3. Adapted from (94).

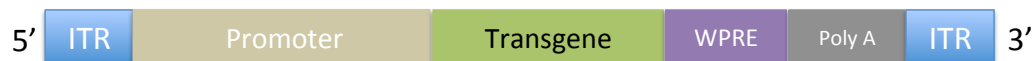


Figure 1.13 Schematic of the recombinant AAV expression cassette used for retinal gene therapy.

Several elements may be optimised including the promoter, transgene, polyadenylation sequence and the inclusion of a Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE).

From the first open reading frame (ORF) containing the rep gene, four rep proteins are produced. The rep gene contains two promoter regions: P5 and P19. One transcript is initiated from the P5 promoter region and in its unspliced form is translated to produce the protein Rep78, and a spliced form produces Rep68. Likewise the transcript from the P19 promoter site is alternately spliced to form Rep52 and Rep40 proteins, figure 1.12b. The second ORF contains the cap gene from which the capsid proteins VP1, VP2 and VP3 are produced. From the P40 promoter region the unspliced transcript is translated to VP1 and the spliced transcript to VP2 and VP3. An ACG start codon initiates translation of VP2 whereas further down the transcript and AUG start codon initiates VP3 translation. The latter is ten times more abundant than VP1 and VP2 (94).

Recombinant AAV (rAAV) vectors for gene therapy are generated by deleting the entire coding sequence of the wild type AAV genome, and replacing it with a reporter gene such as green fluorescent protein (GFP) or a therapeutic transgene. This eliminates the ability of the AAV vector to integrate into the host genome, further increasing its safety as a therapeutic vector. One limitation however is the size of the transgene that can be included as the entire construct including ITRs should be under 5kb for efficient packaging (98).

1.5.2 Optimising AAV for retinal gene therapy

When using AAV for gene therapy in the retina, certain properties may have to be optimised depending on the target cell type and transgene of interest, figure 1.13. These include choice of promoter, whether to include any additional regulatory elements such as the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and whether to use a single stranded or self-complementary virus.

1.5.2.1 Promoter

With regards to a promoter, the choice lies between a ubiquitous promoter or to restrict gene expression using a cell specific promoter. The two most extensively assessed ubiquitous promoters in retinal transduction are the CMV (human cytomegalovirus immediate-early gene) promoter, and the CAG promoter (comprising the CMV early enhancer and chicken β -actin (CBA) promoter)(99), the latter being used in three of the reported human clinical trials (78, 79, 100). Following subretinal delivery, transgene expression in RPE cells, photoreceptor cells, and ganglion cells has been shown with both the CMV (101) and CAG promoters (102). However in other tissues AAV driven transgene expression has been demonstrated to be 137 times higher when using the CBA promoter compared to CMV (103). In brain (104) and muscle (105), there is evidence that the CMV promoter may be silenced due to methylation in the longer term, leading to transient or reduced gene expression.

For certain retinal degenerations, limiting gene expression to a specific cell type may have benefits with respect to safety and functional efficacy, although in some cases overall levels of transduction may be lower than with the use of a ubiquitous promoter. Regarding expression in the RPE, the 1.6kb *RPE65* promoter has been successfully used to limit gene expression to the RPE following subretinal delivery in Briard dogs (106) and has also been used in human clinical trials, although with questionable efficacy (107). In targeting photoreceptors, the 292bp human rhodopsin kinase (*hGRK1*) promoter has been shown to be effective in driving high levels of gene expression and specifically target rods and cones in both mouse models and non-human primates (108). In conditions such as achromatopsia, it may be useful to limit gene expression to cone photoreceptors alone. For this purpose, the 2.1 kb PR2.1 red cone opsin promoter has been shown to specifically drive gene expression in L/M cones in the normal canine

retina (109), and the human cone arrestin promoter has shown expression in S and M cones in mice (110).

Other approaches, such as optogenetic strategies for restoring vision in the degenerate retina by the ectopic expression of light-sensitive proteins to make residual cells photosensitive (see section 1.7), might be optimized through gene delivery to bipolar cells. Previous work has shown that a 200 bp enhancer region from the murine *GRM6* gene fused to a SV40 (Simian vacuolating virus 40) core promoter restricts transgene delivery to rod and cone bipolar cells following subretinal delivery to the *rd10* mouse model of retinal degeneration (111). In specifically targeting ganglion cells, the 2.8kb human connexin 36 promoter produced selective transduction of foveal ganglion cells following intravitreal delivery to the macaque retina (112), although the large size of this promoter may limit its use.

1.5.2.2 WPRE

The Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) facilitates the nuclear export of viral RNA. Incorporation of this sequence into the 3' untranslated part of the AAV genome has been demonstrated to increase gene expression by a factor of 1.8 times in brain tissue (104), and a six fold increase in gene expression in cultured primary human fibroblasts compared to a vector which did not contain WPRE (113). AAV vectors containing WPRE have been used in clinical trials in humans for the treatment of Parkinson's disease (AAV.GAD study)(114).

Of note, the native WPRE contains a promoter and sequence for the first 60 amino acids of the Woodchuck hepatitis virus X protein, which has been linked to the generation of liver tumours (115). Therefore modification of the WPRE sequence to prevent X antigen

expression can be considered, and such a sequence has been used recently in the AAV vector administered in a clinical gene therapy trial to treat choroideremia (79).

1.5.2.3 Self-complementary vectors

Following transduction by AAV, the onset of transgene expression is delayed by several days, and one factor contributing to this is the need to synthesise a complementary strand of DNA from the single stranded AAV genome within the host cell nucleus. In order to bypass this step and improve AAV transduction, self-complementary AAV (scAAV) vectors have been developed. These vectors contain a single-stranded inverted repeat genome, with a wild type ITR at one end and a mutated ITR in the centre. After uncoating, the viral DNA folds to form a double stranded molecule (116). These vectors have been assessed in the retina, and were found to have more rapid onset and more widespread gene expression following subretinal delivery in mice when packaged in rAAV2/2 (117), rAAV2/8 (118) and in the canine retina in rAAV2/5 (119) when compared to their single- stranded AAV counterparts. However the drawback of these vectors is their small packaging capacity, since both forward and reverse copies of the transgene of interest must be included within the 4.7kb AAV vector.

1.5.3 AAV Pseudotyping

Over 100 AAV variants have been described (120), and of these AAV2, AAV3 and AAV5 are endemic to humans (121). The molecular clone for AAV2 was the first to be isolated (122), and therefore recombinant AAV2 has been the most widely studied vector for gene therapy, showing efficient and long term gene expression in a number of tissues. In order to further increase transduction efficiency and tropism to different cell types, vectors have been pseudotyped in which the ITRs of one serotype are packaged in the capsid of another serotype (123). For example, packaging AAV2 ITRs with the required

expression cassette in an AAV5 capsid (generating a rAAV2/5 vector) confers the safety and efficient expression of rAAV2 with the tropism of AAV5.

Regarding rAAV gene therapy in the retina, the choice of which pseudotype to use is important due to differential cell targeting by different AAV serotypes. The ability of pseudotyped vectors to transduce retinal cells has been extensively assessed in wild type mice following both subretinal and intravitreal delivery to the eye, however tropism in primates may vary from the murine models. rAAV2/2 and to a lesser extent rAAV2/8 are able to transduce ganglion cells following intravitreal injection (120), and therefore one of these vectors should be considered if ganglion cells are the target for gene therapy. Following subretinal injection, rAAV2/1 and rAAV2/9 are able to transduce retinal pigment epithelium (RPE); rAAV2/2, rAAV2/5, rAAV2/7 and rAAV2/8 are able to transduce both RPE and photoreceptors (120). Transduction kinetics and efficiency also vary between these vectors; for example rAAV2/5 has a quicker onset with gene expression evident at 5 to 7 days following subretinal injection in a wild type mouse, when compared to rAAV2/2, in which gene expression is evident at 2 weeks. Studies have also demonstrated that photoreceptor transduction is more efficient in mice using rAAV2/5 compared to rAAV2/2 (124), and in non-human primates using rAAV2/8 compared to rAAV2/2 (125).

1.5.4 Capsid mutation

There is ongoing work to improve transduction efficiency by AAV and increase the number of cell types that can be targeted with these vectors. It is known that AAV2 gains entry to a cell by capsid binding to the heparan sulphate receptor (126), which then leads to AAV internalisation into the cell via endocytosis (127) facilitated by coreceptors, including integrins (128) and fibroblast growth factor (FGF) receptors

(129) . Recent work has shown that modification of the capsid VP3 protein, specifically point mutation of tyrosine residues to phenylalanine, can significantly increase transgene expression in Hela cells *in vitro* and murine hepatocytes *in vivo* (130). The exposed tyrosine residues on the capsid surface are believed to be involved in the ubiquitination pathway, which tags the protein for proteasome-mediated degradation. By modifying these residues, vector ubiquitination is thought to be decreased which leads to increased intracellular trafficking and more vector being delivered to the cell nucleus.

There are seven exposed tyrosine residues on the VP3 protein of the AAV capsid, and vectors containing mutations of one to all seven of these residues have been generated and assessed in the murine retina. Single mutations of capsid tyrosine residues in rAAV 2/2, rAAV2/8 and rAAV2/9 in self-complementary AAV vectors have been shown to significantly increase ganglion cell transduction when delivered intravitreally and RPE and outer retinal transgene expression when delivered subretinally in wild type mice (131). Transduction of cells in the inner nuclear layer such as bipolar cells has previously proven to be difficult.. Tyrosine (Y) to phenylalanine (F) capsid mutations also appear to affect the ability of the AAV virus to transduce these cells: a single mutation in rAAV 2/8 (Y733F) has been demonstrated to result in bipolar cell transduction in the *rd10* mouse model of retinal degeneration (111), and the rAAV2/2 quadruple and pentuple mutants are able to transduce bipolar cells when delivered either subretinally or intravitreally in wild type mice (132). Therefore, the ability to generate these capsid mutant vectors may allow more efficient transduction of the retina, targeting a wider range of cell types and requiring a lower titre of virus to be used which would have less risk of generating an immune response.

1.5.5 Directed evolution approaches

In order to further enhance the transfection efficiency of AAV and potentially transduce photoreceptors using intravitreal viral delivery, a directed evolution approach has been utilised to generate new AAV variants. In this process a library of capsid types generated in the laboratory are screened by subjecting them to the same pressure that they would encounter *in vivo*, for example by intravitreal administration. Successfully transduced target cells of interest eg. photoreceptors are then isolated and the capsid proteins of the viruses that have transduced them isolated by PCR. Following repetition of this process the best AAV candidate can be identified. Using this approach, a recent study generated an AAV named 7m8 which was demonstrated to transduce photoreceptors following intravitreal delivery in both mouse and primate retina (133). Further work is now needed to evaluate the long term efficacy and safety of these new AAV vectors.

1.5.6 Clinical trials

The condition in which the greatest developments in retinal gene therapy have been made is Leber's congenital amaurosis (LCA), a rare severe retinal dystrophy that manifests with poor vision at birth or within the first few years of life. Several causative mutations have been identified, one of which is in the *RPE65* (retinal pigment epithelium-specific 65kDa protein) gene (134), and has autosomal recessive inheritance. Despite loss in retinal function the structure of the retina remains largely intact, making this condition an ideal target for gene therapy since potential for functional improvement remains. Gene replacement therapy to the *Rpe65*^{-/-} knockout mouse using subretinal delivery of an adeno-associated viral (AAV) serotype 2 vector expressing RPE65 under the control of a ubiquitous chicken β -actin (CBA) promoter resulted in RPE65 expression in the RPE and electroretinogram (ERG) improvement in 4 out of 10

eyes (135). In addition, a canine model of *RPE65* mutation exists, the Briard dog, which has also been treated with AAV2 expressing RPE65 under a CBA promoter, with restoration of visual function observed (102). These and other animal studies provided proof of principle that AAV retinal gene therapy for *RPE65* mutations could be administered safely and give efficacious results, paving the way for translation to patients.

In 2008, results from three human clinical trials of ocular gene therapy for *RPE65* mutations causing LCA were published (78, 100, 107). These initial reports from three independent groups at University College London, the Children's Hospital of Philadelphia (CHOP) and the University of Florida/University of Pennsylvania each reported outcomes from three patients. All had subretinal injection of an AAV2 vector containing *RPE65* cDNA to target the RPE, although there were some differences between studies such as vector design, injection volume, and use of steroids. In all of the studies, no significant immune response was found to the vector or transgene product. Three patients showed improvement in visual acuity (100), and increased light sensitivity was found in seven out of nine patients.

Further publications from these centres along with a fourth centre in Israel (136) have now detailed a total of 31 patients of age range 8 to 30 that have been treated. Jacobson et al (137) provide up to three years follow up data from 15 patients and show no systemic toxicity or significant immune reactions but some adverse events related to ocular surgery. There was significant improvement in full field sensitivity testing in study eyes compared to control eyes. Three patients have also had vector administration to their second eye, 1.7 to 3.3 years after the initial treatment, with subsequent follow up reported after six months (138). No detectable T cell or neutralising antibody response was found to the vector or transgene product. In terms

of function, all “second” eyes showed improvement in full-field sensitivity testing, pupillometry and functional MRI, suggesting that eventually treatment of both eyes may be safe and efficacious. More recent work however has reported ongoing retinal degeneration in treated patients despite long-term improvements in visual acuity (139).

Clinical trial results have recently been published of phase1/2 trial treating a further retinal degeneration called choroideremia, an X-linked recessive condition in which there are mutations in the CHM gene encoding Rab escort protein-1 (REP1). In this trial an AAV2 vector expressing REP1 under the control of a CBA promoter was delivered subretinally in 6 patients. There were no adverse effects and in two patients visual acuity improvements were reported (79).

These trials show great promise for conditions in which the causative gene defect is known, however in a large proportion of cases there may not be an identified mutation and so a broader gene therapy approach is required. At an early stage of the disease process, one such method could be to induce expression of a neuroprotective protein such as ciliary neurotrophic factor (CNTF) (80) to reduce or arrest cell loss. In conditions where degeneration is advanced, other strategies may be more appropriate to restore vision.

1.6 Strategies for end-stage disease

The electronic implant is the only form of treatment for end-stage retinal degeneration that is currently in clinical use and involves directly stimulating the residual retina using an electrical stimulus. There are two main classes of retinal implant design: one located in the subretinal space and the other on the epiretinal surface.

Subretinal implants (Retina Implant AG, Reutlingen, Germany) consist of a scleral-fixated microchip inserted between the retinal pigment epithelium and outer retina (140). The microchip contains 1500 microphotodiode array (MPDA) elements, each containing a light sensitive photodiode connected to an amplifier and output electrode; therefore every MPDA generates a pixel. The natural focussing power of the eye is used to direct incident light onto the chip and the light intensity on the MPDA determines the output current from the electrode. The resultant voltage is delivered to adjacent bipolar cells, which transmit the signal through the retina, and therefore there is potential for the signal to be processed by the inherent mechanisms present in the inner retina. The implant provides a visual field of 11 degrees and is sensitive to light intensities ranging over 2 log units; the overall sensitivity can also be manually adjusted (83). The microchip is connected to a silicone cable, which exits the eye and is connected to a coil placed behind the ear for wireless power and signal transmission. Following implantation in nine patients, the majority were able to see and localise light, with some being able to detect facial features and objects of daily living such as cutlery and doorknobs and three patients being able to read letters (141). The current version of the implant recently received the CE mark for distribution in Europe.

In epiretinal systems, the retinal implant itself does not contain a light sensitive element. Instead a camera outside the eye detects the image, which is processed and the signal conveyed to an electrode array placed on the inner surface of the retina and secured in place using a retinal tack. This retinal electrode stimulates ganglion cells, which transmit this information to the brain via the optic nerve. The most established epiretinal implant is the Argus II retinal prosthesis system (Second Sight Medical Products Inc, Sylmar, California). It consists of a 60-electrode stimulating microelectrode array made up of 200 μm diameter disc electrodes, and an inductive coil link positioned on the surface of

the globe which transmits power and data to the electrodes. The external component involves a spectacle-mounted camera to capture the image and a video processing unit which digitizes the image into 10x6 pixels. The stimulus pulses created are transmitted wirelessly to the internal part of the prosthesis (84).

Following implantation of the Argus II prosthesis in 27 patients, 96% of patients showed improvement in accuracy of spatio-motor skills with the system on (84). A further report of 28 patients described an improvement in identification of letters in some subjects with the system switched on and a few who could identify words (142). The US FDA recently approved this device for use.

Results from both subretinal and epiretinal implants are exciting and provide proof of concept that stimulating the residual retina can lead to restoration of some visual function. However complex surgery is involved and the visual field achieved is small, and so other strategies should also be investigated.

1.7 Optogenetic therapy

The field of optogenetics uses light to modulate a population of cells that have been made light-sensitive (143). The first reports used expression of a combination of *Drosophila* photoreceptor genes to induce light sensitivity in mammalian neurones and control their activity (143). Further developments have seen the optogenetic tool refined to a single protein (144) and the number of available proteins vastly expanded (145). Optogenetics can be used not only in the control of individual neurones but also animal behaviour in *Drosophila* (146) and *C. elegans* (147) and even for the modulation of mammalian behaviour (148). Optogenetics now has widespread applications in

neuroscience, including as a method to investigate disease processes such as Parkinson's disease (149), Schizophrenia and autism (145).

The aim of optogenetic approaches for the treatment of retinal degenerations is to deliver a light sensitive protein to cells of the intact residual retina using AAV to make them intrinsically light sensitive. In the degenerate retina, along with photoreceptor cell death the second messenger system for rhodopsin and cone opsins are also lost, therefore another photopigment must be used to restore light sensitivity via gene therapy. In considering which protein to use for optogenetic purposes several factors need to be considered such as its sensitivity to light, response kinetics, the range of light over which responses are seen and safety of administration. There are currently three main candidate proteins for this: channelrhodopsin-2, halorhodopsin and melanopsin, figure 1.14.

1.7.1 Channelrhodopsin-2

Channelrhodopsin-2 (ChR2) is a rhodopsin with a microbial-type chromophore and was originally isolated from the green alga *Chlamydomonas reinhardtii* (150, 151). It is a 7-transmembrane protein, but differs significantly from mammalian rhodopsin since its chromophore in the ground state is all-trans retinal, and is not G protein coupled (152). ChR2 is itself a light-sensitive cation channel: incident light isomerises all-trans into 13-cis retinal causing a conformational change in ChR2 allowing cations into the cell resulting in depolarization (150). This response is rapid with an onset of less than 50 microseconds (153). ChR2 responds to light of wavelength under 540nm, with a peak sensitivity of 450nm (150).

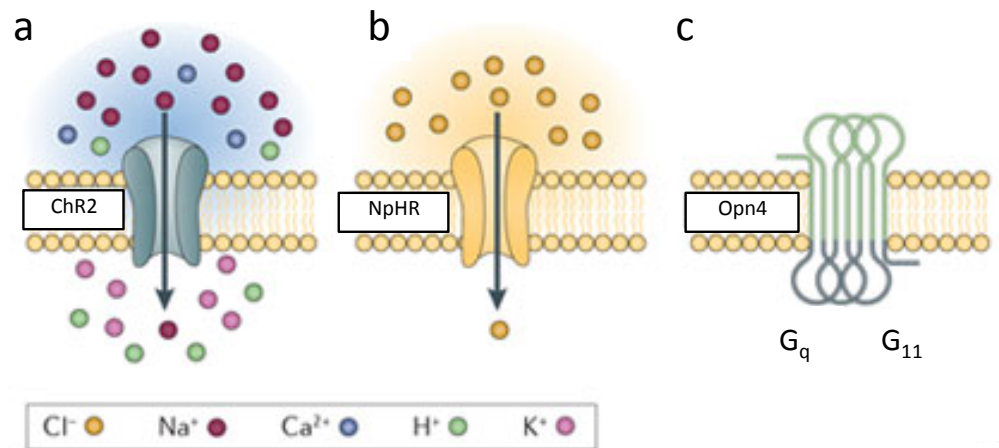


Figure 1.14 Candidate proteins for use as optogenetic tools in retinal gene therapy.

Channelrhodopsin 2 (a) and halorhodopsin (b) are both ion channels the former being a cation channel and the latter an anion channel. Melanopsin (c) acts via activation of a G protein mediated cascade. Adapted from (145).

Thus far, gene therapy has been used to introduce ChR2 into the degenerate retina in animal models. Two types of cells have been targeted: ganglion cells and bipolar cells. Intravitreal injection of AAV containing the ChR2 gene has been shown to successfully transfect retinal ganglion cells in *rd1* mice (86), Royal College of Surgeons rats (154) and marmoset retina with some foveal transduction in this model (155). The transfected cells were demonstrated to depolarize in response to light using whole cell patch clamping (86) and there was subsequent improvement in visual evoked potentials (VEP) as compared to controls (86, 154). Long term transduction of ganglion cells using this method has also been demonstrated (156).

However, there is variation in the literature. A transgenic mouse line has been created with ChR2 present in approximately one third of ganglion cells. Cross-breeding this line with the *rd1* mouse results in a mouse with a degenerate retina and ChR2 present in ganglion cells (157). Electrophysiological testing showed that the ganglion cells did spike in response to light. However, there was no significant difference in activation of the visual cortex in response to light between ChR2-expressing and *rd1* mice. Nor was there any significant difference between these two groups at discriminating gratings or a bright field from a dark field (157).

It is unclear why these mice do not show increased visual cortex responses to light despite having light sensitive ganglion cells. The authors suggest that it may be that inadequate numbers of ganglion cells express ChR2. Alternatively since all types of ganglion cells (ON, OFF and ON-OFF) contain ChR2 in this model and all respond in a similar fashion to a light response, the processing of visual signals and visual channels may be disturbed and the brain unable to interpret them (157).

An alternative and more specific means could be to selectively target ON bipolar cells: in the physiological state when photoreceptors hyperpolarise in response to light, ON bipolar cells depolarise, and OFF bipolar cells hyperpolarise (158). Using a plasmid with ChR2 expression under the control of a mouse GRM6 (metabotropic glutamate receptor subtype 6) enhancer and SV40 promoter, ChR2 expression was restricted to ON bipolar cells (87). This plasmid was administered via a subretinal injection and *in vivo* electroporation to *rd1* mice pups and transfected approximately 7% of ON bipolar cells. Ganglion cells in the treated mice spiked in response to light in a time-dependent manner whereas those not in control *rd1* mice did not. These ganglion cells were shown to receive lateral inhibitory inputs from amacrine cells, which is important for contrast sensitivity. Treated mice also showed larger VEP amplitudes and improved behavioural testing using a light avoidance test and optokinetic drum when compared to control *rd1* mice (87).

Further work has used rAAV2/8 with a single tyrosine to phenylalanine capsid mutation (Y733F) to deliver ChR2 to ON bipolar cells using this promoter, showing gene expression at 10 months and behavioural improvement in a water maze test compared to untreated animals (111). Bipolar cells have also been targeted with cell-specific promoters using an AAV capsid derived by a direct evolution approach (159) and also a synthetic AAV capsid (160), with functional improvement.

A disadvantage of ChR2 is the high stimulus intensity required to obtain a response compared to rhodopsin or cone opsins (10^{15} photons/cm²/s required for ChR2 versus 10^{10} photons/cm²/s for cones and 10^6 photons/cm²/s for rods) (86, 87). It has been suggested that a higher density of ChR2 would be needed to compensate for this (161), but the question of what effect the high light intensities needed to stimulate ChR2 would have on the remaining retina has not been answered. Another potential limitation is the

range of intensities of light to which ChR2 is sensitive. Healthy photoreceptors are able to respond to a large range of light intensities, spanning 8 log units, whereas ChR2 responses are seen over 2 log units of light intensity (87).

Therefore work is ongoing to optimize the sensitivity and kinetics of ChR2. One variant calcium translocating channelrhodopsin (CatCh), has a voltage response that is seventy times more sensitive to light than ChR2 (162), and another has red-shifted spectral sensitivity (163). However, neither has the sensitivity or range of response of conventional photopigments and so some form of image or light processing device may be required to stimulate ChR2 in the optimal range.

1.7.2 Halorhodopsin

Another candidate for re-introducing light sensitivity into the degenerate retina is Halorhodopsin (HR), a light-sensitive chloride ion pump derived from *Natronomonas pharaonis* halobacteria (164). Stimulation of halorhodopsin in response to light causes hyperpolarisation of the cell (as do photoreceptors in the physiological state in response to light). Its peak sensitivity is to light of wavelength 570nm and its activation and de-activation are very fast (165) giving halorhodopsin good temporal characteristics for use in gene therapy. However the stimulus intensity required in order to obtain a light response is even higher than that for ChR2 (10^{16} photons/cm²/s) (89).

In retinitis pigmentosa, rods degenerate initially with a secondary loss of cones. Despite their lack of light sensitivity, there is evidence that cone cell bodies do remain for some time (166) and could be a target for optogenetic gene therapy. AAV vectors have previously been used to transduce these residual cones in *rd1* mice and also a slower retinal degeneration model (*Rho*^{-/-} and *Cnga3*^{-/-}) with enhanced *Natronomonas*

pharaonis halorhodopsin (eNpHR) (89). Appropriate light responses were measured in both ON and OFF retinal ganglion cells, along with lateral inhibition (which is important for contrast enhancement) and directional selective responses. There was also improvement in visually guided behaviour using a light avoidance test and optokinetic drum in treated versus control mice. The results of this paper are exciting, but clinically there may be a limited window of opportunity when these residual cone cells are present and could be targeted.

Other groups have utilised halorhodopsin to create an OFF processing pathway. When light stimulates the retina, the visual signal is processed by an ON pathway in which ganglion cells depolarize in response to light, and an OFF pathway in which cells hyperpolarize (167). Following co-administration of ChR2 and Halorhodopsin to *rd1* mice via intravitreal injection, ChR2 and HR colocalised in the majority of transfected ganglion cells. Light stimuli with wavelength up to 500nm elicited an ON response and wavelengths of 540-620nm created an OFF response (168). Therefore, there is potential for the two photopigments to be used in conjunction and the wavelength of incident light to be modulated with a view to processing the visual input signal.

1.7.3 Melanopsin

As described in section 1.3, melanopsin is an opsin protein found in mammals and other species, in which photoisomerisation of 11-*cis* retinal results in activation of a G protein and downstream signalling cascade causing cell depolarisation. The foundations of its use as an optogenetic tool are from *in vitro* studies demonstrating that expression of melanopsin in non-photoreceptor neuronal cells induces photosensitivity (55, 169, 170). Since it is already present in the eye, melanopsin gene therapy is unlikely to provoke an immune response and furthermore, the G protein coupled nature of

melanopsin is advantageous as an optogenetic tool, since one melanopsin molecule may activate a number of G proteins causing signal amplification. In fact melanopsin may be capable of coupling to ubiquitous cell signaling pathways (51), thus it is likely to be functional when introduced into a new cell type. The ability of melanopsin to regenerate its own chromophore (169) also facilitates its use over other mammalian opsins, which require chromophore regeneration by the RPE.

Melanopsin is more sensitive to light than other optogenetic tools such as ChR2 or NpHR, and studies have shown that melanopsin can respond to light in the physiological light range (88). Therefore it could be used for optogenetic therapy at ambient light levels without the need for augmentation of the light stimulus required by ChR2 and NpHR. In addition, melanopsin is a native protein in humans and so in theory is much less likely to incur an immune response when expressed within the eye compared to microbial opsins.

The disadvantage of using melanopsin is its slow kinetics. In certain conditions stimulus onset has been demonstrated to have a time constant of 0.4 seconds (58), which is slower than that seen with ChR2 and NpHR. This could however still be physiologically acceptable, since for example the subretinal electronic implant stimulates the retina at a frequency of approximately five Hertz (83). The time constant of decay is at best 6.4 seconds (58), making the effective response duration about 20 times that of mouse rods. However, it may be that plasticity of the brain allows better interpretation of these signals than can be predicted on the basis of kinetics alone.

Only one study to date has used melanopsin as an optogenetic tool for retinal degeneration. Lin et al, 2008 administered rAAV2 containing the melanopsin gene driven by a CMV promoter into *rd1* mice via intravitreal injection (88). They showed

successful transduction of ganglion cells of a variety of morphologies compared to controls and these cells were demonstrated to be light sensitive using whole cell patch clamping. Following assessment using a twenty second white light stimulus, the pupillary light response significantly improved at a range of light intensities in treated mice versus controls. Treated mice also showed significantly better performance in two behavioural tests: light-dark discrimination and two-choice visual discrimination.

This study shows proof of principle that melanopsin could be used as an optogenetic tool in the eye. However, by stimulating ganglion cells directly and making them all ON cells, the complex processing of the visual signal that occurs in the retina is bypassed. Light may be detected, but processing such as that mediated by amacrine cells in the inner retina would not occur. Furthermore, the intravitreal approach might lead to ganglion cell axons expressing melanopsin, making them light sensitive and resulting in the loss of a retinotopic map (171).

Optogenetic therapy with melanopsin has great promise since it is a native protein rendering it unlikely to generate an immune response and is G protein coupled permitting signal amplification. However a subretinal approach which has been validated as safe in current clinical trials (77, 79) may allow image processing by remaining retinal cells and deliver better visual outcomes than intravitreal treatment. Following further optimisation melanopsin gene therapy could play an important role in treating patients with end-stage retinal degeneration.

1.8 Thesis aims

The overall aim of this project was to develop melanopsin gene therapy using subretinal delivery of an adeno-associated viral (AAV) vector. In order to do so, the AAV vector for transducing cells of the degenerate retina via a subretinal approach was optimised: a suitable capsid identified, promoter chosen and optimisation of the melanopsin sequence investigated. Subsequently expression of the melanopsin AAV vector was tested, along with establishing functional activity by *ex vivo* and *in vivo* testing to examine whether visual function could be restored in a model of end-stage retinal degeneration.

Chapter 2 Materials and Methods

2.1 Plasmid preparation

2.1.1 Preparation of CMV.CBA-OPN4-IRES-DsRed construct

Using polymerase chain reaction (PCR), the CMV enhancer/CBA promoter sequence was isolated from a pUf6.1 plasmid (gift from Vince Choido, University of Florida, USA) and human OPN4 was isolated from an existing plasmid produced by our laboratory. The IRES sequence had previously been isolated in our laboratory from pIRES2-AcGFP plasmid (Clontech, USA) and joined to DsRed (from pSIREN-DNR-DsRed-Express plasmid, Clontech, USA). PCR primers were designed manually and manufactured by Sigma-Aldrich (Dorset, UK), table 2.1. KOD polymerase (Millipore, USA) was chosen due to its proofreading activity and low PCR mutation frequency.

Target	Forward Primer	Reverse primer*
CBA (with Mfe1 restriction site inserted at 5' end)	GGCCAATTGCTCAGATCTGAATT	AAGGAGGGTTCATGGTGGTCGCCCCGCCGCG
OPN4	CGCGGCGGGCGACCACCATGAACCCTCCTT	GGGAGGGAGAGGGGCCTACATCCTGGGGTC
OPN4 fragment 1 (OPN4 ¹)	CGCGGCGGGCGACCACCATGAACCCTCCTT	GGGAGCCCAGGAGAGCACGAAGAGGAGG
OPN4 fragment 2 (OPN4 ²)	GGGGTTGCTGACATCCTGCTCCTGGG	GGGAGGGAGAGGGGCCTACATCCTGGGGTC
IRES DsRed (with Not1 restriction site inserted at 3' end)	GACCCCAGGATGTAGGCCCTCTCCCTCCC	AAAGCGGCCGCTACAGGAACAG
CHX10 (with Mfe1 restriction site inserted at 5' end)	AACCAATTGCTGATGCTGCCAA	CCGAAGGAGGGTTCATGGTGGTCTCCCTGTT
4xMg6 (with Mfe1 restriction site inserted at 5' end)	CCCCAATTGACGCGTGATCTCC	AAGGAGGGTTCATGGTGGTGGATCCGGTAC

* Reverse primers are written in standard notation (5' to 3')

Table 2.1 Primers used for generation of wild type OPN4 constructs

The standard PCR mix used was 1.5 µl forward primer, 1.5µl reverse primer, 1µl DNA, 5µl 10x reaction buffer, 5µl dNTPs, 3µl MgSO₄, 1µl KOD polymerase and 32µl molecular biology grade water. Standard PCR conditions were an initial denaturation step of 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 65°C for 10 seconds and extension at 70°C for 30 seconds. PCR products were identified by size using gel electrophoresis.

PCR SPLICE reactions (172) were performed to join the CMV enhancer/CBA promoter to human *OPN4* and IRES-DsRed, and to insert MfeI and NotI restriction sites. Since the IRES sequence in particular can form a complex secondary structure figure 2.1, 1µl DMSO was added to SPLICE reactions to reduce secondary structure formation in the template DNA. For generation of the full length construct, PCR conditions were an initial denaturation step of 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 10 seconds and extension at 70°C for 150 seconds, followed by a final extension step of 10 minutes.

The full CMV.CBA-*OPN4*-IRES-DsRed construct was inserted into an AAV production plasmid, containing wild type AAV2 inverted terminal repeat (ITRs) and a WPRE sequence (gift from Vector Biolabs) using the MfeI and NotI restriction sites, and ligated using T4 DNA ligase (all New England Biolabs (NEB), UK).

After cloning, the presence of the construct was confirmed by MfeI and NotI restriction enzyme digests and the entire CMV.CBA-*OPN4*-IRES-DsRed construct was sequenced using primers detailed above to ensure no mutations were created during cloning (sequencing was done by Source Bioscience, Oxford UK).

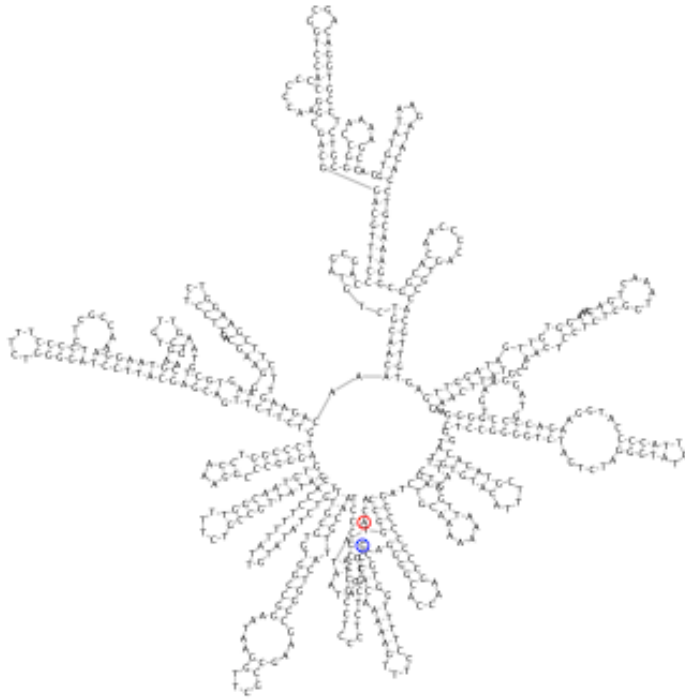


Figure 2.1 Secondary structure formed by the IRES sequence

Predicted mRNA secondary IRES structure using RNA fold, Geneious v5.6.

2.1.2 CHX10-Opn4-IRES-Dsred construct

The human CHX10 promoter was isolated from human genomic DNA, using primers detailed in table 2.1 and the CHX10-*OPN4*-IRES-DsRed construct generated as above.

2.1.3 4xMg6-*OPN4*-IRES-DsRed construct

The 4xMg6 bipolar cell specific promoter (gift from Botond Roska, FMI, Switzerland) was isolated using primers listed in Table 2.1. In order to generate the 4xMg6-*OPN4*-IRES-DsRed construct, PCR was performed to isolate *OPN4* in two parts (herein called *OPN4*¹ and *OPN4*²), with a 200 base pair overlap. Using PCR, the 4xMg6 promoter was joined to *Opn4*¹ and *Opn4*² joined to IRES-DsRed. A further PCR SPLICE reaction was used to join these two sequences, and the construct inserted into the AAV production plasmid as detailed above.

2.1.4 Codon optimised *Opn4* constructs

Codon-optimised (co) human *Opn4* sequence was custom-made by Genscript, USA. The CMV.CBA enhancer/promoter sequence was isolated by PCR, and *OPN4* was isolated in two fragments (*OPN4*¹ and *OPN4*² with a 200bp overlap) as above, using primers detailed in table 2.2. PCR SPLICE reactions were used to join the CMV.CBA enhancer/promoter to *OPN4*¹ and also *OPN4*² to IRES-DsRed, before the two generated constructs were joined.

Target	Forward Primer	Reverse primer*
CBA (with Mfe1 restriction site inserted at 5' end)	GGCCAATTGCTCAGATCTGAATT	CTTGGGGGGTTCATGGTGGTCGCCCCGCCG
coOPN4 Fragment 1 (CBA construct))	GCGGCGGGCGACCACCATGAACCCCCAAG	TTTtagccatctTACACTCGCTCT
coOPN4 Fragment 2	GGGACTGCTGACATCCTGCTCTTGGG	GGGAGGGAGAGGGGCTTACATTCTTGGGT
IRES DsRed (with Not1 restriction site inserted at 3' end)	ACCCAAGAATGTAAGCCCCTCTCCCTCCC	AAAGCGGCCGCCTACAGGAACAG

* Reverse primers are written in standard notation (5' to 3')

Table 2.2 Primers used for generation of (codon optimised) coOPN4 constructs

2.1.5 Electrophoresis and gel extraction

DNA products were separated using gel electrophoresis on gels consisting of 1% agarose in 1x TAE buffer containing 0.5µg/mL ethidium bromide. A 1kb DNA ladder (NEB, UK & Norgen, Canada) was run to provide size markers. Samples were loaded using gel loading dye (NEB, UK). Gels were run at 180 volts, viewed on an ultraviolet transilluminator and relevant bands were excised using a clean scalpel.

DNA was isolated using a gel extraction kit (Qiagen, UK). In brief, the agarose section excised was weighed and dissolved in the appropriate volume of buffer at 55°C. After addition of isopropanol, the mixture was placed in a binding column and centrifuged. After further washes in buffer to remove residual agarose and contaminants, the DNA was eluted in tris buffer. DNA concentration was determined by spectrophotometry using Nanodrop apparatus (Thermo Scientific). The ratio of absorbance at 280nm and 260 nm was also examined, with a ratio of 1.7 to 1.9 indicating acceptable purity.

2.1.6 Amplification in bacteria and recovery of DNA

75µl XL-10 bacteria (Agilent Technologies) were thawed on ice and 3 µl β-mercaptoethanol added. Cells were incubated with 5µl ligation product on ice for 30 minutes prior to a 30 second heat shock at 42 °C, followed by a further 2 minutes on ice. 1ml of SOC medium (Invitrogen) was added and the culture incubated at 37°C on a shaker rotating at 225 rpm for one hour. The transformation mix was spread on LB-agar plates containing 100µg/mL ampicillin and incubated at 37°C for 16 hours. Single colonies were picked, cultured in 4ml LB/ampicillin for 16 hours and the plasmid isolated using a Miniprep kit (GenElute plasmid Miniprep Kit, Sigma-Aldrich, UK). In brief, cultures were centrifuged and the cell pellet resuspended and lysed with appropriate buffer. The cell lysate was added to a binding column and DNA adsorbed onto a silica membrane within the column. This was then washed to remove contaminants, and the DNA eluted in Tris buffer.

For virus preparation, plasmid was cultured overnight in 2 litres of LB/ampicillin and isolated using a Megaprep kit (Qiagen, UK). Cultures were centrifuged, resuspended and lysed and the lysate filtered to remove cell debris. The filtered lysate was treated with endotoxin removal buffer and run through an anion-exchange resin column to bind DNA. The column was washed, DNA eluted using the appropriate buffer and precipitated using isopropanol. Following wash with ethanol, the DNA pellet was dissolved in Tris buffer. DNA concentration was determined as above.

2.1.7 Checking ITRs

In its native form, the 145bp AAV ITR forms a stable secondary structure and is therefore susceptible to deletion in the cloning process. Two *Xma I* restriction sites are

present within the ITR sequence, and therefore, the presence of ITRs was confirmed via a restriction digest. 500ng plasmid was added to 4µl *Xma I*, 2µl *NotI*, 2.5µl buffer, 2.5µl BSA, made up to 25 µl with sterile water and incubated for 2 hours at 37°C. The products were separated using agarose gel electrophoresis and the size of bands seen indicated the presence of both ITRs and the inserted transgene.

2.2 AAV production

Production of AAV involves co-transfecting a stable cell line such as HEK 293T with 3 plasmids:

- (1) A plasmid containing AAV ITRs and the reporter or therapeutic gene of interest. This was produced as detailed above.
- (2) A plasmid containing AAV rep and cap genes.
- (3) A plasmid containing helper genes identified from adenovirus (helper plasmid).

2.2.1 Site directed mutagenesis

In order to produce a capsid mutant vector, site-directed mutagenesis of the AAV rep cap plasmid was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), which is suitable for large plasmids. The steps involved are detailed below and are based on the manufacturers guidelines.

Primer sequences for rAAV2/2 single capsid mutation were used as per the published literature (130). These primers contain the desired capsid mutation and anneal to the same sequence on complementary strands of the plasmid. In addition to the capsid mutation, a second point mutation was also introduced to create an AflIII restriction site

not present elsewhere in the plasmid, which would subsequently allow rapid screening of the presence of successful mutagenesis by restriction enzyme digestion, rather than the need for sequencing all clones.

Synthesis of the mutant DNA strand was generated by thermal cycling, in which the plasmid is denatured, primers containing the mutation to be created anneal and are extended using *PfuUltra* DNA polymerase. Cycling parameters used were: 95°C for one minute, 18 cycles of 95°C for 50 seconds, 60°C for 50 seconds and 68°C for 5 minutes, ending with a final seven minutes at 68°C. After thermal cycling, samples were placed on ice for two minutes, 1µl of DpnI restriction enzyme added to each reaction and samples incubated at 37°C for one hour. This enzyme digests methylated DNA: since the template plasmid was generated in bacterial cells it is methylated whilst the new synthesized plasmid containing the desired mutation is not. Therefore digestion by DpnI degrades any remaining original plasmid.

2µl of the DpnI treated DNA were subsequently used for transformation of XL-10-Gold cells. Colonies were picked, cultured and plasmid isolated by Miniprep. Restriction enzyme digestion using AflIII was used to screen samples for the presence of the desired mutation. Positive clones were then sequenced prior to use for viral preparation.

2.2.2 Transfecting cells for virus production

Transfecting the large number of cells needed for virus production was done in a hyperflask (Corning). This is a multi-layered flask with a total growth area of 1720 cm². Two 75cm² tissue culture flasks were seeded, each with 17x10⁶ HEK 293T cells in Dulbecco's Modified Eagle Medium (DMEM) with 4.5g/litre glucose, 2mM L-glutamine, 10% foetal bovine serum, 100 units/ml Penicillin and 100 µg/ml Streptomycin. After

approximately 72 hours or when cells were 80% confluent, cells were loosened, resuspended in 200ml media and gently transferred from one 75cm² flask into a hyperflask. Hyperflasks were filled with media and incubated at 37°C in a 5% CO₂ environment. Two hyperflasks were prepared per virus produced.

After 72 hours, the cells were transfected with a total of 500µg DNA. The ratio of helper plasmid: Rep-cap plasmid: transgene plasmid is dependent on the size of the plasmids. Quantities used are detailed in table 2.3.

Plasmids	Size (kb)	Amount per transfection (µg)
pHelper	18.7	283
pRep-Cap	7	106
CMV.CBA plasmid	7.4	110
CHX10 plasmid	7.2	
Total		500

Table 2.3 Plasmids used for transfection for AAV production

The 3 plasmids were pipetted into a 50ml Falcon tube and the volume made up to 10ml with 150mM NaCl. In a separate Falcon tube, 1.125ml 10mM Polyethylenimine (PEI) transfection reagent was measured out, and made up to 10ml with 150mM NaCl. PEI was added drop by drop into the Falcon tube containing the DNA and the mixture incubated at room temperature for 20 minutes. This was then added to a 500ml bottle of DMEM containing 4.5 grams/l glucose, 2% foetal bovine serum, 2mM L-glutamine, 100 units/ml Penicillin and 100 µg/ml Streptomycin. Media in the hyperflask was replaced with that containing DNA and transfection agent as prepared above and incubated at 37°C for 72 hours.

2.2.3 Harvesting and lysing cells

Cells from each hyperflask were loosened by shaking vigorously and collected into two Corning 500ml centrifuge tubes. Tubes were spun at 1000rpm for 10 minutes at room temperature. The residual cell pellet was resuspended in approximately 50ml media, transferred to a 50ml Falcon tube and spun for a further 10 minutes at 1000 rpm at room temperature. The supernatant was aspirated and 15ml lysis buffer (4.38 g NaCl (0.15M), 25 ml 1M Tris.Cl pH 8.5 (50mM) in 500ml dh20; autoclaved) added per tube. Four freeze-thaw cycles were carried out and the lysate stored at -80°C until ready for purification.

2.2.4 Virus purification by iodixanol gradient

The lysate was defrosted for 15 minutes at 37°C, Benzonase (purity grade I for biotechnology, VWR) added to give a final concentration of 50U per ml, to break down any residual free DNA. Following incubation for 30 to 45 minutes at 37°C, the lysate was centrifuged at 3700g for 20 minutes at room temperature.

Iodixanol fractions were made up in 50ml Falcon tubes using Iodixanol OptiPrep Density Gradient Medium (60%) (Sigma), Phenol Red solution (Sigma) and 5X PBS-MK (250 ml PBS 10X (Gibco), 2.5 ml 1M MgCl₂, 6.25 ml 1M KCl, in 500ml H₂O), table 2.4. Fractions were then loaded into Quick-seal Ultra-Clear 25 x 89 mm centrifuge tubes (Beckman).

Per 4 AAV ultracentrifuge tubes:

Fraction	Iodixanol	5M NaCl	5X PBS-MK	H ₂ O	Phenol Red
15%	8 ml	6.4 ml	6.4 ml	11.2 ml	-
25%	10 ml	-	4.8 ml	9.2 ml	40 µl
40%	13.4 ml	-	4.0 ml	2.6 ml	-
60%	20 ml	-	-	-	40 µl

Table 2.4 Preparation of Iodixanol fractions

The following amounts of each fraction were layered in the Beckmann centrifuge tube using a Pasteur pipette: 7.2 ml of iodixanol 15%, 4.8 ml of iodixanol 25%, 4 ml of iodixanol 40% and 4 ml of iodixanol 60%. Subsequently the cell lysate was added above the uppermost fraction, and the tubes spun in a Beckman 70Ti rotor at 59000 rpm for 90 minutes at 20°C. After ultra-centrifugation, the wall of the centrifuge tube was pierced at the junction between the 40% and 60% fractions with an 18 gauge needle on a 5ml syringe. The 40% fraction containing virus was aspirated and stored overnight at 4°C.

2.2.5 Virus concentration

Amicon Ultra-15 tubes (Millipore) were used for virus concentration. The filter was rinsed with 5 ml of PBS and centrifuged at 3000g for 15 minutes at room temperature. 5 ml PBS was added to the iodixanol fraction, then the mixture placed in the filter tube and centrifuged at 3000g at room temperature until the residual volume was approximately 500µl. The filter was washed three times by adding 15 ml PBS and centrifuging for 45-60 minutes. On the final centrifugation, the tube was spun until the remaining volume was between 250 and 500µl. This volume contained purified AAV.

Using this volume, the filter was washed approximately twenty times on each side to loosen any AAV stuck to the filter. The virus was then removed and stored at -80°C.

2.2.6 Virus titre using qPCR

Primers that bind to a 266 base pair region of OPN4 or codon optimised OPN4 were used, table 2.5. A dilution series (10ng, 1ng, 100pg and 10pg) of the OPN4 containing plasmid was used as positive template control. The virus was diluted 1:100 and triplicate reactions were set up per sample, adding 1µl virus to each reaction. Standard SYBR green methods were used, where increase in fluorescence intensity is measured as the SYBR green dye binds to increasing amounts of DNA in the plate. When the reaction was complete a consistent threshold was set incorporating the log phase of each reaction. This selected the comparative Ct value for each sample at that threshold.

	Forward	Reverse*
Wild type OPN4	GGGGTTGCTGACATCCTGCTCCTGGG	GGGAGCCCAGGAGAGCACGAAGAGGAGG
Codon optimised OPN4	GGGACTGCTGACATCCTGCTCTTGGG	TTTtagccatcttactactcgctct

* Reverse primers are written in standard notation (5' to 3')

Table 2.5 Primers used for qPCR

To determine titre, a standard curve was generated for the positive template control by converting the positive template control from nanograms into copies of amplicon (divide mass of template (g) by the molecular weight of amplicon (g/mol), and multiply by Avogadro's constant to generate the number of molecules). Following conversion into log values, this was plotted against the Ct values from the dilution series to generate the standard curve. Ct values of the diluted virus samples were then compared to the standard curve and equivalent molecules determined. The log value was converted back

to the actual number of molecules, then the dilution factor was accounted for and the number of molecules multiplied by 1000 to give viral titre as genome particles per ml.

2.3 Cell culture

2.3.1 Cell culture

SH-SY5Y cells were cultured in complete RPMI-1640 media. HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). All media contained L-glutamine (2mM), penicillin (100units/mL), streptomycin (100µg/mL) all from Sigma-Aldrich UK, and 10% fetal calf serum (GIBCO, Invitrogen, UK). Cells were maintained at 37°C in a 5% CO₂ environment.

2.3.2 *In vitro* testing of plasmids

For testing plasmids, HEK 293T cells were plated on coverslips in 6 well plates at a density of 1×10^5 cells per well. When 60-70% confluent, the appropriate volume of plasmid DNA was added to 3 µl Genejuice (Merck, UK) and 100 µl serum free RPMI media, incubated at room temperature for 30 minutes and added to the cell culture dish. After 48 hours, cells were fixed using 4% paraformaldehyde in PBS and stained for human melanopsin with Hoechst 33342 used for nuclear staining as detailed in section 2.8.1. Coverslips were mounted with an antifade reagent (Prolong Gold; Invitrogen) prior to imaging.

2.3.3 *In vitro* testing of AAV

2.3.3.1 Comparison of rAAV2/2, rAAV2/5, rAAV2/Rec2 and rAAV2/Rec 3

SH-SY5Y cells were seeded in 96-well culture dishes at a density of 5×10^4 cells/well. One day after plating, media was changed to that containing 1.6×10^{-8} M Tetradeanoylphorbol-13-acetate (TPA) and 10^{-5} M retinoic acid (RA) to induce neuron-specific differentiation and morphological changes in the SH-SY5Y cells (173). After 48 hours, media was changed and 1 μ L of virus (1.0×10^{12} vg/mL) added per well, giving an overall multiplicity of infection (MOI) of approximately 2×10^4 vg/cell. Media was changed every 48 hours and RA and TPA were maintained in the media throughout. Cells were imaged daily to detect GFP expression using an inverted epifluorescence microscope (Leica DM IL) and QImaging Retiga 2000R camera with Q Capture pro 6.0 software. Images were obtained at x20 magnification using identical acquisition settings including exposure time, and were saved at a resolution of 1200x1600 pixels. 9 days after transfection, cells were fixed using 4% paraformaldehyde in PBS and Hoechst 33342 was used for nuclear staining.

2.3.3.2 Assessing the effects of proteasome inhibitors on viral transduction

SH-SY5Y cells were seeded as above, but no TPA or RA was used. After 24 hours, media was changed and proteasome inhibitors (Doxorubicin & MG-132, Calbiochem, USA; Bortezomib, LC Laboratories, Woburn, USA) and virus were added. Cells were imaged and fixed as detailed above.

2.3.3.3 *In vitro* testing of Opn4 expressing viruses

HEK 293T or SH-SY5Y cells were seeded on 13mm coverslips in 24-well culture dishes at a density of 1×10^5 cells/well. One day after plating, media was changed and the

appropriate volume of virus added to give an overall multiplicity of infection (MOI) of approximately 1×10^4 vg/cell. Media was changed every 48 hours. Cells were imaged to detect DsRed expression using an inverted epifluorescence microscope (Leica DM IL) and QImaging Retiga 2000R camera with Q Capture pro 6.0 software. Images were obtained at x20 magnification using identical acquisition settings including exposure time, and were saved at a resolution of 1200x1600 pixels. 6 days after transfection, cells were fixed using 4% paraformaldehyde in PBS and stained for human melanopsin with Hoechst 33342 used for nuclear staining as detailed in section 2.8.1. Coverslips were mounted with an antifade reagent (Prolong Gold; Invitrogen) prior to imaging.

2.3.4 MTT assay

SH-SY5Y cells were plated as above and after 24 hours, PIs of various concentrations were added. After a further 24 hours, culture media was replaced and 10 μ l of 12mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Invitrogen, UK) added to each well. Dishes were incubated in the dark for 4 hours at 37°C. Subsequently, media was discarded and 50 μ l DMSO added to each well, and incubated at 37°C for 10 minutes. 570nm absorbance was read by a fluorescence plate reader (Multiskan Ex, Thermo scientific, USA).

2.3.5 Immunocytochemistry and Fluorescence-activated cell sorting (FACS)

HEK 293T cells were plated in 6 well plates at a density of 1×10^5 cells per well, and transfected after 24 hours with 1 μ g DNA and 3 μ l Genejuice (Merck, UK) in 100 μ l RPMI medium. After 72 hours, cells were loosened, suspended in cold PBS and fixed by adding 250 μ l 4% paraformaldehyde (PFA, Thermo Fisher, Loughborough, UK) to each well for

30 minutes. All further steps were done at 4°C. Cells were centrifuged at 1000rpm for 5 minutes, the supernatant was carefully aspirated without disturbing the cell pellet, and the latter was resuspended in 10% Donkey serum in 0.1% Triton X and incubated for 30 mins. Cells were spun as above, supernatant aspirated and the pellet resuspended with primary antibody (1:200 human melanopsin antibody in 2% donkey serum, 0.1% Triton X), and incubated at 4°C overnight. Cells were washed three times. Subsequently, cells were resuspended in secondary antibody (1:250 Donkey anti-Rabbit Alexa Fluor® 488, in 2% donkey serum in 0.1% Triton X) for thirty minutes at room temperature in the dark. Cells were subsequently washed three times by centrifugation at 1000 g for 5 min and resuspended in ice cold PBS. After the final spin, cells were counted using a haemocytometer and resuspended to a count of 10^7 cells per ml in PBS for FACS sorting.

Green fluorescent cells were isolated by FACS (Beckman Coulter MoFlo™ XPD) at room temperature. FACS gates for each sample were determined using control untransfected HEK293T cell suspensions that had been stained using the protocol above to establish background fluorescence levels. The overall fluorescence and number of green cells per sample were measured.

2.4 Culture of human retinal explants

Ethical approval was previously obtained to culture samples of retina *ex-vivo* from patients undergoing retinectomy for complex retinal detachment surgery (REC reference no.10/H0505). Samples used were obtained from three patients with their written consent. A routine 23-gauge pars plana vitrectomy was done for retinal detachment surgery (performed by Professor Robert MacLaren). Discs of scarred retina

were cut with a vitrectomy system (Ocutome; Alcon Surgical, Irvine, TX), refluxed into the eye, aspirated with a flute needle and placed in balanced salt solution.

Within one hour of removal from the eye, retinectomy samples were transferred using a 5 ml pipette into organotypic culture inserts (BD Falcon, Bedford, MA) and placed in a 24 well plate. 700 μ L culture media was added per well consisting of Neurobasal A, L-glutamine (0.08 mM), penicillin (100 U/mL), streptomycin (100 U/mL), B27 supplement (2%), and N2 supplement (1%), all obtained from Invitrogen, UK. Explants were maintained at 34°C in a humidified, 5% CO₂ environment. After 24 hours, media was changed and 10 μ L virus (titre 1×10^{12} vg/mL) was added to each well, with 2 wells tested per virus. Media was changed every 48 hours and explants were imaged daily on an inverted epifluorescence microscope (DMIL; Leica, Germany). Eleven to fourteen days post-transfection, explants were fixed overnight in 4% PFA, cryoprotected in 20% sucrose for 1 hour, embedded in OCT compound and frozen on dry ice. Each explant was cut into 16 μ m sections using a cryostat, counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, UK) and viewed on a confocal microscope (LSM710; Zeiss, Germany).

2.5 Western blot

HEK 293T cells were plated in 6 well plates at a density of 1×10^5 cells per well, and transfected after 24 hours with 1 μ g plasmid DNA, 3 μ L Genejuice and 100 μ L serum free media. 48 hours after transfection, cells were washed in ice cold PBS and loosened from the well using 1ml ice cold PBS. Cells were then centrifuged at 4°C at 1000g for ten minutes, the supernatant discarded and the resulting cell pellets resuspended and washed in a further 500 μ L PBS. After further centrifugation at 4°C at 1000g for ten

minutes, the supernatant was removed and the cell pellets stored at -80°C until further use.

Cell pellets were thawed on ice and resuspended in 200 µl of Tris lysis buffer (50mM Tris, 200mM NaCl, 0.25 mM sucrose pH 8.5 with mini complete protease inhibitors, Roche diagnostics, Germany) before homogenisation using a vibrating pestle. The lysate was incubated at 4°C for 15 minutes and then centrifuged at 25200g at 4°C for 30 minutes. The resulting supernatant was aspirated the protein content quantified by spectrophotometry using Nanodrop apparatus (Thermo Scientific).

Subsequently 20µg total protein was combined with an appropriate volume of Laemmli buffer (Sigma Aldrich UK) and incubated at room temperature for 20 minutes. The latter contains beta mercaptoethanol and SDS, which denatures proteins and neutralises their positive charge so that they are sorted by size and not shape or charge when resolved by electrophoresis.

Samples were resolved on a 10% polyacrylamide gel (10% Criterion™ TGX™ Precast Gel, Bio-Rad, UK) and transferred onto a PVDF membrane (Bio-Rad). Using the SNAP id system (Millipore, MA, USA) the membrane was blocked in 1% BSA in Tris-buffered saline (BioRad, UK) with 1% Tween-20 (TBST) and incubated for 10 minutes at room temperature with primary antibody diluted in 1% BSA in TBST, table 2.6. Blots were washed three times in TBST and incubated with HRP linked secondary antibody for 10 minutes at room temperature, table 2.7. The blots were then washed three times in TBST and developed using an ECL system (Thermo Scientific).

Staining	Antigen	Host	Source (product code)	Dilution
Human Opn4	C-terminus of human Opn4	Rabbit	Santa cruz (sc-32870)	1:200
GAPDH	Full-length human recombinant GAPDH protein produced in HEK293T cell.	Mouse	TRUEMAB Antibody Clone 2D9, Origene, Rockville USA	1:10,000

Table 2.6 Primary antibodies used for Western blot

Reactivity	Host	Label	Source (product code)	Dilution
Rabbit	Donkey	Horse radish peroxidase	Abcam (ab 98503)	1:10,000
Mouse	Donkey	Horse radish peroxidase	Abcam (ab 98799)	1:10,000

Table 2.7 Secondary antibodies used for Western blot

2.6 Physiological testing

2.6.1 Calcium imaging of cells *in vitro*

For imaging of plasmid transfected cells, HEK 293T cells were plated in 35mm dishes at a density of 1×10^5 cells per dish, and transfected after 24 hours with 1 μ g plasmid DNA, 3 μ l Genejuice and 100 μ l serum free media. For AAV transfection, SH-SY5Y cells were plated at 1×10^5 cells per 35 mm dish and after 24 hours were treated with TPA and RA as above. After a further 48 hours, media was changed and AAV added with an MOI of 2×10^4 viral genomes per cell.

On the day of imaging, the following were added to the 35mm dish: Fluo4 (Invitrogen, UK) (prepared in 10% pluronic acid in DMSO) to a final concentration of 5 μ M; Probenecid to a final concentration of 1.25mM and 9-cis retinal to a final concentration of 20 μ M. Dishes were incubated in the dark at 37°C for 40 minutes, cells were then washed with indicator-free media containing 1.25mM Probenecid prior to imaging.

The Fluo4 calcium-sensitive indicator was excited at 480 nm via a monochromator (Cairn Research), controlled using ImagePro software (Media cybernetics), with a 500ms stimulus every 2 seconds for 60 seconds. Emitted fluorescence of 510 $\pm$ 20nm was captured using an Olympus IX71 inverted microscope, with a x40 objective lens every 2 seconds. For analysis, each cell in the field of view was manually identified, and the average fluorescence level over time measured using the Time Series Analyser V2.0 plugin on Image J software. Cells that showed a 5% or greater change in fluorescence in response to the light stimulus were counted as responders.

2.6.2 Multi-electrode array recordings

Following enucleation, retina were dissected from eyes under dim red light (>610nm) at room temperature in Ames media bubbled with 95% O₂ 5% CO₂ (pH 7.3) (Sigma Aldrich, UK). Retina were placed ganglion cell side down onto glass bottomed MEA chambers containing 60 electrodes each 30 μ m in diameter and spaced 200 μ m apart (Multi Channel Systems) and anchored in place with glass coated metal harps (ALA Scientific Instruments). MEA chambers were placed into the MEA recording device (MEA1060-Inv, Multi Channel Systems), fitted with a gas permeable perfusion manifold (ALA Scientific Instruments), and mounted onto the stage of an inverted Olympus IX71 microscope so that the recording electrodes were positioned in the microscope light path. Retina were continuously perfused with AMES media bubbled with 95% O₂ 5%

CO₂ (pH 7.3) and maintained at 34°C using a combination of 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 in the sample chamber. Recorded signals were collected, amplified and digitized at 25 kHz using MC Rack software (Multi Channel Systems).

Following isolation, retinæ were perfused for 30 minutes in the dark prior to recording of light responses. For all recordings baseline activity was recorded for 60 seconds prior to light stimulation. The duration of light stimuli ranged from 2 to 60 seconds as stated. Retinæ were dark adapted for 20 minutes between recordings. A monochromatic light stimulus of 480nm (bandwidth 20nm) was generated by a Xenon arc light source with a slit monochromator (Cairn Optoscan) and delivered via a x10 microscope objective beneath the MEA chamber. Duration and wavelength of light stimuli were controlled via Metafluor software (Molecular Devices). Intensity of light stimuli were adjusted using neutral density filters (0 to 4 log units, Thor Labs) and controlled via an automated filter wheel placed into the microscope light path (Prior Scientific). Power of light stimuli ($\mu\text{W}/\text{cm}^2/\text{s}$) were measured at the sample focal plane using an in-line power meter (PM160T, Thor Labs).

Further recordings were done in the presence of:

- (i) a melanopsin antagonist (10 μM AA92593, Sigma Aldrich, UK)(174) or
- (ii) rod/ cone blocking drugs (a combination of 100 μM L-(+)-2-Amino-4-phosphonobutyric acid, 30 μM D-(-)-2-Amino-5-phosphonopentanoic acid and 40 μM 6,7-Dinitroquinoxaline-2,3-dione, all from Tocris UK) (175) or
- (iii) NMDA and AMPA receptor antagonists (10 μM 3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid and 10 μM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide, both from Tocris UK) (87).

Data was analysed using MC Rack software (Multi Channel Systems). Action potentials were counted as electrical activity greater than 3 standard deviations of baseline activity and the number of action potentials in one second bins was plotted for analysis of responses. A light response was defined as a change in firing rate of 5Hz or greater between the ten seconds prior to light onset compared to the ten seconds after light onset.

After measuring change in firing rate at 5 different light intensities (1.20×10^{12} photons/cm²/s, 1.33×10^{13} photons/cm²/s, 1.37×10^{14} photons/cm²/s, 3.99×10^{14} photons/cm²/s and 1.34×10^{15} photons/cm²/s) irradiance response curves for each electrode were fitted with sigmoidal dose response function (variable slope and EC50). The maximal response for these curves was set as response at the brightest light intensity and the sum of least squares was calculated to find the curve with the best fit to measured data. Only electrodes where good curve fitting was obtained ($R^2 > 0.8$) were included in the analysis.

2.7 *In vivo* procedures

2.7.1 Mouse strains

Wild type (WT) C57BL/6 mice were provided by the Biomedical Sciences division, University of Oxford. C3H/HeNHsd-Pde6b^{rd1} (herein referred to as *rd1* mice) were purchased from Harlan Laboratories (Hillcrest, UK). Non-rd C3H mice were a gift from the group of Prof Russell Foster, University of Oxford. Animals were kept in a 12 hour light (<100 lux) / dark cycle, with food and water available *ad libitum*. All procedures were performed under the approval of local and national ethical and legal authorities.

and in accordance with the Association for Research in Vision and Ophthalmology statements on the care and use of animals in ophthalmic research.

Rd1 mice were genotyped prior to use for presence of the *rd1* and *gpr179* alleles. A small tissue biopsy was removed from the animal's ear, to which 100 µl of 50 mM NaOH was added. This was incubated at 95°C for 1 hour, vortexed and then centrifuged at 13,000 rpm for 2 minutes. Subsequently 10 µl of 1 M Tris-HCl (pH 6) was added, the mixture vortexed and centrifuged at 13,000 rpm for 2 minutes. 500 µl of sterile water was then added and an aliquot of this used for genotyping using a duplex PCR protocol, table 2.8-10.

Allele	Primer	Sequence
rd	1	TGACAATTACTCCTTTTCCCTCAGTCT
rd	2	GTAAACAGCAAGAGGCTTTATTGGGAA
rd	3	GCATTAATTCTGGGGCGCATG
Gpr179	1	TGTGCCTGGGTATCTGTTGA
Gpr179	2	GCATGTGCCAAGGGTATCTT
Gpr179	3	GCTTACACACTTACACACAGATAGATG

Table 2.8 Primers used for genotyping

Temperature	Time
95°C	5 min
94°C	30 seconds)
60°C	30 seconds) x40 cycles
72°C	60 seconds)
72°C	10 minutes
10°C	hold

Table 2.9 PCR conditions for *rd1* genotyping

Temperature	Time
94°C	5 min
56°C	30 seconds)

72°C	30 seconds) x 35 cycles
94°C	30 seconds)
56°C	90 seconds
72°C	5 minutes
10°C	hold

Table 2.10 PCR conditions for gpr179 genotyping

2.7.2 Anaesthesia and pupil dilation

For surgery and *in vivo* imaging procedures, animals were anesthetized by intraperitoneal injection of 1 mg/kg medetomidine (Dormitor 1 mg/ml, Pfizer, Sandwich, UK) and 60mg/kg ketamine (Ketaset 100mg/kg, Fort Dodge, Southampton, UK). Pupils were fully dilated with tropicamide 1% eye drops (Bausch & Lomb, Kingston-Upon-Thames, UK) and phenylephrine 2.5% eye drops (Bausch & Lomb, Kingston-Upon-Thames, UK).

2.7.3 Intraocular injections

At the time of intraocular injection, mice were between 6 and 8 weeks old. Intra-ocular injections were performed tangentially through the sclera with a 10mm 34-gauge needle (Hamilton AG, Bonaduz, Switzerland) mounted on a 5µl syringe (65 RN, Hamilton AG) under direct visual control using a surgical microscope. A circular cover glass (Ø6mm, VWR International, Lutterworth, UK) was applied onto the cornea with a carbomer coupling gel (Viscotears, Novartis, Frimley, UK) to ensure good visualization of the fundus. The eye position was controlled and stabilized by holding the superior or inferior rectus muscle with notched forceps. 1.5µl viral vector (1.0×10^{12} vg/mL) or an equivalent volume of PBS (sham-injection) was administered to each eye, and complete subretinal delivery was confirmed by direct visualization. After injection, the needle was left in position for an additional 20-30 seconds and then withdrawn quickly to minimize

reflux and to allow self-sealing of the scleral tunnel. Different syringes and needles were used for the different viruses and the needle was flushed with sterile water between individual injections.

2.7.4 Fundus imaging using a confocal scanning laser ophthalmoscope

Three weeks after intraocular injection, confocal scanning laser ophthalmoscope (cSLO; Spectralis HRA, Heidelberg Engineering, Heidelberg, Germany) imaging was performed under general anaesthesia. Pupils were fully dilated and a contact lens applied on the cornea. The near-infrared (NIR) reflectance mode (820 nm laser) was used for camera alignment. After switching to the autofluorescence mode (excitation wavelength: 488 nm, emission recorded between 500 and 700 nm) and slight focus adjustments (due to the dioptric shift between the different wavelengths), images were recorded with a standardized detector sensitivity of 70 using the automated real time (ART) mode, without image normalization (176). All images were recorded in the high-resolution mode (1536x1536 pixels) using a 55 degree lens.

2.7.5 Light pulsing for cfos induction

3 days before light pulsing, mice were transferred into open top cages and placed in a light tight coffin where they were maintained on a 12 hour light-dark cycle. On the day of testing, 4 hours into the dark phase (i.e. after lights off) mice were exposed to a 30 minute pulse of 400 lux intensity white light from an overhead fluorescent light source, following which mice were maintained in darkness for a further 30 minutes. A sham group was handled in an identical manner but did not receive a light pulse. 60 minutes after onset of the light pulse, mice were culled for tissue collection and the eyes were placed in 4% paraformaldehyde.

2.7.6 Laser speckle cortical imaging

Transcranial imaging was performed using the Speckle Contrast Imager (moorFLPI, Moor Instruments, UK). Mice were dark adapted for at least two hours prior to the procedure, which took place in a dark room. Following anaesthesia the temperature of the mouse was monitored via a rectal temperature probe, which fed back to a heat pad on which the mouse was placed. Recordings were only performed when the core body temperature of the mouse was 36-37°C to ensure optimal conditions for recording physiological blood flow changes. Pupil dilation was administered as above, the head was fixed in a stereotaxic frame and a midline incision made in the scalp to reveal the cranium. Two blue (480nm) light emitting diodes (LEDs), each positioned on either side of the mouse's head were used as a light stimulus, of total intensity 2000 lux. A trial run was done without the mouse in situ to ensure that this light stimulus did not affect the image captured by the Speckle Contrast Imager.

Both eyes were stimulated simultaneously by a two second 480nm light pulse with a one minute inter-stimulus interval. Ten stimuli were administered to each animal and the signal averaged. Treated mice (n=6) and sham injected mice (n=5) were tested. Data were processed using moorFLPI Review software and analysed with MATLAB R2012a version 7.14.0.739 (MathWorks, MA USA) and Origin Pro 8.6 (OriginLab, MA USA). Percentage changes in cerebral blood flow were calculated in comparison to baseline measurements and trials averaged for each mouse.

2.7.7 Pupillometry

The pupillometry apparatus used involved stimulating the left eye with light and recording the pupil response in the right (contralateral) eye using an infrared digital camera (Cohu, San Diego, USA). Experiments were done during the light phase of the light-dark cycle. Following dark adaptation for approximately two hours, an unanaesthetised mouse was held so that its right eye was in front of an infrared camera and left eye was in front of a Ganzfeld sphere connected to a 100W xenon arc lamp (LOT-Oriel, Germany) via a light pipe. A 2 second white light stimulus was used and the Ganzfeld sphere ensured uniform illumination over the retina. Using neutral density filters, four different intensities of light were used with the dimmest stimulus being assessed first: 5.5×10^{13} , 5.0×10^{14} , 2.0×10^{15} and 2.0×10^{16} photons/cm²/s. An interval of at least twenty minutes was left between the different stimulus intensities for each mouse, with the animal kept in darkness between stimuli.

For analysis, the pupil area was determined by manually identifying the pupil margin and subsequently measuring the area of the region of interest using Image J. Pupil area was measured at 2 seconds after the onset of light and normalised to baseline pupil size for generation of irradiance response curves. For generation of time course curves, pupil area was measured at 500ms intervals and normalised to baseline.

2.7.8 Behavioural light dark avoidance

Mice were tested in a 26cm x 26cm x 26cm box, containing equally sized light and dark chambers connected by a 4 x 5cm opening via which animals could move freely between chambers. The bright half of the box was illuminated by a white fluorescent light

suspended above it, with intensity of 200 lux measured at floor level. Mice were light adapted and tested during the light phase of the light dark cycle. Animals were initially placed in the bright half facing away from the opening to the dark half, the lid of the chamber closed and movement recorded using a camera suspended above the bright half (Logitech HD webcam, Logitech USA). A trial lasted 5 minutes and after this time, the animal was returned to its home cage and the testing apparatus dismantled and cleaned with 70% ethanol. Analysis was done using ANY-maze tracking software, and validated by comparing to manual video analysis. Time spent in the light chamber and number of four paw transitions between chambers (a measure of anxiety) were recorded. Mice that did not transition between chambers within the first three minutes of the trial were excluded from analysis.

2.7.9 Object recognition testing

This test relies on the principle that recognition of an object is context specific, i.e. if an animal encounters a familiar object in a new visual environment it is less likely to recognise the object. Animals were tested in the light phase of the light-dark cycle, in an environment illuminated by a white fluorescent light of 50 lux intensity at floor level. In the first (sample) phase of the test animals were placed in a 30cm x 30cm box containing two identical copies of an object placed in each corner. The walls of the acrylic box were either white or had a black and white pattern on 2 walls. The mouse was allowed to freely explore these objects for the duration of the sample phase and movement was recorded using a camera suspended above the box (Sentient Mini-night vision CCTV camera, Maplin, UK). The mouse was then placed in its home cage for 5 minutes (delay phase). The mouse was subsequently placed in an identical copy of the 30cm x 30cm white acrylic box, with a new copy of the previously encountered object in one corner and a completely novel object in the other corner. For half the animals the

novel object was placed in the top corner of the box, and for the others the novel object was placed at the bottom corner. The mouse was allowed to explore the objects for 3 minutes (test phase) and its movement recorded.

After at least 3 days the test was repeated: mice that had previously been placed in the white box for the sample phase (same visual context for sample and test phases) were placed in the black and white box (change in visual context for sample and test phases) and vice versa. A new set of previously unencountered objects was used for the repeat test. For analysis, ANY-Maze software was used to track the animal's head and measure time spent exploring each object and distance travelled within the box.

2.7.10 Odour testing

This test was used to explore whether old mice that have end-stage retinal degeneration have altered olfactory sensation. Animals were tested in the light phase of the light-dark cycle and were not dark adapted prior to testing. Testing was carried out in darkness to eliminate any visual cues from the task. Under dim red light a mouse was placed in a 30cm x 30cm white plastic box. In each corner of the box was a small glass containing 0.5ml of an identical scented liquid. In half the trials this was a banana scent and in the other half a chocolate scent. The mouse was allowed to freely explore these objects for the ten minute duration of the sample phase and movement was recorded using an infrared camera suspended above the box (Sentient Mini-night vision CCTV camera, Maplin, UK). The mouse was then placed in its home cage for 5 minutes (delay phase) and then returned to an identical copy of the 30cm x 30cm white box, with a new glass containing 0.5ml of the previously encountered scent in one corner e.g. banana and a novel scent e.g. chocolate in the other corner. For half the animals the novel scent was placed in the top corner of the box, and for the others the novel scent was placed at the

bottom corner. The mouse was allowed to explore the objects for 3 minutes (test phase) and its movement recorded. For analysis, ANY-Maze software was used to track the animal's head and measure time spent exploring each scent and distance travelled within the box.

2.7.11 Optomotor response testing

A custom-built optokinetic drum was used which consisted of a rotating cylinder of 30cm diameter, whose speed of rotation could be accurately controlled. The drum was lined with card marked with black and white stripes subtending 0.1 cycles per degree from the centre of the cylinder and was illuminated by white fluorescent light source of 1000 lux intensity suspended above it. In the centre of the drum was a stationary, elevated platform on which the mouse could be placed, and a camera suspended above the drum recorded behaviour of the mouse (Sentient Mini-night vision CCTV camera, Maplin, UK).

Mice were light adapted and tested in the light phase of the light dark cycle. Each trial was 1 minute long consisting of 30 seconds clockwise drum rotation followed by 30 seconds anti-clockwise rotation at a speed of two rotations per minute. Three independent trials were recorded per mouse and responses averaged. The number of head tracks (a slow head movement following the direction of drum rotation) was manually counted from video footage.

2.8 Histological analysis

2.8.1 Tissue collection and processing

For histological analysis, mice were perfusion-fixed using 2-4% paraformaldehyde (PFA, Thermo Fisher, Loughborough, UK) in PBS. After enucleation, the cornea and lens were removed under direct visualisation with an operating microscope in 4% PFA in PBS. Following fixation overnight, the eyecups were cryoprotected using a 10-30% sucrose gradient. Eyecups were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, The Netherlands), frozen on dry ice and stored at -80°C until sectioning. Eyecups were cryosectioned into 16µm sections and affixed to poly-L-lysine coated glass slides (Polysine®; Thermo Scientific, Loughborough, UK). The sections were air-dried and then stored at -20°C until further histological processing.

2.8.2 Immunocytochemistry and Immunohistochemistry

After hydration and 3 x 5 min washes in 0.01M PBS, fixed cells or retinal sections were blocked for 1 hour at room temperature in PBS + 0.1% Triton X-100 + 10% donkey serum. After 3 x 5 min washes, the sections were incubated at 4°C overnight with primary antibody, table 2.11, and subsequently for 2 hours at room temperature with the species-appropriate secondary antibody, table 2.12, both in PBS + 0.1% Triton X-100 + 1% serum. After each step, sections were rinsed for 2 x 5 min in PBS + 0.05% Tween20, followed by 1 x 5 min in PBS alone. All sections were counter-stained with Hoechst 33342 (Invitrogen) 1:5000 and mounted with an antifade reagent (Prolong Gold; Invitrogen).

Immunohistochemistry in some cases was performed using two primary and two corresponding secondary antibodies. If the primary antibodies were raised in different species (e.g. rabbit and mouse) they were applied simultaneously as per the protocol above, with secondary antibodies also being applied at the same time. Staining for cfos and human melanopsin involved labelling with two primary antibodies raised in rabbit. In this case staining with the cfos antibody was carried out as above, Subsequently two further blocking steps were applied to saturate any unbound sites on the secondary antibody (rabbit anti-goat blocking antibody 1:200 for 2 hours at room temperature then Donkey anti-rabbit monovalent fragments 1:100 overnight at 4°C). Sections were then incubated with human melanopsin antibody and the appropriate secondary antibody as per the standard protocol.

2.8.3 Microscopy

2.8.3.1 Confocal microscopy

Fixed cells or retinal sections were viewed on a confocal microscope (LSM710; Zeiss, Jena, Germany). Fluorescent cells were located using epifluorescence illumination before taking a series of overlapping XY optical sections, of approximately 0.5µm thickness. The fluorescence of Hoechst, GFP and Alexa-555, 568, 633 or 647 were sequentially excited using the appropriate wavelength laser. A stack was built to give an XY projection image and image processing was performed using Volocity (Perkin Elmer, Cambridge, UK) and Image J (Version 1.43, National Institute of Health, <http://rsb.info.nih.gov/ij>).

Staining	Antigen	Host	Source (product code)	Dilution
Cone arrestin	Synthetic linear peptide	Rabbit	Millipore (AB15282)	1:1000
UVS opsin	N terminus of human OPN1SW	Goat	Santa Cruz (Sc-14363)	1:1000
Calbindin	28kDa calbindin-D protein purified from rat kidney	Rabbit	Abcam (ab11426)	1:1000
GFAP	GFAP isolated from cow spinal cord	Rabbit	Abcam (ab7779)	1:1000
PKC α	C-terminus of human PKC α	Rabbit	Epitomics (1510-1)	1:1000
PKC α	C-terminus of PKC α	Mouse	Abcam (ab11723)	1:500
Chx10	C-terminus of human Chx10	Sheep	Abcam (ab16141)	1:100
Brn-3a	N-terminus of human Brn-3a	Goat	Santa Cruz (sc-31984)	1:250
Human Opn4	N-terminus of human Opn4	Rabbit	Gift from I. Provencio, University of Virginia (UF028)	1:5000
Human Opn4	C-terminus of human Opn4	Rabbit	Santa cruz (sc-32870)	1:250
cfos	Total human cfos protein	Rabbit	Cell signaling technology (2250S)	1:200

GFAP: glial fibrillary acidic protein, PKC α : Protein Kinase C alpha, Opn4: melanopsin.
 Milipore, Billerica, MA. Abcam, Cambridge, UK. Epitomics, Burlingame, CA. Santa Cruz
 Biotechnology, Santa Cruz, CA

Table 2.11 Primary antibodies used for immunohistochemistry

Reactivity	Host	Fluorescent label	Source (product code)	Dilution
Rabbit	Donkey	Alexa Fluor® 488	Invitrogen	1:250
Rabbit	Donkey	Alexa Fluor® 555	Invitrogen	1:250
Goat	Donkey	Alexa Fluor® 555	Invitrogen	1:250
Sheep	Donkey	Alexa Fluor® 555	Invitrogen	1:250
Rabbit	Donkey	Alexa Fluor® 633	Invitrogen	1:250
Mouse	Donkey	Alexa Fluor® 647	Invitrogen	1:250

Table 2.12 Details of secondary antibodies used

2.8.3.2 Light microscopy

For the purpose of quantitative analysis of fluorescence, images were taken using the Leica DM IL inverted epifluorescence microscope. Images were obtained at x20 magnification using identical acquisition settings including exposure time, and were saved at a resolution of 1200x1600 pixels.

2.8.4 Image analysis

2.8.4.1 Confocal scanning laser ophthalmoscopy (cSLO) images

Image analysis was performed on 8-bit images using ImageJ. Grey levels on cSLO images were measured within a circle of 300 pixel diameter. This area was placed within the region of vector delivery, avoiding the needle entry site where associated retinal damage often led to localized increased transduction.

2.8.4.2 Fluorescence images of transduced cells

Images of fixed cells were analysed by quantifying the total cell number per field (counting Hoechst labelled nuclei). For fluorescence images, mean grey value was calculated for each image using ImageJ software, and background levels of fluorescence subtracted by determining mean grey level of images of untransfected cells.

2.8.4.3 Images of retinae light pulsed for cfos expression

Following immunohistochemistry, for analysis five equally spaced images of two retinal sections per eye were taken at x20 magnification using identical acquisition settings on a confocal microscope (LSM710; Zeiss, Jena, Germany). Using Image J software, 8 bit images were analysed to perform cell counts and quantification of intensity of fluorescence (Protocol: subtract background 50 pixel rolling ball radius; threshold 0-20 and analyse particles).

2.9 Statistical analysis

Data for statistical analysis are presented as mean $\pm$ standard error of the mean (sem). The significance level was set as 0.05 in all cases. The Shapiro-Wilk test was used to determine normal distribution. If two groups of data were normally distributed, a paired or unpaired t-test was used for comparison as appropriate. Otherwise a Mann-Witney test was performed. For more than two groups where the data was normally distributed, one or two way analysis of variance (ANOVA) was done depending on the number of factors to be analysed. Post hoc testing was done as specified in the data. For non-normally distributed data a Kruskal-Wallis test was carried out. All statistical analysis was done using Prism 6 for Mac OS X (San Diego, CA, USA).

Chapter 3 Transduction of the degenerate retina using Adeno-associated viral vectors

3.1 Introduction

In order to efficiently deliver melanopsin to the degenerate retina as an optogenetic tool, the vector for transducing residual retinal cells must be optimised. The use of pseudotyped vectors is well established in gene therapy, in which a transgene cassette flanked by AAV2 inverted terminal repeats (ITR) is packaged into the capsid from another AAV serotype, see section 1.5.3. With regards to the retina, previous studies have shown that AAV capsid type affects transduction properties such as the level of gene expression and cell types targeted (120). Therefore transduction may be enhanced further by modification of the viral capsid.

3.1.1 Hybrid capsid AAV vectors

One strategy for optimisation is to make the capsid protein a hybrid of several different serotypes, possibly combining beneficial effects of each for targeting a specific class of retinal cell. In addition, antigenic capsid sequences that might induce an immune response could be shuffled or removed which could be of use in systemic gene therapy where high vector doses are required e.g. for the treatment of haemophilia, or treating the second eye in ocular gene therapy. Since the discovery of AAV8 through PCR-based screening of human and primate tissues, several further AAV serotypes have been identified (177). Analysis of the infectivity of 15 new serotypes in the mouse brain identified three serotypes (cy5 (cynomolgus macaque-variant 5), rh20 (rhesus macaque-variant 20) and rh39) that had similar tropism to, but greater infectivity than, rAAV2/8 (178).

Hybrid recombinant capsids tested in this chapter were generated by a collaborating group (Professor M During, Ohio State University). Fragments of capsid sequences that matched in cy5, rh20 and rh39 vectors and AAV8 were shuffled around using known restriction sites to generate several novel recombinants termed rec1, rec2, rec3 etc, figure 3.1. Particular attention was made to the N-terminus of the VP1 protein, which is known to affect AAV infectivity. One of the fragments chosen was derived by HindIII-StuI restriction enzyme digest, which codes for the first 80 amino acids of VP1. The fragment derived from cy5 was incorporated into Rec1, 3 and 4, whereas the restriction fragment from rh20 or rh39 (which are identical) created a different VP1 for Rec2, 5 and 6. The C terminus of the VP3 protein contains a region known as the HI loop which is a critical sequence for genome packaging and capsid assembly (179). All of the new hybrid vectors generated contained the C terminus of the VP3 region from rAAV2/8, preserving this critical region in the new vectors.

In a previous study, the rAAV2/Rec vectors were assayed comparing transduction efficiency to rAAV2/8. With intravenous infusion, rAAV2/Rec2 showed the greatest transduction of the target tissue (cardiac muscle), and with direct intracerebral administration, rAAV2/Rec3 led to the greatest neuronal cell transduction (unpublished data, Matthew J. During). These vectors (referred to as rAAV2/Rec2 and rAAV2/Rec3) were therefore selected for assessment of transduction efficiency and cell tropism in the degenerate retina.

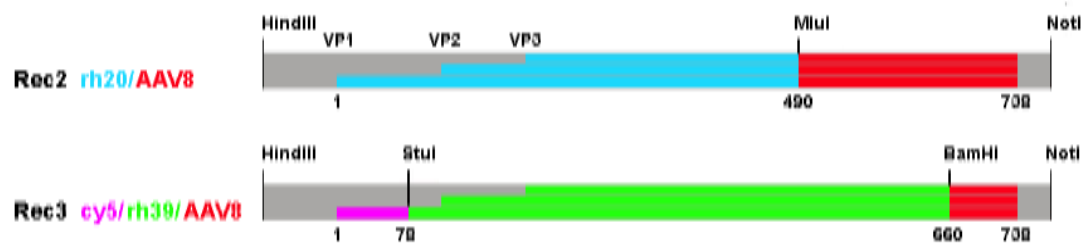


Figure 3.1 Generation of rAAV2/Rec2 and rAAV2/Rec3 vectors

The rAAV2/Rec2 vector is designed so that its chimeric capsid proteins contain amino acids 1 to 490 from rh20 and 491 to 738 from the AAV8 serotype. In the rAAV2/Rec3 vector, amino acids 1 to 78 are of cy5 origin, 79 to 660 are from rh39 serotype and 661 to 738 from AAV8.

3.1.2 Capsid mutant vectors

For optogenetic strategies to restore vision, one target is the bipolar cell however tropism of AAV for these cells is poor. Mutations of exposed tyrosine residues on the surface of VP3 increase transgene expression (130), see section 1.5.4. One previous study obtained adequate transduction efficiency in the degenerate retina with a self-complementary rAAV8 vector with a single Y733F mutation (111), but these vectors have limited packaging capacity and such a vector could not be used if the transgene of interest was large. Further studies have examined multiple (two to seven) capsid mutations of single stranded (ss) rAAV2/2 demonstrating that vectors with four or five tyrosine residue mutations are able to transduce bipolar cells in the wild type retina (132), and a vector with six tyrosine mutations transduced bipolar cells in the degenerate retina (180). Subsequent work has targeted bipolar cells using a synthetic AAV capsid (160) or one developed using directed evolution (159). It is known that AAV capsid mutation can alter antibody binding (181) and thus the increased efficiency of these AAV vectors with multiple capsid mutations must be balanced with the risk of an altered immune response. Thus it would be useful to investigate the retinal transduction efficiency and cell tropism of single stranded AAV with a single capsid mutation in the degenerate retina where high transduction efficiency is required to transduce bipolar cells.

Therefore, the overall aim of this chapter was to investigate the optimal AAV capsid for delivery of melanopsin to the degenerate retina examining both hybrid capsid and capsid mutant AAV vectors.

3.2 Experimental design

Hybrid capsid AAVs (rAAV2/Rec2 and rAAV2/Rec3) were compared to rAAV2/2 and rAAV2/5 both of which are well characterised vectors for retinal gene therapy (120, 125). The effect on transduction of a single tyrosine mutation in single stranded rAAV2/2 Y444F and rAAV2/8 Y733F was also assessed. All vectors expressed green fluorescent protein (GFP). Detailed experimental methods are described in chapter 2.

3.2.1. *In vivo* testing

In order to examine transduction efficiency in the degenerate retina, AAV vectors were injected into the subretinal space of six to eight week old *rd1* mice. This mouse model displays an early onset photoreceptor degeneration resulting in rapid loss of photoreceptors as detailed in chapter 1.4.1. A dose of 1×10^9 viral genome particles was administered to each eye, see section 2.7.3, and all vectors expressed green fluorescent protein (GFP) under control of the CMV-Actin-globin (CAG) promoter. After three weeks, GFP fluorescence of the retina was recorded using *in vivo* confocal scanning laser ophthalmoscopy (cSLO) imaging, see section 2.7.4. This method allows high resolution imaging of the retina *in vivo* and is used clinically to assess levels of fundus autofluorescence. The exciting laser light source is of 488nm wavelength and therefore is suitable for visualising GFP *in vivo*. Subsequently mice were sacrificed, retinas were examined for expression of GFP and cell types were identified using immunohistochemistry, see section 2.8.

3.2.2 *In vitro* testing of hybrid capsid AAV

Since vector efficiency and tropism can differ between species (112), hybrid recombinant AAV vectors expressing GFP were tested on the human neuronal cell line

SH-SY5Y *in vitro*. Vectors were compared to rAAV2/2 and rAAV2/5 under conditions that induce neuron-specific differentiation and stabilise cell proliferation (182). This allowed prolonged culture of cells, which might reveal hybrid capsid AAV transduction if kinetics were slower than those of the other vectors. Nine days after transfection, cells were fixed and counter stained with Hoechst to identify cell nuclei. Images of GFP fluorescence from transfected cells were taken as measure for transduction efficiency with a fixed exposure time and quantified using Image J.

3.2.3 *In vitro* testing and investigating the mechanism of action of capsid mutant AAV

A possible explanation for previous reports describing increased transduction with capsid mutant AAV is altered proteasome-mediated degradation. Therefore transduction of SH-SY5Y cells using rAAV2/2 was compared to rAAV2/2 Y444F and rAAV2/8 Y733F in the presence of three proteasome inhibitors: Doxorubicin, MG-132 and Bortezomib. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay was initially performed to establish the maximum dose of each proteasome inhibitor that was non-toxic to SH-SY5Y cells. Subsequently cells were transduced with AAV, fixed five days after transduction, and images of GFP taken as above.

3.2.4. Transduction of human retinal explants using capsid mutant AAV

In order to assess whether the capsid mutant rAAV types tested in mice would be able to successfully transduce human retina, samples of human retina were obtained from patients undergoing complex surgery for retinal detachment in which a retinectomy procedure was performed as part of the operation. The retinal fragments obtained were maintained in an *ex-vivo* culture system, as described previously (26, 27). Vectors were

administered to explants one day after removal from the eye, and after 14 days the tissue was fixed and histological sections analysed. Since successful human retinal transduction using rAAV2/2 has been demonstrated using this culture system (28), this was used for comparison.

3.3 Results: Hybrid recombinant AAV vectors

3.3.1 *In vivo* Measurement of Fluorescence Intensity

Three weeks after subretinal injection of hybrid recombinant AAV vectors (rAAV2/Rec2 and rAAV2/Rec3) along with rAAV2/2 and rAAV2/5 for comparison, GFP fluorescence was assessed *in vivo* using confocal scanning laser ophthalmoscopy imaging. All vectors tested were capable of driving GFP expression, which did not extend noticeably outside the area of the bleb into which the vector was injected, Figure 3.2a. However, clear differences in GFP fluorescence intensity were observed between rAAV serotypes, ($p=0.002$ ANOVA with Tukey post hoc test), figure 3.2b. GFP fluorescence intensity was four fold higher with the use of rAAV2/2 than with rAAV2/Rec2 and rAAV2/Rec3 ($p<0.01$ for both comparisons). Following transduction by rAAV2/5, fluorescence levels were twice as high compared to rAAV2/Rec2 and rAAV2/Rec3, however neither of these comparisons reached statistical significance on post hoc testing. GFP fluorescence intensities after transduction with rAAV2/2 and rAAV2/5 were similar, as was the case when comparing rAAV2/ Rec2 and rAAV2/Rec3.

3.3.2 Identification of cell types in the degenerate retina

Histology was analysed to assess retinal cell tropism of different rAAV serotypes. Since the degenerate retina is known to undergo remodelling, cell specific markers that could still be effectively used to identify retinal cell types by immunocytochemistry in the *rd1* mouse retina were established. As previously characterised, a population of residual cones persists in the peripheral retina of the *rd1* mouse, which could be identified using cone arrestin, figure 3.3a. Horizontal cells and cholinergic amacrine cells were effectively stained with calbindin, figure 3.3b, rod bipolar cells with protein kinase C

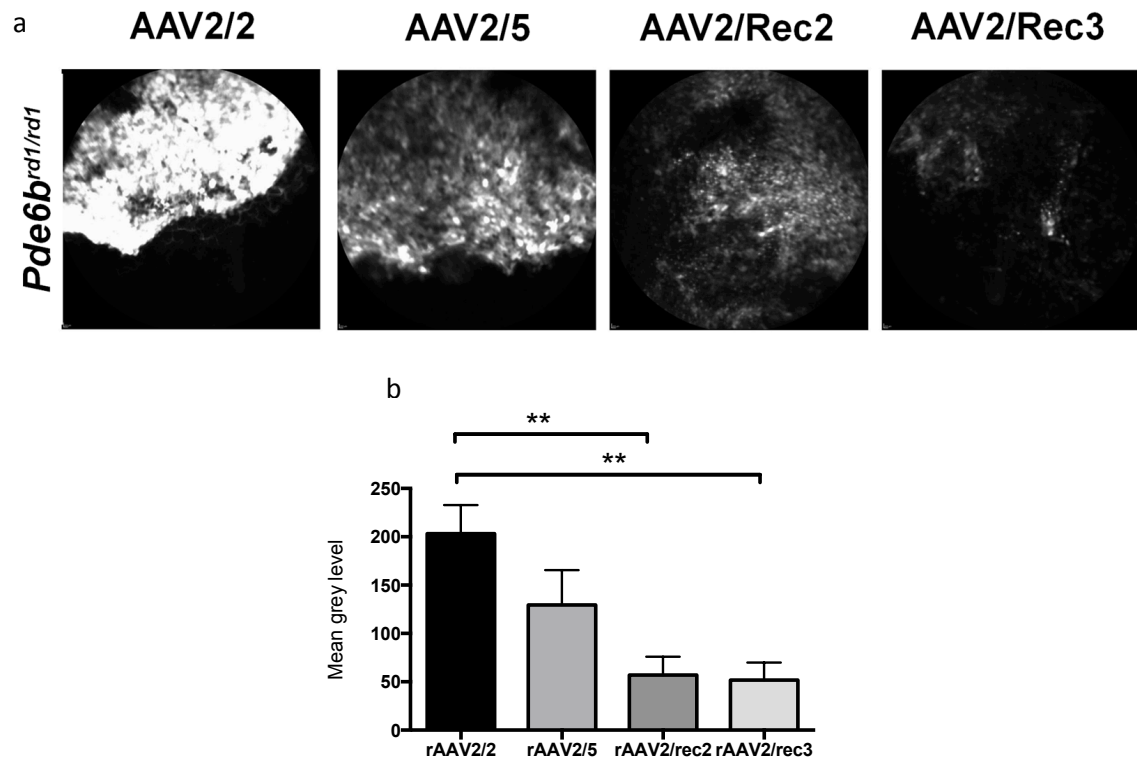


Figure 3.2 *In vivo* measurement of fluorescence intensity 3 weeks after subretinal injection of different hybrid recombinant AAV serotypes expressing green fluorescent protein.

(a) Representative topographical confocal scanning laser ophthalmoscopy (cSLO) 488 nm fluorescence images showing the range of fluorescence intensities that was found in the different groups. (b) Quantification of images using mean grey value revealed significant differences between groups. n=4-8 eyes per group. ** p<0.01, ANOVA with Tukey post hoc test.

(PKC) alpha, figure 3.3c, and all bipolar subgroups with the transcription factor Chx10, figure 3.3d. Müller cells were stained with an antibody for glial fibrillary acidic protein (GFAP), figure 3.3e, and ganglion cells with the transcription factor Brn-3a, figure 3.3f.

3.3.3 Tropism of rAAV2/Rec2 and rAAV2/Rec3 in degenerate retina

In vivo fluorescence intensity measurements using topographic cSLO images reflect the combined fluorescence intensity at each location arising from all retinal layers. In order to assess transduction efficiency and cell tropism within retinal layers, fluorescence was examined on fixed histological section of eyes with subretinal vector delivery, figure 3.4. In the degenerate retina all serotypes transduced RPE, horizontal cells, Müller cells and ganglion cells. GFP fluorescence was less overall when using rAAV2/ Rec3 than other serotypes, especially so for RPE and Muller cells. No bipolar cell transduction was seen with any serotype.

3.3.4 Assessment of transduction in SH-SY5Y Cells in vitro

In order to assess transduction efficiency in human cells, vectors were tested on SH-SY5Y cells in culture. GFP fluorescence intensity was used as a measure of transduction efficiency and was significantly different between rAAV serotypes ($p=0.0006$, one way ANOVA), figure 3.5a. Grey value analysis of images showed a four fold reduction in fluorescence with rAAV2/Rec2 compared to rAAV2/2 ($p<0.05$, Bonferroni post hoc test) and rAAV2/5 ($p<0.05$), figure 3.5b. rAAV2/Rec3 showed an eight fold reduction in fluorescence compared to rAAV2/2 ($p<0.01$) and rAAV2/5 ($p<0.01$). There was no significant difference between rAAV2/2 and rAAV2/5 or between rAAV2/Rec2 and rAAV2/Rec3. Nuclear count was without significant difference between groups, ensuring similar confluence of the cell layers, figure 3.5c.

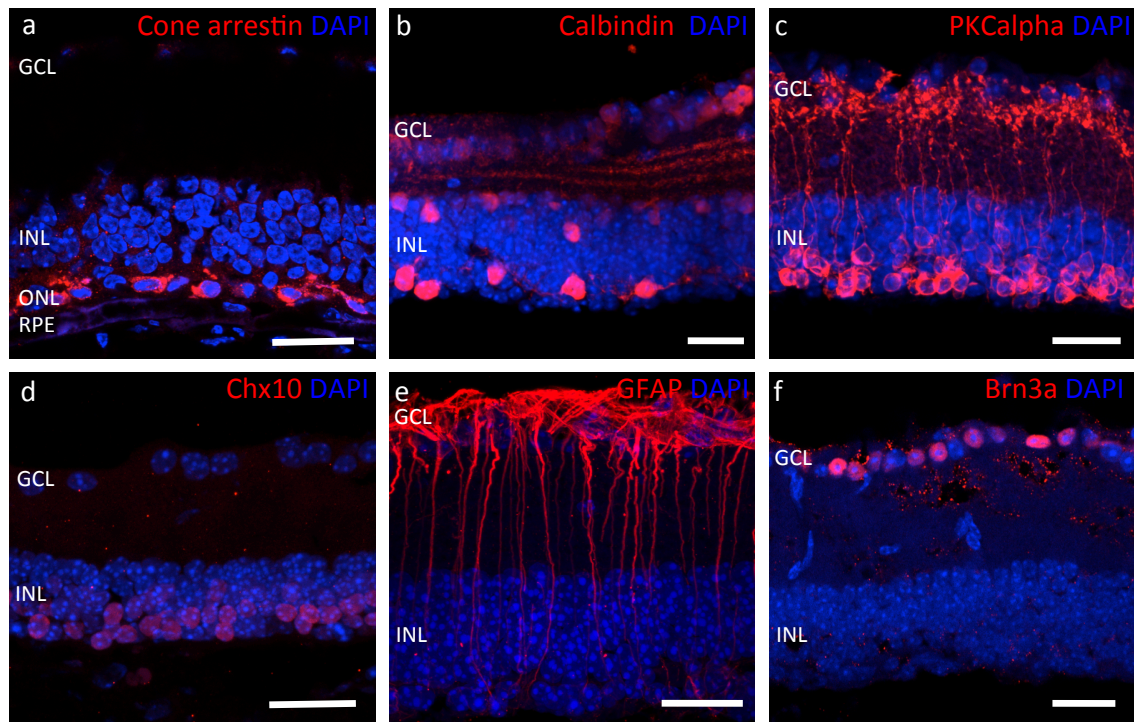


Figure 3.3 Immunocytochemistry in the degenerate retina.

Immunolabelling (red) with (a) cone arrestin for cones, (b) calbindin for horizontal cells and cholinergic amacrine cells, (c) Protein Kinase C alpha for rod bipolar cells, (d) CHx10 for bipolar cells, (e) GFAP for Muller cells and (f) Brn-3a for ganglion cells were all effective in the retina of a 9 week old *rd1* mouse. Nuclei were labeled with DAPI (blue). GCL=ganglion cell layer, INL=inner nuclear layer, RPE=retinal pigment epithelium. Scale bar 25µm.

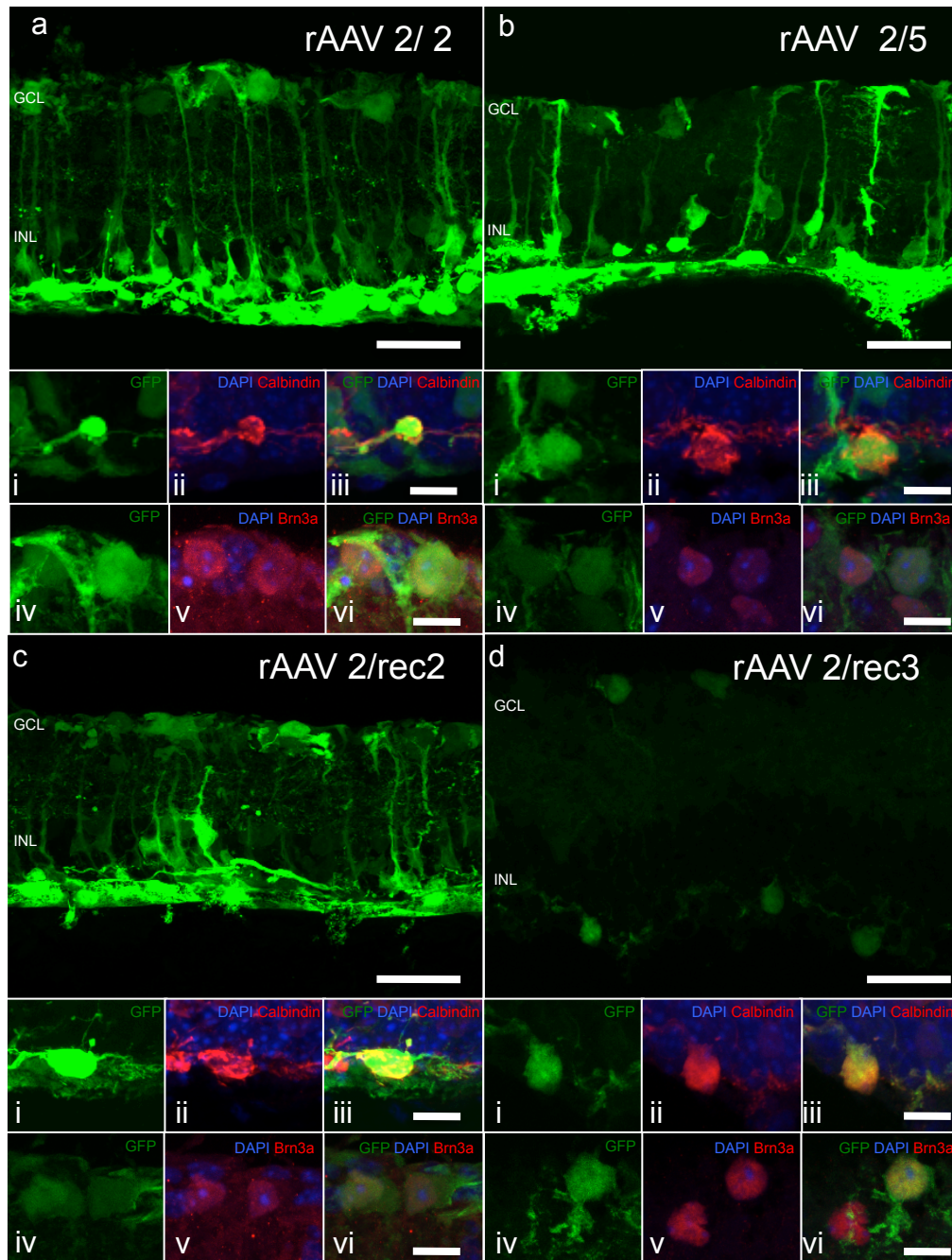


Figure 3.4 Green fluorescent protein (GFP) fluorescence patterns of different recombinant AAV serotypes in the degenerate retina of *rd1* mice following subretinal injection.

The main images a-d are confocal stacks illustrating overall GFP expression patterns. For each serotype, images i-iii are confocal slices showing GFP expression (green), nuclear labeling (blue) and immunostaining with calbindin identifying horizontal cells (red). Colocalisation of signals indicates horizontal cell transduction. Images iv-vi show colocalisation of GFP (green) in ganglion cells identified by Brn-3a immunostaining (red) demonstrating ganglion cell transduction. GCL = ganglion cell layer, INL = inner nuclear layer, RPE = retinal pigment epithelium. Scale bar: 30 μ m for images a-d, 10 μ m for images i-vi.

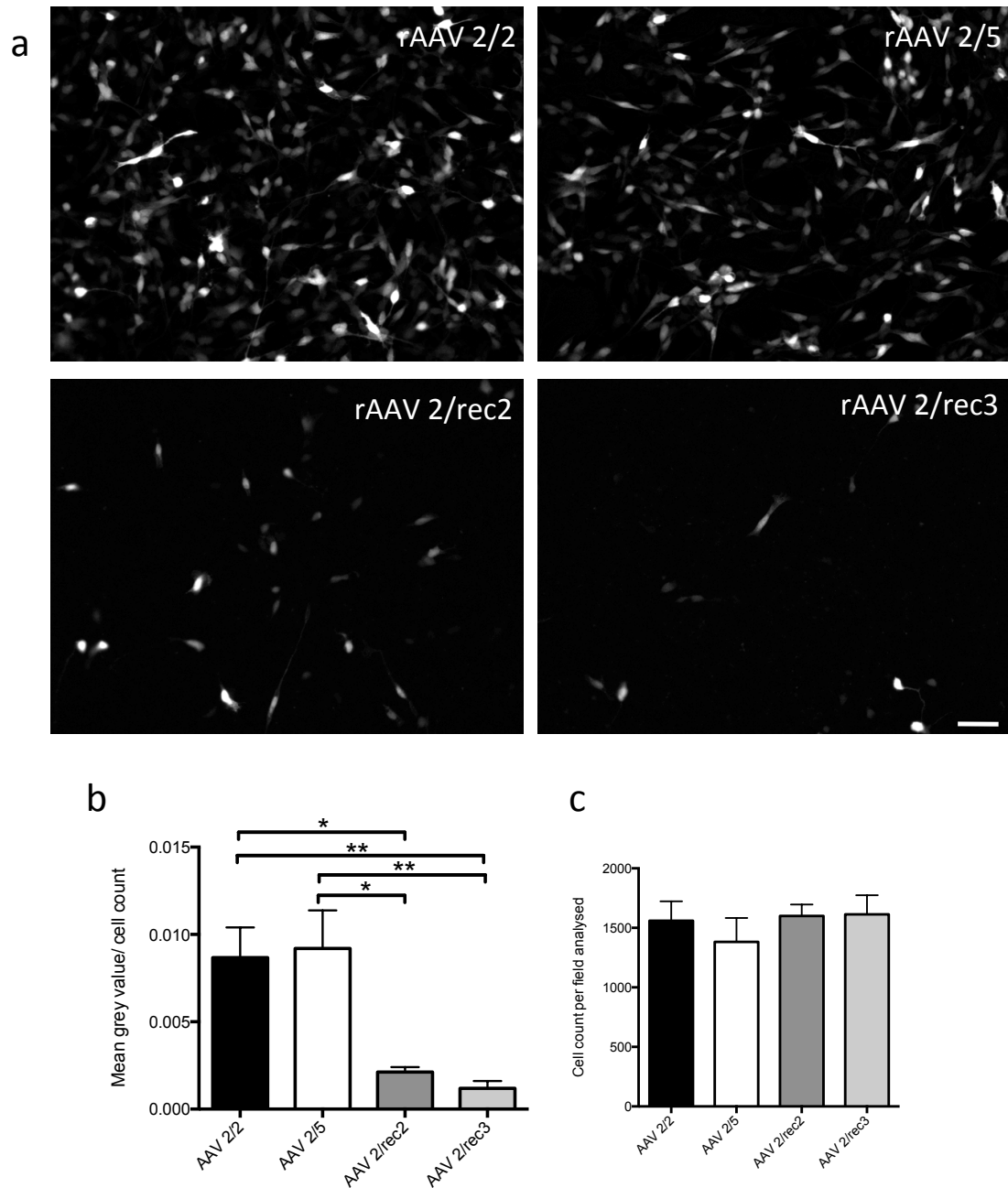


Figure 3.5 Analysis of Green fluorescent protein (GFP) fluorescence following transduction of SH-SY5Y cells *in vitro* with different recombinant AAV serotypes.

Representative images are shown of GFP fluorescence following transduction with rAAV2/2, rAAV2/5, rAAV2/Rec2 and rAAV2/Rec3 (a). Analysis of mean grey value of images (b) demonstrates significant differences between standard rAAV2/2 and rAAV2/5 serotypes and hybrid recombinant vectors (One Way ANOVA, Bonferroni post hoc test * $p < 0.05$, ** $p < 0.01$). In order to ensure levels of cell confluence did not differ between groups and affect transduction, Hoechst-labeled nuclei were counted in each field analysed, with no significant difference between groups (c). Scale bar 50 μm . $n = 6$ (2 wells x 3 biological replicates).

3.4 Results: Capsid mutant AAV vectors

3.4.1 *In vivo* measurement of fluorescence intensity

Using a similar experimental design as the hybrid AAV experiments, rAAV2/2 Y444F and rAAV2/8 Y733F vectors were injected into the subretinal space of six to eight week old *rd1* mice. All vectors expressed green fluorescent protein (GFP) under control of a CAG promoter. Three weeks after subretinal injection, GFP fluorescence was recorded using *in vivo* cSLO imaging and all vectors tested were capable of driving a high level of GFP expression, figure 3.6a. There was no significant difference in fluorescence between the AAV serotypes, figure 3.6b.

3.4.2 Tropism of capsid mutant vectors in the *rd1* mouse

Histological sections of the retina were examined to evaluate transduction and retinal cell tropism by these vectors, with rAAV2/2 used for comparison. All vectors tested were able to transduce RPE, horizontal cells and ganglion cells, figure 3.7 a-c. Müller cell transduction appeared to be reduced with the use of rAAV2/2 Y444F compared to rAAV2/2. Of note, both rAAV2/2 Y444F and rAAV2/8 Y733F capsid mutant vectors transduced bipolar cells effectively as shown by co-labelling for PKC alpha, figure 3.8 a,b. No bipolar cell transduction was seen with rAAV2/2.

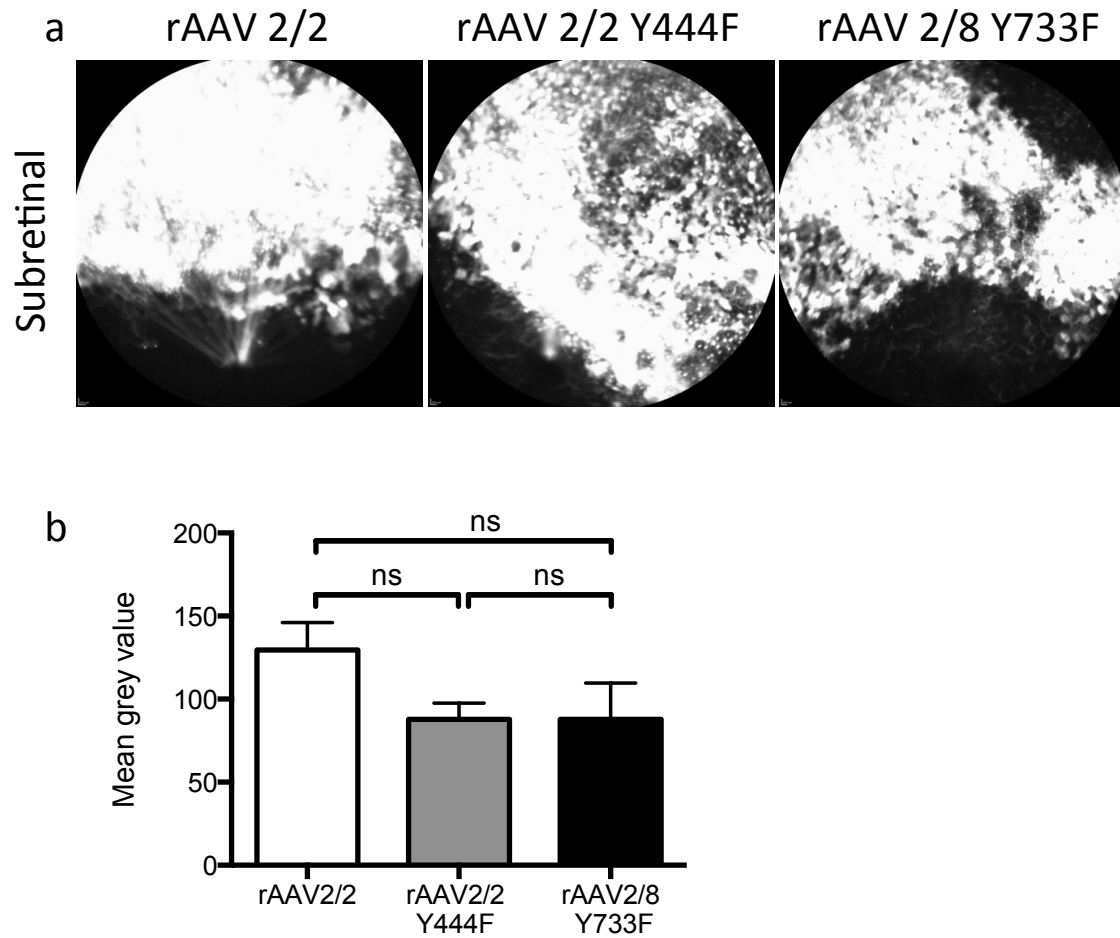


Figure 3.6 *In vivo* assessment of transduction using confocal scanning laser ophthalmoscopy (cSLO)

(a) Representative topographical 488nm-fluorescence images show the range of fluorescence intensities that were found 3 weeks after injection of different recombinant AAV serotypes in *rd1* mice. (b) Analysis of mean grey value of cSLO images (mean±SEM). ns= non significant, ANOVA with Bonferroni post-hoc test; n=6 eyes per group.

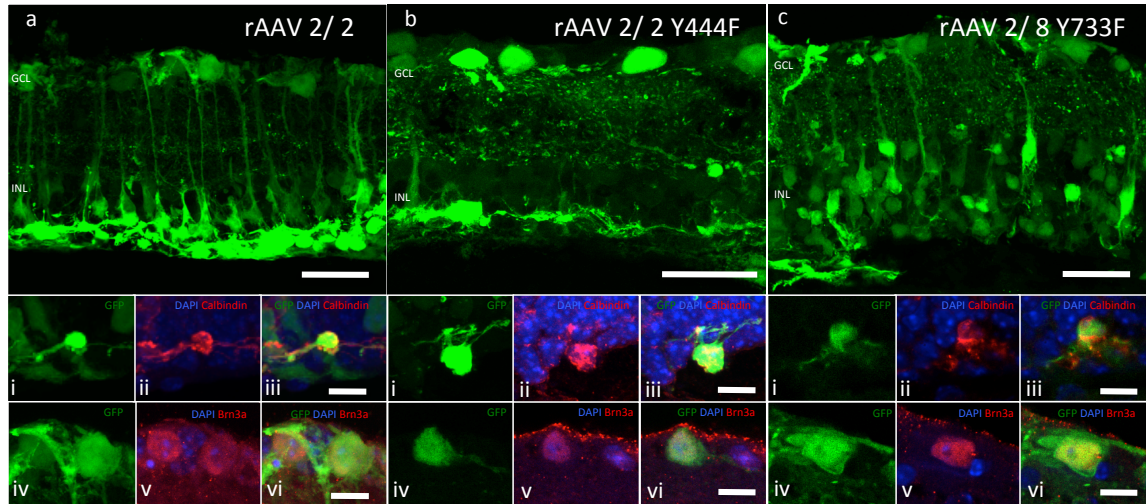


Figure 3.7 GFP fluorescence patterns of rAAV2/2 compared to capsid mutant serotypes following subretinal injection in *rd1* mice.

The main images a-c are confocal stacks illustrating overall GFP expression patterns. For each serotype, images i-iii are confocal slices showing GFP expression (green), nuclear labeling (blue) and immunostaining with calbindin identifying horizontal cells (red). Colocalisation of signals indicates horizontal cell transduction. Images iv-vi show colocalisation of GFP (green) in ganglion cells identified by Brn-3a immunostaining (red) demonstrating ganglion cell transduction. GCL= ganglion cell layer, INL= inner nuclear layer. Scale bar: 30 μ m for images a-d, 10 μ m for images i-vi.

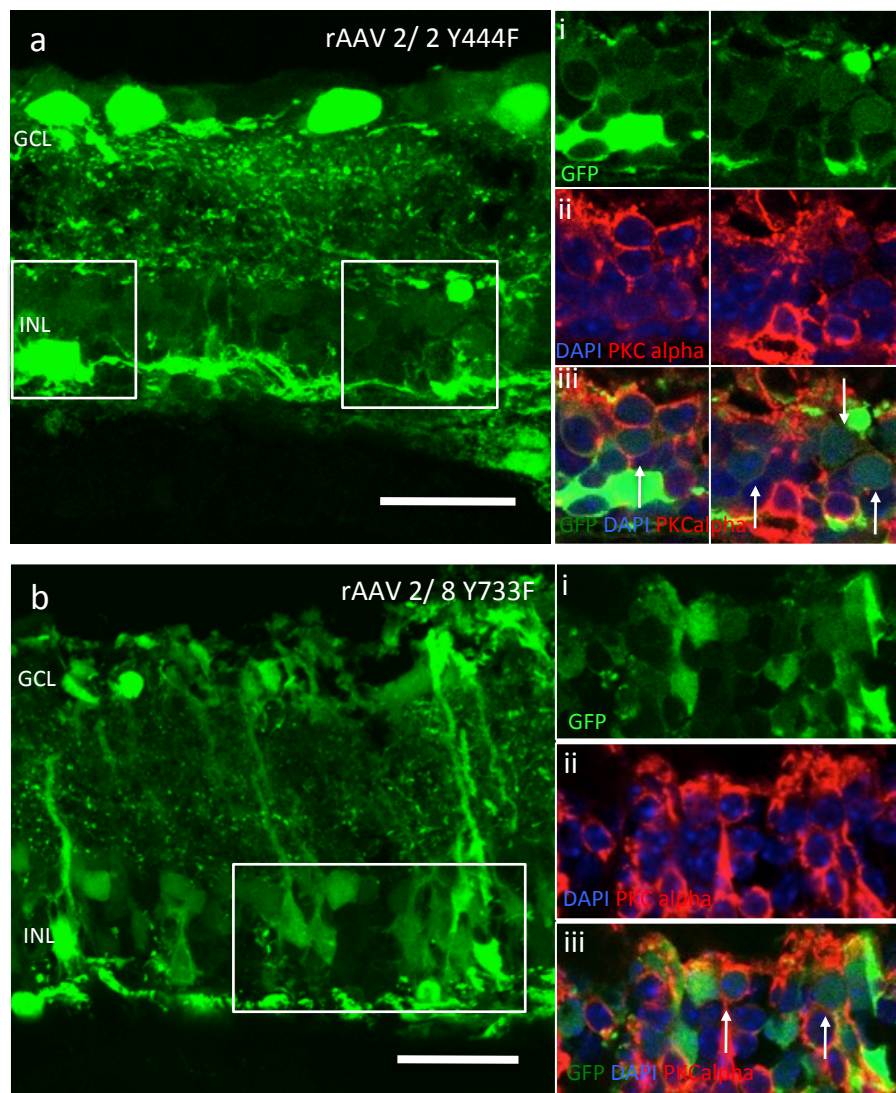


Figure 3.8 Bipolar cell transduction following subretinal injection of capsid mutant AAV in *rd1* mice.

The main images a and b are confocal stacks illustrating overall GFP expression patterns. For each serotype, images i-iii are confocal slices showing GFP expression (green), nuclear labeling (blue) and immunostaining with PKC alpha identifying ON bipolar cells (red). Colocalisation of signals indicates bipolar cell transduction. GCL= ganglion cell layer, INL= inner nuclear layer. Scale bar: 30 μ m.

3.4.3 Tropism of capsid mutant vectors in *ex-vivo* human retinal culture

Retinal explants that had been removed from patients having retinectomy for retinal detachment surgery were cultured *ex-vivo* and transduced with all rAAV types tested, figure 3.9a. This tissue potentially could have had intact outer nuclear, inner nuclear and ganglion cell layers. However, these patients all had retinal detachments for some time before surgery, and thus there may have been varying amounts of retinal degeneration.

GFP expression and therefore successful transduction was observed using all vectors in the human retinal tissue, figure 3.9 b-d. Using rAAV2/2 and rAAV2/2 Y444F, GFP expression was predominantly seen in the ONL consistent with photoreceptor transduction, figure 3.9b,c. Sparse inner retinal transduction was also seen with rAAV2/2 Y444F. Using rAAV2/8 Y733F, the transduction pattern appeared to be different, with less GFP expression in the ONL compared to the other two vectors, but markedly more expression in the INL, figure 3.9d. On the basis of cell morphology, Müller cell transduction was apparent with multiple branching cells that spanned the entire retina. Using PKC alpha to stain ON bipolar cells, immunohistochemistry confirmed transduction of human bipolar cells using rAAV2/8 (Y733F), figure 3.9e.

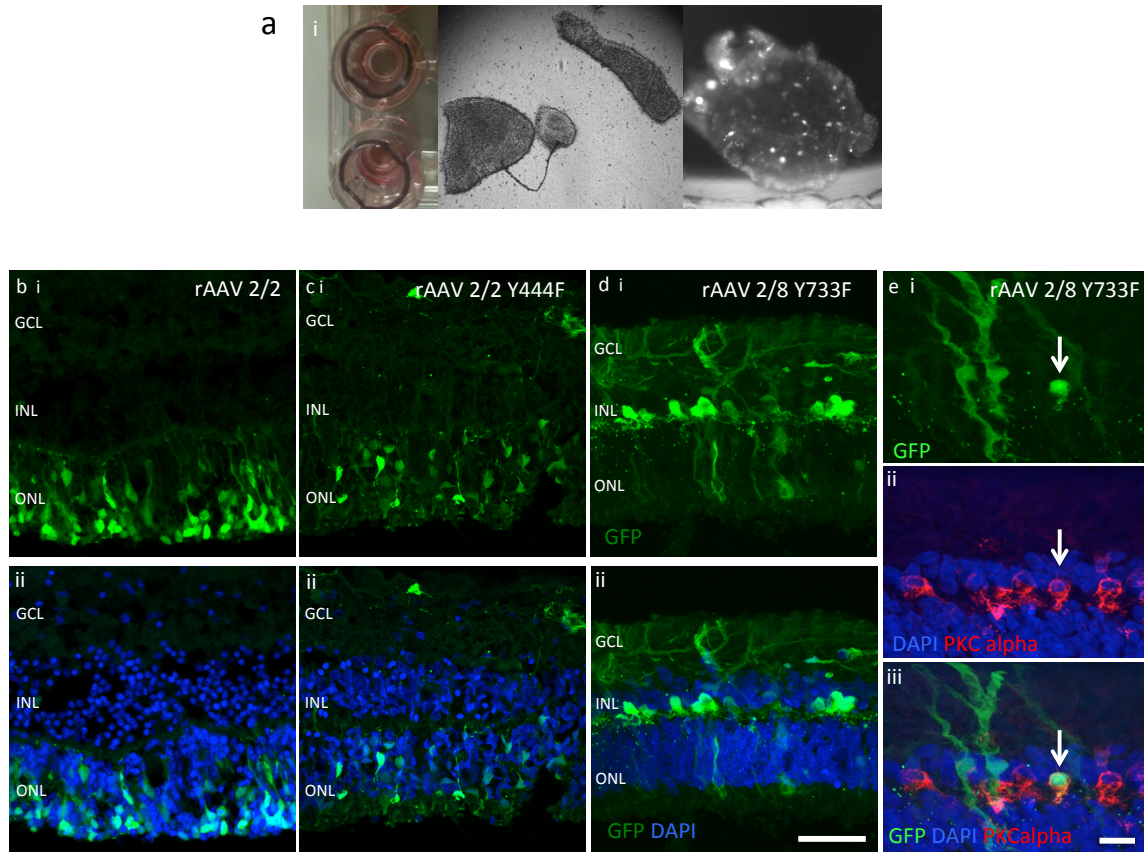


Figure 3.9 Transduction of human retinal explants ex-vivo by Adeno-associated virus (AAV) serotypes.

(a) Ex-vivo culture system for human retinal tissue (i) Tissue culture insert placed in a 24 well culture plate (ii) Retinectomy samples in the tissue culture insert. (iii) GFP expression in retinectomy sample 8 days post addition of rAAV2/2 (b) Images showing human retina transduced with (i) rAAV2/2 and (ii) rAAV2/2 Y444F both at 11 days post-transduction, and (iii) rAAV 2/8 Y733F at 14 days post transduction. (c) Bipolar cell transduction in human retina using rAAV 2/8 Y733F vector. Images showing (i) GFP (green) (ii) PKC alpha staining of bipolar cells (red) and (iii) composite of GFP, PKC alpha and DAPI (blue). Scale bar 50µm in images (b-d) i and ii, scale bar 25 µm in (c) images i-iii. GCL= ganglion cell layer, INL= inner nuclear layer, ONL= outer nuclear layer.

3.4.4 Investigating the mechanism of action of capsid mutant vectors

It has previously been proposed that increased transduction seen with capsid mutant vectors is due to decreased ubiquitin-tagged proteasome-mediated degradation (130). To investigate this further, the effect of proteasome inhibitors (PI) on rAAV2/2 transduction of SH-SY5Y cells was examined.

3.4.4.1 Establishing dose of proteasome inhibitors

Since the effect of PIs on AAV transduction has been shown to be specific to cell type (183) the optimal non-toxic dose of three different PIs: Doxorubicin, MG-132 and Bortezomib on SH-SY5Y cells was established using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay. High doses of all drugs reduced cell survival, figure 3.10a. The highest dose in which there was no significant difference to control in cell survival was 170nM Doxorubicin, 40nM MG-132 and 2.6nM Bortezomib, figure 3.10b. These doses were used for further experiments.

3.4.4.2 Transduction of SH-SY5Y cells *in-vitro* with rAAV2/2 and proteasome inhibitors versus capsid mutant rAAV2/2 Y444F

SH-SY5Y cells were transduced *in vitro* with rAAV2/2. This was compared to cells transduced with rAAV2/2 + 170nM Doxorubicin, rAAV2/2 + 40nM MG-132 and rAAV2/2 + 2.6nM Bortezomib, figure 3.11a. GFP fluorescence intensity was used as a measure of transduction efficiency and was significantly different between groups ($p < 0.01$, one way ANOVA), figure 3.11b. GFP fluorescence was up to 1.5 fold greater in the Doxorubicin treated group ($p < 0.01$) and in the MG-132 treated group ($p < 0.05$) compared to rAAV2/2 alone, indicating higher levels of transduction in the presence of these agents. There was no significant difference in the Bortezomib group.

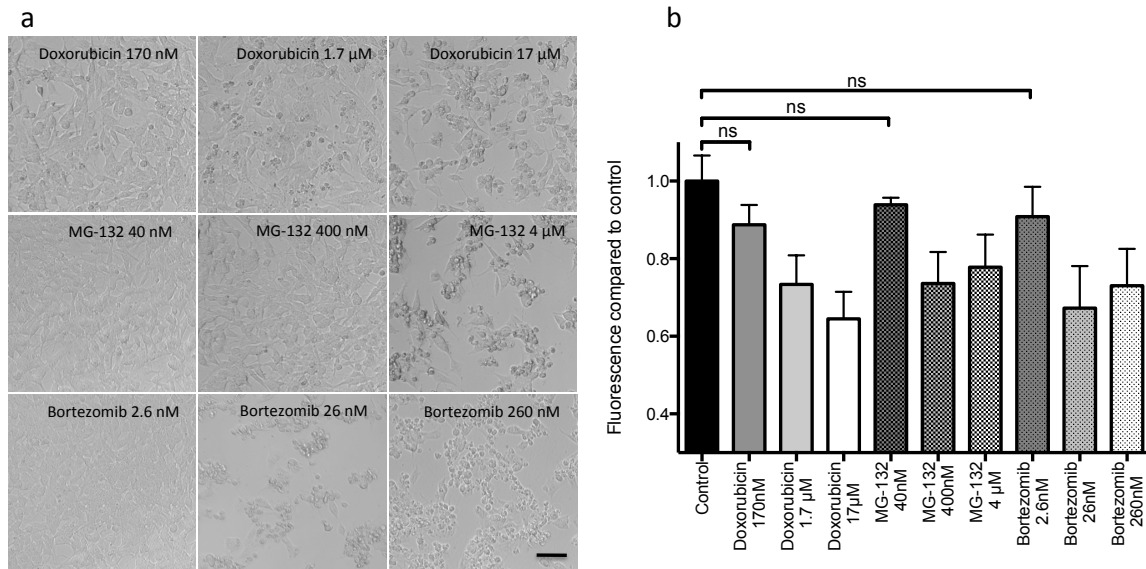


Figure 3.10 MTT assay to establish non-toxic concentrations of proteasome inhibitors (PI) when applied to SH-SY5Y cells in culture.

(a) Representative images showing the effect on cell morphology of different concentrations of 3 proteasome inhibitors: Doxorubicin, MG-132 and Bortezomib. Scale bar 50 μ m. (b) MTT assay showing cell survival compared to control following the application of different doses of PI. n=3 biological replicates. ns= non significant, ANOVA with Bonferroni post-hoc test.

Transduction of SH-SY5Y cells was approximately four times higher with use of capsid mutant rAAV2/2 Y444F compared to rAAV2/2, ($p < 0.001$), figure 3.11 c and d.

3.4.4.3 Transduction of SH-SY5Y cells *in-vitro* with rAAV2/2 Y444F with and without proteasome inhibitors

To assess whether PIs could further augment transduction with capsid mutant rAAV2/2 Y444F, transduction with this virus alone was compared to rAAV2/2 Y444F + 170nM Doxorubicin, rAAV2/2 Y444F + 40nM MG-132, and rAAV2/2 Y444F + 2.6nM Bortezomib, figure 3.12a. There was no significant difference in fluorescence levels between any of these groups ($p = 0.49$) figure 3.12b, indicating that PIs do not further increase transduction with capsid mutant vectors.

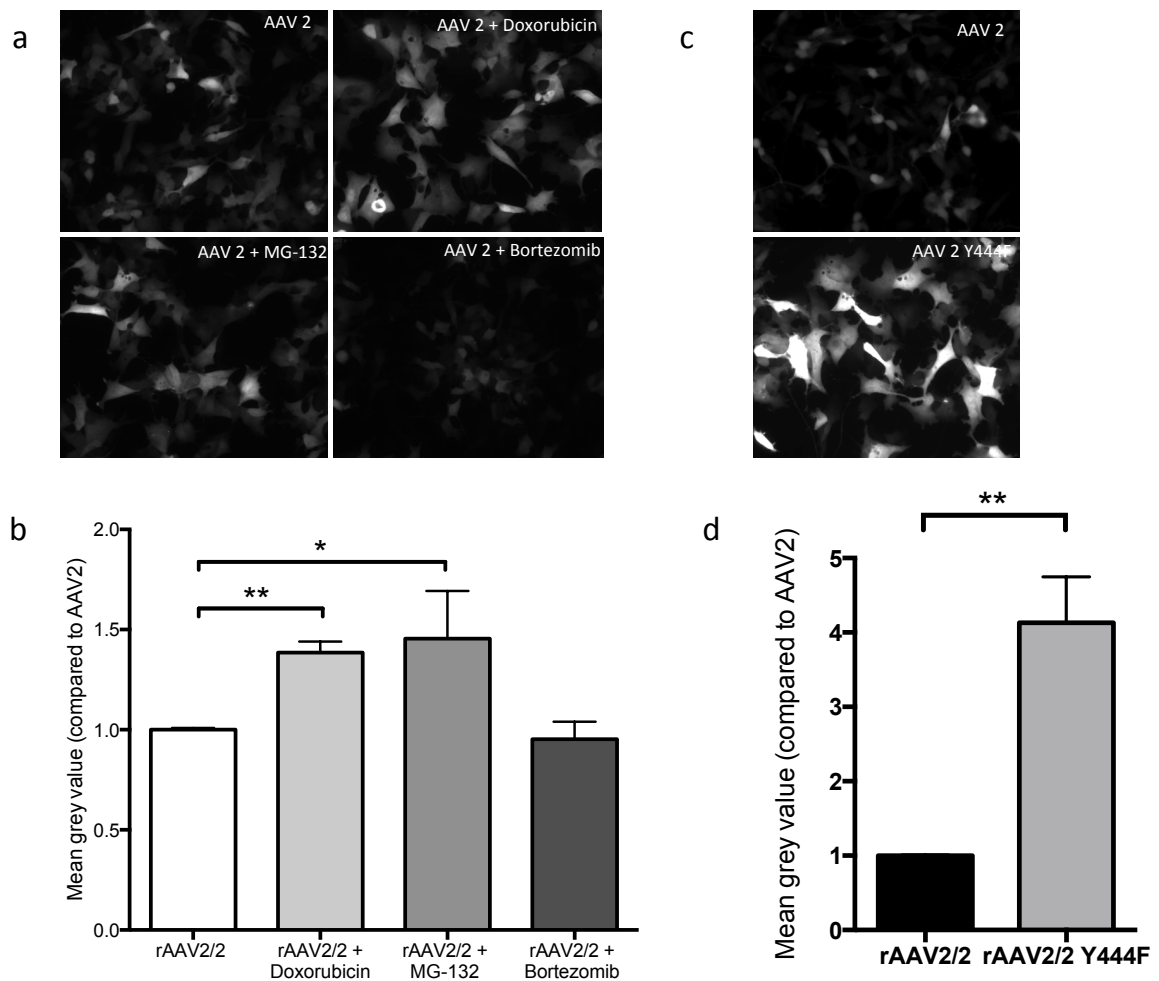


Figure 3.11 Comparing rAAV2/2 transduction of SH-SY5Y cells *in vitro* with proteasome inhibitor treated cells and rAAV2/2 with a single capsid mutation.

(a) Representative images showing GFP expression following transduction with rAAV2/2, and rAAV2/2 with the proteasome inhibitors Doxorubicin, MG-132 and Bortezomib. (c) Representative images showing GFP expression following transduction by rAAV2/2 and rAAV2/2 Y444F (b and d) Grey value analysis at 120 hours (mean $\pm$ SEM). ** $p < 0.01$ * $p < 0.05$, $n = 6$ (2 wells $\times$ 3 biological replicates), Kruskal-Wallis test with Dunn's multiple comparison post hoc test or Mann-Witney test.

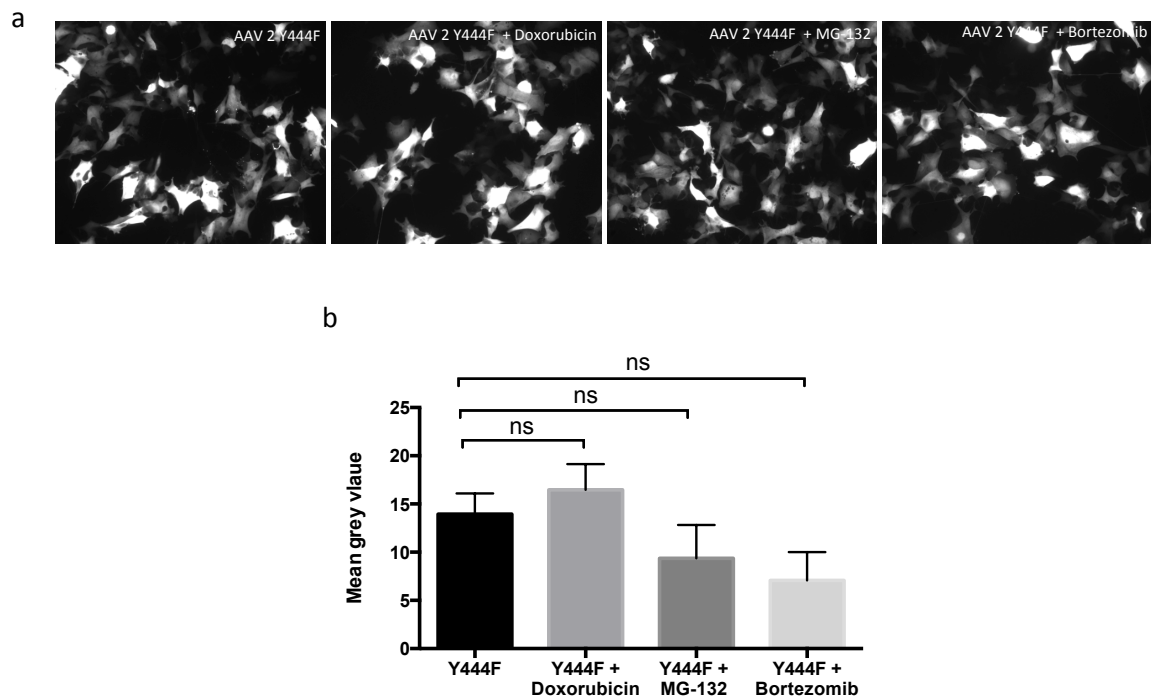


Figure 3.12 Comparing rAAV2/2 Y444F transduction of SH-SY5Y cells *in vitro* with proteasome inhibitor treated cells.

(a) Representative images showing GFP expression following transduction with rAAV2/2 Y444F, and rAAV2/2 Y444F with the proteasome inhibitors Doxorubicin, MG-132 and Bortezomib.. (b) Grey value analysis at 120 hours (mean+/-SEM) representing an estimate for the level of transgene expression within transduced cells. ns= non significant, one way ANOVA with Bonferroni post hoc test. n=9 or 10 per group (3-4 wells x 3 biological replicates).

3.5 Discussion

In this chapter the tropism and transduction efficiency of several AAV vectors has been assessed in the degenerate retina: two novel hybrid capsid vectors (rAAV2/Rec2 and rAAV2/Rec3) and two capsid mutant vectors (rAAV2/2 Y444F and rAAV2/8 Y733F). *In vivo* experiments assessing transduction in the *rd1* mouse retina were combined with *in vitro* testing on a human cell line and *ex vivo* testing of human retinal explants.

The construction of vectors with hybrid capsids involved recombination of structural domains from vectors of different serotypes previously isolated from primate brain together with AAV8. The data presented demonstrates that it is possible to interchange viral capsid protein sequences between different AAV serotypes and that this still results in retinal transduction, albeit at a reduced efficiency compared to rAAV2/2 and rAAV2/5.

The cause of reduced transduction efficiency using the hybrid rAAV/Rec2 and rAAV/Rec3 vectors is unclear. Modification of VP3 surface tyrosine residues are known to affect AAV transduction, however none of the hybrid capsid AAVs included modification of these residues, and the number of exposed surface tyrosine residues on rAAV2/Rec2 and Rec3 capsids is comparable to that of rAAV2/8. The HI loop of the C terminus of VP3 protein is a critical sequence for genome packaging and capsid assembly (179). The HI loop in both rAAV2/Rec2 and rAAV2/Rec3 is the same as that of rAAV2/8 and so this region at least can be excluded as a cause for reduced transduction. It could be possible that rearranging the capsid proteins causes impaired genome packaging and increases the number of truncated transgenes. This might not be detected in the PCR assay used to determine viral titre, therefore overestimating the number of complete viral genomes present. In order to confirm that the hybrid capsids

are able to rearrange into an appropriate AAV structure, it would be necessary to perform more complex analyses such as X-ray crystallography. However, this would only provide details on the structure of fully matured virions and would be unlikely to elucidate improper genome packaging.

Although transduction efficiency was reduced in the retina, this technique may be a useful tool in developing new AAV vectors. Shuffling of specific capsid sequences may be of use in minimizing immune responses to AAV gene therapy. Previous studies have shown that high dose rAAV2/2 administration into the subretinal space in mice leads to production of neutralising antibodies to AAV, which may affect efficacy of treatment to a second eye (184). The novel capsid sequences (cy5, rh20 and rh39) were identified in primate brain and not in the retina and it may be that in future for retinal gene therapy new AAV serotypes should be isolated by PCR of AAV genome particles directly from primate retina.

Neither the hybrid recombinant vectors nor rAAV2/2 or rAAV2/5 were able to transduce bipolar cells. In the *rd1* mouse model used here, virtually the entire ONL (i.e. the photoreceptors) had degenerated and hence failure to target bipolar cells cannot be due to failure of AAV to access this layer. Ubiquitination of AAV particles may be an important mechanism preventing successful AAV transduction of bipolar cells, and hence the next strategy used was to investigate capsid mutant AAV which aims to reduce ubiquitination and proteasome-mediated degradation.

AAV vectors with capsid mutations of a single tyrosine to phenylalanine residue, rAAV2/2 Y444F and rAAV2/8 Y733F were assessed. In the *rd1* model, both capsid mutations enabled bipolar cell transduction in the degenerate retina when delivered subretinally. Bipolar cell transduction was also seen in human retinal explants using

rAAV2/8 Y733F.

A previous study in the *rd10* mouse model of retinal degeneration identified bipolar cell transduction using a self-complementary rAAV2/8 Y733F with transgene expression driven by a bipolar cell-specific promoter (111). The results presented in this chapter show that a single stranded rAAV2/2 Y7444F or rAAV2/8 Y733F vector is able to target bipolar cells in a different mouse model (*rd1*) and using a ubiquitous and different promoter to that which has been previously reported. The ability to do so with a single capsid mutant vector may be advantageous as although capsid mutation increases infectivity, this may be at the expense of altering antibody binding (181) and therefore immune response. Thus for clinical trial applications it may be prudent to limit capsid mutation in order to limit deviation from the presumed optimal AAV capsid structure.

The mechanism by which capsid mutant vectors increase transduction efficiency is likely to be by avoiding proteasome-mediated degradation(130). This hypothesis is supported by the data presented from *in vitro* tests on SH-SY5Y transduction in which addition of the proteasome inhibitors Doxorubicin and MG-132 increased transduction levels of rAAV2/2, but did not further increase transduction using rAAV2/2 Y444F. Bortezomib did not appear to be effective in augmenting transduction by rAAV2/2 in this setting. It has previously been shown to augment AAV transduction in animal models for the treatment of haemophilia when delivered via the portal vein (185), however since the action of proteasome inhibitors is likely to be cell or tissue specific (183), the lack of efficacy of this drug in augmenting AAV transduction in SH-SY5Y cells may be unsurprising.

Another feature seen was reduced Müller cell transduction of rAAV2/2 Y444F compared to rAAV2/2. It has previously been proposed that AAV capsid mutation may alter retinal

penetration (132), however this is unlikely to explain the reduced Müller cell tropism since these span the entire width of the retina. Previous work has also demonstrated that mutations of the AAV capsid may affect heparin sulphate binding capacity (186) and this could contribute to the altered Müller cell tropism seen. In addition, AAV internalization into cells occurs via receptor-mediated endocytosis from clathrin-coated pits (127), and it may be a difference in this process between Müller cells and other cells of the inner nuclear layer affects tropism.

Significantly, rAAV2/8 Y733F was successful at targeting bipolar cells in *ex-vivo* human retinal explants, which would support use of this vector in a future clinical trial. Retinectomy tissue from patients that had chronic retinal detachment was used, which was placed in culture immediately after removal from the eye, making it more likely to be viable for transduction by AAV than post-mortem tissue used in previous studies (187). However the retina may also have had varying degrees of degeneration between patients, since the length of detachment and amount of proliferative vitreoretinopathy (PVR) was different between tissue samples. Therefore, transduction patterns in the human retinal samples may be dependent on these factors, which may explain the limited photoreceptor transduction seen with AAV2/8 Y733F. Also the high level of Müller cell transduction seen in the human retinal explants when transduced with rAAV2/8 Y733F may be related to the extensive PVR in this tissue, in which glial cell proliferation is known to be a feature. Nevertheless some glial cell activation is also likely to occur in end stage retinal degenerations and the observation of efficient AAV transduction in the degenerate human retina may therefore be relevant.

3.6 Conclusions

Subretinal delivery of hybrid capsid AAV showed some transduction of the degenerate retina but with reduced efficiency compared to rAAV2/2 and rAAV2/5. Transgene expression using single capsid mutant rAAV2/2 Y444F and 2/8 Y733F was very high in the degenerate retina, with bipolar cell transduction being observed using both vectors in the murine retina *in vivo* and in human retinal explants *ex vivo* using rAAV2/8 Y733F. On this basis, the AAV2/8 Y733F capsid was selected for generating a vector to deliver melanopsin as an optogenetic tool to the degenerate retina.

Chapter 4 Melanopsin vector generation and promoter optimisation

4.1 Introduction

In order to investigate the potential for human melanopsin expression in the degenerate retina to restore visual function, an appropriate vector must be generated. Since in end-stage retinal degeneration the majority of classical photoreceptors are lost, bipolar cells are likely to be a key target for an optogenetic strategy to restore vision. Light sensitive bipolar cells could then signal to amacrine and ganglion cells sending action potentials to visual areas in the brain.. There are two approaches by which these cells could be targeted: either by transducing bipolar cells specifically to try to maintain the pathway via which visual information is signalled in the healthy retina or by expressing melanopsin ubiquitously in the degenerate retina with the inclusion of bipolar cells.

Bipolar cells are poorly transduced by most AAV serotypes (188) and therefore a virus with high transduction efficiency is required to target these cells. This is probably due to proteasome-mediated degradation of AAV virions since capsid mutation enhances transduction efficiency in bipolar cells, see chapter 3. Limiting gene expression to bipolar cells may have benefits with respect to functional efficacy, although in some cases use of a cell specific promoter can reduce overall levels of transduction compared to a ubiquitous promoter (189). Furthermore, in retinal degeneration where cells are stressed, cell specific promoters may not be as efficient as in the wild type retina (190). Therefore when producing an AAV vector for melanopsin therapy to the degenerate retina, a significant consideration is whether to use a ubiquitous promoter or a bipolar-cell specific one.

One of the most extensively characterised ubiquitous promoters for retinal gene therapy is the CMV-actin-globin (CAG) enhancer/ promoter element. The full-length sequence is a 1.6 kb hybrid of a cytomegalovirus (CMV) early enhancer; the promoter and first exon of the chicken beta actin gene and the first intron of the rabbit beta globin gene (99). With regards to retinal transduction, previous work using this promoter has shown transduction of retinal pigment epithelium (RPE), photoreceptors, Muller cells and ganglion cells using various AAV capsids (120). Data presented in chapter 3 demonstrated bipolar cell transduction in the degenerate retina using the CAG enhancer/promoter with capsid mutant AAV.

Previous work to restrict transgene expression to bipolar cells has focused on the metabotropic glutamate receptor 6 (mGluR6) gene called *grm6*. Retinal ON bipolar cells receive glutamatergic input from photoreceptors and mGluR6 is the principal post-synaptic receptor expressed by ON bipolar cells (191). The most successful bipolar cell specific construct published to date is a 200 base pair enhancer region from the murine *grm6* gene fused to a SV40 (Simian vacuolating virus 40) core promoter. Using a self-complementary capsid mutant AAV vector using this enhancer/promoter, Channelrhodopsin gene delivery was restricted to ON bipolar cells following subretinal delivery in the *rd10* mouse model of retinal degeneration (111). This was also achieved following intravitreal injection using this promoter with a novel AAV capsid in the *rd1* mouse (159). Further work has developed an enhanced version of the Grm6 promoter (4xMg6 promoter) with reported greater efficacy than the previous Grm6 promoter (160). This 1.1kb promoter involves four consecutive repeats of the Grm6 enhancer with the final repeat fused to an SV40 promoter. Therefore use of this promoter to target melanopsin to ON bipolar cells of the *rd1* mouse model of retinal degeneration may be an appropriate strategy.

Chx10 is a homeobox-containing transcription factor expressed in retinal progenitor cells and bipolar cells (192) and loss of Chx10 leads to absence of all types of differentiated bipolar cells (193). One previous study identified a region upstream of the Chx10 gene that directed Chx10 expression to retinal progenitor cells and bipolar cells following electroporation of chick embryos (194). Since Chx10 is expressed in the majority of bipolar cells, this identified region might be suitable to deliver melanopsin to bipolar cells in the degenerate retina using AAV. The strategy would however, transduce all bipolar cells without separation of visual information into ON and OFF pathways.

The overall objective of this chapter was to generate a vector for delivery of melanopsin to the degenerate retina: specifically to identify the optimal promoter for this purpose and characterise the transduction pattern of the vector in degenerate retina.

4.2 Experimental design

4.2.1. Cloning

Human melanopsin was cloned into a plasmid suitable for AAV production under the control of the CMV.CBA, CHX10 or the 4xMg6 (variant of Grm6) promoter. The CMV enhancer and CBA promoter were isolated from an existing AAV plasmid in the laboratory (pUF6.1, gift from Vince Choido, University of Gainesville) using PCR; the CHX10 promoter was isolated from human genomic DNA and the 4xMg6 promoter was a gift from a collaborator (Prof Botond Roska, FMI, Basel). Each was joined to human *OPN4* coding sequence using PCR (172) and this sequence joined to other elements in the construct. Using MfeI and NotI restriction sites, the final construct was inserted into a vector containing AAV2 inverted terminal repeat (ITRs). XL10-Gold cells were used for cloning since they are recombination deficient (*recA*) and therefore encourage construct stability.

4.2.2 *In vitro* testing

Plasmids were tested *in vitro* on two human cell lines: HEK 293T and SH-SY5Y to assess human melanopsin expression.

4.2.3. AAV production

Using the plasmids produced, AAV vectors were generated using standard techniques, see chapter 2. Vector titres were calculated using qPCR (195).

4.2.4 Calcium imaging

This technique was used to assess if melanopsin produced by the AAV vector was functional *in vitro*. Cells were loaded with a calcium indicator dye that becomes fluorescent when bound to calcium. Light activation of melanopsin causes calcium influx into a cell via a G protein mediated cascade. Therefore measuring the change in fluorescence of the indicator dye as calcium enters the cell can be used to assess functional activity of melanopsin. Seven days after transduction of differentiated SH-SY5Y cells by the AAV melanopsin vector, imaging was performed using a Fluo-4 calcium indicator at room temperature. Cells were stimulated with 480nm light pulses lasting 0.5 seconds every 2 seconds for one minute, which allowed both imaging of the calcium dye and stimulation of melanopsin.

4.2.5 *In vivo* testing

AAV vectors were injected into the subretinal space of 6 to 8 week old *rd1* mice. After at least 3 weeks, mice were culled and retinas examined for expression of human melanopsin using immunohistochemistry. Once the optimal vector was identified, further immunohistochemistry was performed to assess transduction patterns and cell types transduced.

4.3 Results

4.3.1 Vector design

Vector optimisation is limited by the packaging capacity of AAV, which should ideally not exceed 4.9kb since packaging efficiency is reduced once this size is exceeded (98). For delivery of melanopsin to the degenerate retina, elements that had to be included were the two AAV ITRs (145 bp each), promoter, melanopsin transgene (1437 bp) and polyadenylation (polyA) sequence (274bp), the latter being important for nuclear export and stability of mRNA.

In order to evaluate gene expression by the vector and especially for assessing melanopsin function *ex vivo* by electrophysiological methods, it was useful to include a reporter gene to indicate areas of successful transduction. There were two methods by which this could be achieved: either to create a fusion protein between melanopsin and the reporter gene or to use an internal ribosome entry site (IRES) sequence. The latter allows internal initiation of translation i.e. away from the 5' end of an mRNA molecule (196) and therefore bicistronic expression of two proteins from one mRNA strand can be achieved. Creation of a fusion protein may affect function of the primary transgene (197) and therefore the strategy of using an IRES sequence from the encephalomyocarditis virus (ECMV) (Clontech, USA) was chosen for the melanopsin vector. The commonest reporter genes included are green fluorescent protein (GFP) from the *Aequorea* jellyfish and red markers such as DsRed from *Discosoma* coral. The excitation wavelength of GFP is 488nm and DsRed is 554nm, the former being very close to the peak sensitivity of melanopsin, which is 480nm. Therefore DsRed was chosen as the reporter gene to be included in the vector construct.

In order to maximise transgene expression in bipolar cells, a Woodchuck Hepatitis Virus post-transcriptional Regulatory element (WPRE) was also included in the construct, incorporation of which into the 3' untranslated part of the AAV genome has been demonstrated to increase gene expression (104), (113). The native WPRE contains a promoter and upstream fragment of coding sequence for the X protein which may be implicated in formation of liver tumours (115) and therefore the WPRE sequence used was modified to prevent X antigen expression, and was an identical sequence to that used in the choroideremia gene therapy trial (79).

4.3.2 CMV enhancer/CBA promoter

The combined construct elements of human melanopsin coding sequence (1437bp), IRES (585bp), DsRed (678bp) and WPRE (593bp) plus the two AAV ITRs, polyA sequence and multiple cloning site present in the AAV production plasmid resulted in a 4kb sequence. Due to the small packaging size of AAV, there was therefore a 900b limit on the size of the promoter that could be included. The full length CAG promoter is 1.6kb and so a shorter version needed to be incorporated in this vector. The CAG promoter used in the choroideremia trial was a 934 bp sequence (79), and another promoter named the small CBA sequence used (smCBA) is 953 bp (198). Since the size of either of these would be greater than that desired, a promoter sequence was chosen comprising the CMV enhancer and CBA promoter alone comprising 664bp, figure 4.1.

4.3.3 Cloning a CMV.CBA-*OPN4*-IRES-DsRed construct

The CMV enhancer and CBA promoter were joined to human *OPN4* coding sequence using the PCR technique SPLICE (172) and this sequence joined to IRES and DsRed to produce a CMV.CBA-*OPN4*-IRES-DsRed construct, figure 4.2a-c.

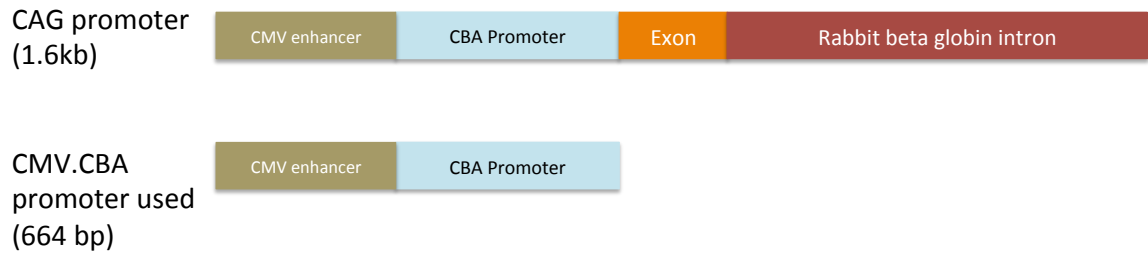


Figure 4.1 CAG promoter

(a) Schematic illustrating the full length CAG promoter and (b) the shortened CMV enhancer-CBA promoter sequence that was incorporated into the melanopsin plasmid vector.

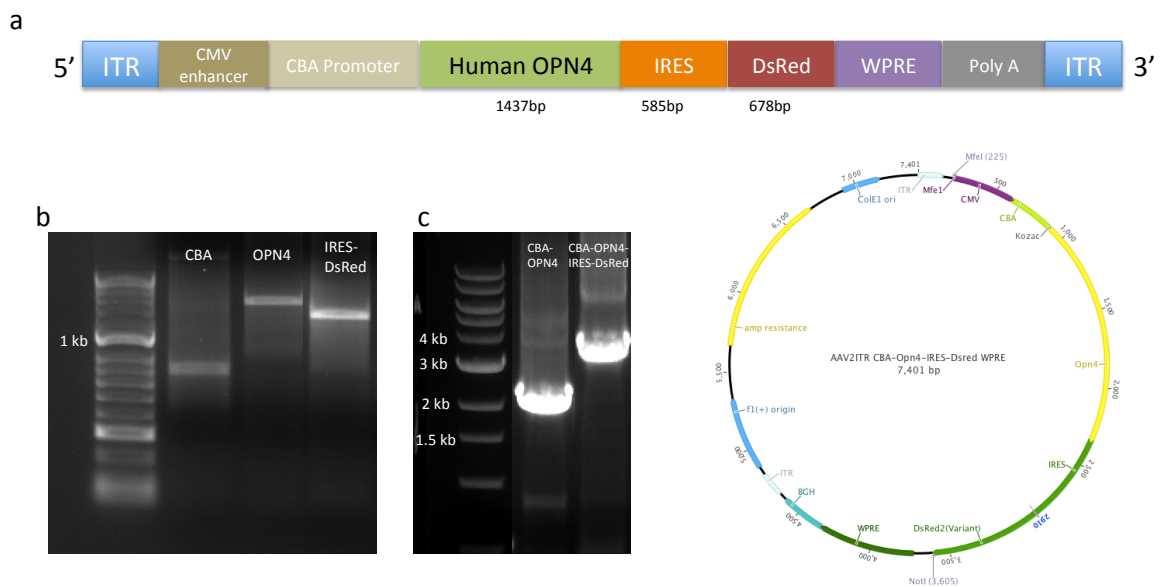


Figure 4.2 Generation of CMV.CBA-*OPN4*-IRES-DsRed construct.

(a) The vector construct contained AAV2 inverted terminal repeats (ITRs), Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) and bovine growth hormone (bGH) polyadenylation (PolyA) signal sequence. The transgene (*OPN4*-IRES-Dsred) was under the control of a CMV enhancer and CBA promoter. (b) Isolation of CMV.CBA enhancer/promoter, human *OPN4* and IRES-DsRed by PCR. (c) The constructs were made by joining CMV.CBA enhancer/promoter to *OPN4*, and joining this to IRES-DsRed. (d) The CMV.CBA-*OPN4*-IRES-DsRed construct was inserted to the AAV vector backbone containing WPRE to produce the plasmid vector shown.

Using MfeI and NotI restriction sites, this was inserted into a vector containing AAV2 inverted terminal repeat (ITRs) and a WPRE sequence, figure 4.2d. After cloning, the presence of the construct was confirmed by MfeI and NotI double digestion, figure 4.3c, and also by sequencing.

4.3.4 Confirmation of the presence of ITRs

Due to the structure of ITRs, plasmid recombination may occur during cloning causing loss of one or both ITRs. Sanger sequencing reactions of the ITRs are rarely successful due to the high GC content and palindromic nature of this region, which results in the formation of tight hairpin secondary structures that affect polymerase efficacy. As expected a sequencing reaction of the 3' ITR in the CMV.CBA-*OPN4*-IRES-DsRed plasmid gave an incomplete result, figure 4.3a. There are two XmaI restriction sites within the ITR hairpin loop, figure 4.3b, and therefore the presence of intact ITRs in the final plasmid was confirmed by XmaI digestion, figure 4.3c.

4.3.5 Melanopsin plasmid transfection of HEK 293T cells

HEK 293T cells were transfected with the CMV.CBA-*OPN4*-IRES-DsRed plasmid. The shortened CMV.CBA enhancer/promoter was able to drive DsRed expression, which was detected by direct fluorescence. Melanopsin expression was confirmed by immunocytochemistry. Two antibodies were used for this purpose: one was a custom made N-terminal antibody (UF006, gift from Ignacio Provencio), figure 4.4 i-iii, and the other was a commercially available C terminal antibody (Santa Cruz), figure 4.4 iv-vi. Both antibodies demonstrated robust melanopsin staining with membrane localisation. For further experiments the commercially produced antibody was used due to ease of availability.

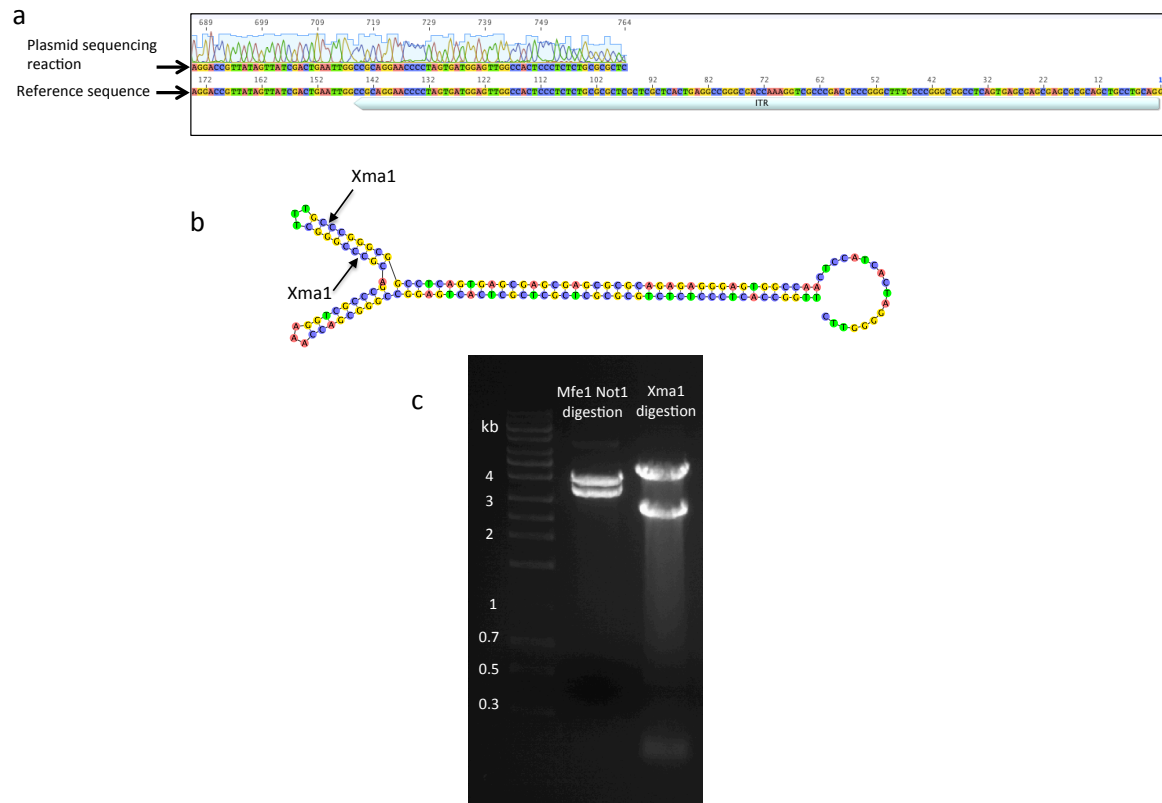


Figure 4.3 Confirmation of Inverted terminal repeats (ITRs)

(a) A Sanger sequencing reaction was incomplete and unable to sequence the hairpin loop of the ITR. (b) Secondary structure of the ITR showing the two XmaI restriction sites. (c) The presence of the construct in the cloned plasmid was confirmed by MfeI and NotI double digestion of the CMV.CBA-*OPN4*-IRES-DsRed plasmid. Expected fragment sizes were 4.0 kb and 3.4 kb. The presence of ITRs was confirmed by XmaI digestion. Expected fragment sizes were 4.5 kb, 2.7kb and 165 bp.

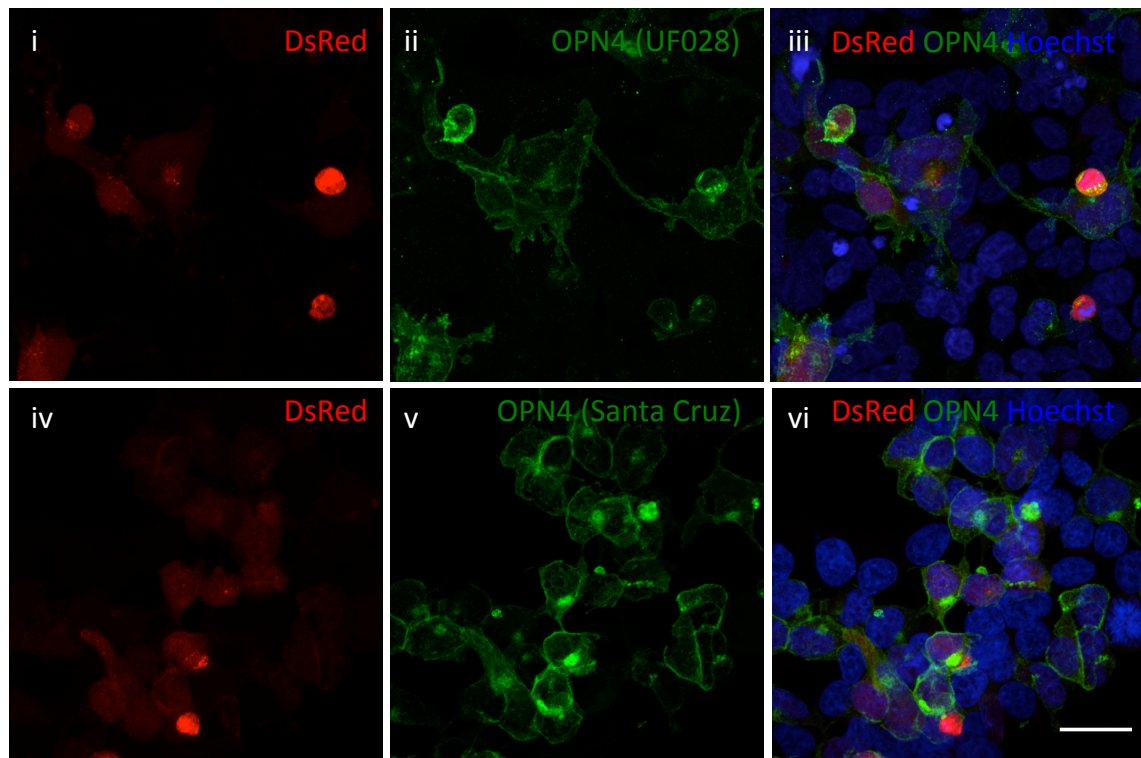


Figure 4.4 Transduction of HEK 293T cells using CMV.CBA-OPN4-IRES-DsRed plasmid.

DsRed fluorescence (red) is shown in panels i and iv. Antibody staining for human OPN4 (green) is shown using the UF028 antibody (ii) and a commercially available human OPN4 antibody (v), both confirming membrane localisation of OPN4. Composite images (iii, vi) show overlay with Hoechst nuclear labeling (blue), demonstrating hOPN4 expression in DsRed expressing cells. Scale bar 25 μ m.

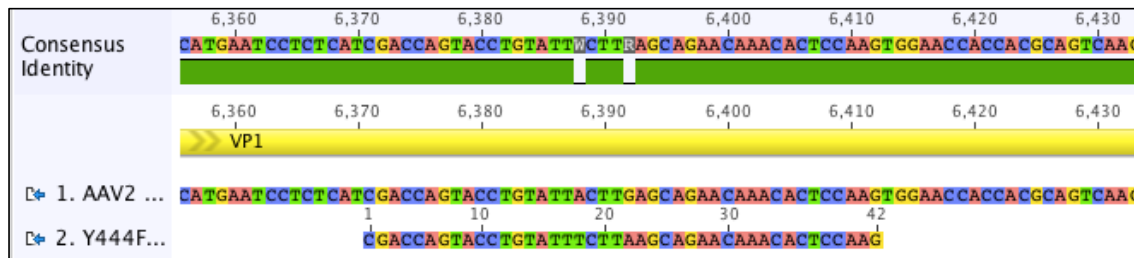
4.3.6 Production of capsid mutant AAV

As demonstrated in Chapter 3, both rAAV2/2 Y444F and rAAV2/8 Y733F serotypes expressing GFP were able to target bipolar cells in the degenerate retina. The GFP vectors were obtained commercially and therefore these capsid mutations had to be introduced into the AAV rep cap plasmid for packaging the melanopsin vector.

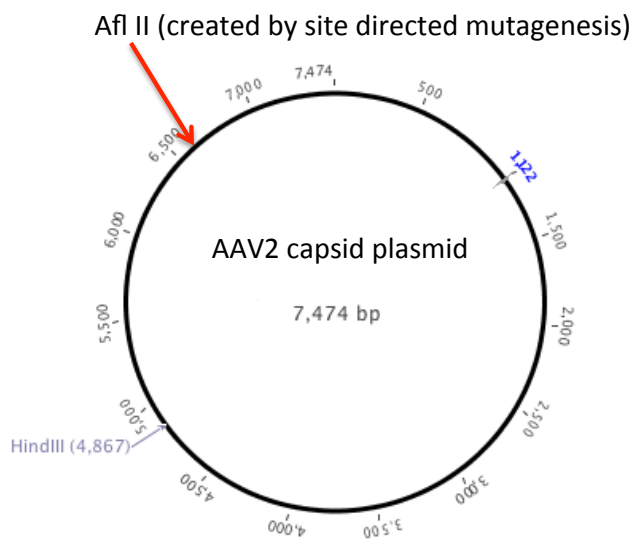
For production of rAAV2/2 Y444F, a change from tyrosine to phenylalanine was induced in the capsid protein at position 444. This was achieved by designing primers to the capsid coding sequence to include this change and also a silent mutation creating an Afl II restriction site to allow confirmation of mutagenesis, figure 4.5a. Following mutagenesis, DNA was extracted from colonies obtained and double digested with HindIII and AflIII, figure 4.5b. The AflIII site was present in colonies screened indicating successful mutagenesis, figure 4.5c, and the presence of the Y444F mutation was confirmed by sequencing.

Subsequently, the CMV.CBA-*OPN4*-IRES-DsRed plasmid produced was packaged in AAV2/2 Y444F using a standard virus production protocol, see Chapter 2, and purified using an iodixanol gradient, figure 4.6a. Viral titres were obtained using qPCR with primers directed to a region within the melanopsin coding sequence, figure 4.6b. The AAV2/8 Y733F capsid mutant was produced in a similar manner by a collaborating group (M During, Columbus State university, Ohio), which was used to package the same CMV.CBA-*OPN4*-IRES-DsRed plasmid.

a



b



c

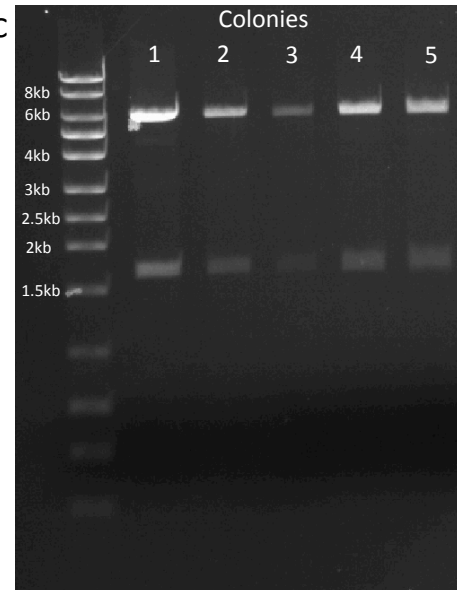


Figure 4.5 Site directed mutagenesis.

(a) Primers to the AAV2 capsid CDS were designed containing 2 point mutations: one to induce an amino acid change from tyrosine to phenylalanine, and a second silent mutation to create an Afl II restriction site. (b & c) Plasmid DNA from colonies obtained by transformation of XL10 cells underwent a double digest using HindIII and AflII, with the presence of the AflII site indicating successful mutagenesis.

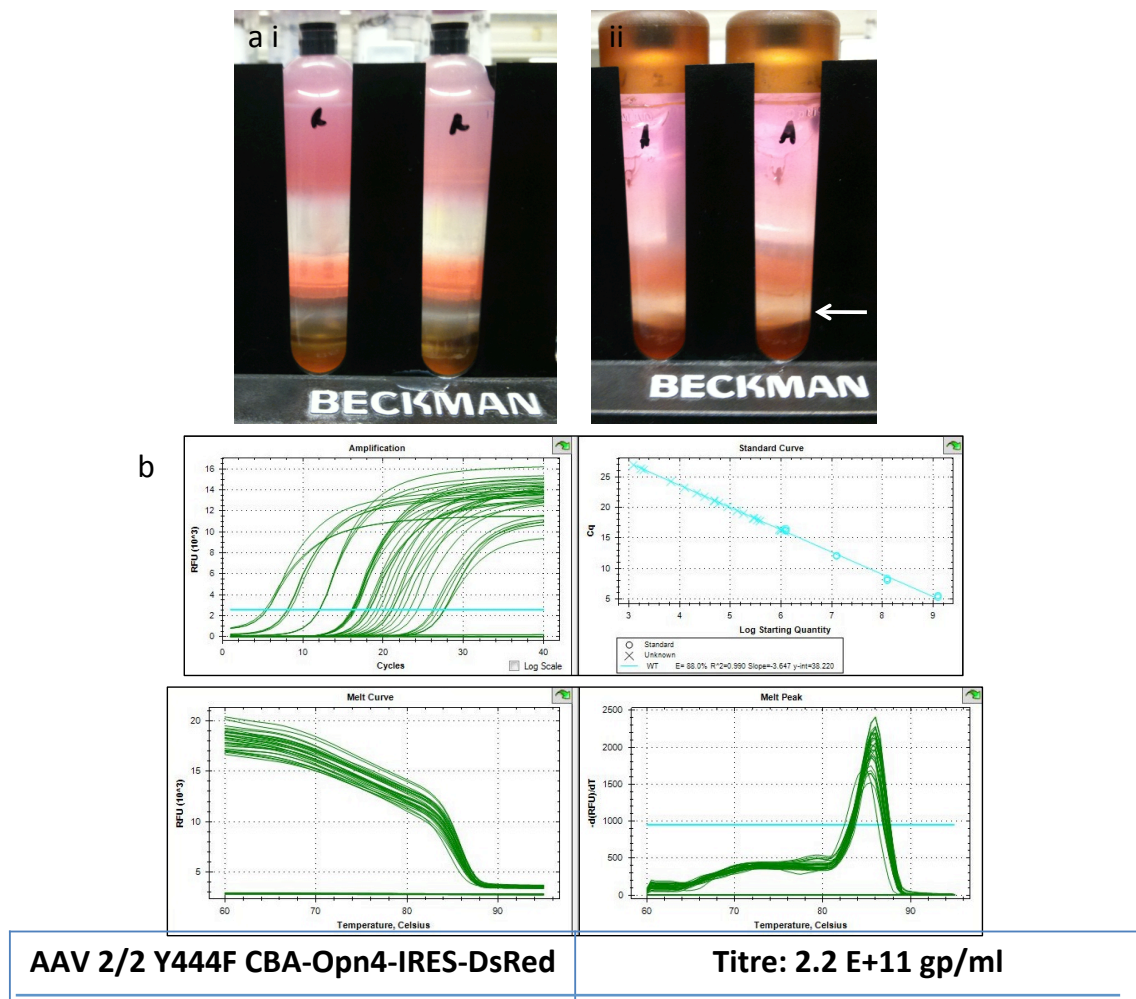


Figure 4.6 Virus production

(a) (i) Iodixanol gradient prior to centrifugation and (ii) Iodixanol gradient after centrifuging, arrow indicating fraction to be aspirated. (b) qPCR for viral titre (i) Amplification curves with a clear exponential phase indicating good amplification of all samples tested, (ii) the standard curve showed efficiency of primer binding at 88% and $R^2 = 0.99$ the latter indicating good reproducibility (iii) the melt curve and (iv) the melt peak was a single peak indicating no additional primer binding sites.

4.3.7 Melanopsin AAV vector testing *in vitro*

The capsid mutant AAV vectors produced were tested *in vitro* to ensure that human melanopsin was expressed. These were tested on two cell lines, HEK293T cells and the human neuronal cell line SH-SY5Y. For the latter, as described in chapter 3, conditions that induce neuron-specific differentiation and stabilise cell proliferation were used to allow prolonged culture of cells, since AAV 2/8 transduction kinetics are slow *in vitro*. Expression in HEK293T cells was assessed after 5 days and SH-SY5Y cells after 9 days.

Following addition of the rAAV2/2 capsid mutant vector, melanopsin expression was detected by immunocytochemistry in both HEK293T cells fixed after 5 days, figure 4.7a and SH-SY5Y fixed after 9 days, figure 4.7b. Appropriate membrane localisation of melanopsin was seen, and this was particularly evident in SH-SY5Y cells, which had branching dendritic processes. Transduction was seen following addition of the rAAV2/8 capsid mutant vector in both cell types, figure 4.8a,b, but melanopsin expression was lower with the use of this vector than rAAV2/2 Y444F *in vitro*.

4.3.8 Calcium imaging of AAV transduced cells

Calcium imaging was performed to assess whether melanopsin expressed by the vectors produced was functional. Since the rAAV2/2 Y444F melanopsin vector worked better *in vitro*, this was used to transduce SH-SY5Y cells for calcium imaging experiments and untransduced cells were used for comparison. For analysis, a light response was defined as a five percent change in fluorescence from baseline after the onset of light. $10.6\% \pm 3.905$ (mean $\pm$ sem) of cells in dishes transduced by AAV showed a light-dependent change in fluorescence consistent with calcium influx, whereas no cells in the control group showed a similar response ($p=0.028$, Mann Whitney test), figure 4.9a.

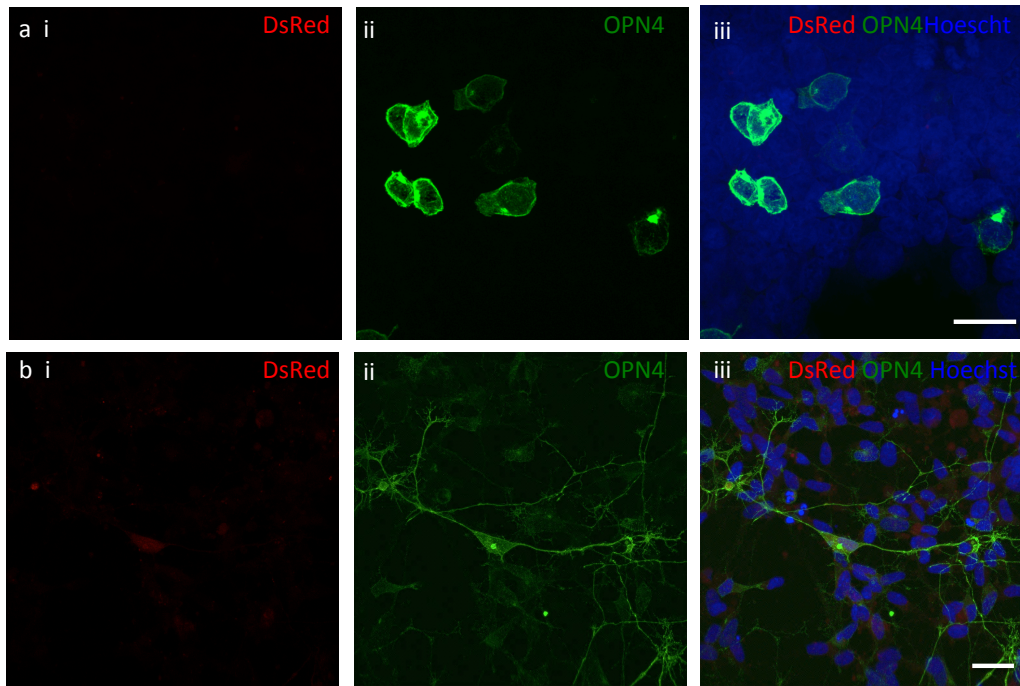


Figure 4.7 *In vitro* assessment of rAAV2/2 Y444F expressing human melanopsin.

(a) HEK 293T cells 5 days after addition of virus and (b) SH-SY5Y cells 9 days after addition of virus. DsRed fluorescence (i), human melanopsin staining (ii) and an overlay with Hoechst nuclear staining (iii) are shown for each cell type. Scale bar 25um.

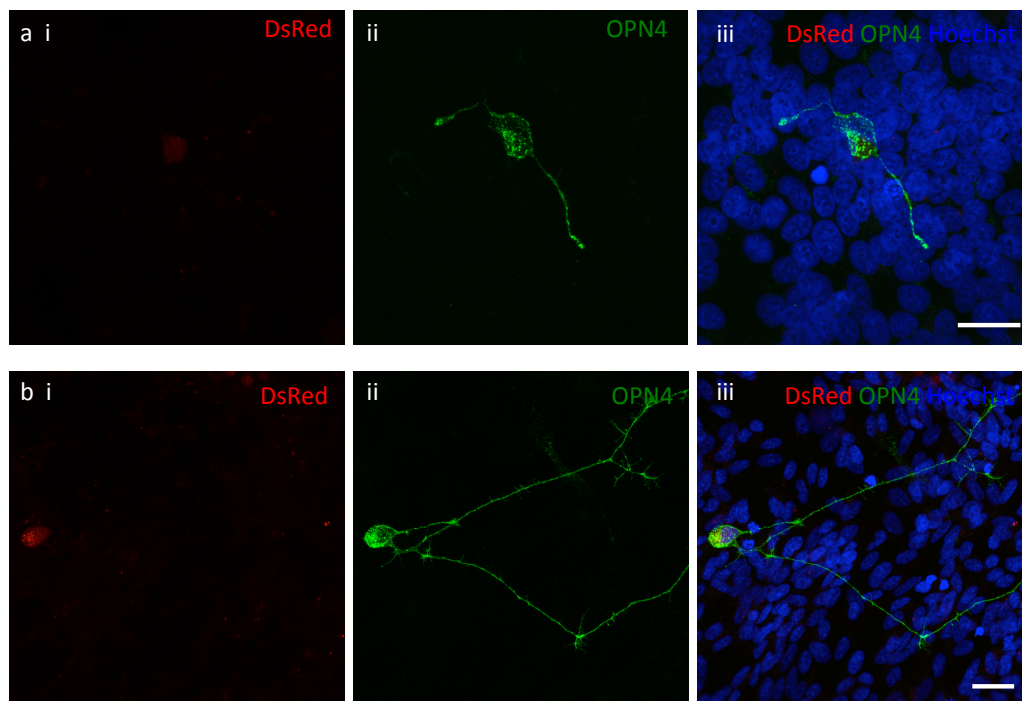


Figure 4.8 *In vitro* assessment of rAAV2/8 Y733F expressing human melanopsin.

(a) HEK 293T cells 5 days after addition of virus and (b) SH-SY5Y cells 9 days after addition of virus. DsRed fluorescence (i), human melanopsin staining (ii) and an overlay with Hoechst nuclear staining (iii) are shown for each cell type. Scale bar 25um.

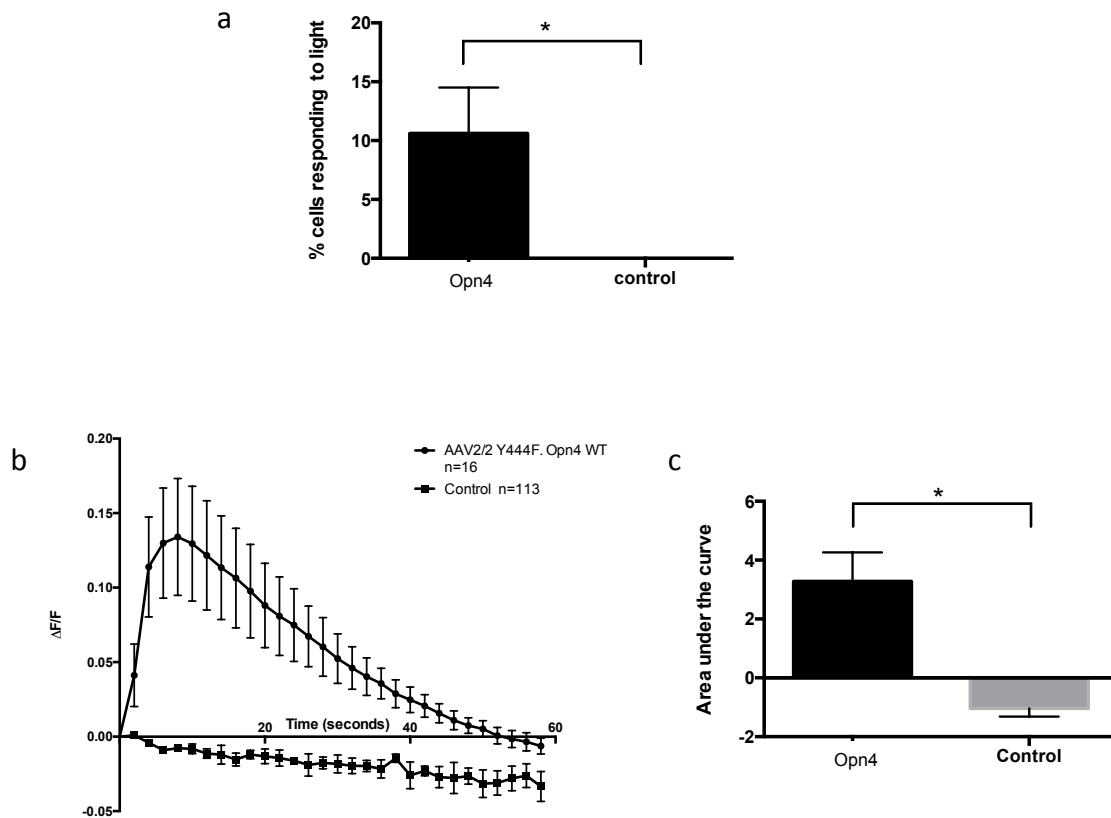


Figure 4.9 Calcium imaging of SH-SY5Y cells transduced with AAV 2/2 Y444F vector expressing human melanopsin

(a) Percentage of cells showing light responses (defined as a greater than 5% change in fluorescence over baseline) in transduced cells versus controls following a 480nm light pulse of 0.5 seconds duration every two seconds for one minute (b) Time course of change in fluorescence of the calcium indicator, F= fluorescence (c) Area under the curve calculated from graph (b) in the two groups tested * $p < 0.05$, Mann-Witney test. N=4 biological replicates AAV.OPN4 group, N=3 biological replicates control group.

Examining the kinetics of responding cells, the peak of fluorescence was at 7 ± 0.86 seconds, figure 4.9b, and the area under the curve was significantly different from untreated controls ($p=0.028$, Mann Whitney test), figure 4.9c. Therefore human melanopsin expressed by the AAV vector tested mediated a light-dependent calcium response *in vitro*.

4.3.9 CHX10 promoter

A previous study identified a 3kb region upstream of the Chx10 (or VSX2) gene that directed Chx10 expression to retinal progenitor cells and bipolar cells and within this, a 500bp region in isolation also drove Chx10 expression (194). The 500bp sequence was assessed for promoter activity using an online tool (www.bimas.cit.nih.gov), and this identified a 251bp predicted promoter region within it, figure 4.10a. Therefore this 500bp region upstream of Chx10 was selected for incorporation into the vector to assess bipolar cell transduction in the degenerate retina.

The CHX10 promoter was joined to human *OPN4* using PCR and this sequence joined to IRES and DsRed to produce a Chx10-*OPN4*-IRES-DSred construct, figure 4.10 c,d. As before, this was inserted into a vector containing AAV2 inverted terminal repeat (ITRs) and a WPRE sequence using MfeI and NotI restriction sites. After cloning, the presence of the construct was confirmed by MfeI and NotI double digestion and also by sequencing, and the presence of ITRs by XmaI digestion, figure 4.10e.

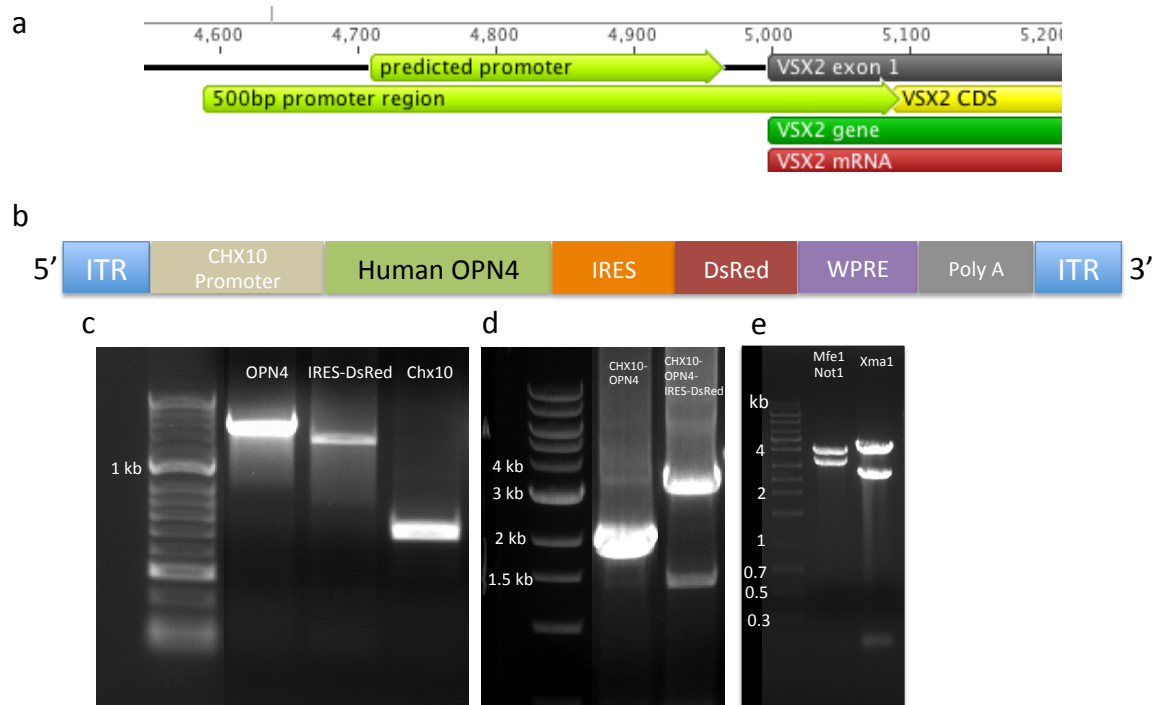


Figure 4.10 Generation of CHx10-OPN4-IRES-DsRed construct.

(a) The 500bp region that was able to drive Chx10 expression identified in the literature was screened for promoter activity and a 251bp predicted promoter region identified within it. (b) The vector construct contained AAV2 inverted terminal repeats (ITRs), Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) and bovine growth hormone polyadenylation (PolyA) signal sequence. The transgene (*OPN4*-IRES-Dsred) was under the control of this CHX10 promoter. (c) Isolation of human *OPN4*, IRES-DsRed and CHX10 promoter by PCR. (d) The constructs were made by joining CHX10 promoter to *OPN4* and then to IRES-DsRed. (e) The presence of the construct in the cloned plasmid was confirmed by MfeI and NotI double digestion in CHx10-hOpn4-IRES-DsRed plasmid, with expected band sizes of 4 and 3.4kb . The presence of ITRs was confirmed by XmaI digestion of the plasmid with expected band sizes of 4.5kb, 2.7kb and 165bp.

4.3.10 4xMg6 promoter

The 4xMg6 promoter was chosen for testing in the melanopsin vector to evaluate if bipolar cell specific expression of melanopsin could be achieved. The length of this promoter is 1.1kb and therefore replacing the shortened CMV.CBA promoter with this in the previously tested vector would exceed the packaging capacity of AAV. For this reason WPRE was not included in this vector. Cloning of the vector was done as previously, except that human melanopsin had to be isolated in two fragments and joined together and also the construct was inserted into the identical AAV backbone but without WPRE, figure 4.11.

4.3.11 Comparison of plasmid transfection of HEK 293T cells

HEK 293T cells were transfected with the CMV.CBA-*OPN4*-IRES-Dsred plasmid, CHX10-*OPN4*-IRES-DsRed plasmid or 4xMg6-*OPN4*-IRES-DsRed plasmid, figure 4.12. Both the CHX10 and 4xMg6 promoters were able to drive melanopsin expression, which was detected by immunocytochemistry, but this was markedly less than that driven by the CMV.CBA plasmid.

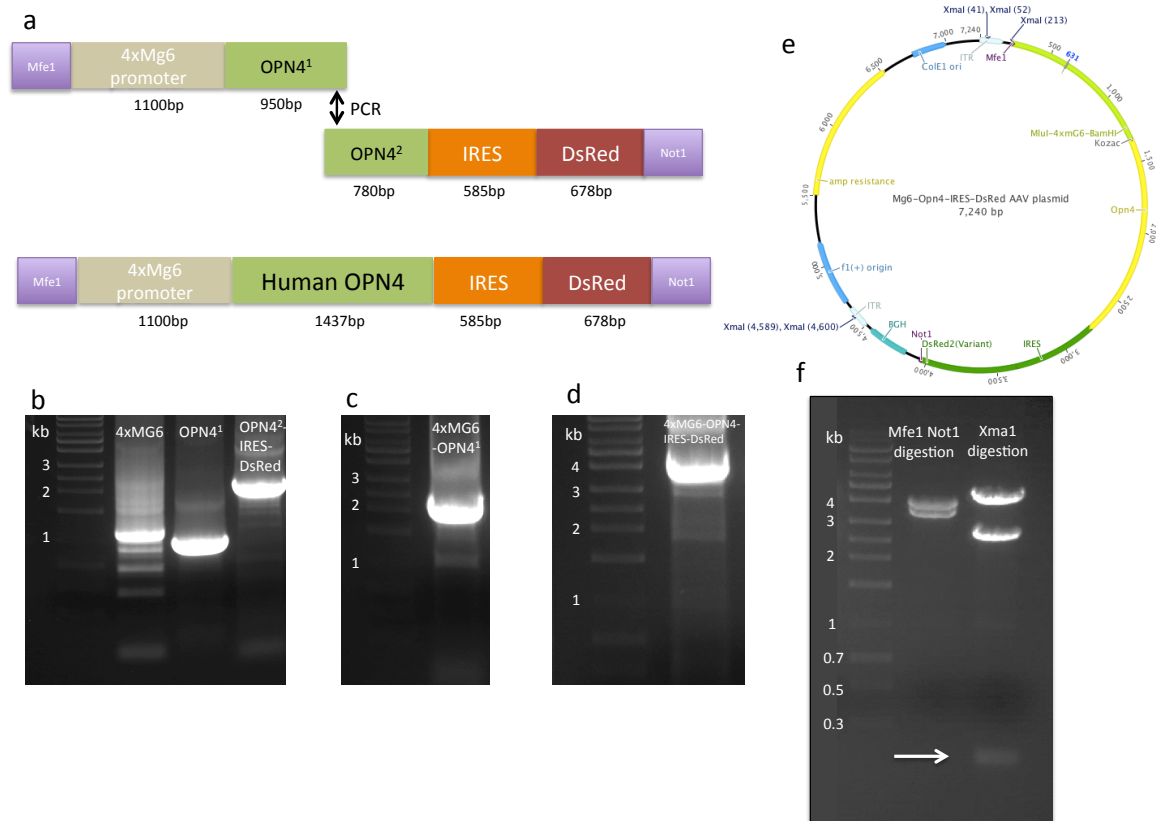


Figure 4.11 Generation of 4xMg6-OPN4-IRES-DsRed construct.

(a) Cloning strategy: Due to the length of the construct that was to be inserted into the plasmid backbone, human melanopsin was isolated in two fragments with a 200bp overlap. The first was joined by PCR to the promoter and the second to IRES-DsRed. The two fragments were then joined by PCR. (b) Isolation of the 4xMg6 promoter, human Opn4 fragment 1, human opn4 fragment 2 joined to IRES-DsRed by PCR. (c) PCR was used to join the promoter to Opn4 fragment 1 (d) The whole construct was created by joining 4xMg6-OPN4 fragment 1 to OPN4 fragment 2-IRES-DsRed by PCR (e) The construct was inserted into a backbone containing AAV ITRs but no WPRE (f) The presence of the construct in the cloned plasmid was confirmed by MfeI and NotI double digestion with expected band sizes of 3.8 and 3.4kb. The presence of ITRs was confirmed by XmaI digestion of the plasmid with expected band sizes of 4.3kb, 2.7kb and 165bp.

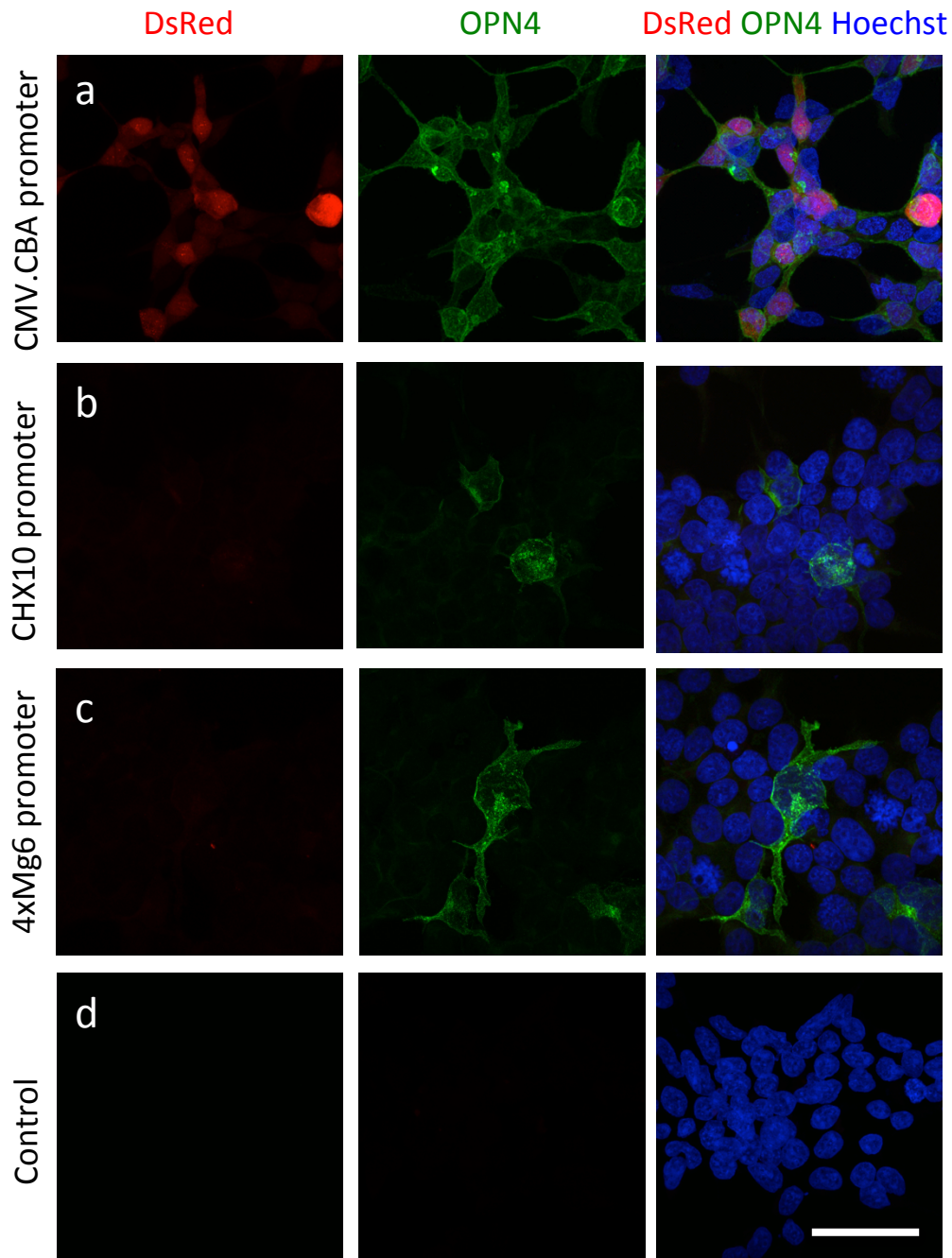


Figure 4.12 Transfection of HEK 293T cells.

Cells were transfected using plasmids expressing melanopsin driven by (a) CMV.CBA enhancer/promoter (b) CHX10 promoter and (c) 4xMg6 promoter. Control untransfected cells are shown for comparison (d) DsRed fluorescence (red) is shown in the left hand panels, antibody staining for human Opn4 (green) is shown in the middle panels and composite images showing overlay with Hoechst nuclear labeling (blue) in the right hand panels. Scale bar 25 μ m.

4.3.12 *In vivo* assessment of melanopsin AAV vectors

Since CHX10 and 4xMg6 are theoretically bipolar cell specific promoters, the likelihood of transcription from these cell specific promoters may be reduced in HEK293T cells. Therefore AAV vectors were generated from these plasmids using standard protocols. Testing of GFP expressing vectors in chapter 3 indicated the rAAV2/8 Y733F capsid was able to transduce bipolar cells in human retinal explants, therefore this capsid was chosen to package the AAV vectors with bipolar cell specific promoters.

The four vectors produced were injected into the subretinal space of six to eight week old *rd1* mice; 1.5 µl virus suspension was injected per eye, table 4.1.

Vector	Titre
rAAV2/2 Y444F CMV.CBA- <i>OPN4</i> -IRES-DsRed	2 x 10 ¹¹ gp/ml
rAAV 2/8 Y733F CMV.CBA- <i>OPN4</i> -IRES-DsRed	1 x 10 ¹² gp/ml
rAAV2/8 CHX10- <i>OPN4</i> -IRES-DsRed	1 x 10 ¹² gp/ml
rAAV2/8 4xMg6- <i>OPN4</i> -IRES-DsRed	8 x 10 ¹¹ gp/ml

Table 4.1 Table of AAV vectors tested *in vivo* indicating construct and vector titre

Three weeks after injection, the retinas were examined for human melanopsin expression using immunohistochemistry, figure 4.13. rAAV2/2 Y444F and rAAV2/8 Y733F vectors containing the CMV.CBA enhancer/promoter were both effective at driving human melanopsin expression. Good RPE transduction was seen with the AAV2

capsid mutant vector and also in the outermost cell layer of the inner nuclear layer, figure 4.13a. Using the capsid mutant 8 vector there was widespread transduction in the RPE and throughout the inner nuclear layer, figure 4.13b. No melanopsin expression was seen using the vector containing the CHX10 promoter, figure 4.13c. In the majority of the retina there was also no expression of melanopsin seen using the 4xMg6 promoter, figure 4.13d, however a few transduced cells were identified at the injection site, figure 4.14. Morphologically, these did not appear to be bipolar cells since the cell bodies transduced were in the ganglion cell layer.

Therefore, based on these experiments, the CMV.CBA enhancer/promoter was most effective at driving human melanopsin expression in the degenerate retina and transduction efficiency was highest using the AAV 2/8 Y733F capsid mutant vector. This vector was thus chosen for further characterisation.

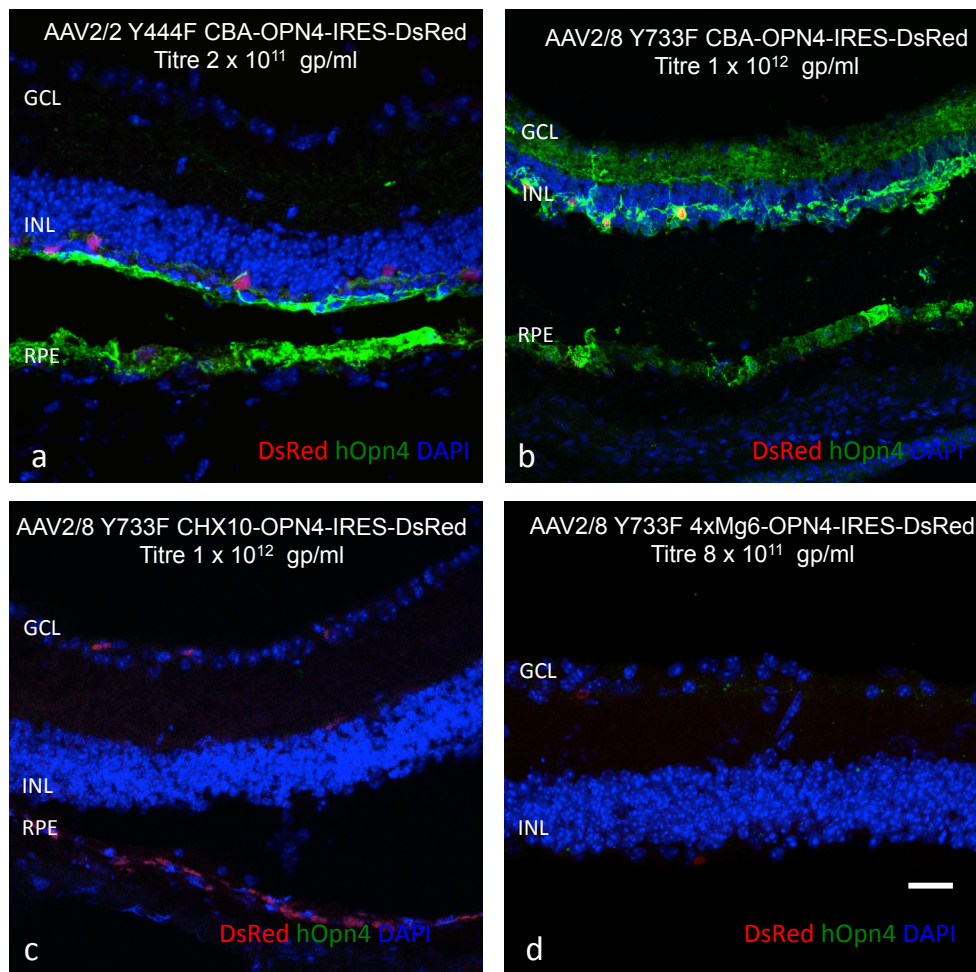


Figure 4.13 *In vivo* testing of AAV vectors.

Three weeks after subretinal injection of one of four AAV vectors in the *rd1* mouse, immunohistochemistry was done on retinal sections using human melanopsin antibody. Composite confocal microscopy images are shown: DsRed (red), human melanopsin (green) and Hoechst nuclear stain (blue). Scale bar 50 μ m.

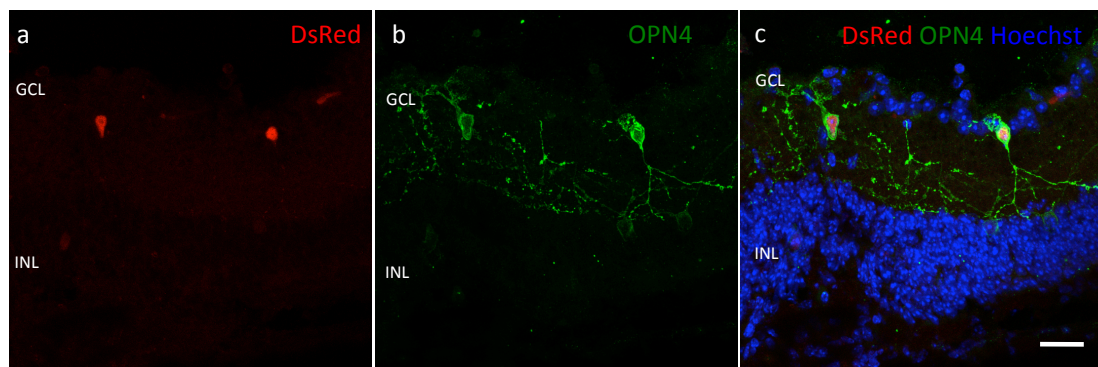


Figure 4.14 *In vivo* testing of AAV 2/8 Y733F 4xMg6- OPN4-IRES-DsRed vector.

Following immunohistochemistry for human melanopsin, images showing (a) DsRed (red), (b) human melanopsin (green) and (c) composite with Hoechst nuclear stain (blue) demonstrate a few transduced cells at the injection site. Scale bar 25 μ m.

4.3.13 Transduction of the degenerate retina by rAAV 2/8 Y733F CMV.CBA-

OPN4-IRES-DsRed

The rAAV2/8 Y733F CMV.CBA-*OPN4-IRES-DsRed* vector was injected into the subretinal space of six to eight week old *rd1* mice. Transduction was assessed after 4 months by immunohistochemistry of retinal flatmounts to obtain information about the area of melanopsin expression, and also by evaluating histological sections.

Examining retinal flatmounts stained for human melanopsin, a wide area of retinal transduction was apparent in treated eyes, but none in age-matched untreated control eyes, figure 4.15a. This was confirmed on histological sectioning which demonstrated widespread human melanopsin expression throughout the layers of the degenerate retina in treated eyes, but none in control eyes, figure 4.15b. Human melanopsin showed appropriate membrane localisation. In order to identify which cell types had been transduced by the vector, further immunohistochemistry was performed.

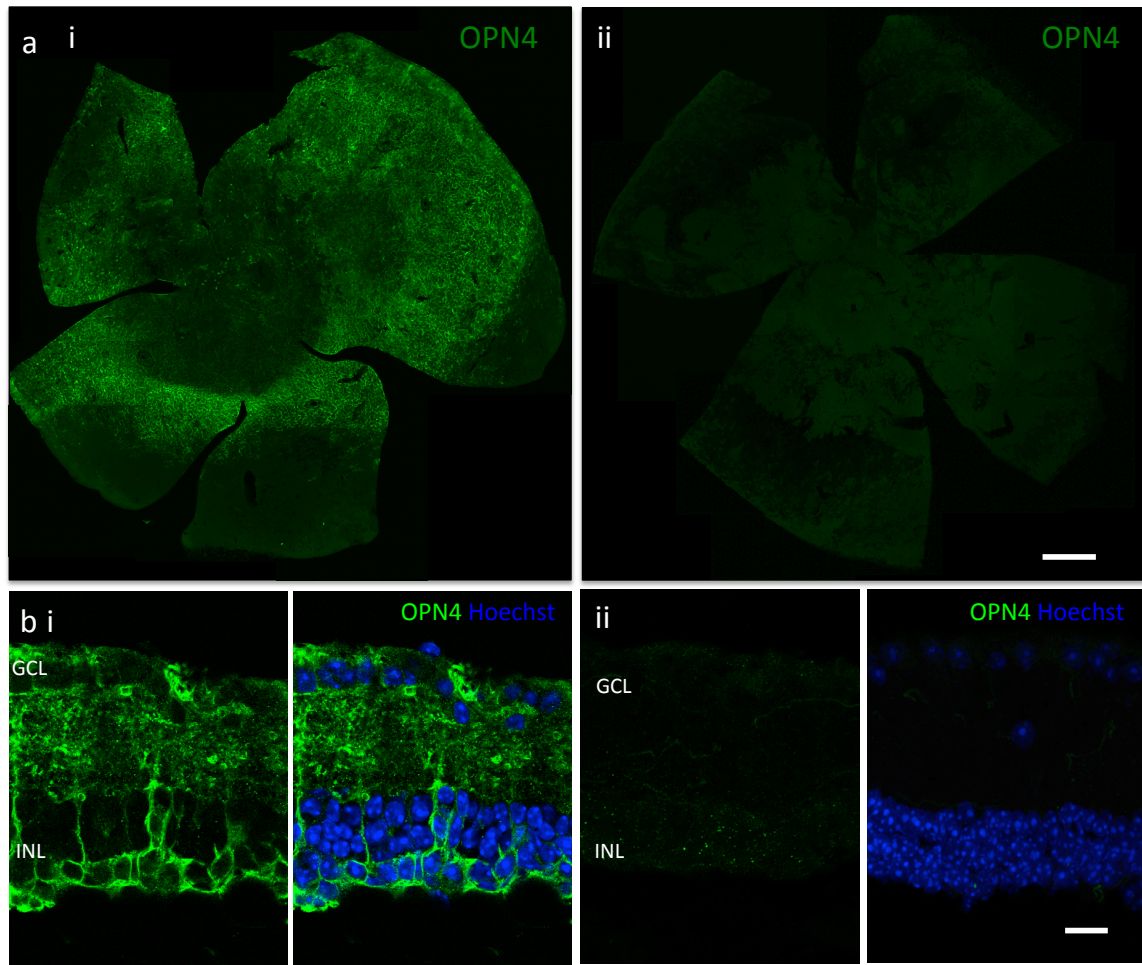


Figure 4.15 Retinal transduction in the *rd1* mouse using Adeno-associated virus (AAV) serotype 2/8 Y733F expressing human melanopsin.

(a) Flatmount of a (i) treated retina stained for human melanopsin showing extent of transduction at 4 months and (ii) an untreated control eye stained for human melanopsin. (b) Cross section of (i) treated retina showing extensive OPN4 expression (green) at 4 months after treatment and (ii) untreated control eye, Hoechst nuclear stain in blue. GCL= ganglion cell layer, INL= inner nuclear layer. Scale bar a=500 μ m , b= 25 μ m.

4.3.13.1 Calbindin antibody

Retinal flatmounts and histological sections were co-labelled with human melanopsin and calbindin antibodies, the latter staining horizontal cells and cholinergic amacrine cells. This demonstrated alignment of calbindin and melanopsin staining, figure 4.16, indicating horizontal cells are transduced by this vector. Staining of flatmounts revealed a large network of transduced horizontal cells: the majority of cell bodies positive for calbindin also co-localised with DsRed expressed by the AAV vector, and horizontal cell membranes and processes colocalised with human melanopsin staining, figure 4.17.

4.3.13.2 Glutamate synthetase antibody

Histological sections stained with glutamate synthetase antibody to identify Muller cells showed some colocalisation of this staining with melanopsin antibody, indicating that some Muller cells are transduced by the vector, figure 4.18. Staining flatmounts with the same antibody, overlapping of glutamate synthetase and melanopsin signals are still apparent but a smaller proportion of Muller cells are transduced than horizontal cells identified previously, figure 4.19.

4.3.13.3 PKC alpha antibody

Histological sections were stained to identify rod bipolar cells with PKC alpha antibody. A few cells in each histological section expressing melanopsin were identified as bipolar cells due to colocalisation with PKC alpha in the cell membrane, figure 4.20. Flatmount staining also revealed several bipolar cells in each field of view being transduced by the vector, but many cells staining positive with PKC alpha were not transduced. Therefore the rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector is able to transduce bipolar cells leading to melanopsin expression with moderate efficiency.

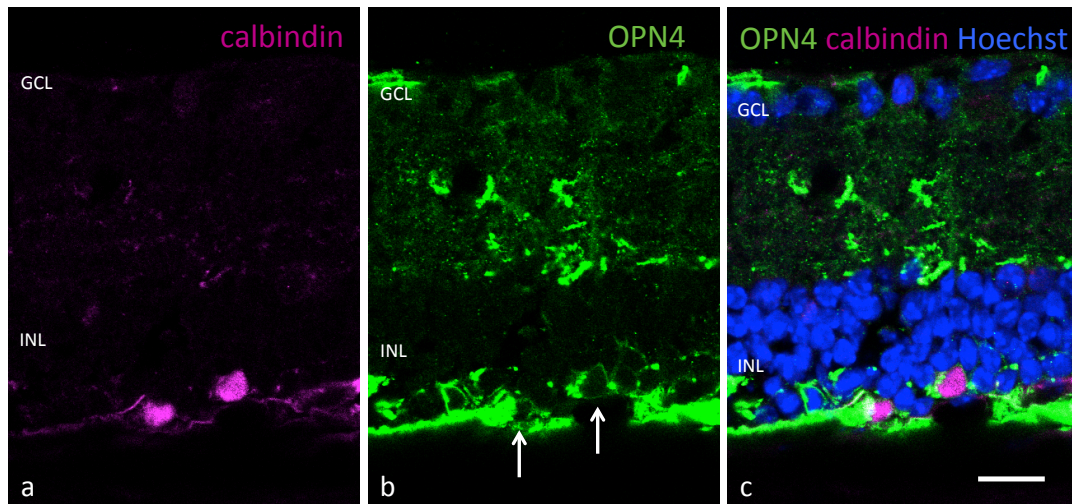


Figure 4.16 Immunohistochemistry of a retinal cross-section with human melanopsin and calbindin antibodies.

(a) Immunostaining using calbindin (purple) overlaid with (b) melanopsin signal (green) to create composite image (c) with Hoechst nuclear stain illustrates horizontal cell transduction. Scale bar 25 μm .

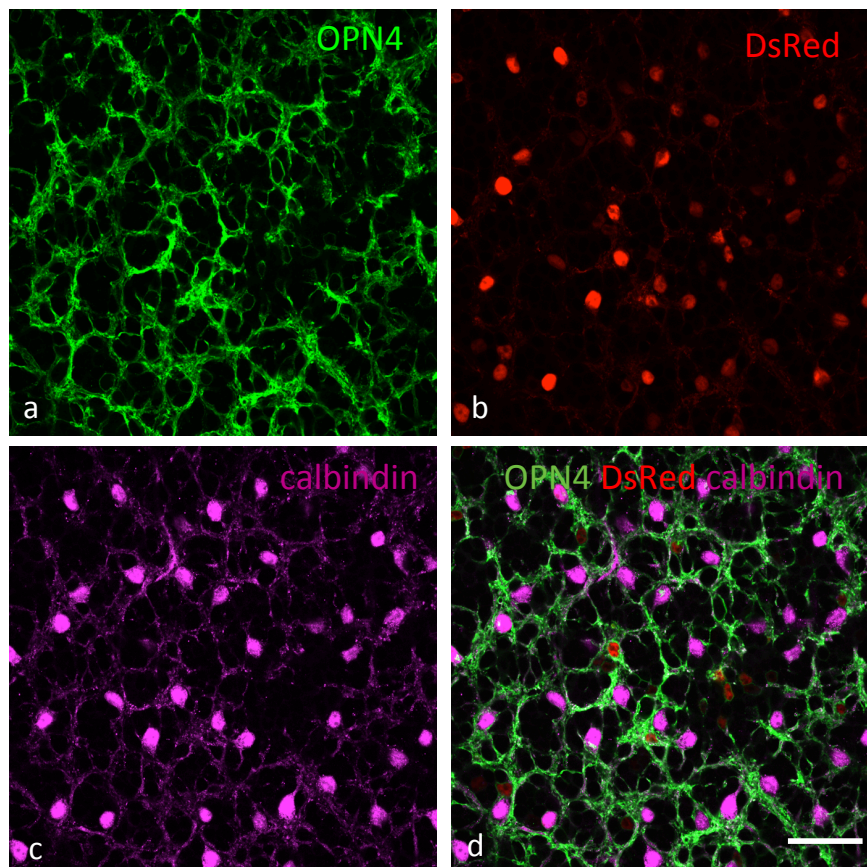


Figure 4.17 Immunohistochemistry of a retinal flatmount with human melanopsin and calbindin antibodies.

(a) Melanopsin signal (green) and (b) DsRed fluorescence (red) overlaid with immunostaining using calbindin (purple) illustrates horizontal cell transduction (d). Scale bar 25 μm .

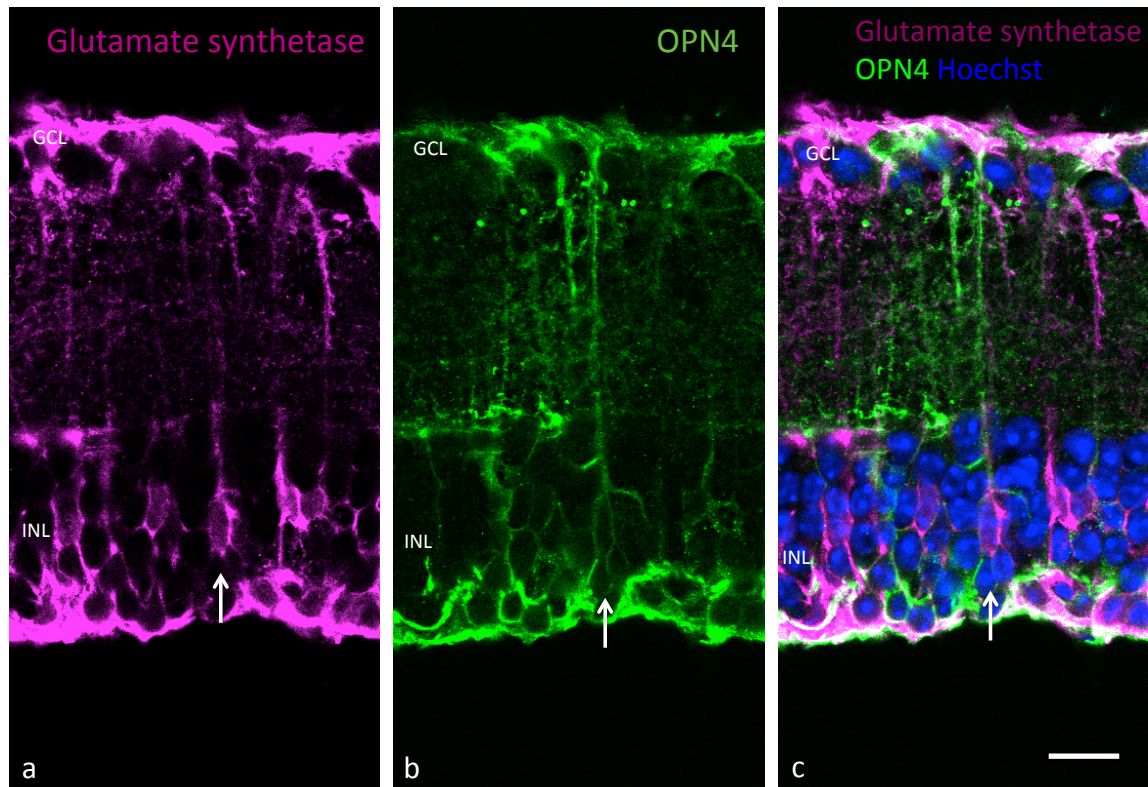


Figure 4.18 Immunohistochemistry of a retinal cross-section with human melanopsin and glutamate synthetase.

(a) Immunostaining using glutamate synthetase (purple) overlaid with (b) melanopsin signal (green) to create composite image (c) with Hoechst nuclear stain illustrates Müller cell transduction. Scale bar 25 μm .

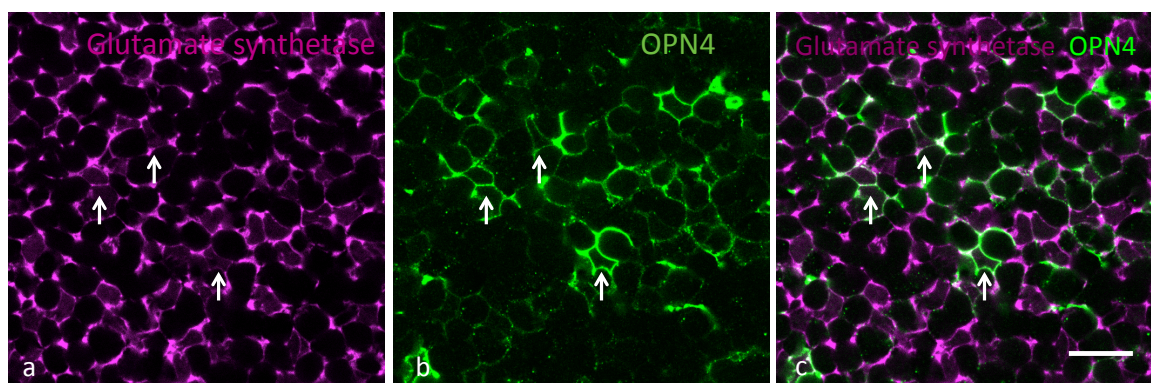


Figure 4.19 Immunohistochemistry of a retinal flatmount with human melanopsin and glutamate synthetase antibodies.

(a) Glutamate synthetase staining (purple) overlaid with and (b) human melanopsin staining to create composite image (c) illustrates Müller cell transduction (a few cells are highlighted with arrows). Scale bar 25 μm .

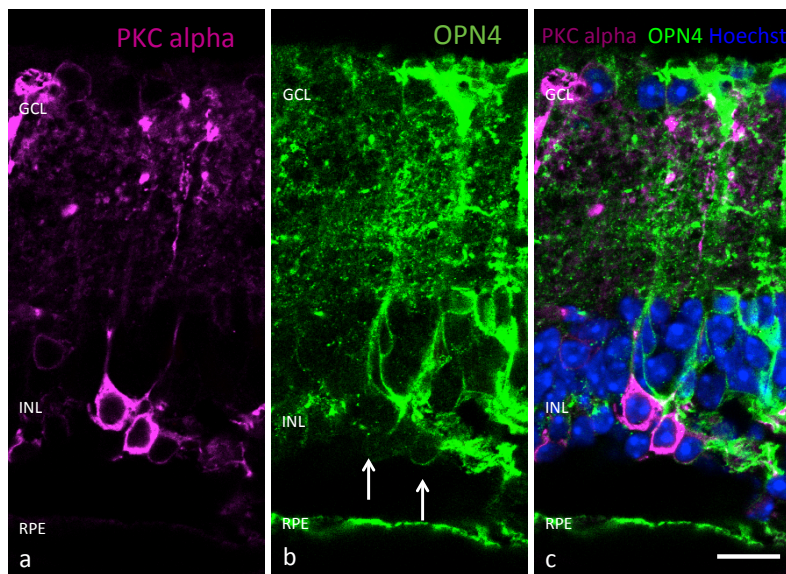


Figure 4.20 Immunohistochemistry of a retinal cross-section with human melanopsin and PkCalpha.

(a) Immunostaining using PKCalpha (purple) overlaid with (b) melanopsin signal (green) to create composite image (c) with Hoechst nuclear stain illustrates bipolar cell transduction. Scale bar 25 μ m.

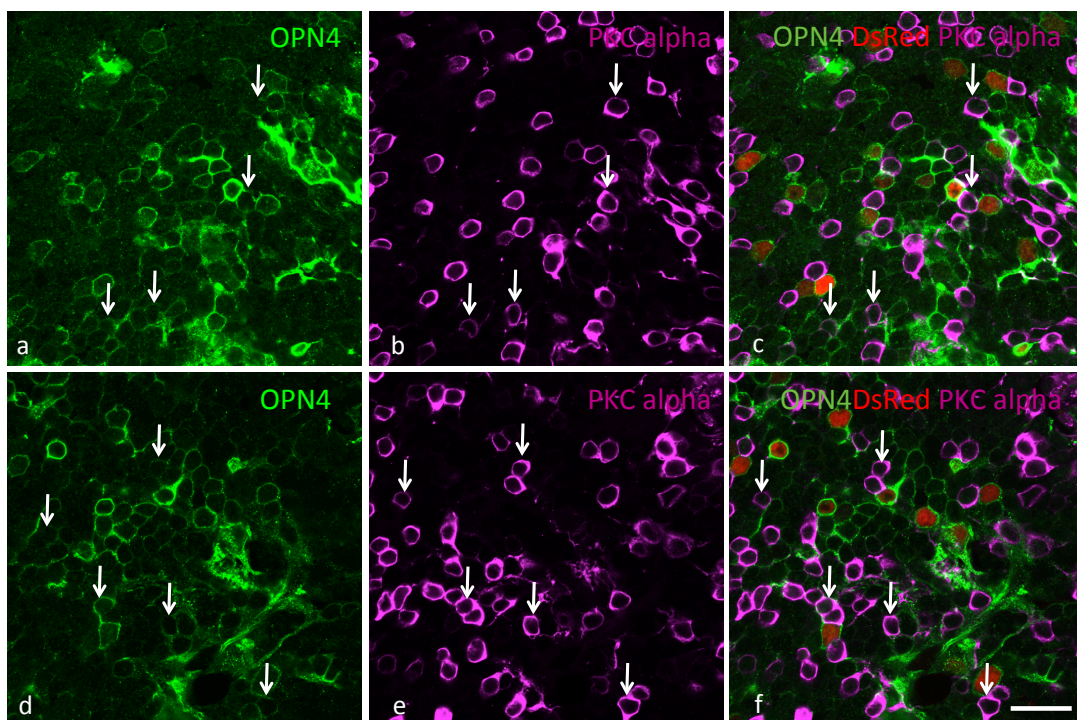


Figure 4.21 Immunohistochemistry of two retinal flatmounts with human melanopsin and PKC alpha antibodies.

(a,d) Human melanopsin staining (green) overlaid with (b, e) PKC alpha staining to create composite image (c,f) illustrates bipolar cell transduction (examples of a few transduced bipolar cells are indicated by arrows). Scale bar 25 μ m.

4.3.13.4 Brn3a antibody

Sections were stained with Brn3a and human melanopsin antibodies to identify whether ganglion cells were directly transduced by this vector. Examination of histological sections showed melanopsin staining within the ganglion cell layer, but it was unclear as to whether this staining was from Muller cell end feet or ganglion cell transduction, figure 4.22. Staining of flatmounts did not show melanopsin staining of ganglion cell membranes. Therefore, there does not appear to be any significant direct transduction of ganglion cells by this vector. Some melanopsin expressing axonal processes did appear to meet Brn3a positive ganglion cells. These could be melanopsin expressing bipolar cells synapsing with ganglion cells, or Muller cell end feet surrounding them.

4.3.14 Transduction of human retinal explants

Small retinal explants taken from patients having retinal detachment surgery were cultured *ex-vivo* and transduced with the rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector. After 10 days, these were fixed and stained for human melanopsin. A few cells in each explant expressed human melanopsin, figure 4.24. Staining of native melanopsin would also be expected with this antibody, however the tissue cultured was very degenerate and there was significant ganglion cell loss. Therefore at least some of the staining seen can be attributed to the melanopsin vector, especially that within the outer nuclear or inner nuclear layers. Due to the degenerate nature of the tissue, it was difficult to make further assessment of the cell types transduced.

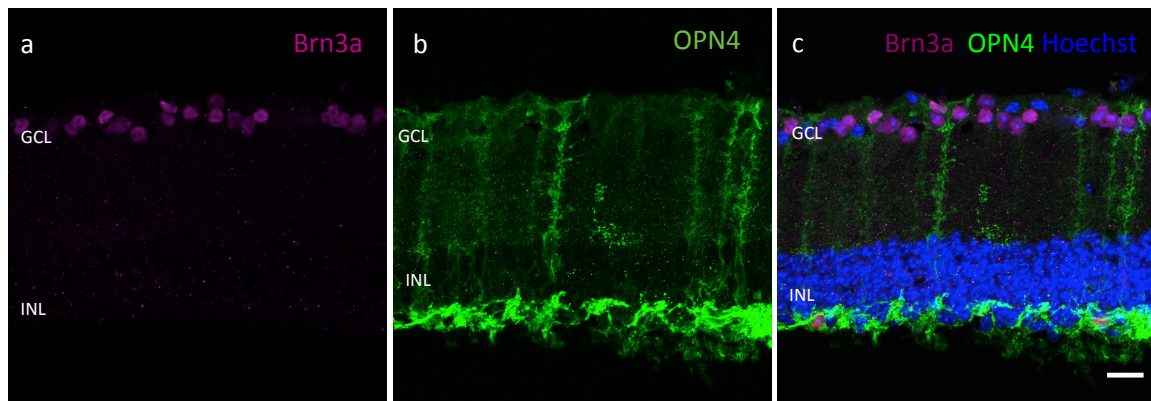


Figure 4.22 Immunohistochemistry of a retinal cross-section with human melanopsin and Brn3a

(a) Immunostaining using Brn3a (purple) overlaid with (b) melanopsin signal (green) to create composite image (c) with Hoechst nuclear stain does not illustrate any clear ganglion cell transduction. Scale bar 25 μ m.

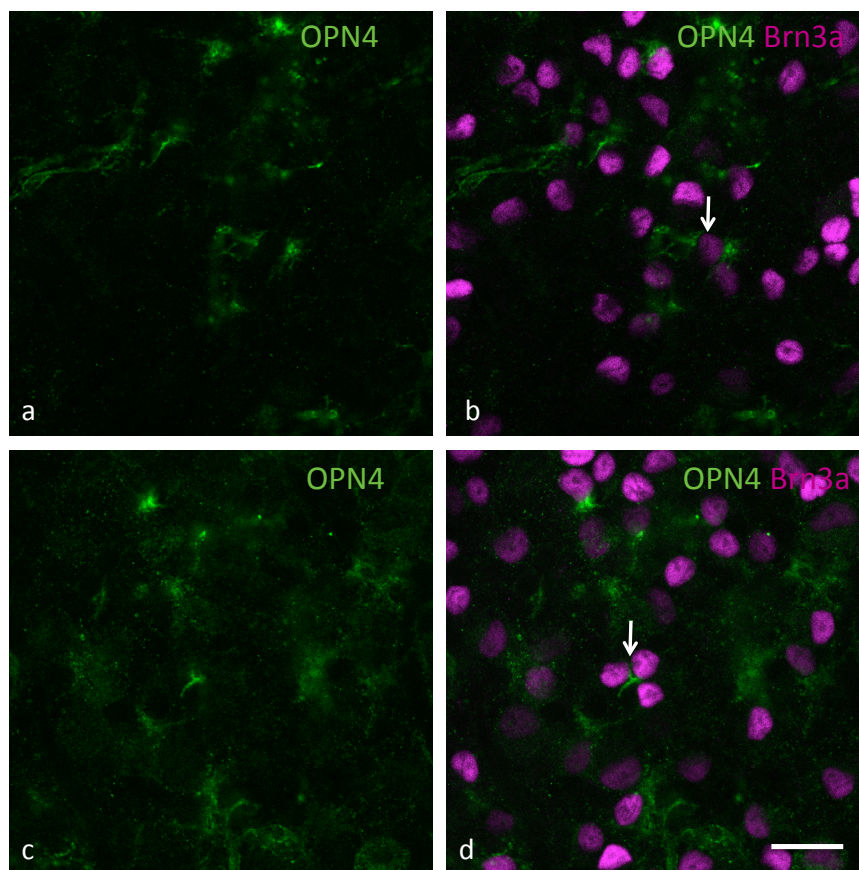


Figure 4.23 Immunohistochemistry of two retinal flatmounts with human melanopsin and Brn3a antibodies.

(a,c) Human melanopsin staining (green) overlaid with (b, d) Brn3a staining to create composite images does not indicate any transduction of ganglion cells. Some melanopsin expressing processes meet Brn3a expressing cells: these could be Muller cell end feet in (b) or bipolar cell synapses in (d) (highlighted with arrows). Scale bar 25 μ m.

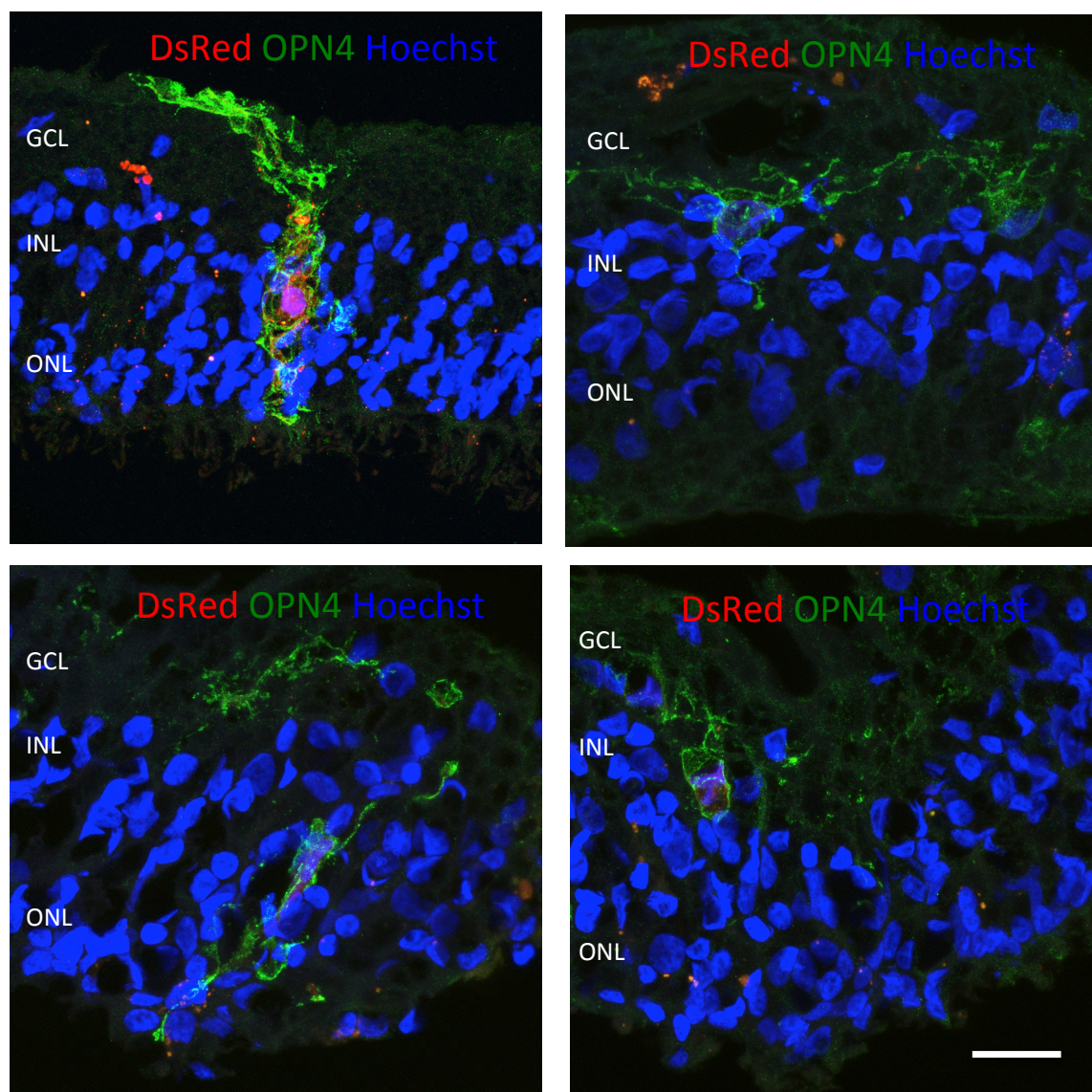


Figure 4.24 Immunohistochemistry of human retinal explants with human melanopsin following *ex-vivo* culture.

All images show overlay of DsRed (red) with human melanopsin (green) and Hoechst nuclear stain (blue). A few cells express human melanopsin in each field of view. Scale bar 25um.

4.4 Discussion

In this chapter a vector for delivery of human melanopsin to the degenerate retina has been produced, optimised and tested. Several promoters were trialled with the ubiquitous CMV.CBA enhancer/promoter driving the highest levels of expression. Optimal transduction was obtained using the AAV2/8 Y733F capsid following subretinal injection in *rd1* mice, using which melanopsin was widely expressed in the cells of the inner nuclear layer including horizontal cells, bipolar cells and some Muller cells. The vector also showed some ability to transduce human retinal tissue, validating its potential use as a therapy for end-stage retinal degenerations.

Despite some evidence of function *in vitro*, the CHX10 promoter was not able to drive human melanopsin expression *in vivo*. This could be due to a number of factors. Firstly, in the potentially hostile environment of the degenerate retina where many genes are being down-regulated (190), the CHX10 promoter may no longer be active. Secondly, the original paper described a 3kb region upstream of the mouse *Chx10* coding sequence which had promoter activity, within which a 500bp fragment was also able to drive *Chx10* expression. This smaller fragment from the human sequence was used in the melanopsin vector, and it may be that human promoter requires the upstream region for binding of transcription factors. Furthermore, melanopsin expression seen *in vitro* may have been driven by the ITRs in the plasmid, which also have weak promoter activity, rather than the CHX10 promoter itself. Further experiments using a plasmid containing the CHX10 promoter but without ITRs are required to answer this question.

The 4xMg6 promoter again showed some activity *in vitro* but using the rAAV2/8 Y733F capsid was able to drive melanopsin expression only in a few cells around the injection site *in vivo*. Bipolar cells are known to be difficult to target using AAV (188) and other

groups have required the use of new generation of AAV capsids such as the synthetic AAV2/8 BP2 capsid in conjunction with this promoter to drive targeted expression (160). Therefore further work is required with this promoter using other AAV capsids to direct melanopsin expression to ON bipolar cells in the degenerate retina.

In this chapter, rAAV2/2 Y44F and rAAV/8 Y733F vectors expressing human melanopsin under the control of a ubiquitous CMV.CBA enhancer/promoter were compared. There was markedly more transduction using the AAV8 capsid mutant compared to the AAV2 capsid mutant, whereas this difference was much less evident using the GFP vectors tested in chapter 3, (except for transduction of human bipolar cells by the AAV8 capsid mutant). The difference between transduction by the melanopsin vectors may be due to titre: the AAV2 mutant vector had a titre of 2.2×10^{11} gp/ml whereas the AAV8 mutant vector had a 1×10^{12} gp/ml. Further work may be required to produce an AAV2 capsid mutant vector with a higher titre and compare this to the AAV8 mutant. Alternatively it may be that the AAV8 capsid mutant is actually better at transducing cells of the degenerate retina being able to bind to remaining cell surface receptors for entry into the cell and also better avoid proteasome mediated degradation.

The AAV 2/8 vector containing a CMV.CBA enhancer/promoter was able to successfully transduce RPE, horizontal cells, some Müller cells and some bipolar cells. No direct ganglion cell transduction was evident. Therefore any potential functional effects seen following treatment with the vector are likely to be transmitted to ganglion cells via synaptic pathways through the retina, and would not be attributed to simply augmenting the amount of melanopsin in ipRGCs alone.

Interestingly, despite high levels of melanopsin expression following plasmid transfection and AAV transduction using the CMV.CBA enhancer/promoter, DsRed expression was variable. Previous studies have shown that expression from an encephalomyocarditis virus IRES sequence, such as that used in the melanopsin plasmid, can be significantly lower than that of the first gene both *in vitro* and *in vivo* (199). This may be due to the requirement of IRES trans-acting factors for IRES-dependent translation, which vary between cell types and species (200).

Finally the vector was tested on human retinal tissue to assess its ability to transduce human cells. Melanopsin expression was detected using immunohistochemistry. The antibody used would also detect native melanopsin expressed in human ipRGCs. However, since this patient had a long-standing retinal detachment, the tissue used was very degenerate and few ganglion cells were evident. Furthermore, melanopsin expression seen in the outer and inner nuclear layers were indicative of viral transduction since native melanopsin would not be found in these layers. Thus the only conclusions that can be made are that the vector was able to transduce some human retinal cells, but the level of transduction may have been higher in healthier tissue. Further work will include testing this vector on further human retinal explants.

4.5 Conclusions

Work presented in this chapter has identified that of those assessed, the CMV.CBA enhancer/promoter and rAAV2/8 Y733F capsid resulted in maximal expression of human melanopsin in the degenerate retina. Following a single subretinal injection, a large area of retina was transduced and immunohistochemistry revealed transduction of RPE, horizontal, Müller and bipolar cells after four months. Melanopsin was also expressed in human retinal tissue *ex-vivo* following vector transduction. This approach

does include transduction of cells that are theoretically 'off target' such as Muller cells, however the effect of this could only be known by functional testing, as will be addressed in Chapter 6. The next question is whether or not the melanopsin itself should be codon optimised to increase expression levels of the protein – this is investigated in the following chapter.

Chapter 5 Codon optimisation of human melanopsin

5.1 Introduction

Previous chapters have discussed optimisation of the AAV capsid and promoter for delivery of human melanopsin to the degenerate retina. A further question is whether the sequence of melanopsin itself should be optimised and if this would be effective at increasing levels of expression.

Translation is the process by which protein is synthesised from a messenger RNA (mRNA) template. It is facilitated by a ribosome, which induces binding of a transfer RNA (tRNA) that has a complementary sequence to the mRNA and carries a specific amino acid (201), figure 5.1. As the ribosome ‘reads’ the mRNA sequence, amino acids held by adjacent tRNAs are linked by peptide bonds and a polypeptide chain is formed. Folding of the polypeptide chain then occurs with the aid of molecular chaperones, giving the protein its functional three-dimensional structure (202). This process may occur alongside translation, with the N-terminal polypeptide chain folding as the C-terminal end of the protein is being synthesised.

A codon consists of three DNA or RNA nucleotides that encode a specific amino acid or stop signal for protein synthesis. There is redundancy in this genetic code since 61 potential codons represent 21 amino acids (the other 3 codons encode stop signals), for example the bases GAA and GAG both encode glutamic acid, figure 5.2. For each amino acid, the frequency at which different codons representing it are utilised is not random but there appears to be a preference for certain codons known as ‘codon usage bias’.

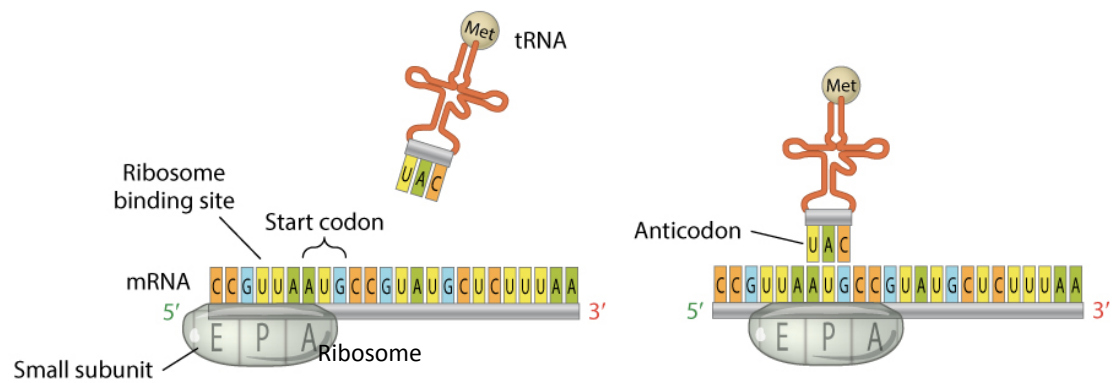


Figure 5.1 Initiation of translation.

A ribosome binds to the appropriate site on a strand of messenger RNA and initiates translation by inducing binding of a transfer RNA with a complementary sequence to the start codon. Adapted from (201).

		Second Letter					
		U	C	A	G		
1st letter	U	UUU Phe UUC UUA Leu UUG	UCU Ser UCC UCA UCG	UAU Tyr UAC UAA Stop UAG Stop	UGU Cys UGC UGA Stop UGG Trp	3rd letter	U C A G
	C	CUU Leu CUC CUA CUG	CCU Pro CCC CCA CCG	CAU His CAC CAA Gln CAG	CGU Arg CGC CGA CGG		U C A G
	A	AUU Ile AUC AUA AUG Met	ACU Thr ACC ACA ACG	AAU Asn AAC AAA Lys AAG	AGU Ser AGC AGA Arg AGG		U C A G
	G	GUU Val GUC GUA GUG	GCU Ala GCC GCA GCG	GAU Asp GAC GAA Glu GAG	GGU Gly GGC GGA GGG		U C A G

Figure 5.2 The genetic code.

This consists of 64 triplets of nucleotides, 61 of which encode an amino acid, with 3 triplets being stop codons. There are only 21 amino acids, and therefore there is redundancy in the code. From (203).

For instance there are six codons for the amino acid leucine, but in *E.coli* the CUG codon is used in more than fifty percent of cases (204). The use of a synonymous codon for the same amino acid can have significant effects on the level of gene expression, RNA processing, protein translation, folding and function (205).

Codon usage bias has largely been studied in prokaryotic organisms and there are a number of possible explanations. There is often variation in codon usage between different genes in the same genome, with codon bias being more marked for highly expressed genes. The classical explanation for this is that codon usage has evolved to match the population of tRNAs present in the cell that display the respective anti-codon sequence for the appropriate amino acids since some tRNAs are more abundant than others. This would allow increased efficiency of translation and protein synthesis (206). However there are likely to be many other additional reasons for codon usage bias since it can vary markedly within a gene itself. Certain codons may undergo selection if they create or disrupt motifs recognised by transcription factors, regulatory elements such as micro RNAs or disrupt splice sites if at an exon-intron boundary (205). In some cases, the use of poorly adapted codons may result in ribosomal pausing and slowing of translation to allow time for post-translational modification and protein folding (207).

The process of codon optimisation adjusts nucleotides of the gene coding sequence so that codons used match the most abundant tRNAs in the host cell. One method by which codon optimisation can be measured is to use the codon adaptation index (CAI), which uses a reference set of highly expressed genes in a particular species to assess the relative benefits of using a particular codon in that species (208). A score can be calculated for a gene of interest based on the frequency of used codons both in the original (wild type) and the codon optimised gene sequence.

Two large studies have demonstrated increased gene expression in approximately sixty percent of genes tested following codon optimisation (209, 210). The latter study describes a mean increase in gene expression of 3.5 fold following codon-optimisation, with a 50 fold increase being demonstrated in one gene tested (210). The technique has also been used in systemic gene therapy in animal models of Duchenne muscular dystrophy (211) and haemophilia B (212) where high gene expression levels are required. A codon-optimised sequence has subsequently been used in a gene therapy clinical trial for haemophilia B (213). In addition, if high transgene expression for gene therapy can be achieved using a codon optimised sequence, it may be possible to reduce the dose of vector administered, helping to minimise any immune response. Therefore, it would be useful to investigate whether codon optimisation of human melanopsin could increase levels of expression for the purpose of optogenetic therapy in the degenerate retina.

5.2 Experimental design

5.2.1. Optimisation of the human melanopsin sequence

Codon optimisation was performed and the usage of rare codons minimised using a computerised algorithm (Genscript, USA). Other parameters optimised were GC content, and AU-rich elements.

5.2.2 Cloning

A plasmid vector was cloned containing codon optimised human melanopsin under the control of the CMV enhancer/ CBA promoter discussed in chapter 4.

5.2.3 *In vitro* testing

Levels of expression of human melanopsin from this plasmid were compared to that from the wild type melanopsin plasmid *in vitro* following transfection of HEK293T cells. Three techniques were used for this purpose: quantification of immunocytochemistry for human melanopsin, fluorescence-activated cell sorting (FACS) and Western blot. For each technique, HEK293T cells were transfected with 1µg plasmid vector containing either wild type or codon-optimised melanopsin. Untransfected cells seeded at the same density were used as controls. Cells were fixed or harvested at 48 hours as required.

5.2.4 Functional testing

Functional activity of codon-optimised melanopsin was compared to wild type using calcium imaging. Cells were loaded with a calcium indicator dye that becomes fluorescent when bound to calcium. Light activation of melanopsin causes calcium influx into a cell, therefore measuring change in fluorescence of the indicator dye was used to

assess functional activity of melanopsin. Imaging was performed 48 hours after transfection of HEK293T cells in the presence of 9-*cis* retinal using a Fluo-4 calcium indicator at room temperature. Cells were stimulated with 480nm light pulses lasting 0.5 seconds every 2 seconds for one minute, which stimulated melanopsin and also allowed imaging of the calcium dye.

5.2.5 *In vivo* testing

AAV vectors were produced containing wild type and codon optimised human melanopsin and injected into the subretinal space of 6 to 8 week old *rd1* mice, a mouse model of end-stage retinal degeneration. After 3 weeks, retinas were harvested and immunohistochemistry done for human melanopsin to assess expression levels.

5.3 Results

5.3.1 Codon optimisation of human melanopsin

Codon optimisation was performed by comparing codon usage in the human melanopsin sequence with a reference database of codon usage in human cells, via a computerised algorithm (done by GenScript, USA). A score was given to each codon in the human melanopsin sequence, with a value of 100 indicating that the codon present had the highest usage frequency for that amino acid in human cells.

Of the 479 codons in human melanopsin, 64% had a score of 91-100 indicating that these codons have a high frequency of usage in human cells, figure 5.3. The overall codon adaptation index for melanopsin was 0.83 (with 1.0 representing the best codon usage for the desired host cell). However, twelve percent of codons had score of 50 or less indicating a low frequency of usage. For the purpose of optogenetic gene therapy, the protein of interest must be highly overexpressed in the target cell. If a rare codon is used then the corresponding tRNA pool may be insufficient to facilitate high levels of translation, restricting the overall gene expression in that cell. Therefore, using a predefined protocol, these rare codons were changed to those with higher usage in human cells without changing the resulting amino acid sequence, figure 5.3.

In the optimised sequence, only two percent of codons had a score of 50 or lower, figure 5.3, and the overall codon adaptation index increased marginally from 0.83 to 0.85. Of note, following optimisation of other elements discussed below, the number of codons with a score of over 90 was also affected, reducing this from 64 to 56%.

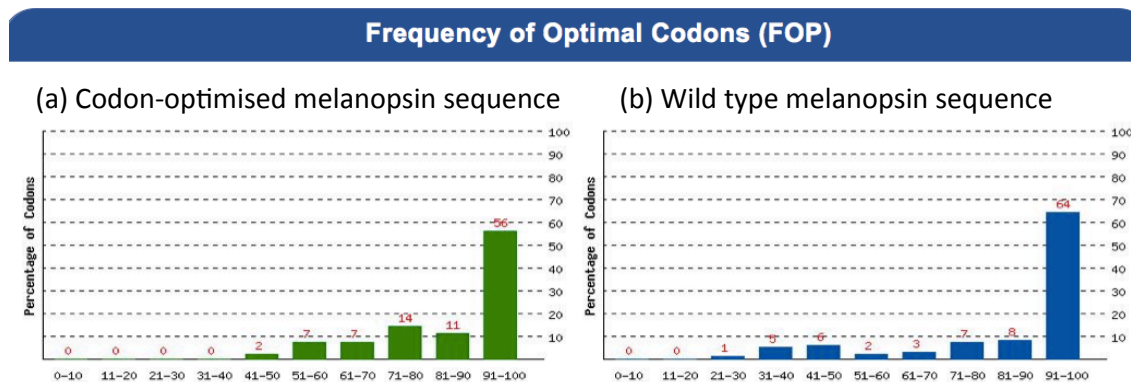


Figure 5.3 Codon optimisation.

A score was given to each codon in the human melanopsin sequence, with a value of 100 indicating that the codon present had the highest usage frequency for that amino acid in human cells. The percentage of codons for each group are shown for (a) the codon – optimised and (b) the wild type melanopsin sequence.

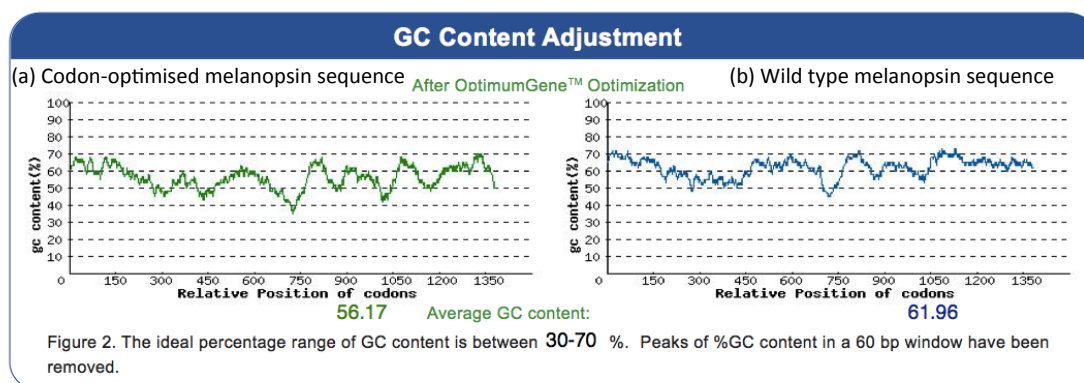


Figure 5.4 Adjustment of GC content.

Percentage GC content shown for (a) codon optimised and (b) wild type human melanopsin sequences.

5.3.2 Adjustment of GC content

In addition to codon usage optimisation, the protocol used allowed GC content to be considered when selecting or modifying codons. GC-rich areas can be difficult to sequence and GC-rich mRNA is known to form strong secondary structures which in bacteria can prevent ribosome binding and therefore initiation of translation (214). GC content of the wild type human melanopsin sequence is 61.96%, which is within the ideal GC range of 30 to 70%. Codon optimisation reduced the GC content to 56.17%, figure 5.4, which is only a small absolute change but may still have a positive effect on mRNA secondary structure.

5.3.3 Adjustment of AU-rich elements

AU-rich elements (AREs) are sequences found in the 3' untranslated region (UTR) of an mRNA molecule and are implicated in mRNA regulation (215). They commonly consist of motifs such as AUUUA or multiples of this within a U-rich region (215). The sequences are targets for ARE-binding proteins, some of which promote RNA degradation whereas others have a stabilising influence (216). MicroRNAs may also bind to this region causing mRNA degradation (216).

Only the coding sequence for human melanopsin was included in the plasmid and AAV vector produced, and therefore the elements present in the native 3'UTR did not need to be considered for optimisation. However, the coding sequence itself was screened for any destabilising elements and one ATTTA motif was found. This was not located in a U-rich region, which would usually be necessary for binding by regulatory ARE-binding proteins. Nevertheless, this motif was modified to ATCTA in case it had any destabilising effect on coding sequence mRNA.

5.3.4 Optimised human melanopsin sequence

The final optimised human melanopsin sequence contained changes in 304 out of 1437 nucleotides (22% of the sequence), figure 5.5. This was likely to result in a change in mRNA secondary structure between wild type and codon-optimised sequences, as predicted by a software model, figure 5.6. The amino acid sequence of the protein produced was unchanged by all modifications, as confirmed by protein alignment, figure 5.7. The codon-optimised nucleotide sequence was synthesised by GenScript, USA.

5.3.5 Cloning of a vector containing codon-optimised human melanopsin

Codon-optimised human melanopsin was cloned into a plasmid vector containing AAV2 ITRs. The vector contained a bicistronic expression cassette with codon-optimised human melanopsin and DsRed under control of a CMV enhancer/CBA promoter linked by an encephalomyocarditis virus IRES sequence, as per the plasmid vector containing wild type human melanopsin produced in Chapter 4. All other elements were also identical including AAV2 ITRs, WPRE and bovine polyA sequence, figure 5.8a.

The CMV.CBA-codon-optimised-*OPN4*-IRES-DsRed construct was produced following amplification of individual elements by PCR. Melanopsin was isolated in two fragments with a 200 base pair overlap, the first part joined to the promoter, and second part joined to the IRES-DsRed sequence using the PCR technique SPLICE (172). Subsequently, these two parts were joined by PCR reaction, figure 5.8 b,c. The construct was inserted into the AAV backbone using *MfeI* and *NotI* restriction sites. After cloning, the presence of the construct was confirmed by restriction digest and sequencing, figure 5.8d, and the presence of ITRs by *XmaI* digestion as described in Chapter 4, figure 5.8e.

Optimized	1	ATGAACCCCCAAGTGGACCCGAGTCCCTCCCTCACCTACCCAGGAACCTCATGTATG
Original	1	ATGAACCTCTCTTCGGGGCCAAGAGTCCCGCCAGCCCAACCAAGAGCCAGCTGCATG
Optimized	61	GCTACCCCGCCCCCAAGCTGGTGGGACAGCTCCAGTCTAGTATTTCAAGCCTGGGC
Original	61	GCCACCCAGCACCACCAGCTGGTGGGACAGCTCCAGAGCAGCATCTCCAGCCTGGGC
Optimized	121	CGCCTGCCCTCTATCAGTCCAACCGCTCCAGGAACATGGGCCGCTGCATGGGTGCCTCTG
Original	121	CGGCTTCATCCATCAGTCCCACAGCACCTGGGACTTGGGCTGCTGCCTGGGTCCCCCTC
Optimized	181	CCAACCGTGGACGTCCCTGATCACGCCATTACACACTGGGCAGTGTGATTCTGCTGGTC
Original	181	CCCACGGTTGATGTTCCAGACCATGCCCACTATACCTTGGGCACAGTGATCTTGTCTGGTG
Optimized	241	GGGCTGACTGGAATGCTGGGCAACCTGACTGTGATCTATACCTTCTGCAGGTACCGAAGC
Original	241	GGACTCACGGGGATGCTGGGCAACCTGACGGTCATCTATACCTTCTGCAGGAGCAGAAGC
Optimized	301	CTGCGAACCCTGCCAACATGTTTATCATTAATCTGGCTGTGAGTGACTTCCTGATGAGC
Original	301	CTCCGGACACCTGCCAACATGTTTATCATCACTCGCGGTGAGCGATTCTCATGTCTC
Optimized	361	TTCACCCAGGCCCCAGTGTTCCTTACCTCCTCTGTACAAGCAGTGGCTGTTCGGGGAG
Original	361	TTCACCCAGGCCCTGTCTTCTTACCAGTAGCTCTATAAGCAGTGGCTCTTGGGGAG
Optimized	421	ACCGGATGCGAATTCTATGCCCTTTGTGGCGCTCTGTTTGGGATTAGTTCAATGATCACC
Original	421	ACAGGCTGCGAGTTCTATGCCCTTCTGTGGAGCTCTTTTGGCATTCTCCATGATCACC
Optimized	481	CTGACAGCTATTGCACTGATCGGTACCTGCTCATCACAAGCCTCTGSCAACTTTCGGA
Original	481	CTGACGGCCATCGCCCTGGACCGCTACCTGGTAATCACACGCCCGCTGGCCACCTTTGCT
Optimized	541	GTCGCCCTCAAACGGAGAGCCGCTTTTGTGCTGCTGGAGTCTGGCTGTACGCACTGSC
Original	541	GTGGCGTCCAAGAGGCGTGGCGCATTTGTCTGCTGGGCGTTTGGCTCTATGCCCTGGCC
Optimized	601	TGGTCTCTGCCCCCTTCTTTGGGTGGAGTGCCTATGTGCCAGAGGGAGCTGTGACATCC
Original	601	TGGAGTCTGCGACCTTCTTTCGGCTGGAGCGCTACGTGCCGAGGGGTGTGACATCC
Optimized	661	TGCTCTTGGGATTACATGAGCTTACACCCGCTGTGAGAGCATATACTATGCTGCTGTGC
Original	661	TGCTCCTGGGACTACATGAGCTTACGCCGCGCTGCGTGCCTACACCATGCTTCTCTGC
Optimized	721	TGTTTTGTCTCTTTCTGCCACTGTGATCATCATCTACTGTACATTTTCATCTTTAGA
Original	721	TGCTTCGTGTCTTCTCCTCTGCTTATCATCATCTACTGTACATCTTCATCTTCAGG
Optimized	781	GCCATTAGGGAGACCGGCAGGGCTCTGCAGACATTGCGGGCATGCAAGGGCAACGGGAG
Original	781	GCCATCCGGGAGACAGGACGGGCTCTCCAGACCTTCGGGCGCTGCAAGGGCAATGGCGAG
Optimized	841	TCTCTGTGGCAGCGACAGCGGCTGCAGAGCGAGTGAAGATGCTAAAAATTATGCTGCTG
Original	841	TCCTGTGGCAGCGGACGGGCTGCAGAGCGAGTGAAGATGGCAAGATCATGCTGCTG
Optimized	901	GTCATCCTGCTGTTCTGCTCTGCTGGGCTCCCTATTCCGCTGTGGCCTGGTGGCCTTT
Original	901	GTCATCCTCTCTTCTGCTCTCTGCTGGGCTCCCTATTCCGCTGTGGCCTGGTGGCCTTT
Optimized	961	GCAGGGTACGCACACGTGCTGACACCTTATATGAGCTCCGTGCCAGCCGTCAATGCCAAG
Original	961	GCTGGGTACGCACACGTCTGACACCTTACATGAGCTCGGTGCCAGCCGTCAATGCCAAG
Optimized	1021	GCTTCCGCAATCCACAATCCATTATCTAGCCATTACTCATCTAAATATCGAGTGGCC
Original	1021	GCCTCTGCAATCCACAACCCCATATTACGCCATCACCCACCCCAAGTACAGGGTGGCC
Optimized	1081	ATCGCTCAGCACCTGCCCTGTCTGCGAGTGTGCTGGCGTCTCCAGGCGACATCTCGA
Original	1081	ATTGCCACGACCTGCCCTGCCTGGGGGTGCTGCTGGGTGTATCAGCCGCGACAGTGC
Optimized	1141	CCCTACCTAGCTATCGTCCACCCACCGAAGCACTCTGACCTCTCATACAAGTAACCTG
Original	1141	CCCTACCCAGCTACCGCTCCACCCACCGCTCCACGCTGACCAAGCACTCTCAACCTC
Optimized	1201	TCAATGATTAGCATCCGACGGAGACAGGAGTCCCTGGGAGTGAGTCAGAAGTGGATGG
Original	1201	AGCTGGATCTCCATACGGAGGCGCCAGGAGTCCCTGGGCTCGGAGTGAGTGGGCTGG
Optimized	1261	ACCACATGGAAGCAGCCGCTGTCTGGGAGCAGCAGCAGCAATGGCAGGAGCCTG
Original	1261	ACACACATGGAGGACAGCTGTGTGGGAGCTGCCAGCAAGCAATGGGCGGTCCCTC
Optimized	1321	TATGGACAGGGCTGGAGGACCTGGAAGCAAAAGCCCCACCCCGCCCCAGGGCCATGAA
Original	1321	TACGGTCAGGGTCTGGAGGACTTGAAGCCAAGGCACCCCCAGACCCAGGGACACGAA
Optimized	1381	GCCGAGACCCAGGAAAACTAAAGGACTGATCCCAAGCCAGGACCCCAAGATGTAA
Original	1381	GCAGAGACTCCAGGGAAGACCAAGGGGTGATCCCCAGCCAGGACCCAGGATGTAG

Figure 5.5 The codon-optimised human melanopsin sequence.

An alignment of the codon optimised (upper) and wild type (lower) sequences to illustrate modified codons which are highlighted in red.

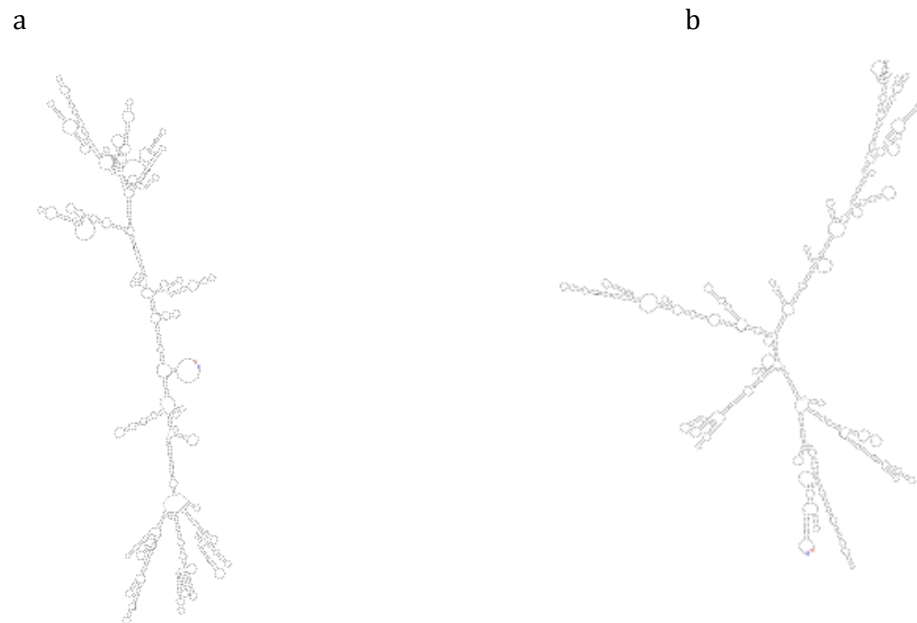


Figure 5.6 Messenger RNA secondary structure.

Predicted mRNA secondary structure from (a) codon optimised and (b) wild type human melanopsin sequences using RNA fold, Geneious v5.6.

Optimized	1	MNPPSGPRVPPSPTQEPSCMATPAPPSWWDSSQSSISSLGRLPSISPTAPGTWAAAWVPL
Original	1	MNPPSGPRVPPSPTQEPSCMATPAPPSWWDSSQSSISSLGRLPSISPTAPGTWAAAWVPL
Optimized	61	PTVDVPDHAHYTLGTVILLVGLTGMLGNLTVIYTFCSRSLRTPANMFIINLAVSDFLMS
Original	61	PTVDVPDHAHYTLGTVILLVGLTGMLGNLTVIYTFCSRSLRTPANMFIINLAVSDFLMS
Optimized	121	FTQAPVFFTSSLYKQWLFGETGCEFYAFCGALFGISSMITLTAIALDRYLVITRPLATFG
Original	121	FTQAPVFFTSSLYKQWLFGETGCEFYAFCGALFGISSMITLTAIALDRYLVITRPLATFG
Optimized	181	VASKRRAAFVLLGVWLYALAWSLPPFFGWSAYVPEGLLTSCSWDYSFPTPAVRAYTMLLC
Original	181	VASKRRAAFVLLGVWLYALAWSLPPFFGWSAYVPEGLLTSCSWDYSFPTPAVRAYTMLLC
Optimized	241	CFVFFLLPLIIICYIIFIRAIRETGRALQTFGACKNGESLWQRQLQSECKMAKIMLL
Original	241	CFVFFLLPLIIICYIIFIRAIRETGRALQTFGACKNGESLWQRQLQSECKMAKIMLL
Optimized	301	VILLFVLSWAPYSAVALVAFAGYAHVLTYPMSSVPAVIKASAIHNPIIYAITHPKYRVA
Original	301	VILLFVLSWAPYSAVALVAFAGYAHVLTYPMSSVPAVIKASAIHNPIIYAITHPKYRVA
Optimized	361	IAQHLPCLGVLGVSRRHSRPYPYSRSTHRSTLTSHTSNLSWISIRRRQESLGSESEVGW
Original	361	IAQHLPCLGVLGVSRRHSRPYPYSRSTHRSTLTSHTSNLSWISIRRRQESLGSESEVGW
Optimized	421	THMEAAAVWGAAQQANGRSLYGQGLEDEAKAPPRPQGHEAETPGKTKGLIPSQDPRM*
Original	421	THMEAAAVWGAAQQANGRSLYGQGLEDEAKAPPRPQGHEAETPGKTKGLIPSQDPRM*

Figure 5.7 Alignment of amino acid sequences

Translation of codon-optimised (upper) and wild type (lower) human melanopsin sequences illustrates identical amino acid alignment.

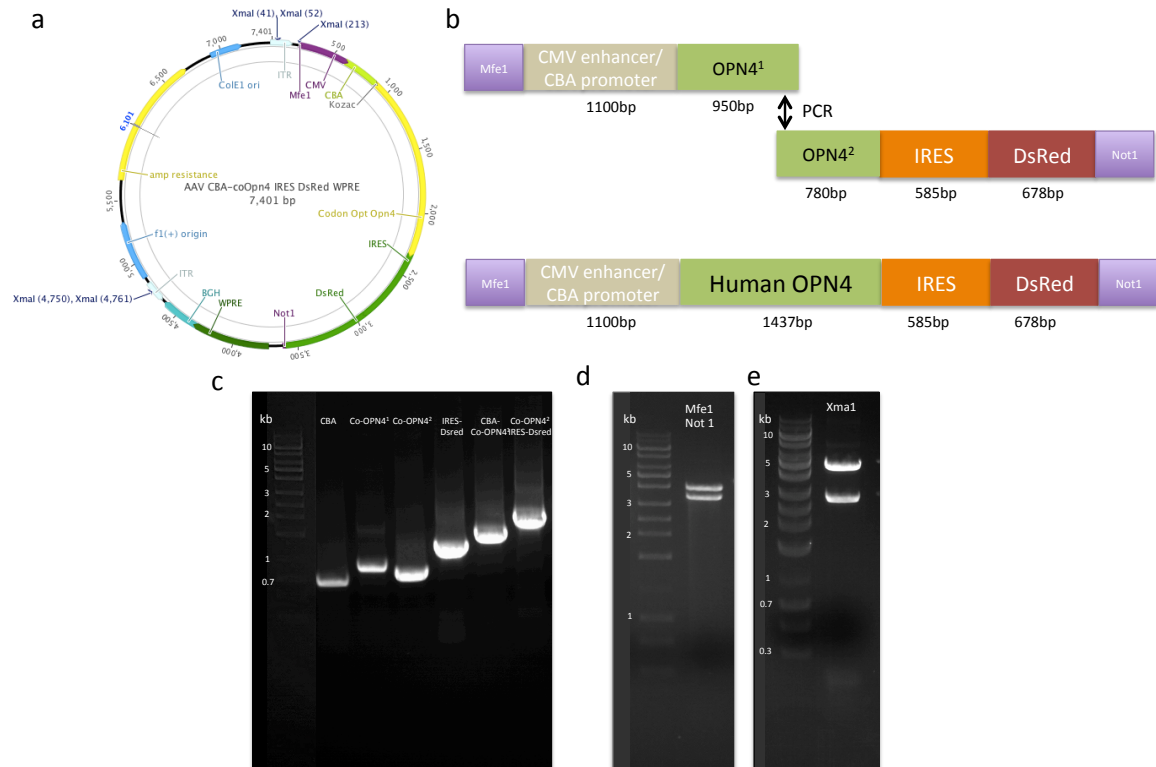


Figure 5.8 Cloning a plasmid vector containing codon-optimised melanopsin.

(a) Plasmid map illustrating the final plasmid to be cloned. Elements present include AAV2 ITRs, a CMV enhancer/CBA promoter, WPRE and bovine polyA sequence. (b) Cloning strategy: melanopsin was isolated in two fragments with a 200 base pair overlap, the first part joined to the promoter, and second part joined to the IRES-DsRed sequence using PCR. Subsequently, these two parts were joined by PCR reaction to form the final construct. (c) Each element to be included in the construct was isolated by PCR reaction, the CMV enhancer/CBA promoter joined to the first melanopsin fragment and IRES-DsRed to the second. (d) The presence of the construct in the cloned plasmid was confirmed by MfeI and NotI double digestion of the CMV.CBA-co-OPN4-IRES-Dsred plasmid. Expected fragment sizes were 4kb and 3.4 kb (e) The presence of ITRs was confirmed by XmaI digestion. Expected fragment sizes were 4.5 kb, 2.7kb and 165 bp.

5.3.6 Plasmid transfection of HEK 293T cells

HEK 293T cells were transfected with the CMV.CBA-codon-optimised-*OPN4*-IRES-Dsred plasmid, figure 5.9. Melanopsin expression was confirmed by immunocytochemistry on cells after fixation, demonstrating melanopsin production by the codon-optimised sequence and appropriate membrane localisation.

5.3.7 Comparison of melanopsin expression by wild type and codon-optimised plasmids using fluorescence quantification

HEK293T cells were transfected with plasmid vector containing either wild type or codon-optimised melanopsin, with untransfected cells used as controls. After 48 hours, cells were fixed and immunocytochemistry performed for melanopsin with a green fluorescent secondary antibody. Using an epifluorescence microscope, images of standard exposure time were taken of native DsRed fluorescence, melanopsin antibody staining and nuclear (Hoechst) staining for cell counting.

After 48 hours, there was good transfection of cells in both wild type and codon-optimised melanopsin groups as assessed by DsRed expression. Fluorescence from melanopsin antibody staining was greater in the wild type than in the codon-optimised group, figure 5.10a. This was confirmed by grey value analysis of fluorescence images which revealed no significant difference in DsRed fluorescence between groups but significantly higher fluorescence from melanopsin staining following transfection by the wild type compared to the codon-optimised plasmid ($p < 0.01$; 2 way ANOVA with Bonferroni post hoc test), figure 5.10b. Counting images of Hoechst stained nuclei in each group revealed no significant difference in cell density between the two groups ($p > 0.05$, unpaired t-test), figure 5.10c.

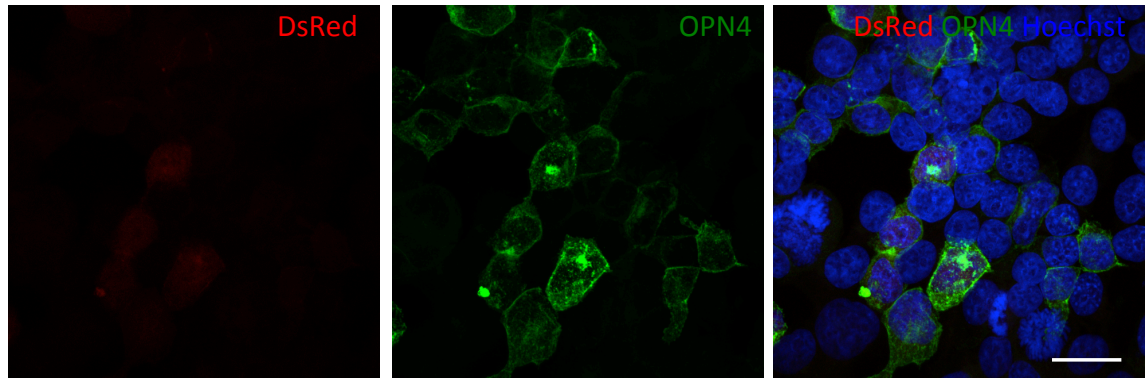


Figure 5.9 Transfection of HEK 293T cells with CMV.CBA-codon- optimised *OPN4*-IRES-DsRed plasmid.

DsRed fluorescence (red) is shown in the left hand panel, antibody staining for human OPN4 (green) is shown in the middle panel and composite images showing overlay with Hoechst nuclear labeling (blue) in the right hand panel illustrating OPN4 expression by the plasmid. Scale bar 25 μ m.

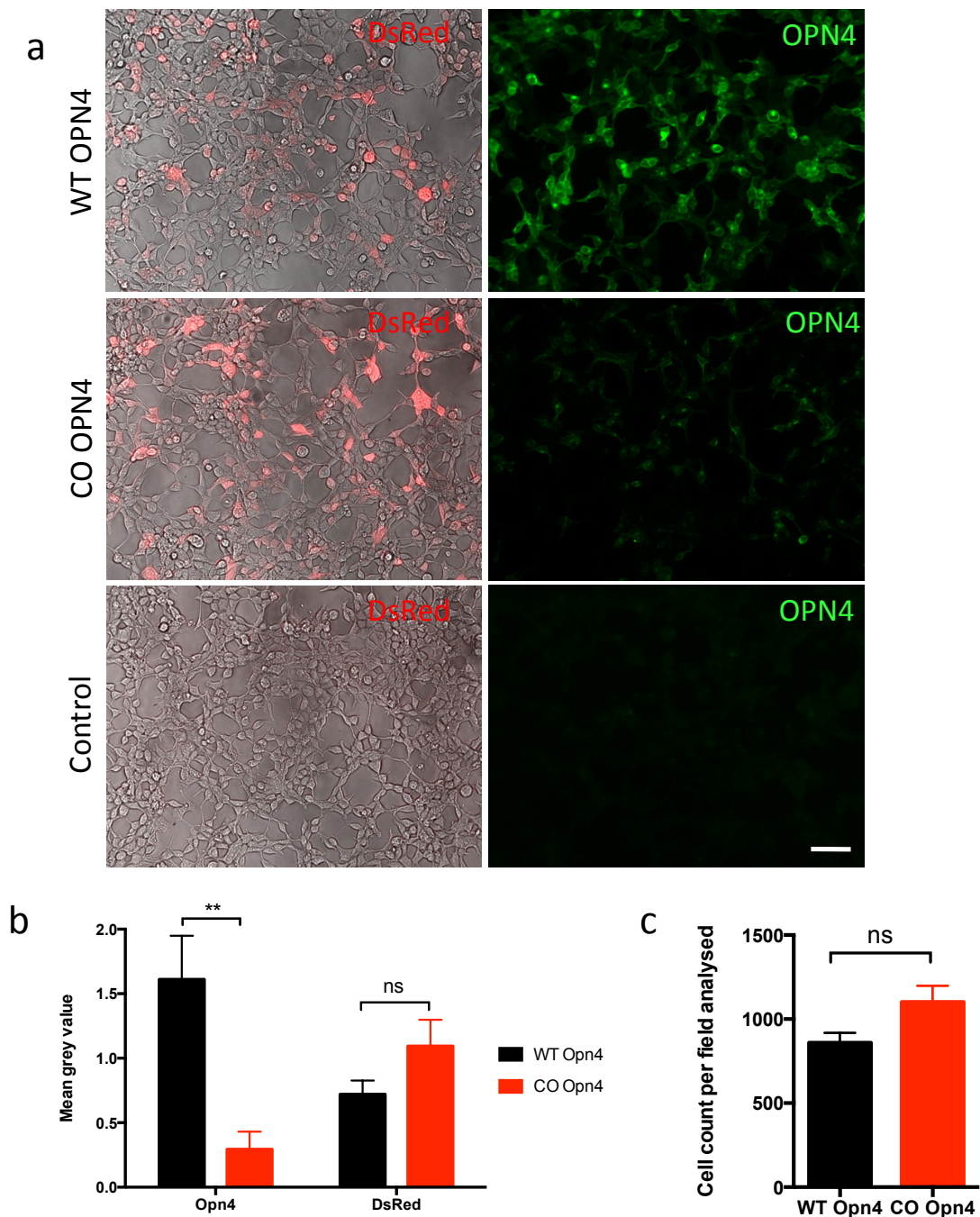


Figure 5.10 Comparison of melanopsin expression from wild type and codon optimised sequences using quantification of fluorescence images.

(a) Representative images for each group showing (left) DsRed fluorescence superimposed on a transmitted light image illustrating levels of DsRed expression and also cell density; (right) fluorescence images following staining with an antibody for human melanopsin. (b) The mean grey value of fluorescence images stained for human melanopsin and DsRed was obtained for each group, ** $p < 0.01$, 2 way ANOVA with Bonferroni post hoc test. (c) Following Hoechst nuclear staining, the number of cell nuclei per image analysed was counted to ensure the cell density between groups was consistent, ns= non significant, unpaired t test. $n=6$ per group (2 wells x 3 biological replicates).

5.3.8 Comparison of melanopsin expression by wild type and codon-optimised plasmids using FACS sorting of transfected cells

In order to compare melanopsin expression using the wild type and codon-optimised plasmids further, fluorescence was measured by Fluorescence-activated cell sorting (FACS). As previously, HEK293T cells were transfected with either the wild type or codon-optimised plasmid and an untransfected control group was used for comparison. After 48 hours cells were fixed, immunocytochemistry for human melanopsin was done on cells in suspension with a green fluorescent secondary antibody (Alexa Fluor 488). Cells were subsequently FACS sorted and FACS gates were set so that less than one percent of untransfected cells were detected in the sample region. Approximately 20,000 cells were sorted per sample and the number of fluorescent cells and median fluorescence intensity were measured, figure 5.11a.

Following FACS sorting, there were significantly more fluorescent cells in the samples transfected by wild type plasmid than codon-optimised plasmid ($p < 0.001$, unpaired t test, $n=6$ (2 samples for each of 3 biological replicates), figure 5.11b). There was no significant difference in median fluorescence between groups ($p=0.57$, unpaired t-test), figure 5.11c.

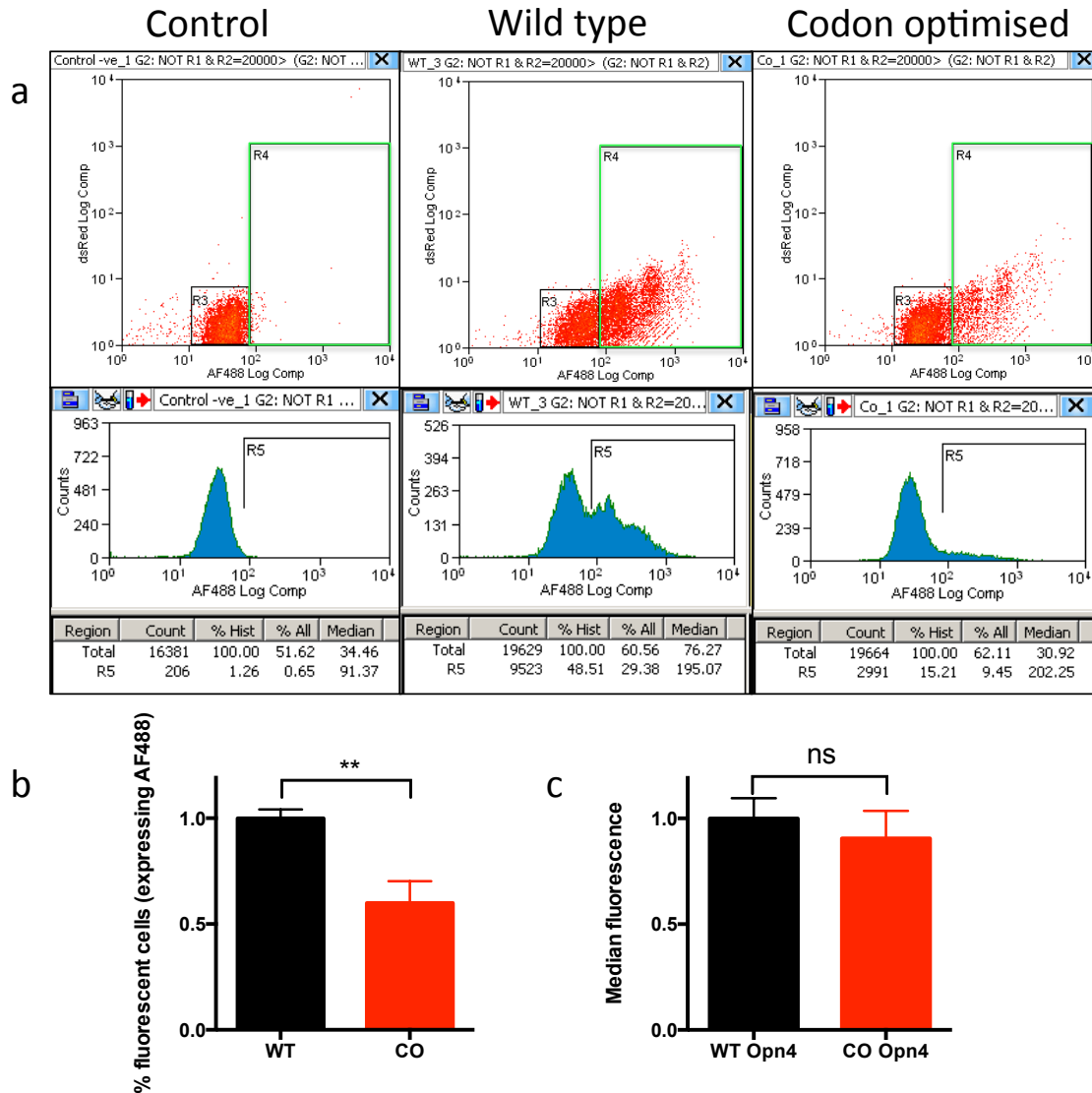


Figure 5.11 FACS sorting of cells transfected with wild type or codon optimised human melanopsin and labeled with Alexa Fluor 488.

(a) Representative plots showing the number of fluorescent cells in each group. Both DsRed and Alexa Fluor 488 fluorescence are shown in the upper panel, with the area delineated in green identifying the population of cells selected by FACS gates, and each red dot representing a single cell. The lower panels illustrate the number of green fluorescent cells of varying intensity, with the R5 gate indicating those selected for analysis. (b) There was a significant difference in the number of cells expressing green fluorescence (Alex Fluor 488) in the wild type versus codon optimised groups. Data was normalised to the level of fluorescence in the wild type group for each sample. ** $p < 0.01$, 2 tailed unpaired t test. (c) Median fluorescence of cells. There was no significant difference in the amount of fluorescence per cell between the two groups. ns= non significant, 2 tailed unpaired t test. $n=6$ per group, (2 samples processed per group $\times$ 3 biological replicates).

5.3.9 Comparison of melanopsin expression by wild type and codon-optimised plasmids using Western blot

Further comparison of melanopsin protein expression following transfection of HEK293T cells by wild type and codon-optimised plasmids was done using Western blot. Transfected cells were harvested after 48 hours and immunoblotting of lysates performed using anti-human melanopsin and anti-GAPDH (loading control) antibodies. Three independent replicates were performed.

Immunoblotting for human melanopsin revealed a band at approximately the expected size of 52 kDa. In each replicate, expression of melanopsin using the wild type plasmid was greater than expression using the codon-optimised plasmid, figure 5.12a. There was variation in the size of this difference between replicates, but all showed the same trend on examination of band density, figure 5.12b. Analysis of mean density revealed a significant difference between melanopsin expression by wild-type versus codon-optimised plasmid with both being significantly higher than control ($p=0.015$, one way ANOVA, Tukey post hoc test), figure 5.13c.

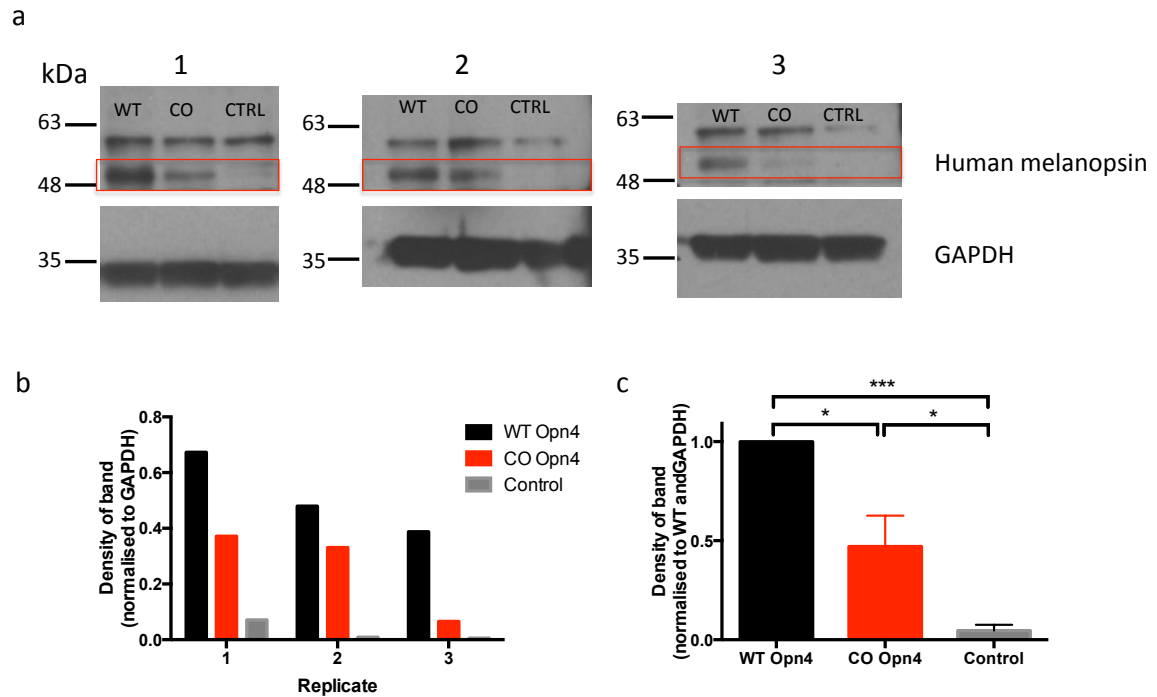


Figure 5.12 Western blot of HEK 293T cell lysates following transfection with wild type or codon optimised human melanopsin.

(a) Images of blots of the three replicates performed illustrating a 52kDa band for human melanopsin (red delineated area, upper panel) and 36kDa band for GAPDH which was used as a loading control (lower panel). (b) Densitometry of bands was performed per replicate for each group as normalised to GAPDH loading control.

(c) Densitometry of bands was performed as normalised to GAPDH and the level of wild type melanopsin to allow comparison between groups. There was a significant difference between the level of human melanopsin expression between wild type and codon optimised plasmids, * $p < 0.05$, *** $p < 0.001$, one way ANOVA, Tukey post hoc test, $n = 3$ per group.

5.3.10 Functional analysis of melanopsin expressed by wild type and codon-optimised plasmids using calcium imaging

Results from image quantification, FACS sorting and Western blot all indicated that expression of melanopsin was reduced following transfection of cells with the codon-optimised plasmid versus wild type. Therefore, calcium imaging was performed to assess if melanopsin expressed by the codon-optimised vector was functional and induced light sensitivity when expressed in a HEK 293T cell. 48 hours after transfection, cells were loaded with 9-cis retinal and Fluo-4 calcium indicator, which exhibits increased fluorescence on binding calcium and stimulated with 480nm light pulses. For analysis, a light response was defined as a five percent change in fluorescence from baseline after the onset of light.

Cells transfected with either the wild type or codon-optimised plasmid showed an increase fluorescence in response to the light stimulus, figure 5.13a, indicative of light-dependent calcium influx into the cell. No such responses were seen in untransfected controls. Kinetics of the change in fluorescence were similar in both transfected groups, figure 5.14a, with no significant difference in area under the curve ($p=0.88$ for wild type versus codon-optimised, one way ANOVA, Tukey post hoc test), figure 5.14b. There was no significant difference in the total number of cells showing light-dependent responses between wild type and codon-optimised groups but both were significantly higher than controls ($p=0.62$ for wild type versus codon-optimised, one way ANOVA, Tukey post hoc test), figure 5.14c.

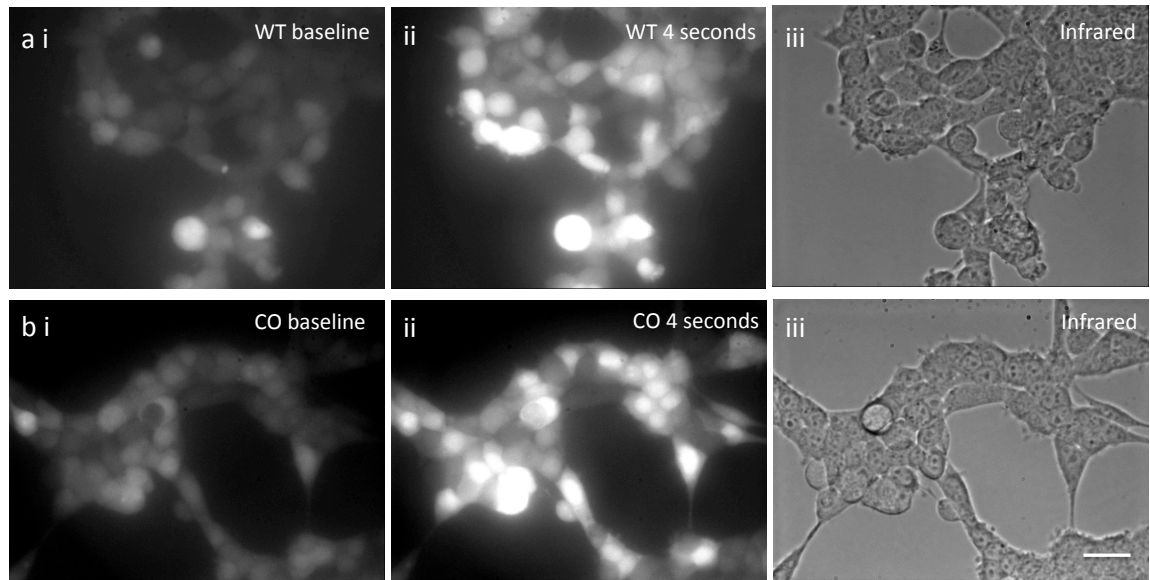


Figure 5.13 Calcium imaging following transfection with wild type or codon optimised human melanopsin plasmids.

(i) Baseline images of HEK293T cells transfected with (a) wild type or (b) codon optimised melanopsin plasmids loaded with Fluo4 calcium indicator dye prior to light exposure, taken with an infrared camera. (ii) Images of cells 4 seconds into the imaging protocol, which involved a 0.5 second stimulus of 480nm light every 2 seconds, illustrating increased fluorescence of cells due to cleavage of the indicator dye following cellular calcium influx. (iii) Representative transmitted light images illustrating similar cells density in both groups.

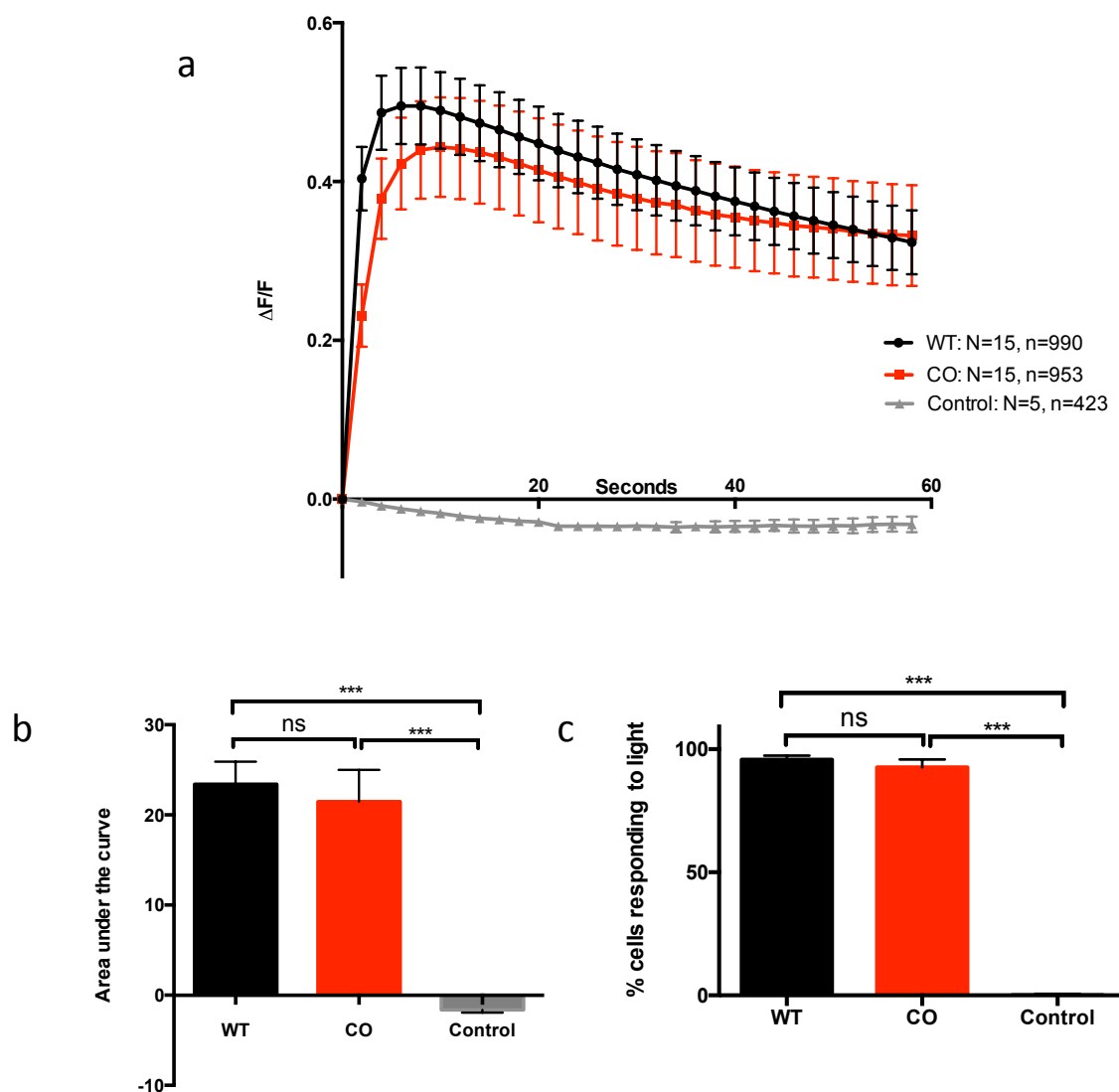


Figure 5.14 Analysis of calcium imaging data following light stimulation of cells transfected with wild type or codon optimised human melanopsin.

(a) Kinetics of the response were similar in both transfected groups, with a rapid rise in fluorescence peaking at approximately 6 seconds, but no such change in untreated controls. (b) Analysis of area under the curve revealed no significant difference between groups transfected with wild type or codon optimised plasmids, but a significant difference between both groups and controls *** p<0.001 ANOVA with Tukey post hoc test. (c) There was no significant difference between the number of light responsive cells in the groups transfected with wild type or codon optimised melanopsin (defined by a 5% change in fluorescence from baseline), whereas there was a significant difference between transfected groups and controls *** p<0.001 ANOVA with Tukey post hoc test. N=15 for transfected groups (5 plates x 3 biological replicates), N=5 for control groups (1-2 plates x 3 biological replicates). n=990 cells analysed for wild type group, n=953 for codon optimised, n=423 cells for control group.

5.3.11 Comparison of melanopsin expression from wild type and codon-optimised sequences *in vivo*

The results presented above demonstrate that melanopsin expression by the codon-optimised plasmid *in vitro* is lower than that from wild type plasmid. However conditions may differ *in vivo*, with altered availability of tRNA pools in cells under stress (217) such as in the degenerate retina. Therefore capsid mutant rAAV2/8 Y733F was produced using the codon-optimised plasmid and compared to the same vector produced using wild type plasmid in Chapter 4. Vectors of equal titre were injected into the subretinal space of six week old *rd1* mice and transgene expression evaluated by histological analysis after three weeks using immunohistochemistry.

Eyes injected with the wild type OPN4 vector showed greater levels of melanopsin expression than eyes injected with the codon-optimised vector. Melanopsin expression was widespread in the degenerate retina using the wild type vector, figure 5.15a, however expression was only detected in retinal pigment epithelium and in the outermost cells of the inner nuclear layer with the codon-optimised vector, figure 5.15b.

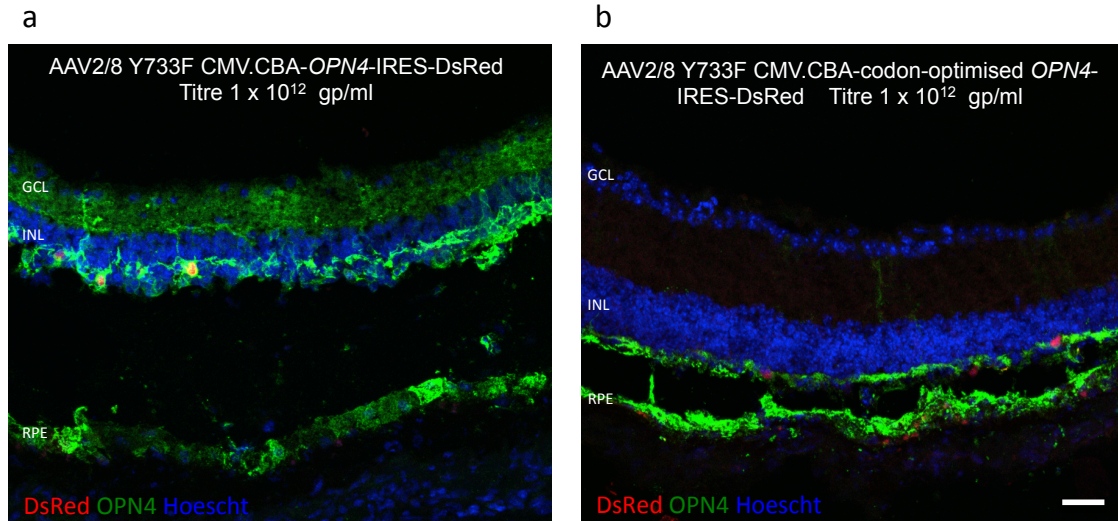


Figure 5.15 Evaluation of wild type and codon optimised melanopsin vectors in the degenerate retina.

Capsid mutant AAV vectors of equal titre were injected into the subretinal space of six week old *rd1* mice. After three weeks, retinas were stained for human melanopsin. Extensive melanopsin expression (green) was evident in the retinal pigment epithelium (RPE) and inner retina using the wild type vector (a) whereas transduction of only the RPE and outermost cell layer of the inner nuclear layer (INL) was seen using the codon optimised vector (b). Composite images are shown with melanopsin staining (green) overlaid with DsRed fluorescence (red) and Hoechst nuclear staining (blue). GCL= ganglion cell layer, Scale bar 25 μ m.

5.4 Discussion

In this chapter, data are presented on codon optimisation of the human melanopsin sequence, aiming to reduce usage of rare codons, GC content and remove destabilising motifs. The synthesised codon-optimised sequence was cloned into a plasmid under control of a CMV enhancer/CBA promoter and compared to an identical plasmid containing the wild type melanopsin sequence. Melanopsin expression was compared *in vitro* using image quantification of fluorescence from immunocytochemistry, FACS sorting, Western blot and calcium imaging. In addition, plasmids were packaged into a capsid mutant AAV2/8 Y733F vector and melanopsin expression from wild type and codon optimised vectors compared in the degenerate retina *in vivo*.

Results from *in vitro* experiments indicate reduced melanopsin expression from the codon-optimised melanopsin plasmid. Western blots confirm the overall reduction in expression but there was variation between experimental replicates as to its magnitude, ranging from 31 to 83% reduction. Image analysis of fluorescence after immunocytochemistry and FACS sorting also confirmed this result. The protein expressed was demonstrated to be functional using calcium imaging.

Reduced melanopsin expression detected following codon optimisation could be due to a decreased number of successfully transfected cells or a reduction in the amount of melanopsin expressed per cell. Analysis of fluorescent images show a similar amount of DsRed fluorescence between cells transfected by wild type and codon optimised plasmids. Therefore it would appear that transfection rates are the same, indicating that reduction in fluorescence intensity from melanopsin immunocytochemistry is due to less protein being expressed per cell. FACS sorting results showed fewer fluorescent cells in the codon optimised group but no significant difference in median level of

fluorescence in those cells detected. This would indicate an opposite result with a reduction in number of cells transduced but a similar level of melanopsin expression per transduced cell. However, it may be that cells with a low level of fluorescence in the codon optimised group could not be detected by the FACS gates set, and so these cells were counted as not fluorescent.

Calcium imaging results showed the number of light responsive cells and magnitude of calcium response to be similar in both groups. The response detected is a measure of calcium influx into the cell and it may be that even a low level of melanopsin expression can saturate the detectable calcium response by this dye. Nevertheless, three methods of quantification *in vitro* along with assessment *in vivo* demonstrated reduced melanopsin expression using a codon-optimised vector, indicating this result is likely to be real.

There are a number of possible reasons as to why codon optimisation of the human melanopsin sequence reduced expression compared to wild type. Codon usage bias has largely been studied for endogenous gene expression, but the situation may be different in heterologous situations where the transgene is massively overexpressed. In this instance, high levels of translation may lead to amino acid deprivation and a change of the available tRNA pool to a starvation-induced tRNA pool (205, 218). Therefore, optimisation protocols may need to change their reference for codon usage to suit heterologous expression.

Secondly, the presence of certain non-optimal codons may be advantageous. They may have a role in temporarily slowing down translation to allow correct protein folding (219). Many genes contain a cluster of poorly adapted codons within the first 90 to 150 nucleotides (220) and it has been proposed that this slows down elongation at the start of translation to allow binding of chaperone proteins and also prevent ribosomal

clustering at the 3' end of mRNA. The codon adaptation index of the wild type human melanopsin sequence was already high at 0.83 before optimisation, and it may be that the 12% of non-optimal codons present have a role in regulating translation.

A further consideration is GC content. Extensive studies in prokaryotes have shown that GC-rich areas form strong secondary structures which, if present near the ribosome binding site can affect initiation of translation (221). In addition, GC rich areas are often difficult to clone and sequence and therefore many optimisation protocols aim to reduce GC content. However, in mammalian cells there is some evidence that a low GC content may reduce expression (222), possibly due to an effect on transcription or mRNA export from the nucleus. The optimisation protocol used only reduced GC content of the human melanopsin sequence from 61.96 to 56.17%, therefore this effect is unlikely to be the explanation for reduced expression from the codon-optimised plasmid but potentially could have had a role.

Another contributing factor could be reduced mRNA stability of the construct following codon optimisation, but again this is less likely since DsRed expression from the bicistronic vector appeared to be the same in both groups. Finally, optimisation of the human melanopsin sequence could have created a new splice site resulting in modification of mRNA post-transcription. A software based analysis of the codon-optimised sequence did not, however, reveal any strong candidates for new splice sites (<http://www.umd.be/HSF>) and in addition full length melanopsin was detectable on Western blot of cells transduced by the codon-optimised plasmid.

5.5 Conclusions

Codon-optimisation of human melanopsin resulted in a reduction of expression compared to the wild type sequence both from a plasmid vector *in vitro* and an AAV vector *in vivo*. The protein produced was of full length as shown by Western blot, and calcium imaging confirmed functional activity. On the basis of the data presented, the wild type human melanopsin sequence in a rAAV2/8 Y733F vector was chosen for further experiments to assess melanopsin as an optogenetic therapy in the degenerate retina.

Chapter 6 Assessment of melanopsin expression by AAV in the degenerate retina: longevity and function

6.1 Introduction

Data presented thus far investigated optimisation of the vector for melanopsin gene delivery to the degenerate retina with regards to AAV capsid, promoter and codon-optimisation. In order to use this vector as a strategy for restoration of vision, further work was required to determine whether the retina transduced showed long-term expression of melanopsin and whether this was able to drive a functional light response.

Once AAV has entered the host cell, its genome exists as double stranded episomes which are known to be stable (97). Previous studies have shown long-term gene expression following a single subretinal injection of AAV2/2 carrying a CMV.CBA enhancer/promoter in non-human primates (223). Since a shortened CMV.CBA enhancer/promoter was used in the generation of the melanopsin vector, which was packaged in a capsid mutant serotype 8, long-term efficacy of this vector also had to be established.

With regards to function, for melanopsin gene therapy to generate a useful visual signal, retinal cells must depolarise in response to light, this information must be transmitted to the brain via action potentials and subsequently be processed by the visual cortex. In order to determine whether these events occur, three different techniques were used: assessment of *cfos* induction in response to light, multi-electrode array recordings of retinas *ex-vivo* and laser speckle cortical imaging.

Calcium influx into the cell and subsequent membrane depolarisation causes expression of a set of 'intermediate early' genes such as the proto-oncogene *cfos*, which is rapidly induced by membrane depolarisation, figure 7.1 (224). Previous work has demonstrated that light stimulation induces *cfos* upregulation in retinal ganglion cells and inner nuclear layer cells in rodents (225, 226). Rods and cones drive the majority of *cfos* expression in the retina, but in the degenerate retina a light stimulus will drive *cfos* expression specifically in ipRGCs. (227). Since the transduction cascade of melanopsin causes calcium influx and cell depolarisation in response to light, *cfos* induction following a light stimulus was measured to assess whether human melanopsin expressed by the capsid mutant AAV vector showed functional photosensitivity.

Following detection of visual information, this must be transmitted from the eye to the brain. In the degenerate retina, two types of electrical activity output are apparent: action potential firing and oscillatory potentials. The former are the principal method of signal transmission to the brain, whereas the latter in the *rd1* mouse are 10 to 20 Hz oscillatory activity believed to arise from AII amacrine cells in the absence of regulation by photoreceptor inputs (228). In order to assess whether expression of human melanopsin was functional following subretinal delivery of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DSred, Multi-electrode arrays (MEA) recordings were used in order to evaluate action potential firing and thus whether this information could be transmitted centrally. MEAs contain an arrangement of approximately sixty titanium nitride electrodes, onto which tissues slices, such as retina can be placed. The MEA electrodes detect activity in retinal ganglion cells as voltage changes (229), and these signals are amplified by a filter amplifier and sent to the data acquisition computer. This technique allows recordings to be made from multiple areas of tissue simultaneously.

In order to be useful for image-forming vision, action potentials transmitted must activate neural circuitry that reaches the visual cortex, which must in turn process this information. To investigate this, the technique of laser speckle cortical imaging was used. This measures real time *in vivo* changes in cortical blood flow using laser speckle flowmetry, and has previously been used to assess activation of the visual cortex following transplantation of rod precursor cells into the degenerate retina (85).

6.2 Experimental design

The overall objectives were to ascertain whether melanopsin expressed by capsid mutant rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed in the degenerate retina was sustained in the long-term, and if this was able to mediate a light response. Full details of methodology are given in Chapter 2.

6.2.1 Characterisation of model

For functional testing the *rd1* mouse model was used, which shows rapid photoreceptor degeneration. All mice were genotyped prior to use for both the *rd* allele and also *Gpr179*, mutations of which are known to be prevalent in the *rd1* model (230), see section 2.7.1. The presence of any residual cones was also evaluated following immunohistochemistry using an antibody for UVS opsin and manually counting the number of residual cones in three complete histological sections per eye.

6.2.2 Long term expression

To determine whether melanopsin expressed by the vector was sustained in the long term, two cohorts of *rd1* mice were assessed. In each cohort six to eight week old mice had subretinal injections of the rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector with age-matched mice having sham-injections of phosphate buffered saline and untreated controls also being included, see section 2.7.3. One cohort of mice was assessed by behavioural tests (data presented in chapter 7), functional tests and immunohistochemistry, see section 2.8.2, from two to six months after injection. The other cohort of mice was assessed at thirteen to fifteen months after injection, figure 6.1.

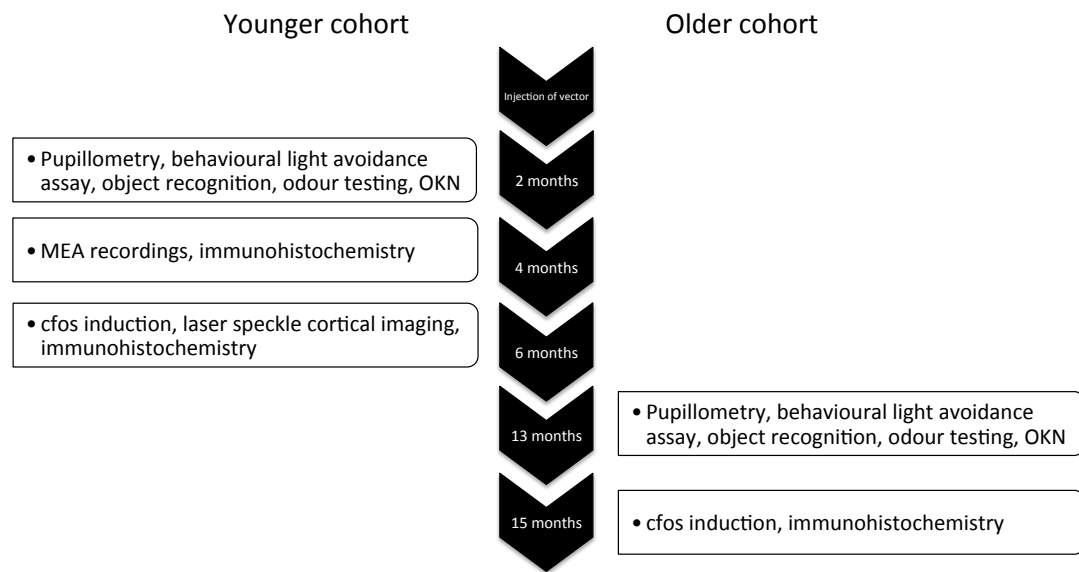


Figure 6.1 Schematic illustrating timeline of experiments

Two cohorts of *rd1* mice were treated with subretinal injection of AAV melanopsin vector, with age-matched sham-injected and untreated controls. One cohort was examined at 2 to 6 months after treatment whereas the other was examined at 13 to 15 months after treatment.

6.2.3 Retinal *cfos* expression

Four hours after lights were turned off on a twelve hour light-dark cycle (i.e. ZT 16) mice were pulsed with a white light stimulus produced by LED light sources of 350 or 400 lux intensity for 30 minutes, see section 2.7.5 for full methods. Two cohorts were assessed, one that was six months after treatment (350 lux stimulus) and another that was 15 months after treatment (400 lux stimulus) with melanopsin AAV vector. Sham-injected or untreated controls were used for comparison. In the older cohort a further group of animals was not exposed to light as a control for the light stimulus. Thirty to sixty minutes after the light stimulus ended, mice were sacrificed and eyes fixed, figure 6.2a. Subsequently levels of *cfos* expression in the retina were assessed using immunohistochemistry. For analysis, five equally spaced images of each of two retinal sections per eye were taken at x20 magnification using identical acquisition settings, figure 6.2b. Cell counts and quantification of intensity of fluorescence were performed using Image J software, see section 2.8.3.4.

6.2.4 Multi electrode array recordings

Freshly dissected retinas were placed ganglion cell side down on a multi electrode array (MEA) and secured in position with a wire harp, figure 6.3, see section 2.6.2 for detailed methods. A perfusion manifold was fixed to the MEA which allowed constant perfusion of the tissue with Ames solution bubbled with 95% O₂ 5% CO₂. The MEA was placed in its amplifier and positioned on the stage of an inverted fluorescence microscope connected to a monochromatic light source. After a period of acclimatisation of 30 to 60 minutes the retina was stimulated with 480nm light for 60 seconds at a light intensity of 1.3×10^{15} photons/cm²/s unless otherwise stated, and resulting electrical activity was recorded. At least twenty minutes was allowed between stimuli unless otherwise described. Data was analysed using MC Rack software (Multi Channel Systems).

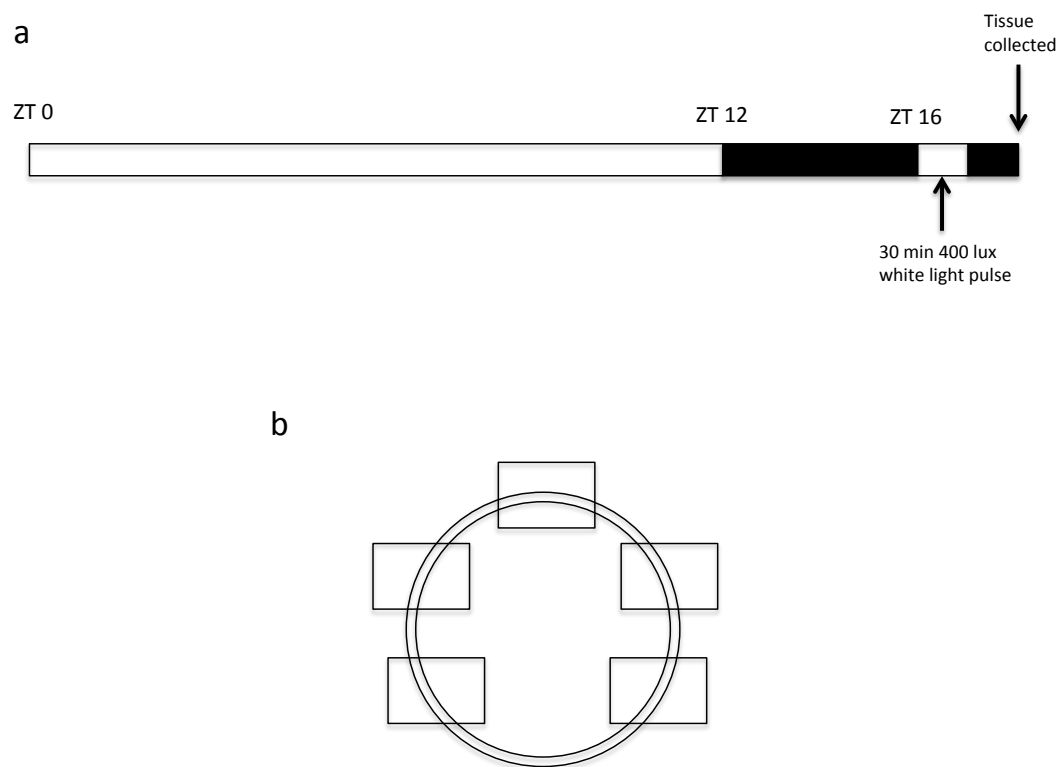


Figure 6.2 Schematic illustrating methods for *cfos* induction by light.

The diagram (a) illustrates the time course of experiments, with a 30 minute light pulse being administered 4 hours after lights have been switched off on a 12 hour light/ dark cycle. Tissue was collected 30-60 minutes after the end of the light pulse. The schematic (b) illustrates images taken for analysis, with 5 equally spaced x20 images being taken per retinal section.

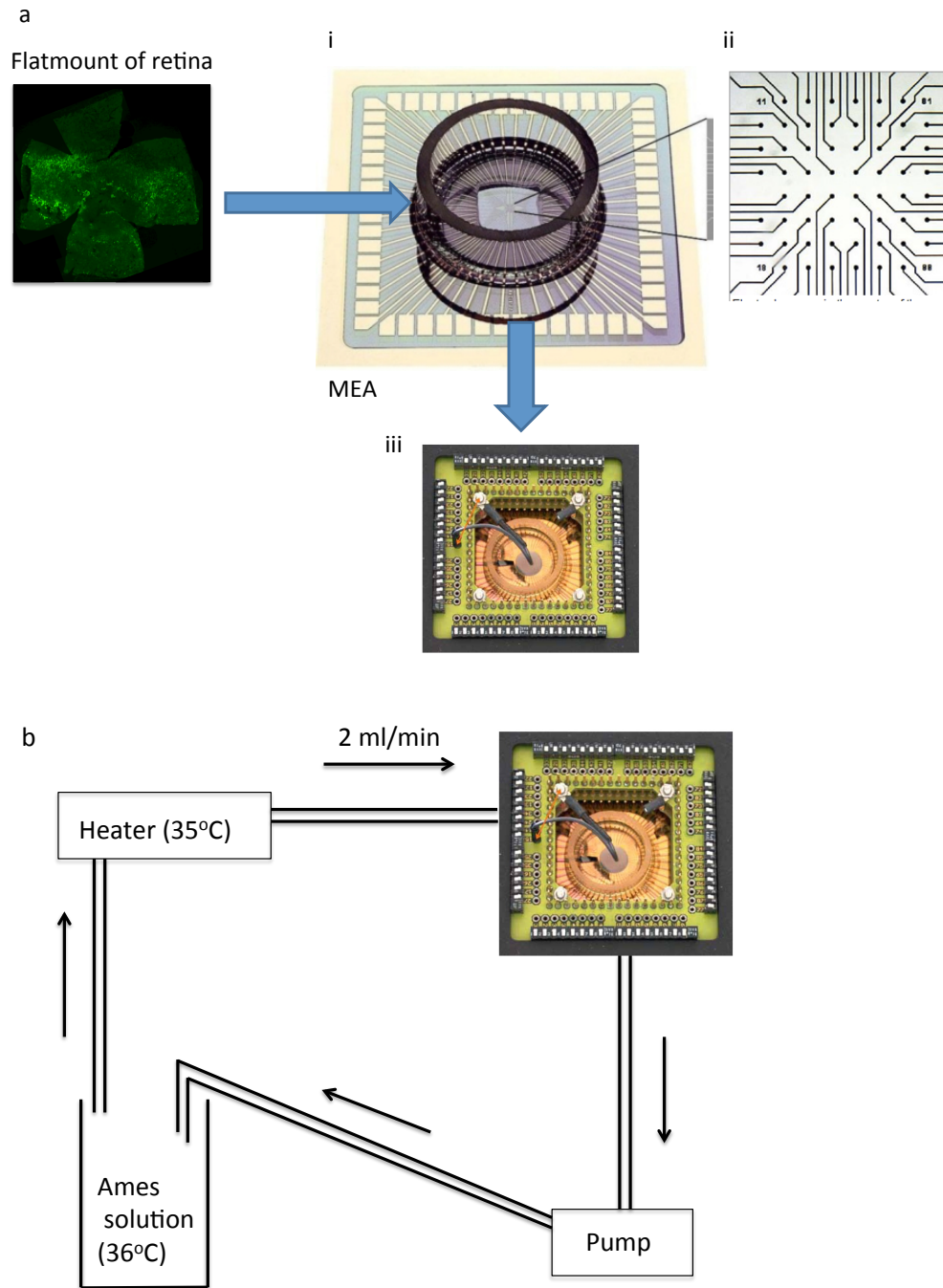


Figure 6.3 Multi-electrode array (MEA) recordings

(a) Freshly dissected retinas were placed ganglion cell side down in a multi electrode array chamber (i), an enlargement of electrodes to illustrate their arrangement is shown in (ii). MEAs were placed in an amplifier (iii) and positioned on the stage of an inverted fluorescence microscope connected to a light monochromator. (b) Illustration of the perfusion system: retinæ were continuously perfused with AMES media bubbled with 95% O₂ 5% CO₂ (pH 7.3) and maintained at 34°C using a combination of 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 in the sample chamber.

Further recordings were done in the presence of (i) a melanopsin antagonist (10 μ M AA92593)(174) or (ii) rod/ cone blocking drugs (a combination of 100 μ M L-(+)-2-Amino-4-phosphonobutyric acid, 30 μ M D-(-)-2-Amino-5-phosphonopentanoic acid and 40 μ M 6,7-Dinitroquinoxaline-2,3-dione) (175) or (iii) NMDA and AMPA receptor antagonists (10 μ M 3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid and 10 μ M 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide) (87).

6.2.5 Laser speckle cortical imaging

Mice that had been dark adapted for at least two hours were deeply anaesthetised. Their heads were secured in a stereotaxic frame and scalps incised to reveal the cranium over the visual cortices. Cranial sutures and landmarks could be clearly visualised following haemostasis, enabling the area anterior to lambda i.e. the location of the visual cortex to be identified, figure 6.4a. The temperature of the mouse was monitored via a rectal probe, which fed back to a heat pad to maintain physiological conditions, figure 6.4b. A 480nm light stimulus lasting 2 seconds was applied bilaterally via a light emitting diode in front of each eye, with total intensity of 2000lux. This was followed by 58 seconds of recording in darkness and repeated a further nine times per animal. The results were averaged to optimise the signal to noise ratio. Blood flow changes were captured using the Speckle Contrast Imager (moorFLPI, Moor Instruments, UK), figure 6.4c. This used a 785nm laser with 50mW laser power for measurement of blood flow. Images were acquired at 580 x 752 pixels at a rate of 5 frames per second. Data were processed using moorFLPI Review software and analyzed with MATLAB.

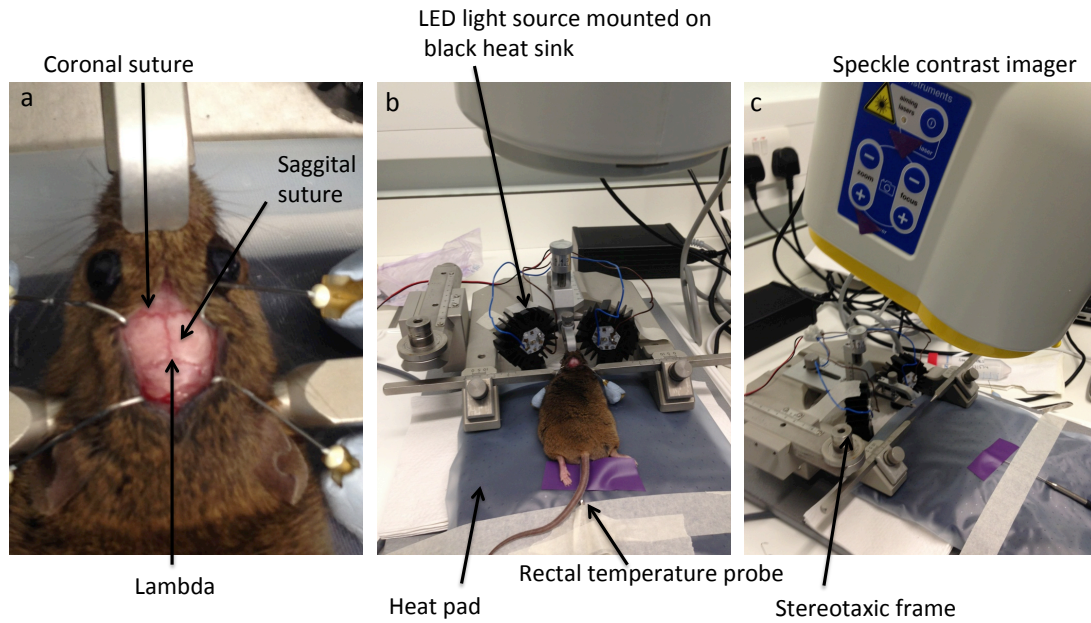


Figure 6.4 Experimental set up for laser speckle cortical imaging

Following deep anaesthesia, the head of the mouse was placed in a stereotaxic frame and scalp incised to reveal the cranium with underlying visual cortex anterior to lambda (a). The temperature of the mouse was monitored via a rectal probe, which fed back to a heat pad to maintain physiological conditions. A 480nm light stimulus lasting 2 seconds was applied bilaterally via a light emitting diode in front of each eye (b). Blood flow changes were captured using the Speckle Contrast Imager (c).

6.3 Results

6.3.1 Assessing long-term vector expression

6.3.1.1 Genotyping

All *rd1* mice used were genotyped for the causative mutation in rod-specific cGMP phosphodiesterase- β -subunit (PDE β) to ensure that there were no heterozygote or wild type mice in the cohort that would affect functional testing. All mice tested were homozygous for the *rd1* mutation, figure 6.5a.

Furthermore, mice bred on the C3H background may have mutations in *gpr179*, an orphan G protein receptor present on bipolar cell dendritic terminals (230, 231). Mutations in *gpr179* result in abnormal bipolar cell function and loss of the b-wave on electroretinography (231) and this mutation has been shown to be prevalent in the *rd1* mouse model (232). Therefore mice to be included were also genotyped for the *gpr179* mutation, to ensure that there were no differences between mice that might affect interpretation of data. All mice in both cohorts were found to be homozygous for the mutation, figure 6.5b.

6.3.1.2 Residual cones

Degeneration of cones in the *rd1* model is slower than that of rods with 1.5% of the cone population still being present in the *rd1* retina at age 18 months (75). In order to assess whether any functional differences seen between groups might be attributed to residual cones, retinal sections were immunolabeled with antibody for ultraviolet/S-cone (UVS) opsin.

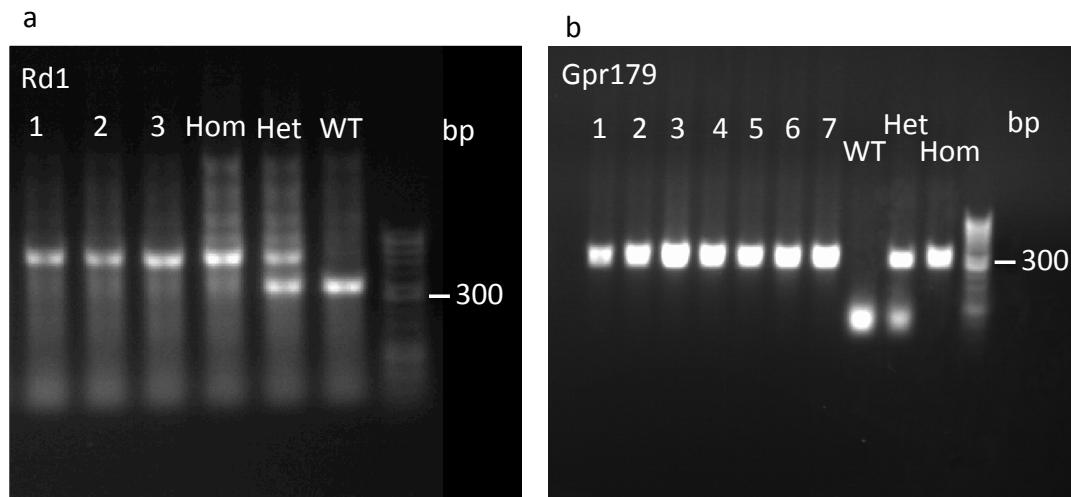


Figure 6.5 Genotyping of *rd1* mice.

(a) Genotyping for the *rd1* mutation using a duplex PCR. Samples from three different mice are shown (1-3), with homozygous, heterozygous and wild type controls. Expected band sizes are 550bp for the mutant allele and 400bp for the wild type allele. (b) Genotyping for the *Gpr179* mutation, with samples from 7 different mice shown (1-7) alongside wild type, heterozygous and homozygous controls. Expected band sizes are 400bp for the mutant allele and 100bp for the wild type allele.

Some residual UVS cones were present in treated, sham-injected and untreated retinas 4 months after treatment (i.e. 6 month old mice), figure 6.6a, with a small number of cones also present in all groups at 15 months after treatment (17 month old mice), figure 6.6b. In all cases, cones were morphologically abnormal: UVS cone opsin was mislocalised to the entire cell membrane with inner and outer segments being absent.

Following antibody labelling, the number of residual UVS cones in retinal sections was quantified for each group. There was a significant effect of age of mice ($p < 0.0001$, 2 way ANOVA) and treatment ($p = 0.0013$) on the number of residual cones with a significant interaction between the two factors ($p = 0.0087$). Examining groups in more detail, there was a trend for more cones to be present in all groups in the younger cohort, with this being statistically significant in the sham-injected (36.5 ± 4.0 cones per section young versus 2.0 ± 0.8 cones per section old, $p = 0.023$, Bonferroni post hoc test) and untreated groups (65.4 ± 16.8 cones per section young versus 7.4 ± 1.6 cones per section old, $p < 0.0001$). This effect did not reach statistical significance in the treated group (21.0 ± 10.1 cones per section young versus 3.8 ± 1.3 cones per section old, $p = 0.82$). There were also significantly more residual cones in the untreated versus the treated group at 4 months ($p = 0.001$), with no significant difference in other comparisons at this time point. Of note, there was a wide variation in the number of residual cones across the different eyes examined in the untreated group, figure 6.7. Very few cones remained in the cohort examined 15 months after treatment, with no significant difference seen between groups.

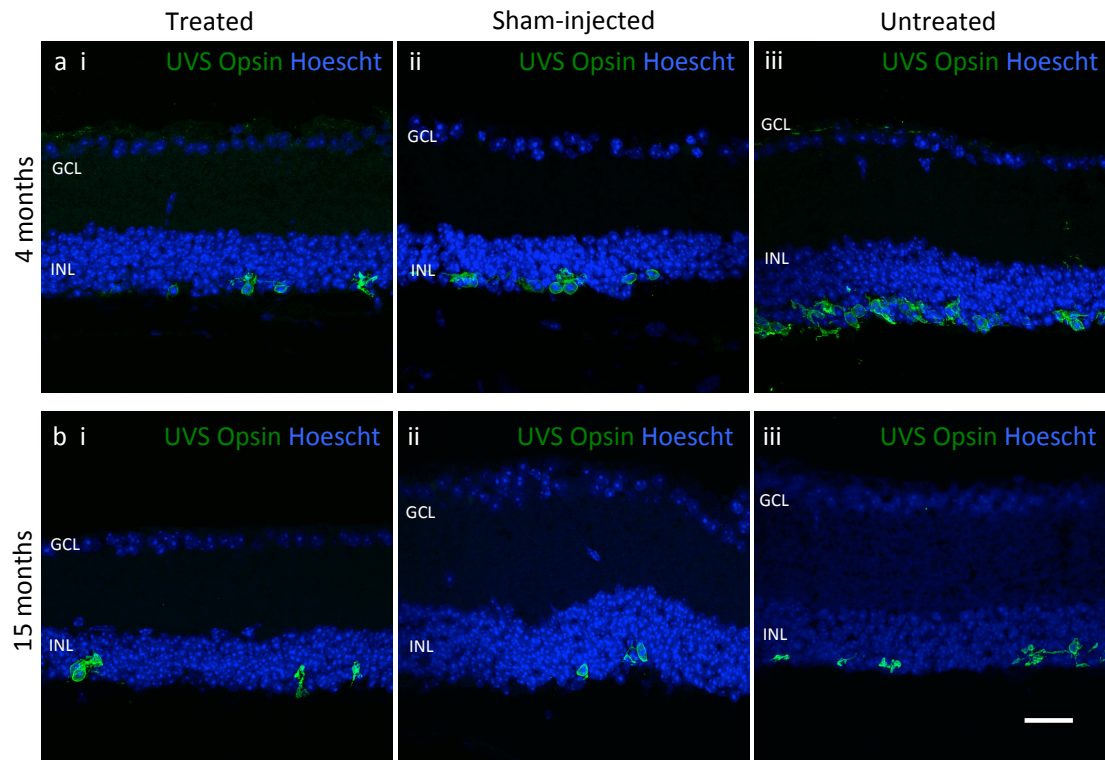


Figure 6.6 Residual cones

Residual cones were labelled with UVS opsin antibody (green) in treated (i), sham-injected (ii) and untreated (iii) eyes from cohorts of mice 6 months after injection (a) and 15 months after injection (b). Images are overlaid with Hoechst labelling of nuclei (blue). Scale bar 25 μ m.

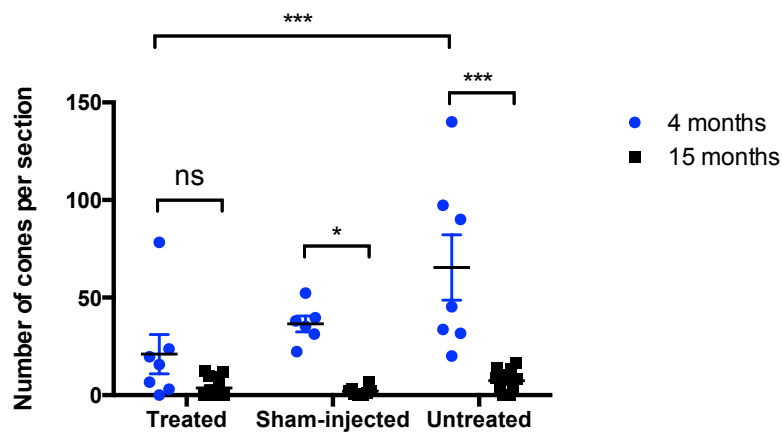


Figure 6.7 Quantification of residual UVS cones

Following immunolabelling with UVS antibody, the number of residual cones in 3 circular histological sections was manually counted per eye assessed. Treated, sham-injected and untreated eyes were compared at both 4 and 15 months after treatment. (4 month cohort: treated $n=7$, sham-injected $n=6$, untreated $n=7$; 15 month cohort: treated $n=14$, sham-injected $n=8$, untreated $n=12$). * $p<0.05$, *** $p<0.001$ 2 way ANOVA with Bonferroni post hoc test.

6.3.1.3 Immunohistochemistry

Immunohistochemistry using human melanopsin antibody was performed on retinal flatmounts four months after subretinal injection of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector in the first cohort and fifteen months after injection in the second cohort to assess melanopsin expression. Sham-injected and untreated retinas were also processed for comparison. In treated eyes, there was a large area of expression of human melanopsin in the degenerate mouse retina, but no such expression was seen in sham-injected or untreated eyes, figure 6.8a. At 15 months there was also significant melanopsin expression, indicating sustained efficacy of the vector/promoter combination even at this much later time point, figure 6.8b. Examining the retina more closely at the 15 month time point, a wide network of cells expressing human melanopsin was evident with appropriate membrane localisation of the antibody, figure 6.9.

Staining retinal sections using human melanopsin antibody also revealed widespread melanopsin expression of inner nuclear layer cells, with cellular processes extending into the inner plexiform layer after 4 months, see chapter 4 for further characterisation. A similar pattern of staining was seen at 15 months, figure 6.10.

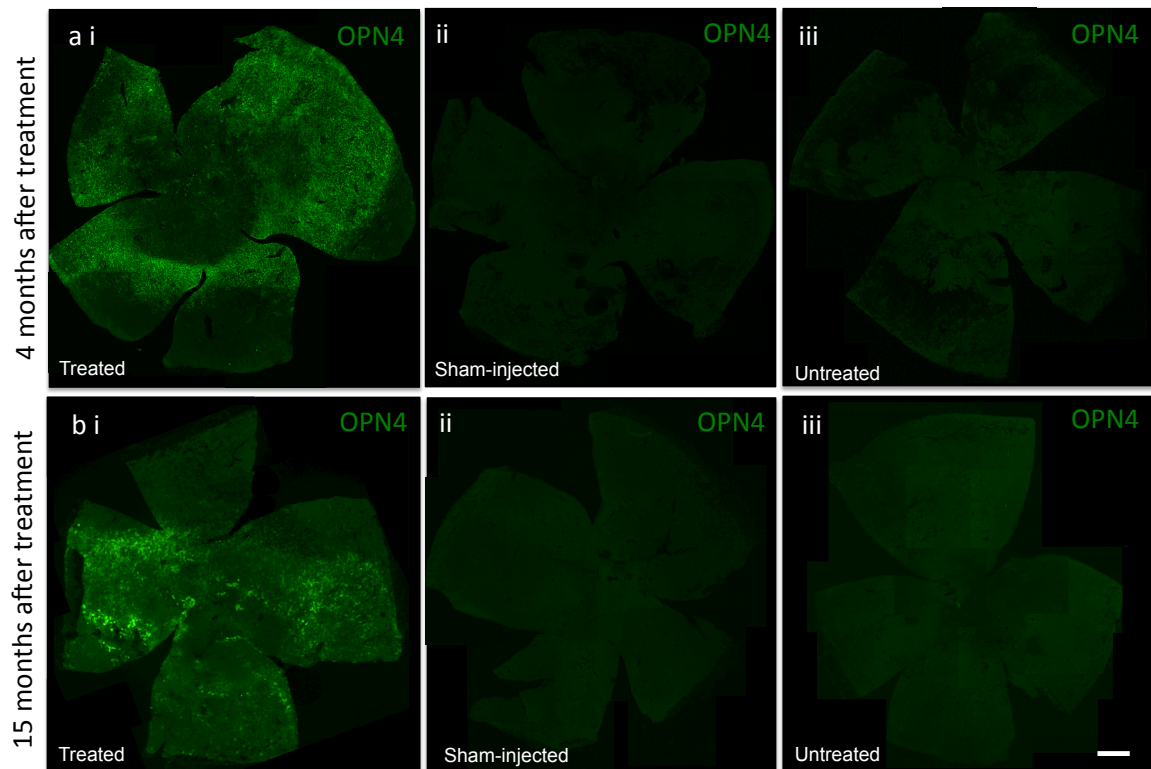


Figure 6.8 Long term retinal transduction in the *rd1* mouse following subretinal injection of rAAV 2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed.

(a) Flatmount of (i) treated retina stained for human melanopsin showing extent of transduction at 4 months with (ii) a sham-injected and (iii) an untreated control eye stained for human melanopsin for comparison. (b) Flatmount of (i) treated retina 15 months after subretinal injection with age-matched (ii) sham-injected and (iii) untreated controls stained for human melanopsin. Scale bar 500 μ m.

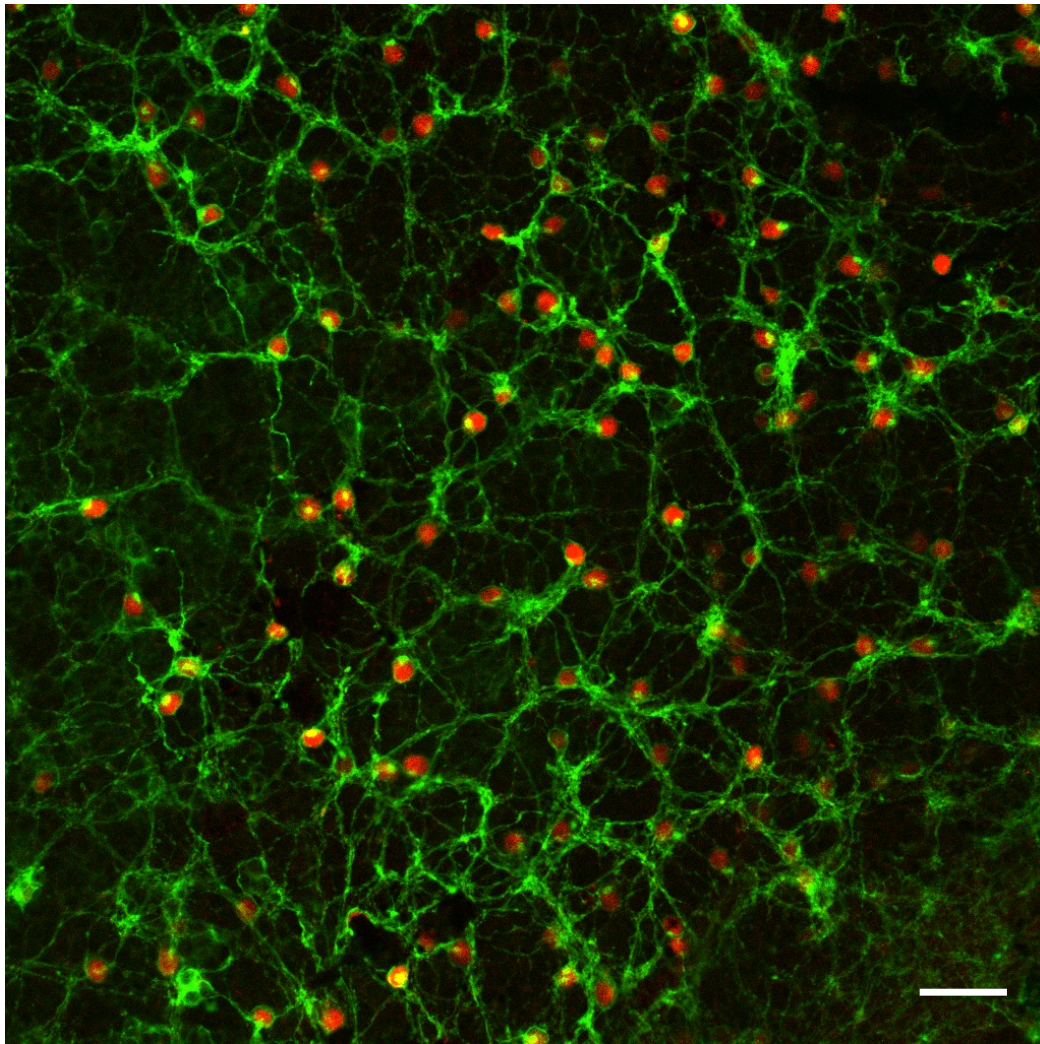


Figure 6.9 High powered view of treated retina following immunohistochemistry using human melanopsin antibody.

A network of cells expressing human melanopsin (green) is evident with appropriate membrane localisation. Direct fluorescence from DsRed (red) is overlaid, confirming AAV transduction of this area of retina. Scale bar 25 μm .

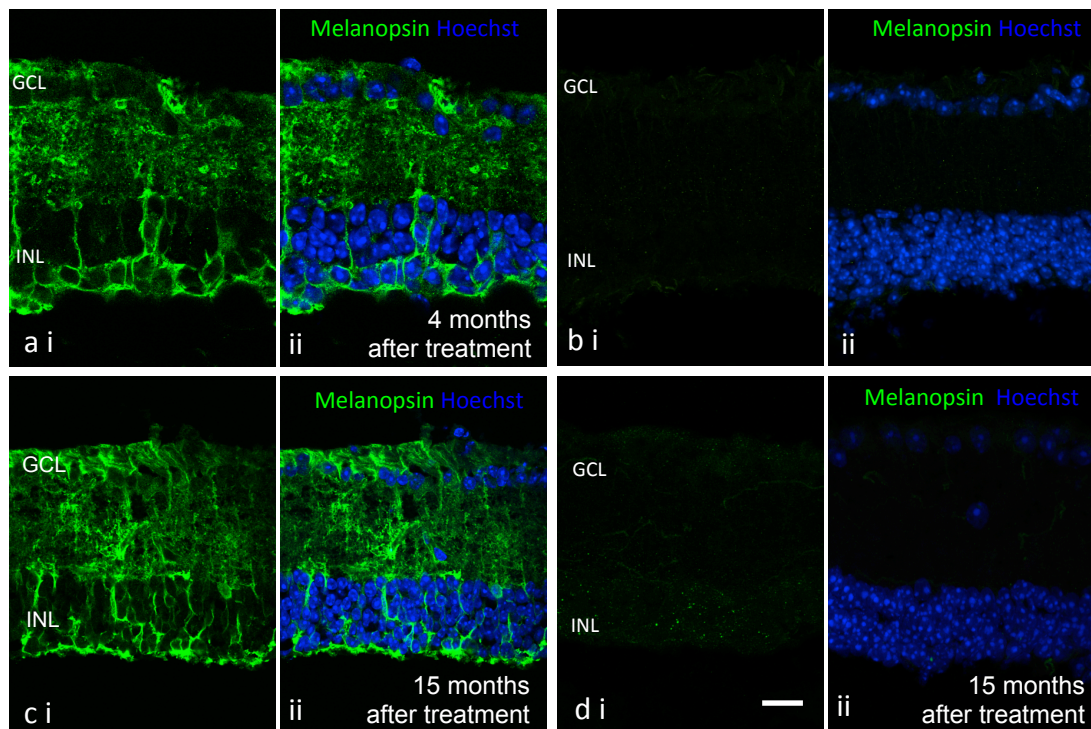


Figure 6.10 Immunohistochemistry using human melanopsin antibody of retinal sections following subretinal delivery of rAAV 2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed versus untreated controls.

Sections of (a) treated retina stained for human melanopsin showing extent of transduction at 4 months with (b) an untreated control eye stained for human melanopsin for comparison. (c) Section of a treated eye 15 months after subretinal injection with age-matched (d) untreated control stained for human melanopsin. Panels show (i) human melanopsin antibody staining alone, and (ii) overlay with Hoechst nuclear stain. Scale bar 25µm.

6.3.2 Light induced cfos expression

In order to assess whether human melanopsin expressed following vector delivery resulted in cell depolarisation, cfos induction following a light stimulus was examined. Inspecting histological sections of eyes treated with rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector 6 months previously, areas of transduction were identified by DsRed fluorescence. In transduced areas, increased cfos expression was seen in cells within the inner nuclear layer compared to sham-injected control eyes. There was some colocalisation of DsRed fluorescence and cfos expression suggestive of depolarisation of transduced cells, figure 6.11. A similar increase in cfos expression in the inner nuclear layer was seen in a cohort of mice treated 15 months previously in comparison to untreated control mice, figure 6.12.

Since the expression of DsRed from the vector tended to be less than that of human melanopsin (see Chapter 4), immunohistochemistry was performed using antibodies for both cfos and human melanopsin. This revealed nuclear cfos expression in some cells that expressed human melanopsin in their cell membrane. In addition, other cfos positive cells were seen adjacent to these but did not appear to also express melanopsin, figure 6.13, indicative of depolarisation of neighbouring cells. This would suggest that there is cell to cell signalling as a result of ectopic expression of human melanopsin in the degenerate retina.

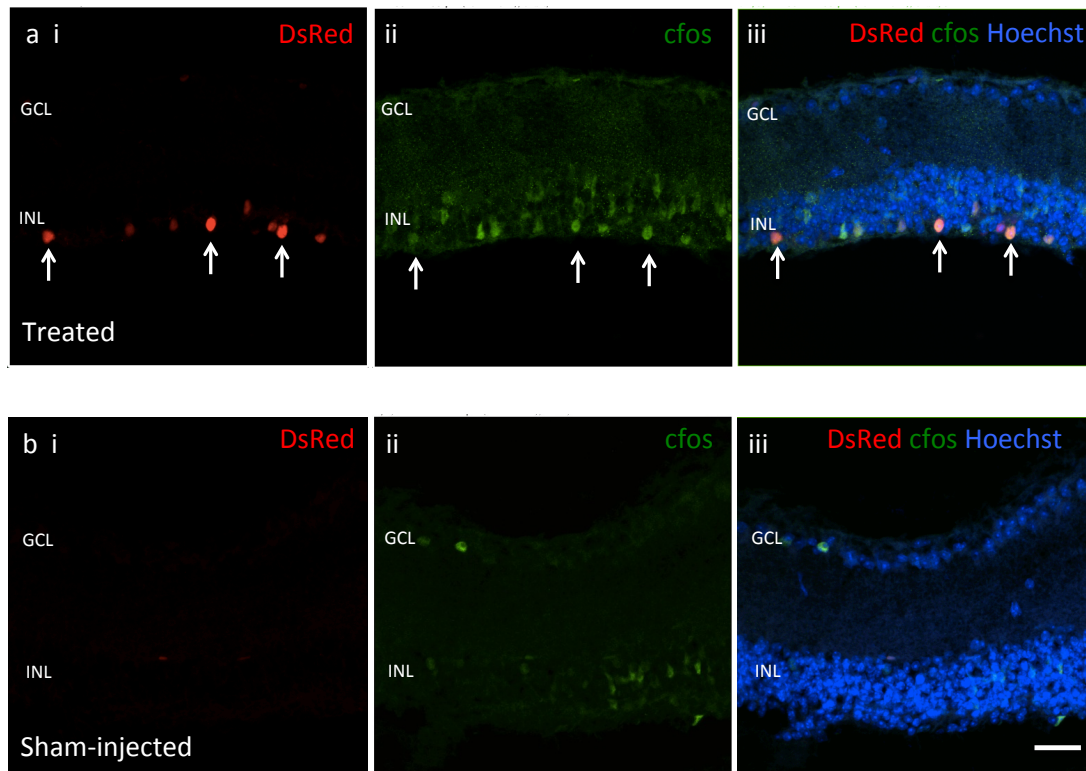


Figure 6.11 Light-induced *cfos* expression in the degenerate retina 6 months after treatment with AAV melanopsin.

DsRed fluorescence (red) is shown in panel i, antibody staining for *cfos* (green) in (ii) and composite images (iii) show overlay with Hoechst nuclear labelling (blue), demonstrating *cfos* expression in areas of DsRed fluorescence in the treated retina (a), with levels of *cfos* greater in this retina than a sham-injected control (b). Arrows indicate examples of cells that express both DsRed fluorescence and are labeled with *cfos* antibody. Scale bar 25 μ m.

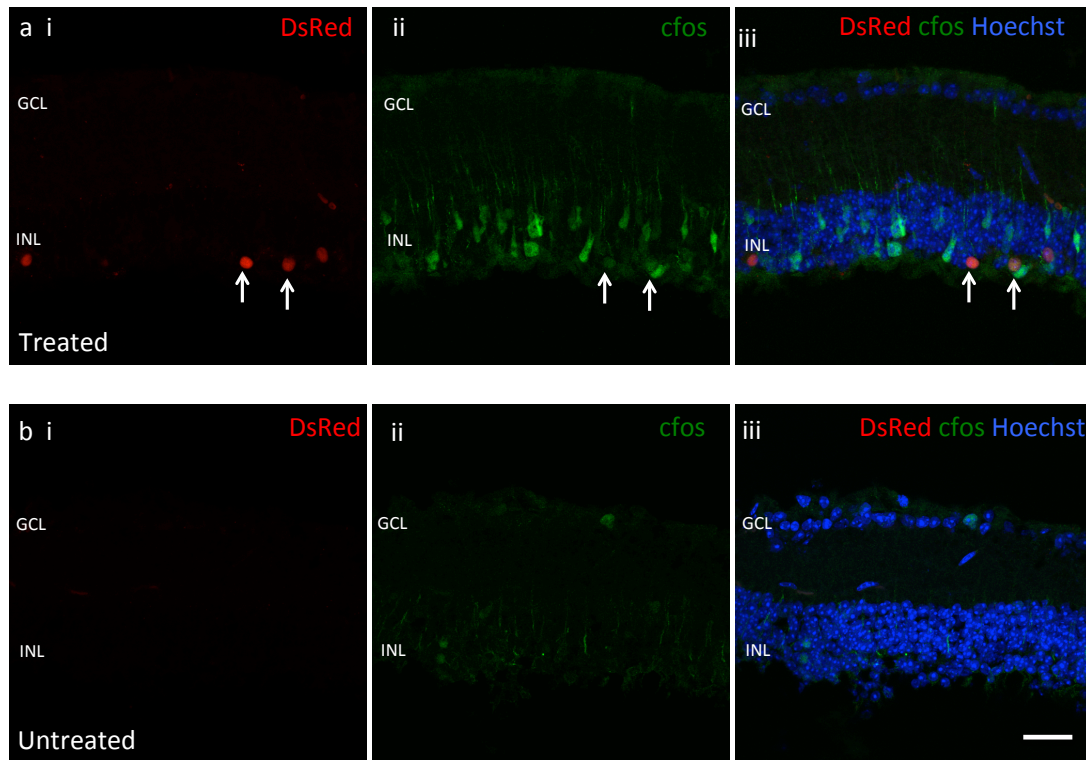


Figure 6.12 Light-induced *cfos* expression in the degenerate retina 15 months after treatment with AAV melanopsin.

DsRed fluorescence (red) is shown in panel i, antibody staining for *cfos* (green) in (ii) and composite images (iii) show overlay with Hoechst nuclear labelling (blue), demonstrating *cfos* expression in areas of DsRed fluorescence in the treated retina (a), with greater levels of *cfos* in (a) compared to an untreated control (b). Arrows indicate cells that express both DsRed fluorescence and are labeled with *cfos* antibody. Scale bar 25 μ m.

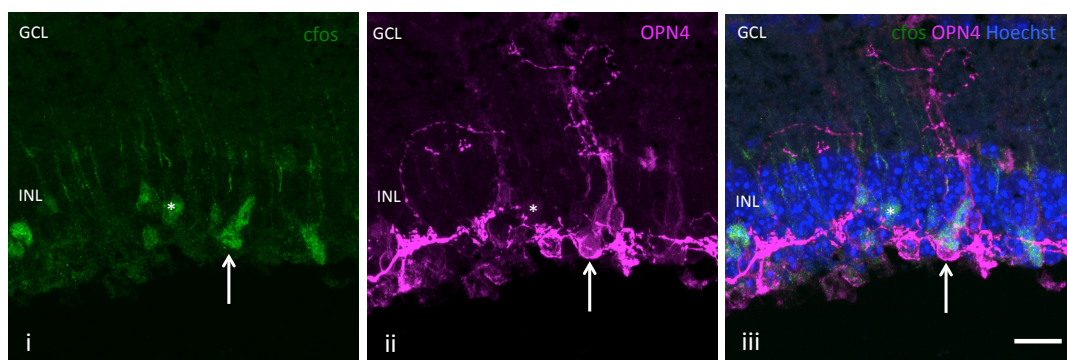


Figure 6.13 Light-induced *cfos* and human melanopsin expression in the degenerate retina 15 months after treatment with AAV melanopsin.

Antibody staining for *cfos* (green) is shown in panel i, antibody staining for human melanopsin (purple) in (ii) and the composite image (iii) shows overlay with Hoechst nuclear labelling (blue). Arrows demonstrate colocalisation of *cfos* in the cell nucleus and human melanopsin in the membrane of some cells. The asterisk indicates a *cfos*

expressing cell that does not colocalise with human melanopsin expression. Scale bar 25 μ m.

Cfos expression was quantified from images of retinal sections 6 months and 15 months after subretinal delivery of vector compared to controls. There was a significant effect of treatment on the number of cfos positive cells per retina ($p=0.0089$, two way ANOVA), but no significant effect of length of treatment ($p=0.84$) or interaction between the two factors ($p=0.36$). In the cohort 6 months after treatment, there was a greater than 10-fold increase in the number of cfos expressing cells in treated (13.5 ± 3.0 cells per image) compared to control eyes (1.2 ± 0.1 cells per image; $p=0.015$, Bonferroni post hoc test; total number of cfos positive cells counted 623 treated and 44 control group), figure 6.14a. A similar trend was seen in the group 15 months after treatment with a mean of 10.1 ± 5.4 cfos positive cells per image in the treated group versus 3.4 ± 0.7 cells per image in the control group. However this difference did not reach statistical significance ($p=0.35$ Bonferroni post hoc test; total number of cfos positive cells counted 302 treated and 103 control group).

Examining mean fluorescence intensity of cfos expressing cells identified above, there was also a significant effect of treatment ($p=0.0018$, two way ANOVA) with no significant effect of length of treatment ($p=0.24$). Interaction between factors did not reach significance ($p=0.054$). In the group 6 months after treatment, there was a three fold increase in the intensity of cfos expression in treated retinae compared to controls ($p=0.0009$, Bonferroni post hoc test), figure 6.14b. A similar trend was seen in the cohort 15 months after treatment, although again this did not reach significance, ($p=0.47$).

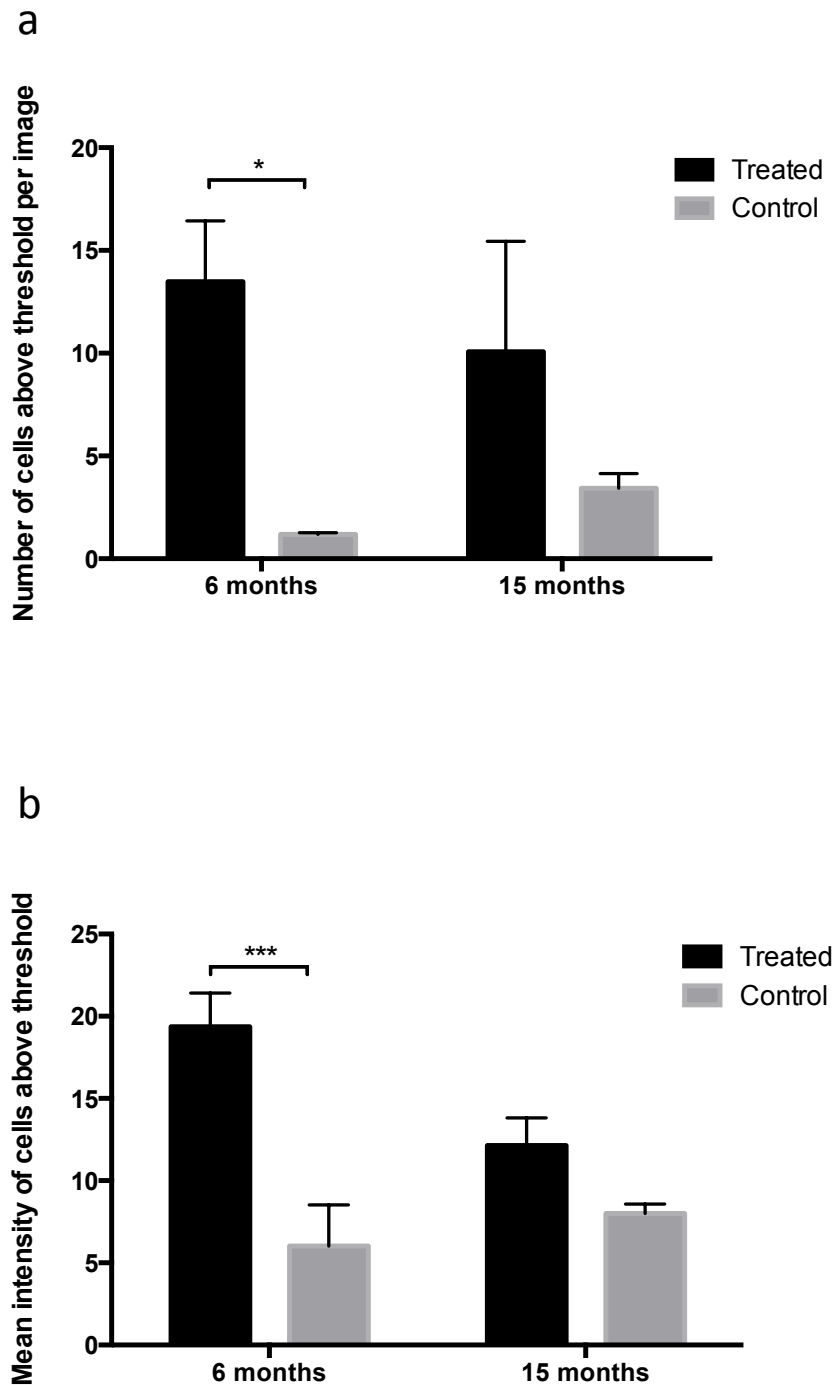


Figure 6.14 Quantification of light induced *cfos* expression in eyes that had been treated with AAV melanopsin 6 or 15 months previously in comparison to controls.

Images of retinal sections were processed using an automated method (Image J) and following thresholding, the number of *cfos* expressing cells was counted (a) or signal intensity of cells above threshold was measured (b). 6 month cohort: treated n=5, control n=4; 15 month cohort treated n=3, control n=3. * $p < 0.05$, *** $p < 0.001$ 2 way ANOVA with Bonferroni post hoc test.

In order to examine whether the cfos expression seen was induced by light, a pilot test was done in the older cohort where treated animals and untreated animals were handled in an identical manner but did or did not receive a light pulse. Examining retinal sections, there was a trend for more cfos expressing cells to be present in light pulsed retinas compared to non-pulsed retinas, figure 6.15. However following quantification of the number of cfos positive cells, the effect of light did not reach statistical significance (treated light pulsed group 10.1 ± 5.4 cells per image (total n=302), treated no light 2.4 ± 0.5 cells per image (total n=73); untreated light pulsed group 3.4 ± 0.7 cells per image (total n=103), untreated no light 1.7 ± 0.5 cells per image (total n=51); effect of light $p=0.12$, two way ANOVA, effect of treatment $p=0.21$), figure 6.16a.

There was however a significant effect of light pulsing ($p=0.0002$, two way ANOVA) and treatment ($p=0.035$) on the intensity of fluorescence of cfos expressing cells identified above, but no significant interaction between the two factors ($p=0.22$). In the treated group, mean fluorescence intensity was three-fold higher following light pulsing (12.1 ± 1.7 units after light pulse versus 3.9 ± 0.5 units no light pulse, $p=0.0012$ Bonferroni post hoc test). This effect was also seen in the untreated group (8.0 ± 0.6 units after light pulse versus 2.7 ± 1.0 units no light pulse, $p=0.015$ Bonferroni post hoc test) indicating a light dependent increase in levels of cfos within cells, figure 6.16b.

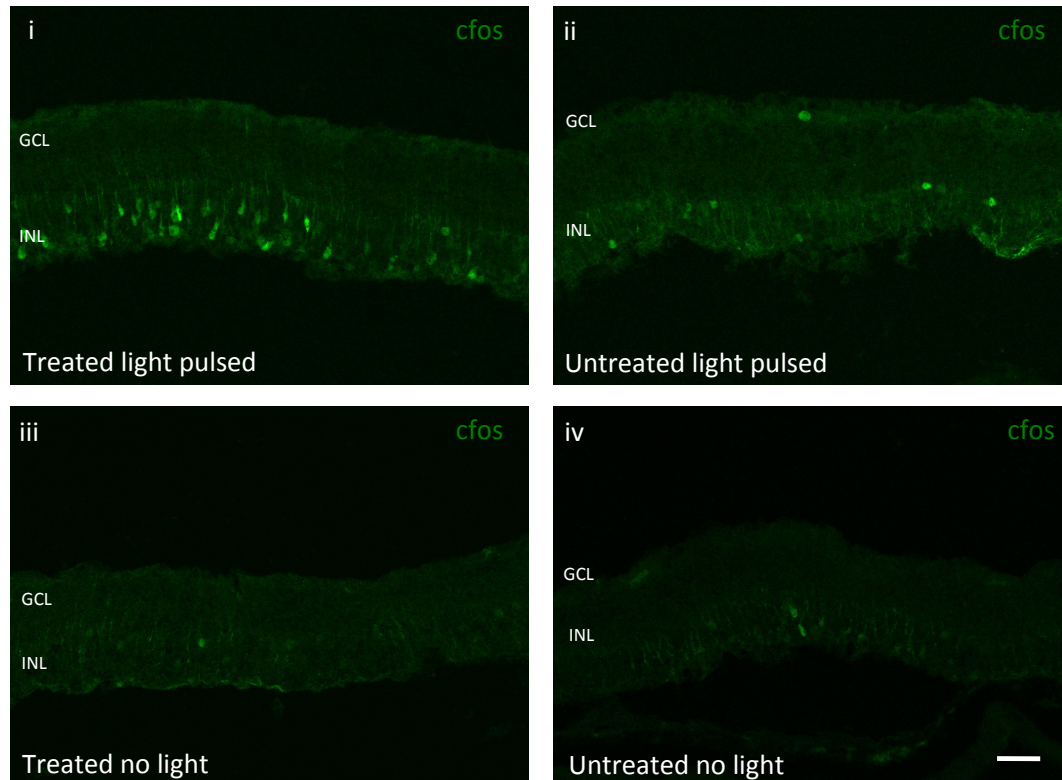
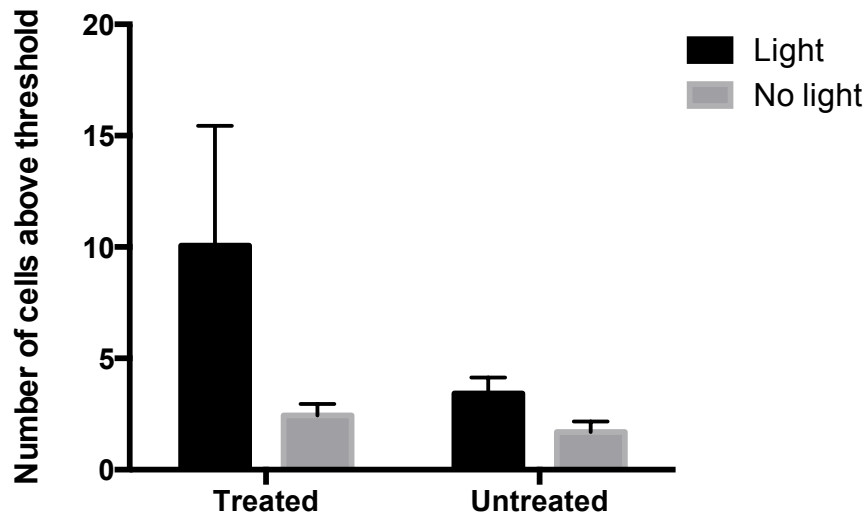


Figure 6.15 Light induction of cfos expression

Antibody staining of cfos (green) was compared in *rd1* mice treated with AAV melanopsin 15 months earlier that received a light pulse (i) with treated animals that did not receive a light pulse (iii). Age-matched untreated mice also did (ii) or did not (iv) receive a light pulse to establish background levels of cfos expression. Scale bar 50 um.

a



b

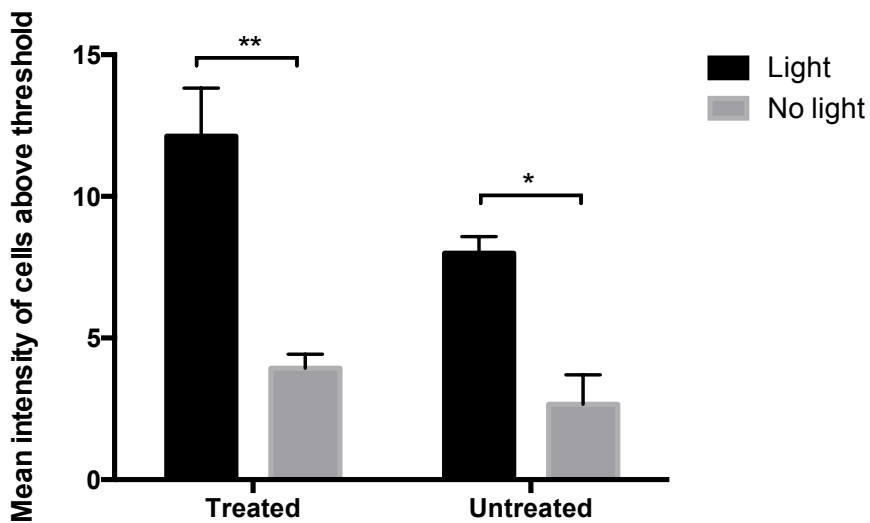


Figure 6.16 Quantification of light induction of *cfos* expression

Cfos expression was analysed in eyes that had been treated with AAV melanopsin 15 months previously following a 30 minute light pulse (n=3) in comparison to treated animals that did not receive a light pulse (n=3). An age-matched untreated control group was also included, and these animals were also divided into two groups: one group received an identical light pulse (n=3), and the other group did not (n=3). The number of *cfos* expressing cells was counted (a) or signal intensity of cells above threshold was measured (b). * $p < 0.05$, ** $p < 0.01$ 2 way ANOVA with Bonferroni post hoc test.

6.3.3 Multi Electrode Array recordings

In order to assess whether the cell depolarisation inferred from increased *cfos* expression in treated retinas was able to drive ganglion cell action potential firing, multi-electrode array recordings were performed. Since intrinsically photoreceptive ganglion cells (ipRGCs) were intact and potentially able to respond in untreated retinae, responses in a few electrodes would also be expected in this group, but any difference between treated and untreated groups would be attributable to ectopic expression of human melanopsin.

In the dark, baseline recordings revealed some spontaneous action potential firing, figure 6.17a, and also oscillatory potentials at a frequency of 7-10Hz, figure 6.17b, which are known to be a feature of degenerate retina. For the purpose of analysis, action potentials/ spikes were counted as electrical activity greater than 3 standard deviations of baseline activity, figure 6.17c, and the number of action potentials in one second bins was plotted. A light response was defined as a change in firing rate of 5Hz or greater between the ten seconds prior to light onset compared to the ten seconds after light onset. The effect of light on oscillatory potentials was not included.

Retinae from eyes that had subretinal injection of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector four months previously were compared to untreated controls. For each recording, electrical activity was not seen in every electrode either due to the retinal explant not covering the electrode or a lack of good contact. Therefore the number of electrodes that showed electrical activity out of a potential 60 are stated per experiment.

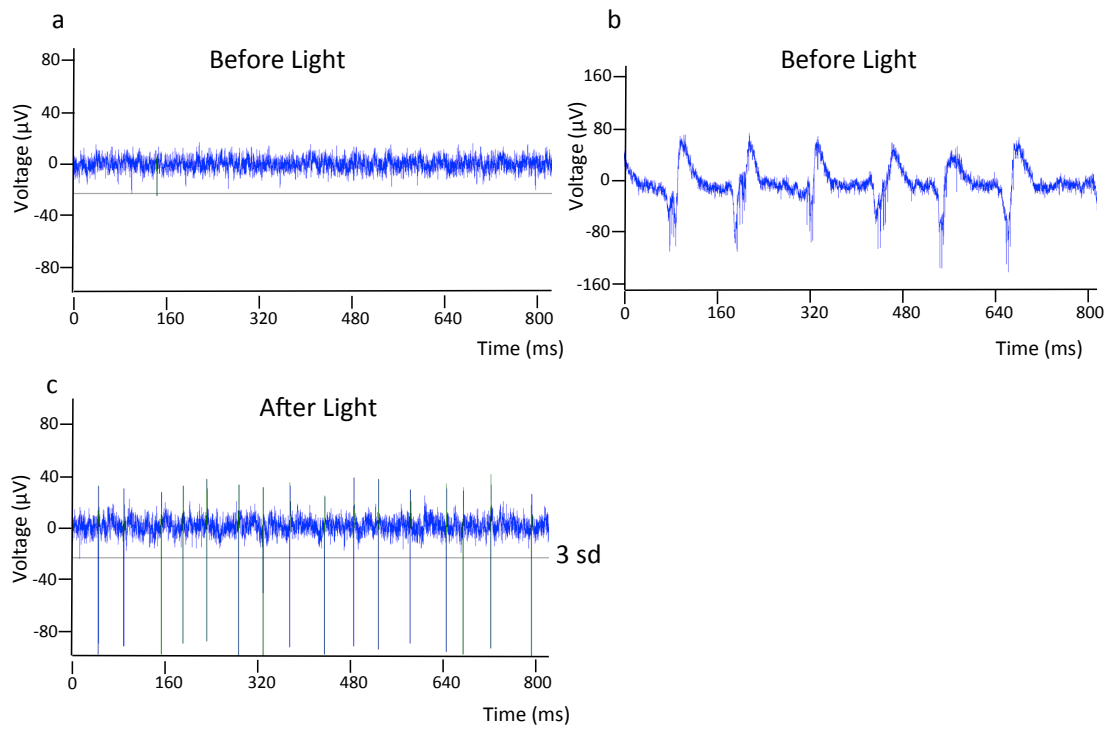


Figure 6.17 Data acquired during MEA recordings

Baseline recordings in the dark either revealed spontaneous firing activity (a) or oscillatory potentials (b). After light onset responses were of larger voltage and for analysis, an action potential was counted as a voltage change greater than 3 standard deviations of baseline activity (c).

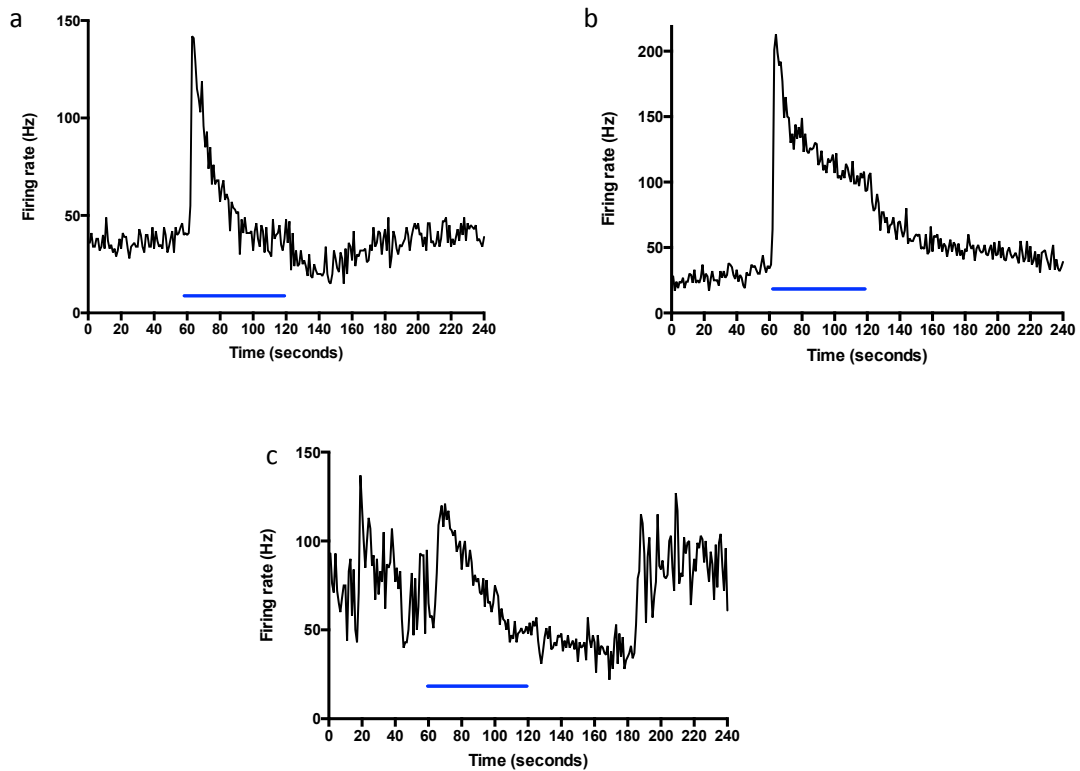


Figure 6.18 Examples of light-induced responses in a degenerate retina recorded by a multi-electrode array (MEA)

Baseline recordings in darkness were followed by a 60 seconds 480nm light stimulus (blue line) followed by a further 2 minutes of recording in darkness. Transient (a) and sustained (b) responses were seen. In a few electrodes there was an initial increase in firing in response to light followed by a reduction in firing rate (c).

Previous work using calcium imaging has documented three types of response from intrinsically photoreceptive ganglion cells: transient, sustained and repetitive, the latter showing successive transient responses after termination of the light stimulus (54). In retinæ following subretinal delivery of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector and also untreated controls, transient and sustained responses were seen with regards to action potential firing, figure 6.18a,b. No repetitive responses were seen. In a few electrodes in treated retinæ and also in one untreated retina, an initial increase in action potential firing was seen in response to the light stimulus followed by a subsequent reduction in firing rate, figure 6.18c. These electrodes tended to have high levels of spontaneous activity at baseline.

6.3.3.1 Spatial distribution of responses

Areas transduced by the rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector in treated retinas were identified by visualisation of DsRed fluorescence using 540nm light. Electrodes that showed light responses largely corresponded with areas of retina that expressed the most DsRed indicative of these being responses from ectopically expressed melanopsin, figure 6.19.

6.3.3.2 Percentage of responsive electrodes

The percentage of electrodes that showed light-dependent responses was compared in treated (N=6 retinæ, n=116 electrodes) and age-matched untreated retinæ (N=9, n=99 electrodes). In treated retinas, $34.7 \pm 14.5\%$ electrodes showed light responses in comparison to $19.7 \pm 23.4\%$ electrodes in untreated controls ($p=0.043$, Mann-Whitney test), figure 6.20. This indicates an increase in overall light-dependent action potential firing in treated retinæ compared to controls.

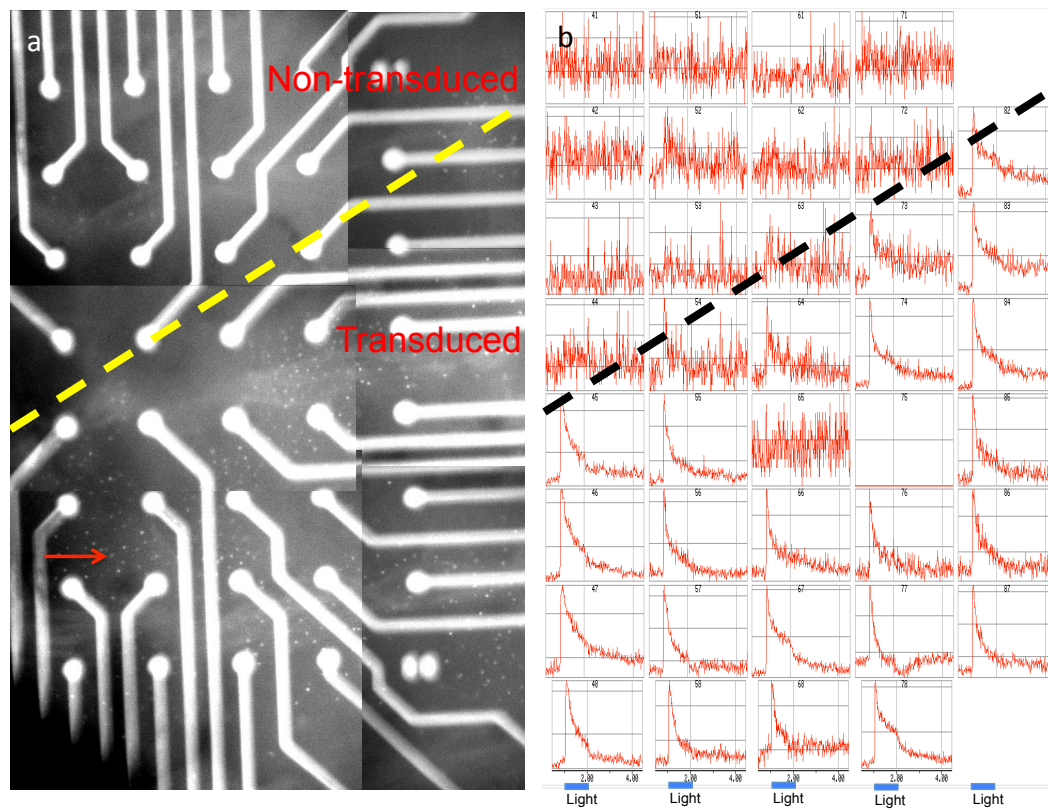


Figure 6.19 Spatial distribution of responses in a treated retina

Image (a) illustrates the MEA recording electrodes with a treated retina placed ganglion cell side down onto it. Areas of AAV transduction were detected via visualisation of DsRed fluorescence (red arrow). (b) Analysis trace of MEA recordings with action potential firing grouped into one second bins, illustrating change in ganglion cell firing in response to a 60 second 480nm light stimulus in transduced areas.

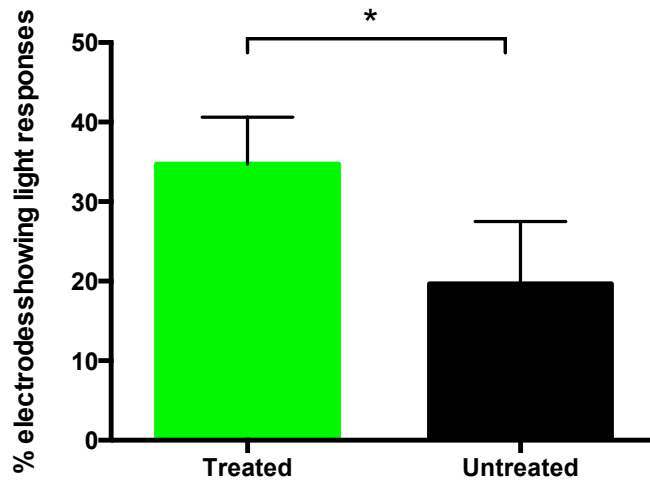


Figure 6.20 Percentage of responsive electrodes

The percentage of electrodes that showed a 5Hz or greater change in firing rate in response to a 480nm light stimulus were counted in treated (N=6 retinae, n=116 electrodes) and untreated (N=9, n=99 electrodes) retinae. There were significantly more responsive electrodes in the treated group * $p < 0.05$, Mann-Whitney test.

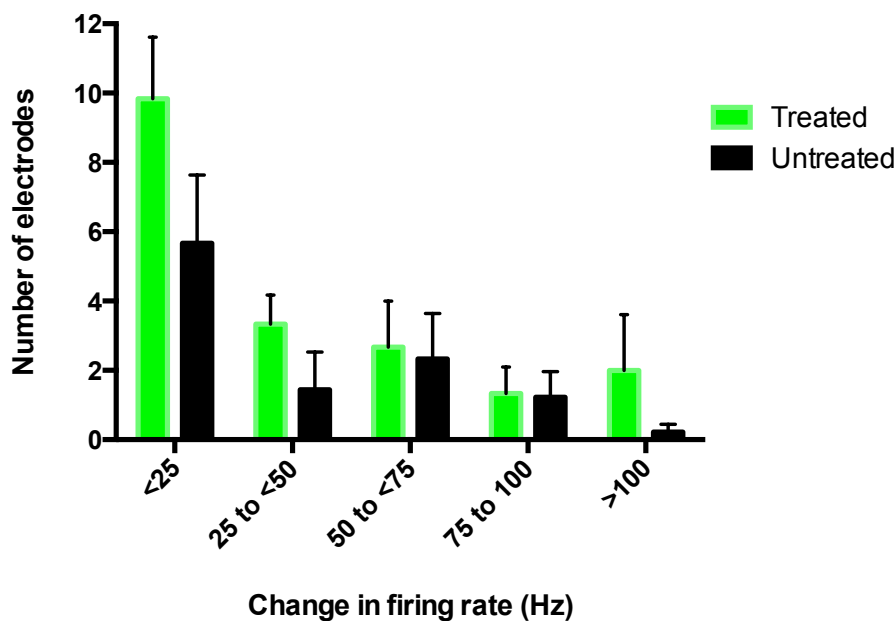


Figure 6.21 Number of responsive electrodes according to magnitude of response

The number of responsive electrodes were counted according to magnitude of light-induced change in firing rate, arranged into 25Hz bins in treated (n=6) and untreated (n=9) retinae. The number of responsive electrodes varied according to treatment ($p = 0.04$, two way ANOVA) and magnitude of response ($p < 0.0001$), with no significant interaction between the two factors. Post hoc analysis did not reach statistical significance for comparison within any of the 25Hz bins.

6.3.3.3 Magnitude of response

A response was defined as a light-induced change in firing rate of 5Hz or greater between the ten seconds prior to light onset versus the ten seconds after light onset for each electrode. In electrodes that showed light responses, the magnitude of this change in firing rate was compared in treated (n=6) versus untreated retinae (n=9).

Overall, the mean change in firing rate in response to light was 33.2 ± 8.7 Hz in the treated group versus 20.9 ± 6.7 Hz in the untreated group; this difference did not reach statistical significance ($p=0.14$, unpaired t-test). Further analysis was done by examining the number of electrodes that demonstrated different magnitudes of change in firing rate grouped into 25Hz bins, figure 6.21. The number of responsive electrodes varied according to treatment ($p=0.04$, two way ANOVA) and magnitude of response ($p<0.0001$), with no significant interaction between the two factors. There was a greater range of response sizes in treated versus untreated retinae, with more electrodes showing both smaller i.e. 5-25Hz responses (9.8 ± 1.8 electrodes in treated retinae, versus 5.7 ± 2.0 electrodes in untreated retinae) and large i.e. >100Hz responses in the treated group; although post hoc analysis did not reach statistical significance for these comparisons.

6.3.3.4 Irradiance response curves

Irradiance response curves (IRCs) were generated for responsive electrodes in treated (n=64, N=3) and untreated (n=32, N=3) retinae. For each electrode, the change in firing rate comparing ten seconds prior to light onset versus ten seconds after light onset was normalised to the response at the highest light intensity. Examining the mean IRC for electrodes from treated retinae versus untreated retinae, there was no significant effect of treatment ($p=0.87$, 2 way ANOVA). There was a significant effect of light intensity ($p<0.0001$) and the interaction between the two factors did not reach significance

($p=0.08$), figure 6.22. Examining individual IRCs of electrodes in the untreated group, there appeared to be distinct groups of responsive cells, one with high sensitivity to light and a second less sensitive group, figure 6.23a. This corresponds with previous reports regarding intrinsically photosensitive ganglion cells, which can be divided into two classes based on sensitivity (233).

In contrast, IRCs in the treated group appear to be more varied, with many more cells of intermediate sensitivity, figure 6.23b. In order to assess this difference further, the light intensity at which half maximal response was reached (effective concentration 50, EC50) was calculated for each electrode, figure 6.24. There was a significant effect of treatment on the number of responsive electrodes ($p=0.04$, 2 way ANOVA), but no significant effect of sensitivity (EC50, $p=0.24$) or overall interaction between treatment and sensitivity ($p=0.25$). Post hoc analysis revealed a trend for more electrodes with an EC50 of 13.5 to 14 to be present in the treated group versus the untreated group but this did not reach significance ($p=0.07$, Bonferroni post hoc test).

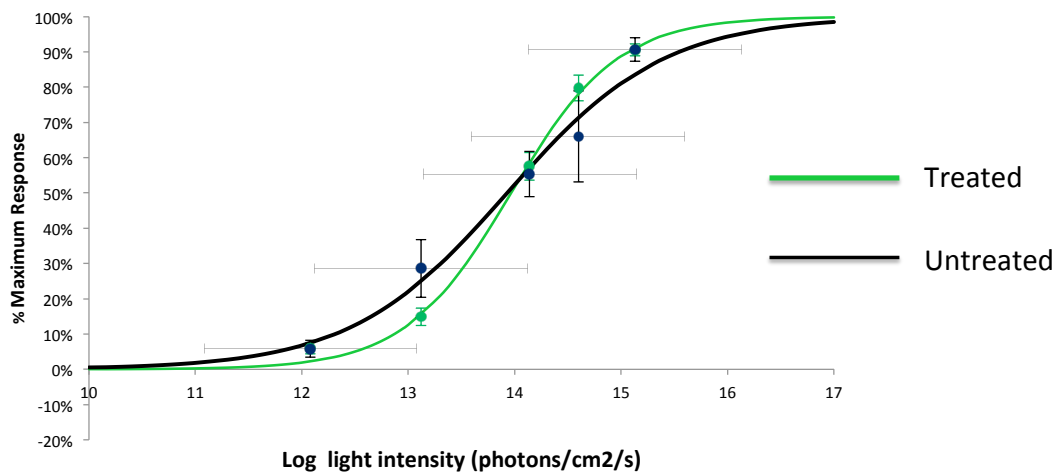


Figure 6.22 Irradiance response curves for responsive electrodes

Irradiance response curves were generated for responsive electrodes in treated (n=64, N=3) and untreated (n=32, N=3) retinæ. For each electrode, the light-dependent change in firing rate was normalised to the maximal response. There was no overall significant effect of treatment on response (treatment factor $p=0.88$, 2 way ANOVA).

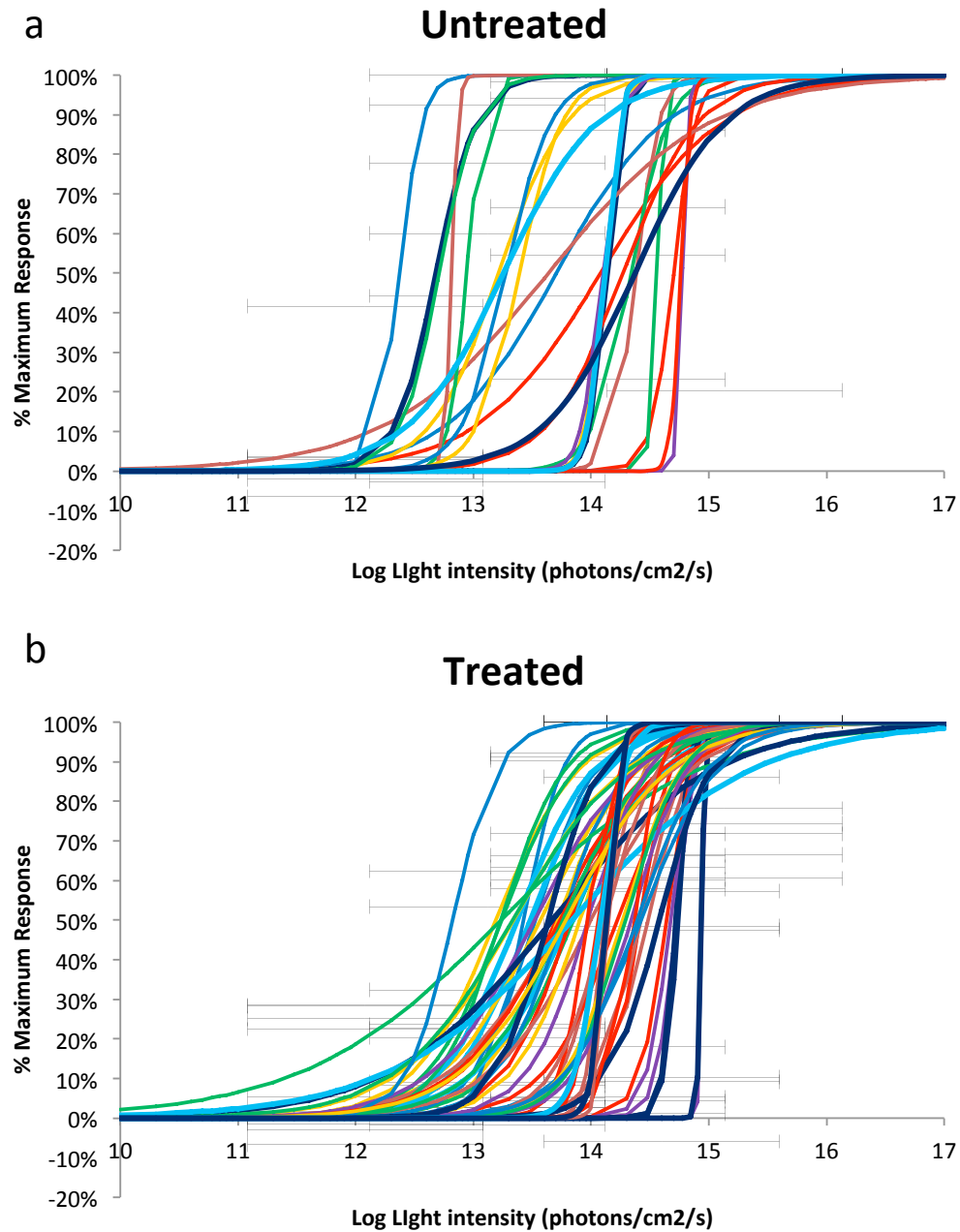


Figure 6.23 Irradiance response curves for individual electrodes

Irradiance response curves for electrodes in (a) untreated retinæ (n=32, N=3) and (b) treated retinæ (n=64, N=3) illustrating an increased number of electrodes with intermediate sensitivity in the treated group.

6.3.3.5 Time course of response

Time taken to reach maximal response following onset of the light stimulus was compared in responsive electrodes between treated and untreated retinae (both N=3) across different light intensities, figure 6.25a. There were more responsive electrodes in both groups with increasing light intensity, figure 6.25b. There was a significant effect of treatment ($p=0.019$, 2 way ANOVA) and intensity ($p<0.0001$) on the time to peak response with a significant interaction between the two factors ($p=0.0045$). At the lowest light intensity of 1.20×10^{12} photons/cm²/s, responses were slow in both groups with mean time to peak response being 21.2 ± 2.5 seconds in the treated group versus 26.0 ± 5.7 seconds in the untreated group. At the highest light intensity of 1.34×10^{15} photons/cm²/s, responses were significantly faster with mean time to peak response being 4.9 ± 0.4 seconds in the treated group versus 6.2 ± 0.5 seconds in the untreated group. Post hoc analysis revealed a significant difference between treated (5.1 ± 0.4 seconds) and untreated (8.9 ± 1.2 seconds) groups at a light intensity of 3.99×10^{14} photons/cm²/s, ($p=0.0102$, Bonferroni post hoc test), figure 6.25c.

Time to half decay ($t_{1/2}$) was calculated for responsive electrodes in treated and untreated retinae, figure 6.26a. At the highest light intensity of 1.34×10^{15} photons/cm²/s, mean $t_{1/2}$ was 12.5 ± 1.0 seconds in the treated group versus 16.0 ± 2.0 seconds in the untreated group. There was no significant effect of treatment ($p=0.72$) or light intensity ($p=0.29$) on time to half decay, with no significant interaction between factors ($p=0.29$), figure 6.26b.

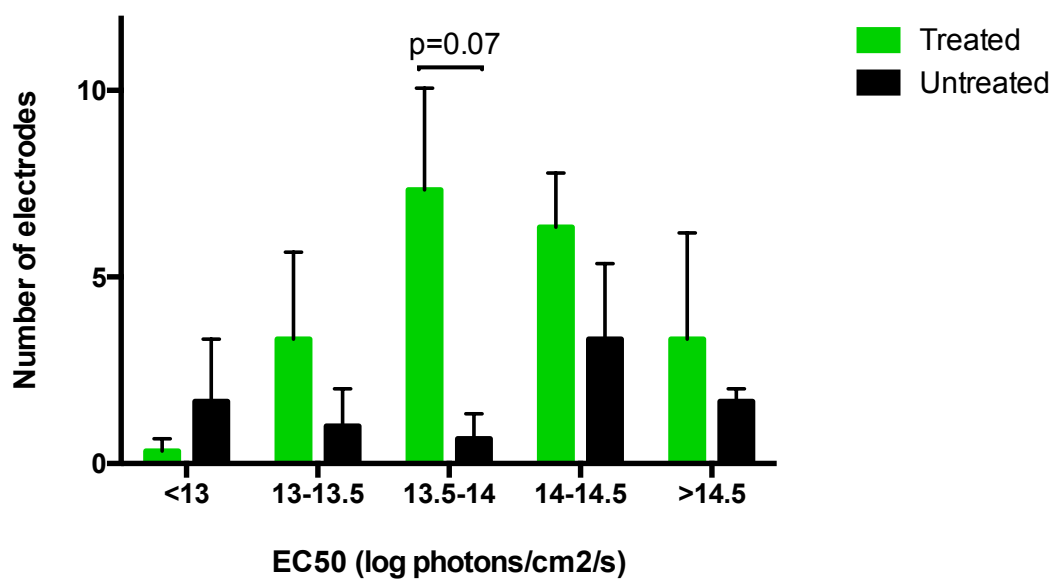


Figure 6.24 Distribution of photosensitivities of individual electrodes

The light intensity at which half maximal response (EC50) was calculated for each electrode in treated (n=64, N=3) and untreated (n=32, N=3) retinal. Treatment had a significant effect on the number of responsive electrodes (p=0.04, 2 way ANOVA). Bonferroni post hoc analysis did not reach statistical significance for comparison within any of EC50 subgroups.

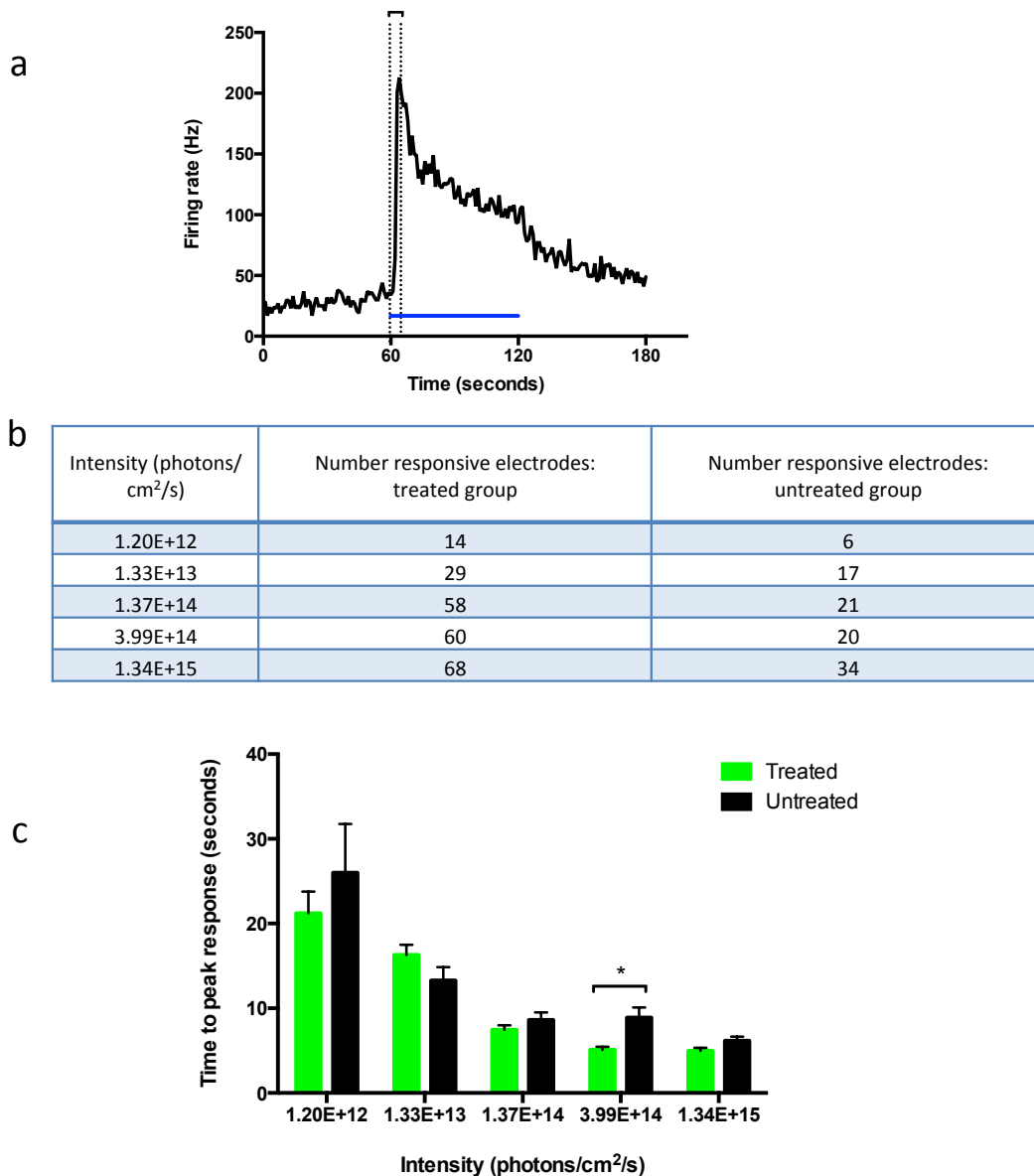


Figure 6.25 Time to peak response

(a) Time (seconds) from light onset to the maximal change in firing rate was calculated across different light intensities for each responsive electrode in treated and untreated retinæ. The blue line indicates the duration of the light stimulus. (b) The number of responsive electrodes at different light intensities is shown for treated and untreated retinæ (both N=3). (c) The time to peak response is shown for responsive electrodes (n numbers as per table in (b)) in treated and untreated retinæ according to light intensity. There was a statistically significant effect of treatment ($p=0.019$, 2 way ANOVA) and intensity ($p<0.0001$) on the time to peak response with a significant interaction between the two factors ($p=0.0045$). * $p<0.05$, Bonferroni post hoc analysis.

6.3.3.6 Short duration light stimuli

To assess whether shorter duration light pulses that are more likely to drive image-forming vision were able to stimulate melanopsin in these retinae, a pilot test was done using a two second light stimulus. In the treated retina, there were 16/54 (29.6%) electrodes that showed responses to light, whereas in the untreated retina 5/56 (8.9%) electrodes showed light responses. Change in firing rate between the ten seconds prior to and after the light stimulus was significantly higher in the treated retina (44.1 ± 8.7 Hz, $n=16$) compared to the untreated retina (9.9 ± 1.6 Hz, $n=5$; $p=0.015$ Mann Whitney test), figure 6.27a.

The treated retina was further tested by stimulation using two second pulses every sixty seconds for five minutes to assess repeatability of the response, figure 6.27b. A few electrodes stopped showing light responses over the first four repetitions, however by the fifth stimulus there were no responsive electrodes, figure 6.27c. There was a significant reduction in the magnitude of response over the successive stimulus repetitions (44.1 ± 8.7 Hz first stimulus, 27.0 ± 4.7 Hz second stimulus, 22.4 ± 4.2 Hz third stimulus, 14.0 ± 3.7 Hz fourth stimulus, -2.4 ± 0.9 Hz fifth stimulus; $p<0.0001$, one way ANOVA). However, in absolute terms a moderate sized response was maintained over the first four stimulus repetitions, figure 6.27d.

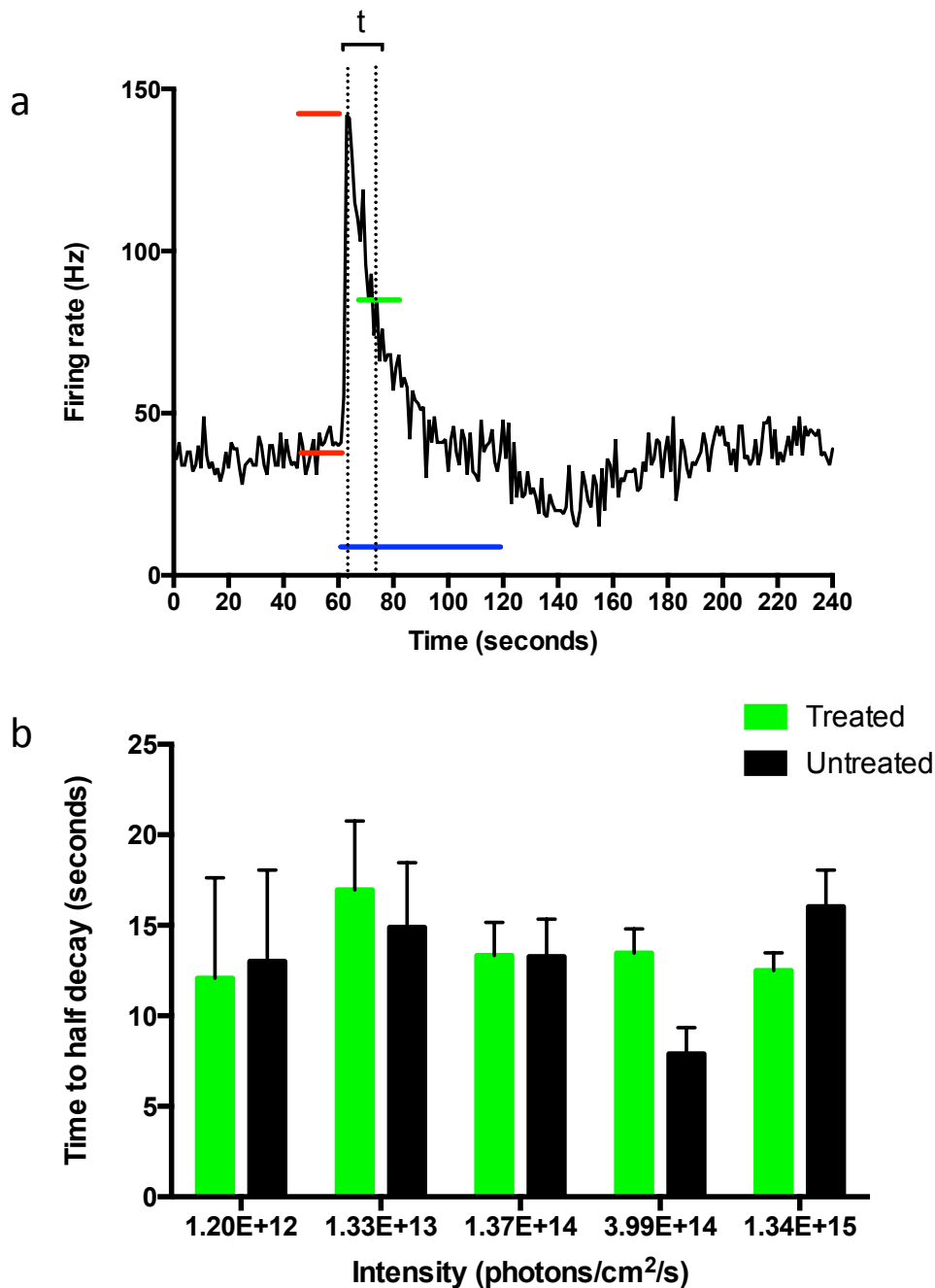


Figure 6.26 Time to half decay

(a) The time (seconds, t) to half decay was calculated for each responsive electrode in treated and untreated retinæ across different light intensities. This was calculated as the time from peak reponse (upper red line) to reach half the value between peak response and baseline firing rate (green line), where the latter was calculated as the average firing rate in the ten seconds preceding the light stimulus (lower red line). The blue line indicates the duration of the light stimulus. (b) The time to half decay is shown for responsive electrodes in treated and untreated retinæ according to light intensity (number of electrodes as per figure 6.25b, $N=3$ per group). There was no significant effect of treatment or light intensity on time to half decay (2 way ANOVA).

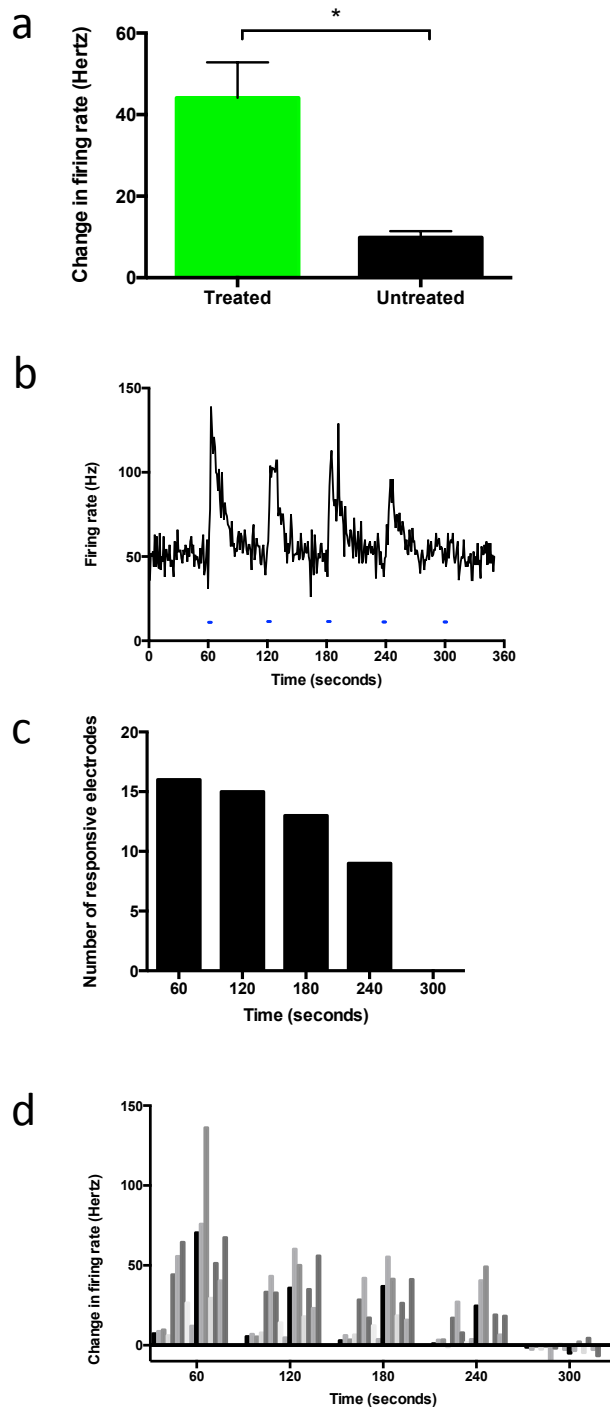


Figure 6.27 Short duration light stimuli

(a) The change in firing rate of responsive electrodes following a two second light stimulus was compared in a treated (n=16 electrodes) and untreated (n=5 electrodes) retina *p<0.05, Mann Whitney test. (b) An example of an electrode response following repeated two second light stimuli to a treated retina every 60 seconds (c) The number of electrodes in a treated retina that showed light responses on successive two second stimulations every 60 seconds (d) The magnitude of response of individual electrodes on successive two second stimulation every 60 seconds.

6.3.3.7 Effect of rod/cone blockers

In order to determine whether any of the responses seen originated from residual cone cells in the degenerate retina, a pilot experiment was run to assess the effect of rod/cone blockers (175). In the treated retina, there was a 67.5% increase in the number of electrodes that showed light responses in the presence of rod/cone blockers. The change in mean firing rate of electrodes in response to light increased from 17.7 ± 7.0 Hz without drugs to 30.5 ± 6.3 Hz in the presence of rod/cone blocking drugs, however this difference did not reach statistical significance ($p=0.06$, two-tailed paired t-test), figure 6.28a. In the untreated retina tested, there was a 245.9% increase in the number of electrodes that showed light responses in the presence of rod/cone blockers. The change in mean firing rate in response to light increased four fold from 11.2 ± 4.8 Hz in the absence of rod/cone blocker to 43.5 ± 8.5 Hz in its presence, ($p<0.001$, two-tailed paired t-test), figure 6.28b.

6.3.3.8 Effect of Melanopsin antagonist

A melanopsin antagonist (10 μ M AA92593, (174)) was used in a pilot test to assess its effect on light-induced responses. In the treated retina, there was a 17.6% decrease in the number of electrodes that showed light responses in the presence of Opn4 antagonist. Mean firing rate in response to light was halved from 48.9 ± 6.05 Hz in the absence of drugs to 23.3 ± 3.5 Hz in the presence of Opn4 antagonist, ($p<0.001$, two-tailed paired t-test), figure 6.29a. In the untreated retina, there was a 69.5% decrease in the number of electrodes that showed light responses in the presence of Opn4 antagonist. The mean firing rate in response to light was reduced almost 10 fold from 51.3 ± 5.3 Hz at in the absence of Opn4 antagonist to 4.6 ± 1.0 Hz in its presence ($p<0.001$, two-tailed paired t-test), figure 6.29b.

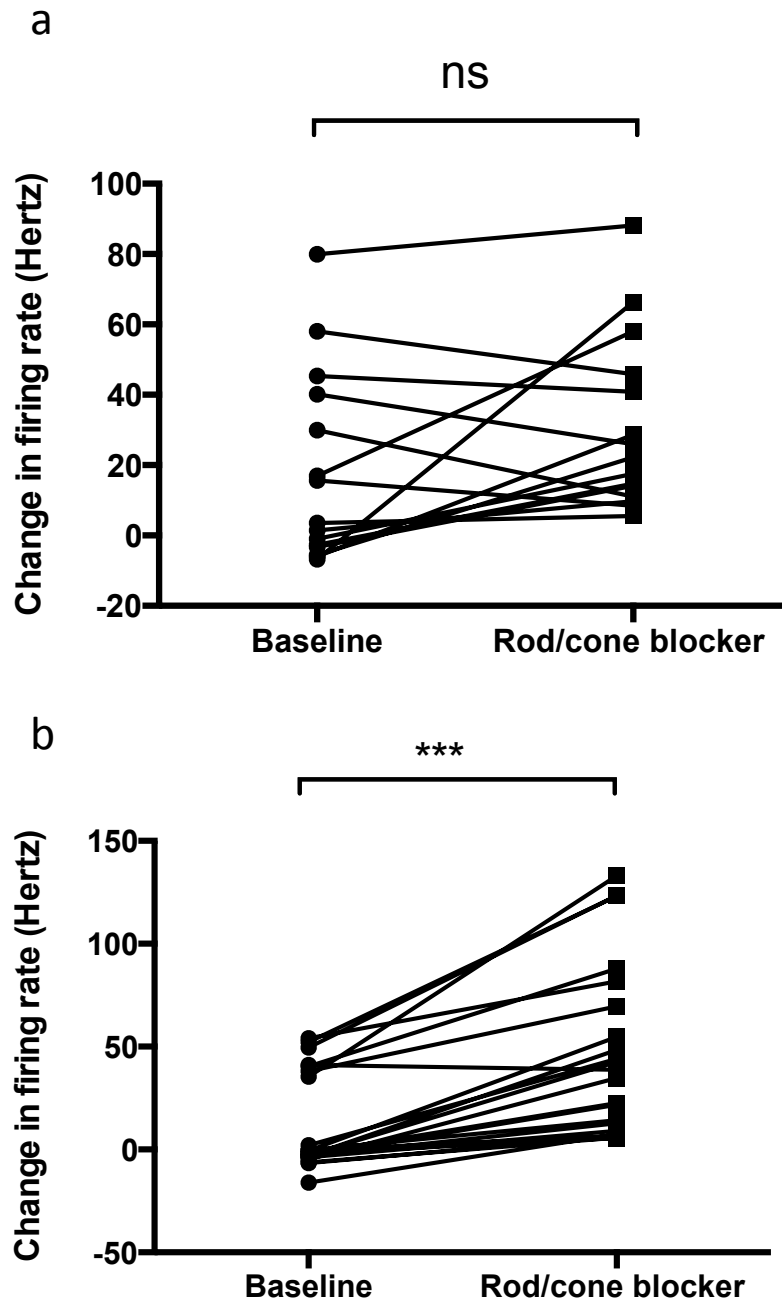


Figure 6.28 Effect of rod/cone blockers

There was no significant effect on the change in firing rate in response to light before and after the addition of rod/cone blockers in a (a) treated retina (n=15 electrodes, ns=non-significant, 2-tailed paired t-test). Addition of rod/cone blockers did significantly increase the change in firing rate in response to light in an untreated retina (b) (n=23 electrodes, *** p<0.001, 2-tailed paired t-test).

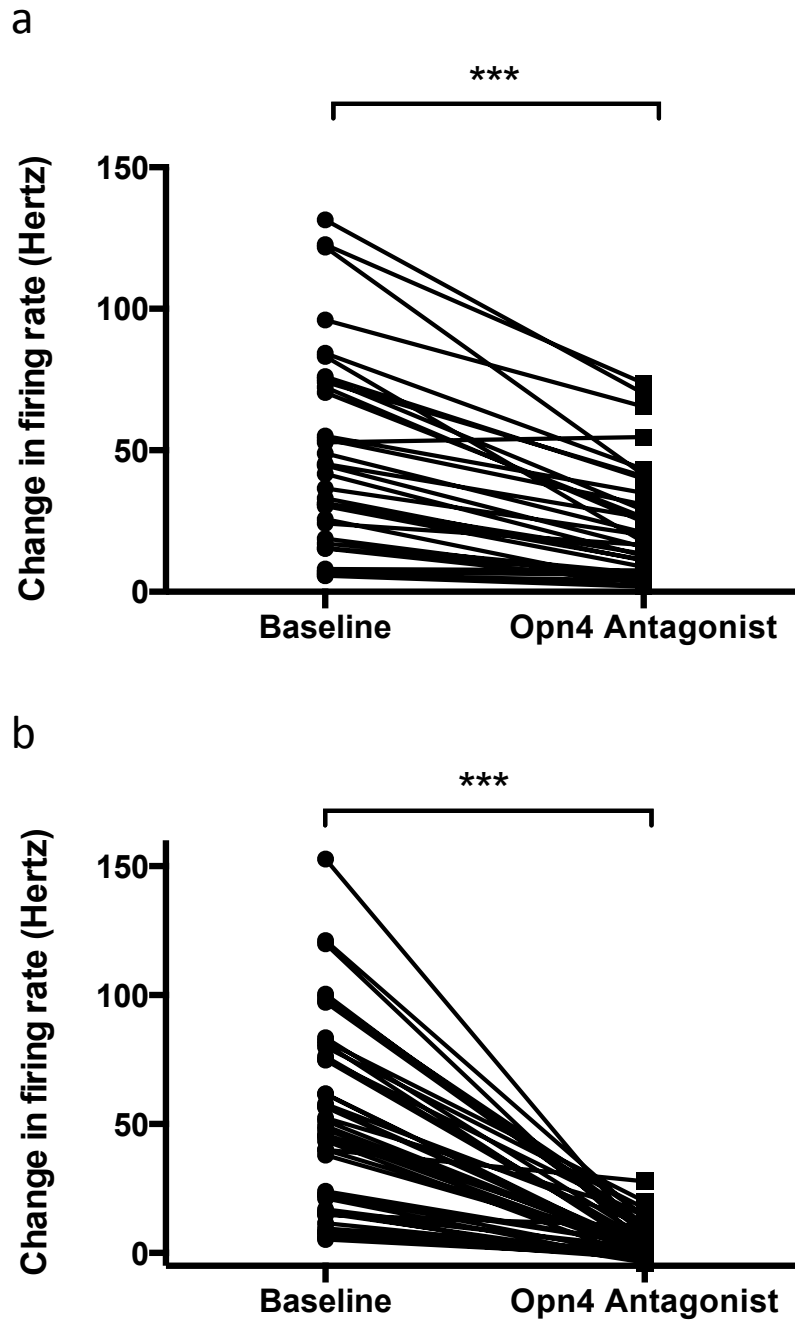


Figure 6.29 Effect of melanopsin antagonist

There was a significant effect on change in firing rate in response to light before and after the addition of melanopsin antagonist to (a) treated (n=34 electrodes) and (b) untreated retina (n=46 electrodes). *** $p < 0.001$, 2-tailed paired t-test.

6.3.3.9 Effect of CPP/ NBQX

In order to try to establish the pathway via which ganglion cells were stimulated in treated retinae, a pilot test was done in the presence of 10 μ M 3-(2-carboxy- piperazin-4-yl)propyl-1-phosphonic acid (CPP) and 10 μ M 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX) (87). These block NMDA and AMPA/kainate ionotropic glutamate receptors, and therefore synapses made via rod ON bipolar cells to AII amacrine cells and cone ON and OFF bipolar cells to corresponding ganglion cells. There was a 5.4% decrease in the number of electrodes that showed light responses in the presence of CPP/NBQX. The mean firing rate in response to light was reduced from 24.9 ± 5.3 Hz in the absence of drugs to 17.9 ± 3.8 Hz in the presence of CPP/NBQX, ($p=0.0012$, two-tailed paired t-test), figure 6.30.

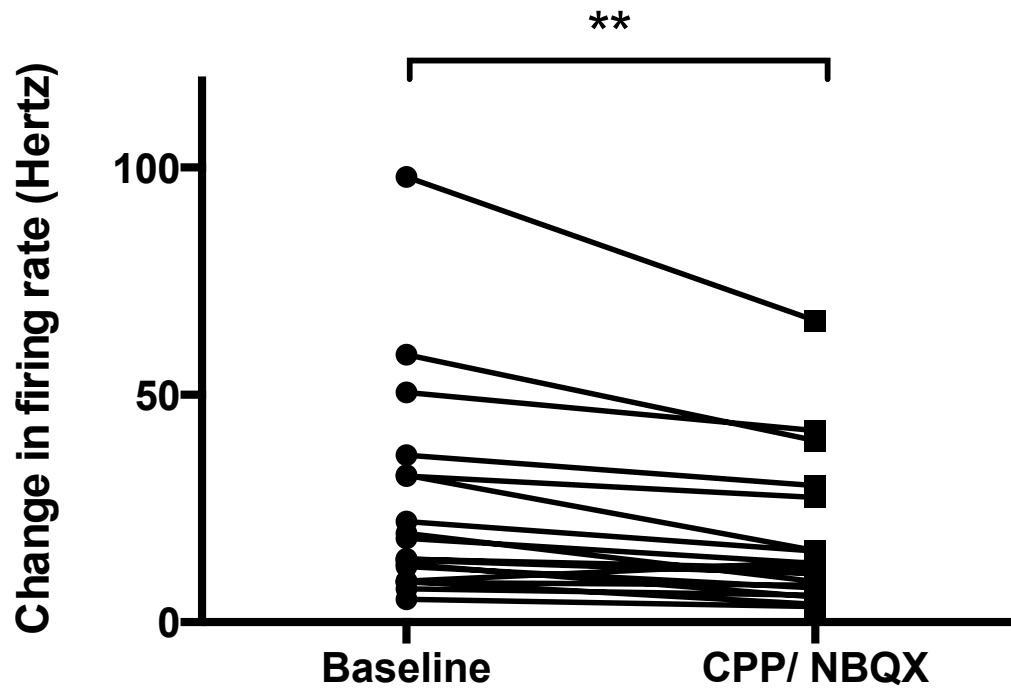


Figure 6.30 Effect of CPP/NBQX

There was a significant effect on change in firing rate in response to a light stimulus in the presence of CPP and NBQX ionotropic glutamate receptor blockers in a retina following subretinal delivery of AAV melanopsin (n=19 electrodes). ** p<0.01, 2-tailed paired t-test.

6.3.4 Laser speckle cortical imaging

To assess whether action potentials generated by ganglion cells could reach central targets and be processed by the visual cortex, the technique of laser speckle cortical imaging was used. Following bilateral two second stimulation with 480nm light, cortical blood flow was imaged over the visual cortices through the cranium using the Speckle Contrast Imager, figure 6.31.

Examining mean change in cortical blood flow (CBF) in treated versus sham animals, mice in the treated group showed an increase in cortical blood flow with an initial peak at 5.4 seconds after light onset, figure 6.32. This is consistent with the expected peak in cortical blood flow, which occurs at five to seven seconds after onset of light in wild type animals (85). There was no clear corresponding peak in the sham-injected group.

The change in CBF between groups was quantified by calculating the area under the curve for the time series data within the first 10 seconds after onset of light. This was higher in the treated group versus the sham-injected group (152.6 ± 94.5 versus 40.2 ± 21.6), although this did not reach statistical significance ($p=0.14$, unpaired t-test), figure 6.33a. The two groups were also compared by examining peak change in CBF within the first seven seconds after onset of light. Again this was higher in the treated group than in the sham-injected group ($1.08\% \pm 0.47$ versus 0.43 ± 0.08) but the difference did not reach statistical significance ($p=0.10$, unpaired t-test), figure 6.33b.

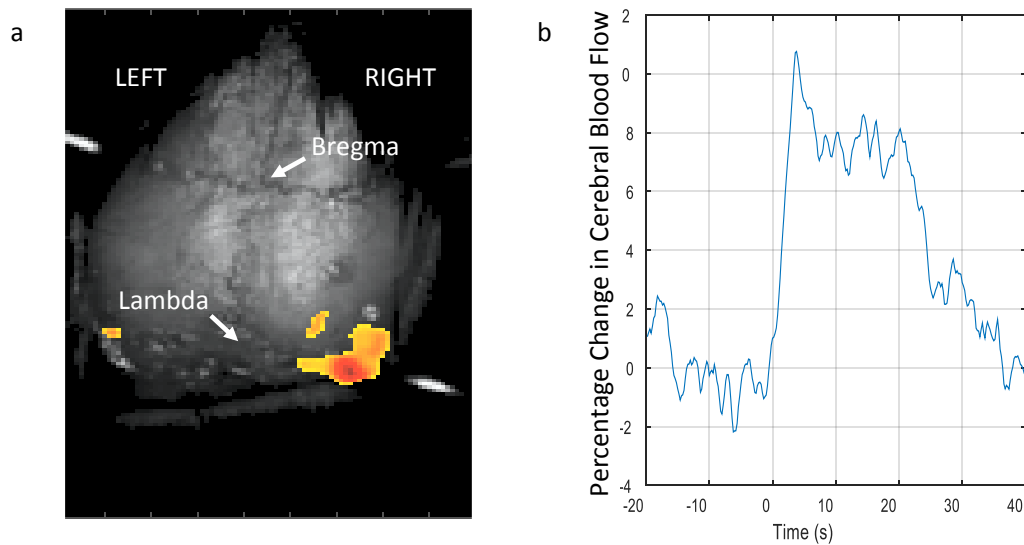


Figure 6.31 Laser speckle cortical imaging

(a) Photograph of exposed skull, indicating positions of Bregma and Lambda as well as left and right directions. Activation in response to visual stimulation is overlayed, with a large response corresponding to right visual cortex, and a small response corresponding to the left visual cortex. (b) Time series of percentage change in cerebral blood flow following a 2 second 480nm light stimulus to both eyes from the region of right cortex highlighted in the activation map.

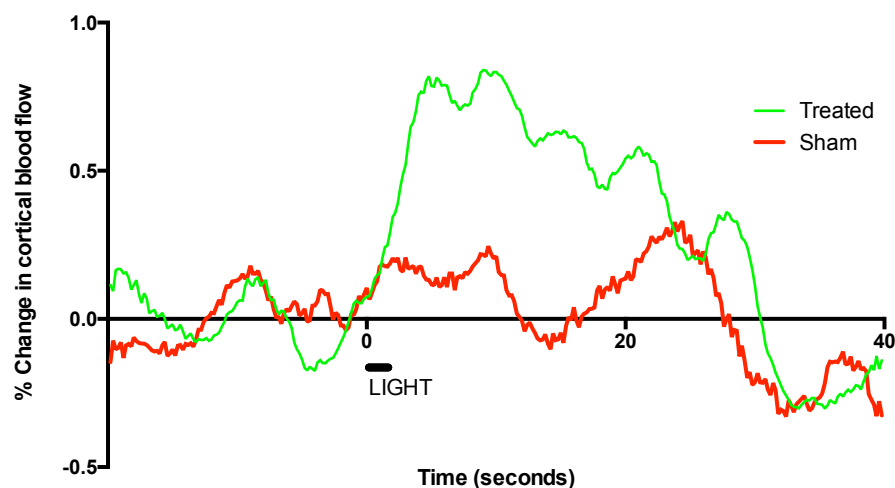


Figure 6.32 Mean blood flow responses from laser speckle cortical imaging

Blood flow changes in the visual cortex were measured for 10 seconds in darkness and then following a 2 second 480nm light stimulus to both eyes of treated (n=6) and sham-injected (n=5) mice. Mean responses in both groups are shown.

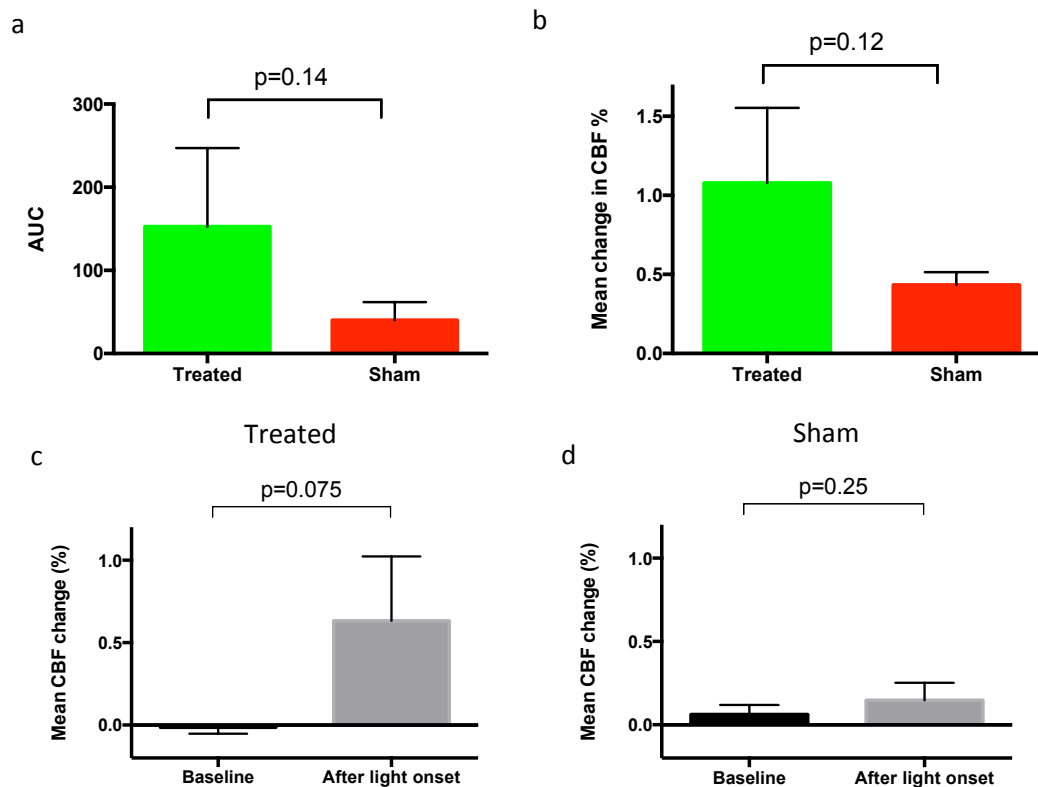


Figure 6.33 Quantification of laser speckle cortical imaging responses

Responses were quantified using (a) area under the curve of the first 10 seconds after light onset and (b) maximal change in cortical blood flow after light onset for the treated (n=6) and sham-injected (n=5) mice tested. Further analysis was done comparing mean blood flow change in the ten seconds before and after light onset in (c) treated and (d) sham-injected mice. p values indicate those for (a,b) unpaired and (c,d) paired t-tests.

Further analysis was done comparing mean blood flow in the 10 seconds preceding light onset versus the 10 seconds after light onset for each animal. In the treated group there was a large change in CBF after light onset ($-0.014\% \pm 0.04$ before light versus $0.63\% \pm 0.39$ after light, $p=0.075$ paired t-test). In the sham-injected group there was no difference in blood flow comparing before and after light ($0.06\% \pm 0.05$ before light versus $0.14\% \pm 0.10$ after light, $p=0.25$ paired t-test), figure 6.33 c,d.

All three methods of comparison indicate a trend for light-induced responses in the treated group, although none reached statistical significance. To evaluate this further, individual animal data were inspected. This revealed separate groups of 'responders' and 'non-responders' in the treated group, figure 6.34a. CBF data were therefore converted to standard scores (z-statistics) and animals were classed as responders if the mean CBF change in the 10 seconds following light onset exceeded $z=2.333$ (corresponding to $p<0.01$), figure 6.34b. This identified three responders in the treated group and none in the sham-injected group. These three responders showed appropriate peaks in cortical blood flow within the first ten seconds after onset of light. Analysis of area under the curve and peak CBF change in the treated responders versus the sham-injected animals revealed a statistically significant difference between groups via both methods ($p=0.015$ and $p=0.017$ respectively, unpaired t test), figure 6.35.

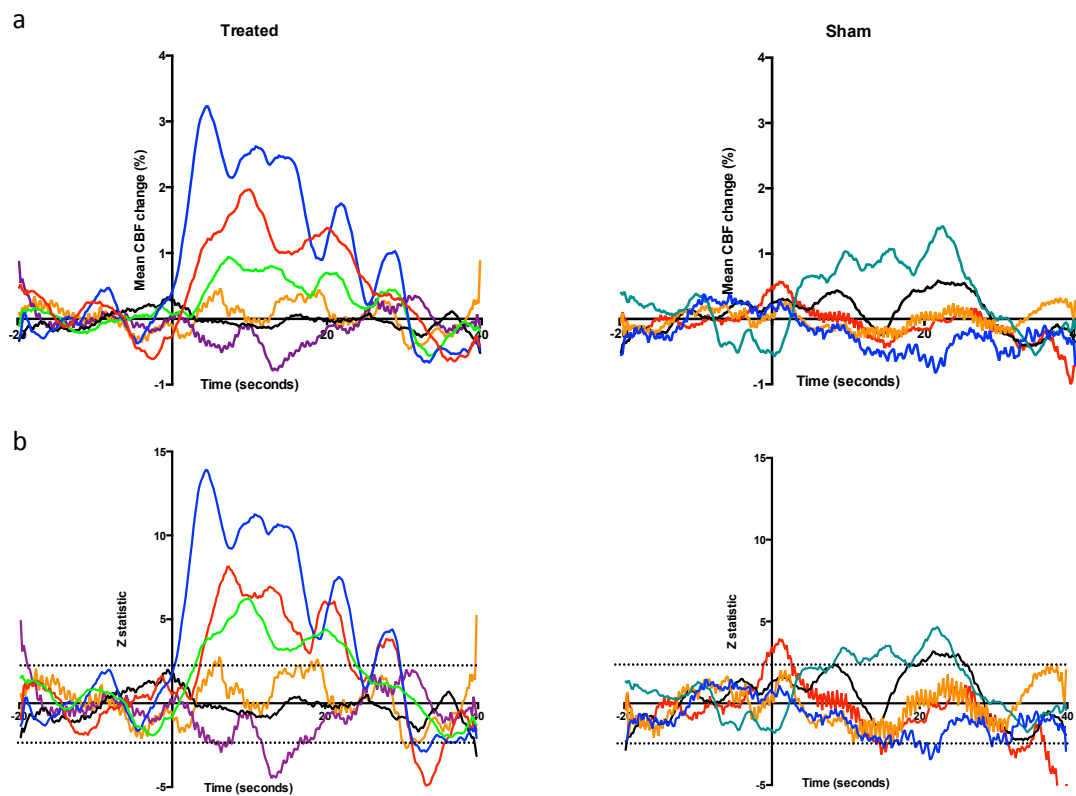


Figure 6.34 Responses of individual animals

Traces show cortical blood flow changes for individual mice tested in (a) treated (n=6) and (b) sham-injected (n=5) groups. Subsequently data were converted to standard (Z) scores and animals classed as responders if the mean response was greater than $z=2.33$ in the first 10 seconds after light onset (corresponding to $p<0.01$) in treated (c) and sham-injected (d) animals.

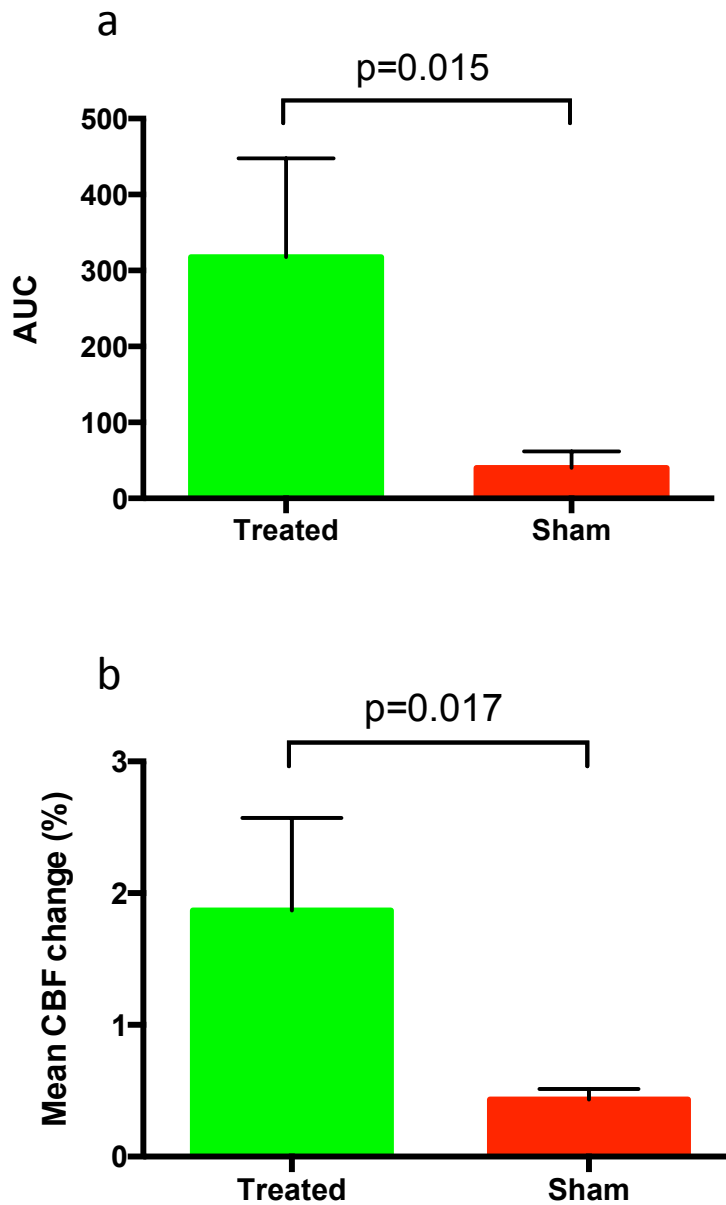


Figure 6.35 Quantification of cortical blood flow changes between responders and sham-injected mice

Animals identified as responders (n=3) were compared to sham-injected mice (n=5) by analysing area under the curve (a) and peak change in cortical blood flow (b). Both showed a significant difference between groups, p values indicate those for unpaired t-tests.

6.4 Discussion

In previous chapters the vector for human melanopsin gene delivery to the degenerate retina was optimised with regards to AAV capsid type, promoter and codon-optimisation. In this chapter, experiments were performed to determine whether human melanopsin expressed in the degenerate retina following subretinal injection of this vector was sustained in the long term. Further work was done to establish whether this was able to mediate cell depolarisation, drive ganglion cell action potential firing and assess if these signals could mediate a physiological change in the visual cortex.

The degree of degeneration within the *rd1* retina was assessed by comparing the number of residual cones (75) in treated versus control retinæ to ensure any responses were not related to the presence of these cells. In all groups these cones were morphologically abnormal with no detectable outer segments and opsin mislocalised to the entire cell membrane, as previously reported (166). In the cohort assessed at 15 months, very few cones remained in any group and therefore this factor was unlikely to have affected results. In the cohort assessed at 6 months, there was variation within each group as to the number of cones remaining but overall there were significantly more residual cones in the untreated versus the treated group. Therefore responses seen in the treated group in excess of those seen in untreated mice cannot be attributed to residual cones. In addition, all mice were homozygous for the *gpr179* mutation, and therefore had abnormal photoreceptor to ON bipolar cell signalling (232). Further supporting that functional responses seen in the treated group were not initiated by residual cones

Immunohistochemistry demonstrated human melanopsin expression at 4 months and also at 15 months after administration of AAV vector. Extensive transduction of the

degenerate retina was seen at both time points, indicative of longevity of gene expression, consistent with previous reports using other AAV vectors (223). The experiments performed involved assessment of each eye at a single time point, and so conclusions cannot be drawn as to whether any reduction in expression was seen over this time period. Comparison of expression between different retinae is difficult since surgical factors probably have the greatest influence on the amount of retinal transduction, with variation between eyes due to the extent of retinal detachment and any reflux of the vector after administration. Therefore, to establish levels of gene expression over time, *in vivo* imaging such as measurement of DsRed fluorescence over time using the confocal scanning laser ophthalmoscope (cSLO) could be performed.

In order to determine if human melanopsin expressed was able to mediate cell depolarisation, *cfos* induction following a light stimulus was assessed (224, 227). Treated retinae were compared with controls, both containing native ipRGCs. Following antibody labelling, treated retinas in the cohort evaluated at 6 months showed a greater number of *cfos* expressing cells and a higher intensity of fluorescence i.e. more *cfos* expression per cell in comparison to controls. A similar trend was seen in mice examined at 15 months. The majority of *cfos* positive cells in treated retinae were seen in the inner nuclear layer consistent with the pattern of cell transduction using the melanopsin vector and distinct from *cfos* expression expected in ipRGCs in degenerate retinae (227). Some *cfos* positive cells also expressed human melanopsin on co-labelling. This indicates that human melanopsin expressed in these cells was able to mediate cell depolarisation. In addition, some cells adjacent to those expressing human melanopsin were also *cfos* positive, suggestive of cell to cell signalling and depolarisation in neighbouring cells. This is well documented in the ganglion cell layer where ipRGCs are coupled to other cells via gap junctions, the latter also showing light

responses (54). It is therefore possible that gap junctions between horizontal cells (235) or bipolar cells (236) in the inner nuclear layer could mediate a similar effect.

Multi-electrode array recordings were performed to determine whether delivery of human melanopsin to the degenerate retina by AAV could generate ganglion cell action potential firing. A greater percentage of electrodes showed light-dependent changes in action potential firing in treated retinæ compared to untreated controls, light responses in the latter being attributable to ipRGCs. The data presented in this chapter probably underestimates this effect since in the treated group, the sample of retina placed on the electrodes may have included some untreated areas, figure 6.19.

The data presented also provide evidence that the nature of responses were different in treated versus untreated retinæ. There was a trend for a greater variety to be seen in the magnitude of responses in treated retinæ, with more electrodes showing small (<25Hz) but also large (>100Hz) responses. The time to reach peak response was shorter in treated versus untreated retinæ across a range of light intensities. Examining irradiance response curves of responding electrodes, in untreated retinæ there appeared to be two populations of cells, one with high and another with low sensitivity to light consistent with previous reports of ipRGCs (228). In treated retinæ there was a much greater range of sensitivities, with more electrodes showing intermediate sensitivity than untreated controls. This may be indicative of action potential firing from different populations of ganglion cells transduced by the melanopsin vector.

Pilot studies were performed to try to elucidate the influence of various cell types or pathways on the response. In the presence of rod/cone blockers (175), a greater number of responsive electrodes and a larger change in firing rate was seen compared to in the absence of drugs in both treated and untreated retinæ but only reaching

statistical significance in the latter. This is consistent with previous work that has also demonstrated increased ganglion cell responses to light in degenerate retina in the presence of synaptic blockers, due to reduced spontaneous firing in the dark in the presence of these drugs (237).

A pilot experiment was also performed using a melanopsin antagonist (AA92593) which acts via competitively inhibiting binding of chromophore i.e. retinal to melanopsin (174). When administered, light-induced change in firing rate was reduced by over half in the treated retina and by ten fold in the untreated retina in presence of the drug. This is consistent with its description as an incomplete inhibitor (174). The difference between treated and untreated retina may be due to the density of melanopsin expressing cells in the inner nuclear layer of treated retina, which may have been harder to access than ipRGCs in the ganglion cell layer of untreated retina. In addition when dissecting the retina, patches of retinal pigment epithelium are often stuck to the neuroretina, which would have provided a source of chromophore to melanopsin expressing cells of the INL in treated retina, which may have reduced the efficacy of the melanopsin antagonist. Furthermore, it may be that different doses are required to inhibit human melanopsin in the treated retina versus mouse melanopsin in the control retina. Further work is therefore required to optimise the dose of melanopsin antagonist administered and establish whether this can fully attenuate responses in the treated retina.

A pilot study was also performed using the ionotropic glutamate receptor blockers CPP and NBQX on a treated retina to establish whether blockade of the rod ON bipolar to amacrine cell synapse or cone bipolar ON or OFF synapse to ganglion cells had an effect on the response. A reduction in response of about 20% was seen. It is difficult to draw conclusions from this pilot study, and further replication of this experiment is required.

It may be that the concentration of drug administered was not adequate to fully inhibit this synapse, or that the response is mediated via an alternative pathway following remodelling in the degenerate retina.

Laser speckle cortical imaging was used to assess whether ganglion cell generated action potentials could result in a physiological change within the visual cortex. Three out of six treated mice showed significant increases in cortical blood flow in the visual cortex following a light stimulus. The kinetics and magnitude of these responses were consistent with previous reports of light-dependent changes (85). No such early responses were seen in sham-injected animals. The fact that only three animals responded in the treated group could be explained by differences in levels of transduction: these animals may have had more human melanopsin expression compared to the animals that showed no responses and further work is required to determine this. Alternatively, the lack of response seen in these animals may be related to the depth of anaesthesia required for the procedure. This may affect physiological responses (C. Martin, personal communication), resulting in a change in cortical blood flow not being generated and is a limitation of the technique used.

6.5 Conclusions

The data presented demonstrates long-term protein expression following subretinal delivery of the human melanopsin gene via an AAV vector. Several experiments support that this melanopsin expressed is functional: transduced cells demonstrated increased *cfos* expression in response to a light stimulus suggestive of cell depolarisation. Using multi-electrode array recordings, an increased percentage of electrodes showed light-dependent changes in ganglion cell action potential firing in treated retinas compared to

ipRGC mediated responses in untreated controls. The characteristics of responses were also different in treated versus untreated retinæ, suggesting firing of different types of ganglion cells in the former. Laser speckle cortical imaging showed evidence of a light-dependent increase in blood flow in the visual cortex in treated versus control animals, indicative of a physiological response in the visual cortex driven by expression of human melanopsin in the degenerate retina. Further work was then undertaken to ascertain whether these responses are able to generate functionally useful visual information.

Chapter 7 Assessing restoration of visual function

7.1 Introduction

Subretinal delivery of human melanopsin to the *rd1* mouse using a capsid mutant AAV vector results in sustained expression at 15 months after injection, with *ex-vivo* multi-electrode array recordings and *in vivo* light pulsing highly indicative of functional photosensitivity mediated by the expressed melanopsin. Furthermore, laser speckle cortical imaging demonstrates a light-dependent change in blood flow in the visual cortex of treated mice. However, this signalling to the visual cortex does not necessarily indicate the presence of physiologically relevant information that can be used by the animal to mediate a light-induced response. Therefore, the next stage of assessment sought to assess whether the increased photosensitivity derived from melanopsin gene therapy to the degenerate retina is useful for driving the pupil light reflex and also visually guided behaviour.

In a healthy eye, light detected by rods, cones and ipRGCs mediates the pupil light reflex (PLR). Action potentials carried from the optic nerve of one eye travel bilaterally to the olivary pretectal nuclei and are then bilaterally passed on to the Edinger-Westphal nuclei. The latter contains parasympathetic preganglionic neurons, which pass to the ciliary ganglion, synapse and postganglionic axons travel via the short ciliary nerve to innervate the iris sphincter muscles of each eye, figure 7.1 (240). Thus light stimulation of one eye results in bilateral pupil constriction (the consensual response). In the absence of rods and cones, a pupil light reflex can still be triggered via ipRGCs by a stimulus of high intensity and long duration (53). We sought to assess whether gene

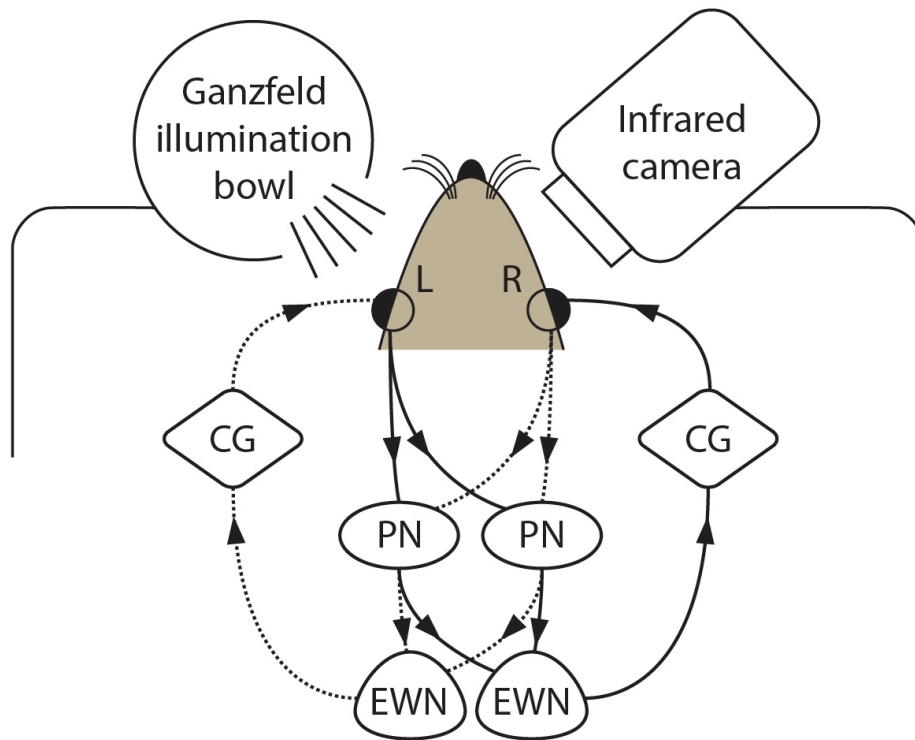


Figure 7.1 Schematic of the pupil light reflex illustrating the ipsilateral and consensual light response.

Retinal illumination results transmission of signals to both pretectal nuclei (PN) and Edinger–Westphal nuclei (EWN). Efferent impulses are transmitted via the ciliary ganglia (CG) and both pupils constrict. Adapted from (85).

therapy using human melanopsin increased sensitivity of the PLR in the *rd1* mouse, indicating that the retinal light sensitivity conferred was functionally useful.

The PLR is a brainstem reflex, whereas restoration of functional vision requires more complex processing. In order to assess whether the photosensitivity mediated by melanopsin gene therapy could drive a behavioural response, a light avoidance assay was used. Rodents, being nocturnal animals, exhibit behavioural light avoidance (BLA), in that they have a tendency to avoid light exposure in an open environment (241). On this basis, the “light/ dark box test” has been developed where mice are able to move freely between a light and dark environment and the amount of time spent in each compartment tracked, which has been used extensively to investigate anxiety and anxiolytic drugs (242). More recently the test has been utilised to assess recovery of visual function after potential treatments for end-stage retinal degeneration (85, 87, 88), since a mouse must be able to detect light in order to exhibit BLA.

A further level of assessment is to examine whether treatment with melanopsin gene therapy can restore image forming vision, rather than light detection alone, in treated mice. For this purpose a visual context recognition task was used. This is based on the understanding that mice encode the background context in which they encounter an object. For example, when a familiar object is encountered in an unfamiliar environment, the object is seen as novel and so more time is spent exploring it compared to when the object is re-encountered in its original environment (243-246). Using this principle, a visual context task assesses whether an animal is able to detect a change in visual environment with all other factors being constant. Previous work has shown that rodless-coneless (*rd/rd cl*) mice perform poorly in this visual context recognition test, indicating that these mice are not able to encode visual information regarding their environment (Tam et al, manuscript in preparation). We aimed to assess

whether melanopsin gene therapy could affect performance in treated *rd1* mice compared to controls.

In order to attempt to quantify visual acuity following melanopsin gene therapy, the optomotor response (OMR) was assessed. In response to a moving repetitive stimulus pattern, characteristic compensatory eye movements are seen: a slow smooth pursuit eye movement in the direction of movement of the pattern and a fast saccadic eye movement in the opposite direction (247). The neural circuitry involved in these eye movements includes retinal projections that innervate the contralateral nucleus of the optic tract (NOT), which generates ipsilateral smooth pursuit eye movements via connections to the medial vestibular, VIth and IIIrd nerve nuclei, figure 7.2. In primates, there is also a cortical contribution via ipsilateral retinal fibres, which project to the lateral geniculate nucleus and in turn feed back to the NOT (248). However in rodents lesions of the visual cortex do not affect the OMR implying that it is driven by a subcortical pathway in these animals (249). Horizontal movement of an image only in the temporal to nasal direction seems to stimulate the response (250) and so each eye drives smooth pursuit eye movement in a different direction. In rodents there is also a characteristic slow head movement which tracks the direction of motion (251), the generation of which does not seem to involve the cortical pathway. Therefore clockwise movement of an image stimulates the left eye to generate head tracking, and anticlockwise movement stimulates the right eye to generate head tracking (249).

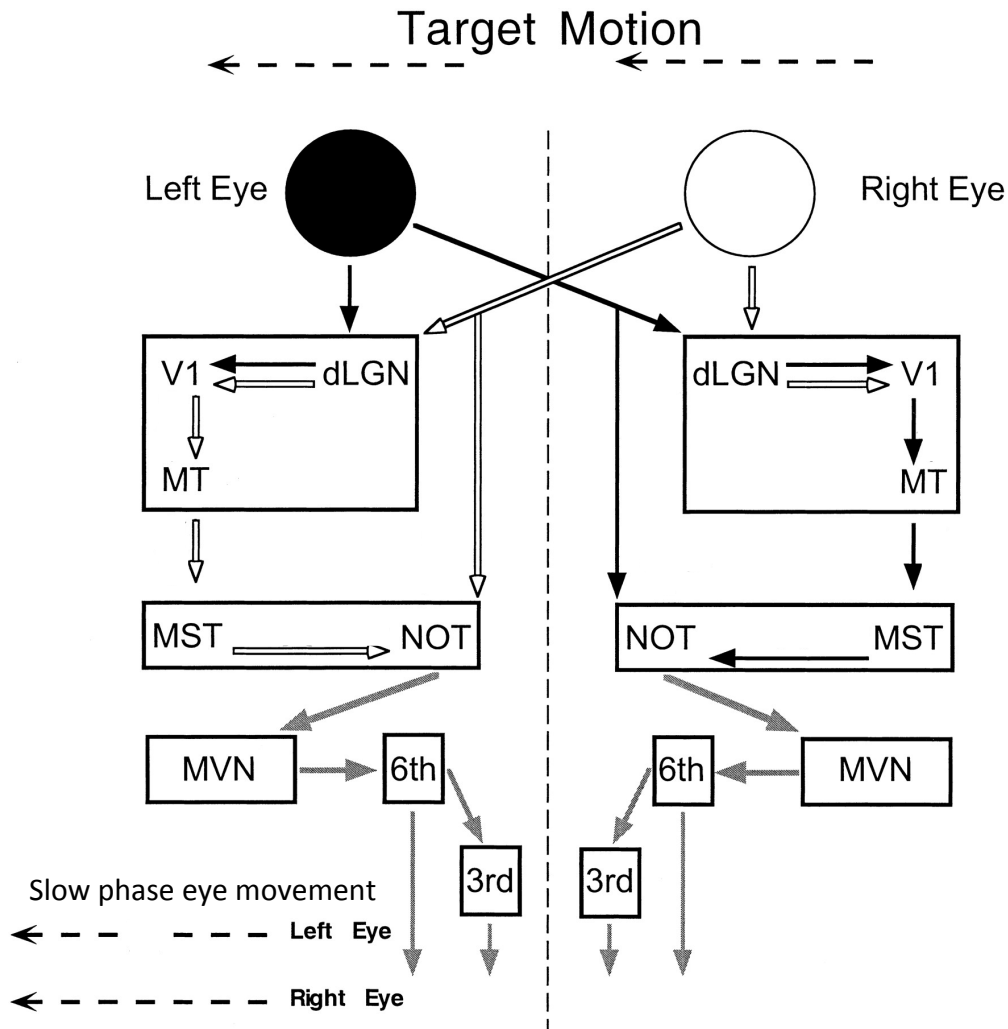


Figure 7.2 Diagram of the circuit mediating optokinetic nystagmus.

The nucleus of the optic tract (NOT) receives a direct retinal projection from the contralateral eye. There is also a cortical afferent system which includes a projection from the lateral geniculate nucleus (LGN) to the primary visual cortex (V1) to the middle temporal area (MT). Each NOT generates ipsilateral smooth pursuit eye movements via a projection to the medial vestibular nucleus (MVN), 6th nerve nucleus (6th) and 3rd nerve nucleus (3rd). Adapted from (248).

There is evidence that blindness from an early age leads to superior olfactory abilities in humans and improved odour discrimination than in sighted controls (252). Functional MRI has also demonstrated a stronger activation of the occipital cortex in blind compared to sighted individuals when performing an odour detection task (253). Therefore, an odour test was performed to assess whether long-term blindness in mice led to increased olfactory discrimination, and whether treatment using melanopsin gene therapy had an effect on this cortical remodelling. A modification of the object recognition test was used to evaluate odour discrimination (254), and previous work has shown that young mice lacking rods and cones (*rd/rd cl*) perform similarly to wild type mice in an odour discrimination task (Tam et al, unpublished data). We therefore compared performance of two cohorts of *rd1* mice of different ages including groups treated with melanopsin gene therapy, sham-injected mice and untreated aged-matched controls to assess whether there was a difference between groups in odour detection ability and therefore potentially altered cortical remodelling.

7.2 Experimental design

The overall objective was to ascertain whether human melanopsin gene delivery to the degenerate retina via an AAV vector could restore light-dependent behavioural responses. A mouse model of retinal degeneration, the *rd1* mouse, was used and mice were six to eight weeks old at the time of injection. Three groups were compared: the first had subretinal injection of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector, the second had a sham injection of phosphate buffered saline to account for any surgical effects and a third untreated natural history control group was included, see section 2.7.3 for detailed methods. All testing was done on two separate cohorts of mice; one assessed two months after subretinal injection and the other twelve to thirteen months after treatment. A number of tests were used for this purpose, full methodology is detailed in Chapter 2.

7.2.1 Pupil light reflex (PLR)

The apparatus used allowed stimulation of the mouse's left eye using a white light stimulus, the intensity of which could be varied using neutral density filters, see section 2.7.7 for detailed methods. The pupil response from the right (contralateral) eye was simultaneously recorded using an infra-red camera, figure 7.1. Since the native melanopsin system in ipRGCs is intact in these mice, a light stimulus of high intensity and long duration would be expected to cause pupil constriction in all groups (47). Therefore a brief white light stimulus of two seconds duration was chosen to minimise stimulation of ipRGCs. A range of intensities (5.5×10^{13} , 5.0×10^{14} , 2.0×10^{15} and 2.0×10^{16} photons/cm²/s) was used to assess sensitivity of the PLR and constriction kinetics.

7.2.2. Behavioural light aversion (BLA)

This assay was used to assess whether treated mice showed a change in behaviour based on an increased level of ambient light, see section 2.7.8 for detailed methods. The test involved placing each mouse into a box divided into two halves, one of which was in darkness and the other illuminated by a white light of 200 lux intensity at floor level, and these were joined by an aperture to allow movement between the two chambers, figure 7.3a. A relatively low intensity light stimulus was chosen to minimise stimulation of ipRGCs. The movement of each mouse was recorded via a camera placed over the bright half for five minutes, after which time the mouse was returned to its home cage. Movement was analysed by automated ANY-maze software for the first three minutes of the test, as per previous reports (87). The software tracked position of the mouse in the bright half of the test arena, figure 7.3b, or dark half, figure 7.3c.

7.2.3. Visual context recognition

This task was used to assess whether treated mice could detect a change in visual environment independent of ambient light levels. The basis of this test is an object recognition task comprising a sample phase, delay phase and test phase. In the sample phase, a mouse is placed into an open top box and allowed to explore two identical replicates of an object for a limited period of time. After a short delay, the mouse is placed into a further open top box with one replicate of the object previously encountered and another novel and distinct object. In this, the test phase, the animal is allowed to explore the familiar and novel objects. Animals often spend more time exploring the novel object, and this novelty preference is taken as evidence for recognition of the previously encountered object as being familiar (255, 256).

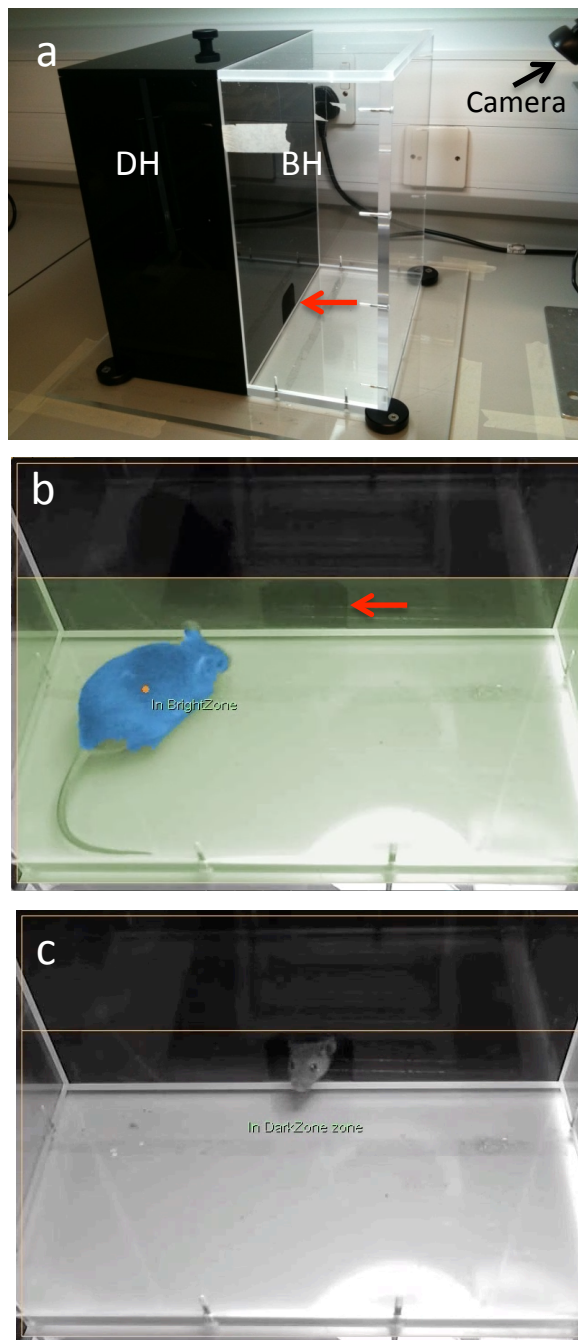


Figure 7.3 Behavioural light aversion assay.

(a) The test is a box divided into two halves, one of which was in darkness, dark half (DH), and the other bright half (BH) illuminated by a 200 lux white light, and these were joined by an aperture to allow movement between the two chambers (red arrow). Movement of the mouse during the test was recorded using a camera. ANY-maze software was used to track the position of the mouse in the bright half (b) and time not spent in the BH (i.e. spent in the dark half) was also recorded (c).

A visual context recognition task can be incorporated into this test to assess image-forming vision, based on the understanding that animals detect the background context in which they encounter an object. For example, when a familiar object is encountered in an unfamiliar background context with all other factors being constant, animals tend to spend more time exploring the object than when it is encountered in its original context (243-246).

In the first test or 'Same' condition, the trials are performed as described above with the sample and test phases both being carried out in an open top box made of white acrylic i.e. the visual environment is constant throughout the test, figure 7.4a . The same mouse was then tested at least 4 days later in a similar test but with a change in visual environment during the test ('Change' condition). For this test, the sample phase is carried out in an open top box with a checkerboard pattern with alternating 4 cm × 4 cm black and white squares on one wall and a white symmetrical five-point star on a black background on another wall, figure 7.4b. The test phase is then carried out in an open top box made of white acrylic as previously, and therefore creating a change in visual environment between the sample and test phases. All other aspects of the object recognition test are identical under the 'Same' and 'Change' conditions, and the test arenas were illuminated by white light of 50 lux intensity throughout. For half of each group tested, the 'Same' condition was assessed first, and for the other half the 'Change' condition done first. Movement of the mouse was recorded using a camera suspended over the box, see section 2.7.9 for detailed experimental protocols.

Videos of behaviour during the test were analysed using automated ANY-maze software and movement of the mouse's head was tracked. Two outcome measures were assessed: distance travelled during the test phase and recognition ratio.

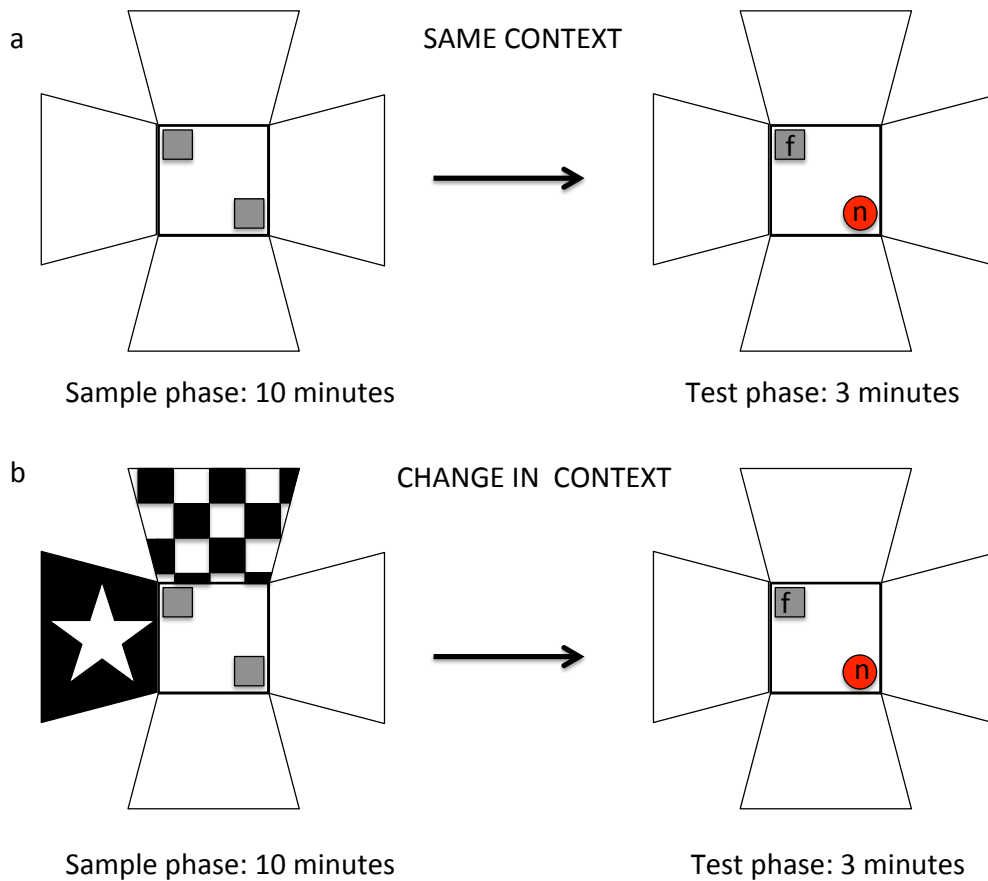


Figure 7.4 Visual context recognition testing.

In the sample phase, a mouse is placed into an open top box and allowed to explore two identical replicates of an object for 10 minutes. After a short delay, the mouse is placed into a further open top box with one replicate of the object previously encountered (grey square, f) and another novel and distinct object (red circle, n). In the same context condition (a) the visual environment is the same in both phases. In the change in context condition (b) there is a change in visual environment between the two phases but all other factors are constant. The recognition ratio is calculated as $n/(n+f)$, ie the time spent exploring the novel object as a fraction of the total time spent exploring objects.

Distance travelled is that in metres travelled by the mouse during the test phase when the novel and familiar object are encountered. Since a mouse travels less in a familiar environment (257), distance travelled can be used as a measure of visual environment recognition. Recognition ratio is expressed as $n/(n+f)$, where f and n represent the average time per minute spent in contact with the familiar versus new objects (245). A preference for the novel object would give a ratio of greater than 0.5, and preference for a familiar object would give a ratio less than 0.5. A ratio of 0.5 would suggest that the animal did not differentiate between familiar and novel objects during the test.

7.2.4. Optomotor response

The optokinetic reflex was evaluated to investigate whether the spatial resolution of vision in treated mice could be measured. This test involved placing an unrestrained mouse on a platform in a large rotating cylinder with black and white stripes of defined width and contrast on its walls, figure 7.5 a,b. For this assessment, a vertical grating of 0.1 cycles per degree was used, which was the grating of lowest spatial frequency available and therefore was most likely to be detected. A white light of 1000 lux intensity at the level of the platform illuminated the drum and three trials, each of one minute duration, were run per mouse, see section 2.7.11 for full experimental details. Characteristic head tracking movements matching the direction and speed of movement of the drum (251) were counted manually from video footage.

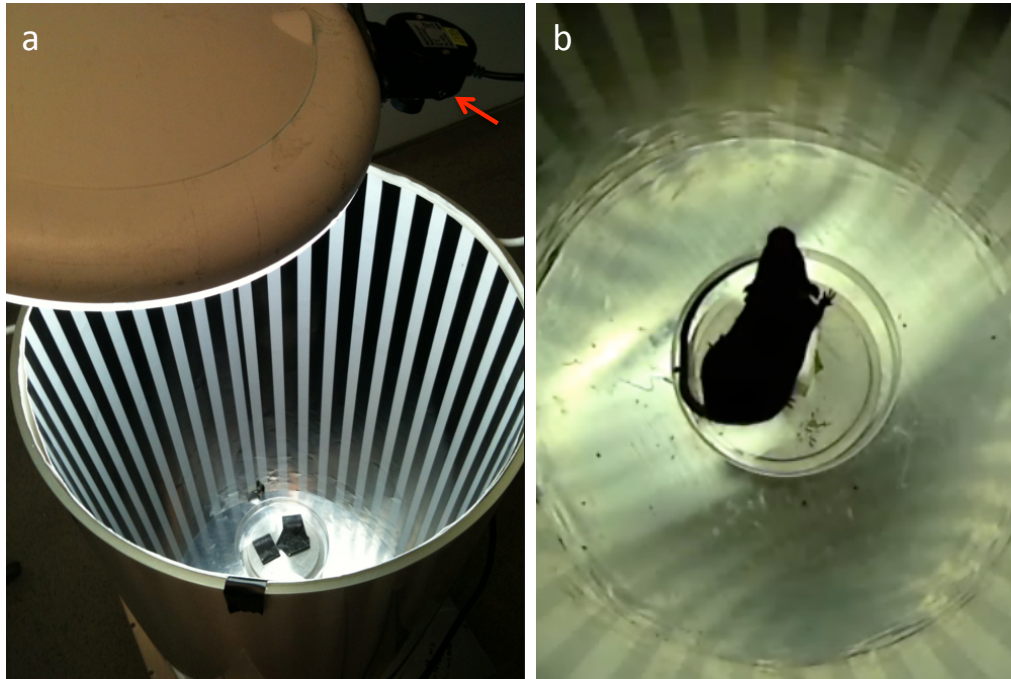


Figure 7.5 Evaluating the optomotor response.

(a) The apparatus used was a rotating drum lined with black and white stripes of a defined grating. The arena was illuminated by a 1000 lux white light, and movements of the mouse tested were recorded using a camera attached to the light (red arrow). (b) Mice were placed unrestrained onto an elevated platform in the centre of the drum. 3 x 1 minute trials were run per mouse and the results averaged.

7.2.5. Odour discrimination testing

This was performed to investigate whether aged *rd1* mice had a heightened sense of smell compared to younger animals and whether there was any difference between sham-injected or untreated controls and the treated group.

A modification of the object recognition test was used to assess the ability to discriminate odours (254). In these trials the stimulus was a small glass containing a scented liquid (1 ml of chocolate or banana extract). In the sample phase, the mouse was placed in a test arena with two replicates of the same odour stimulus e.g. chocolate extract, and allowed to acclimatize to this odour, figure 7.6. In the test phase the mouse was placed in a new arena with two different stimuli eg. chocolate in one corner and banana in the other corner, and its behaviour monitored. Different replicates of the shot glass were available, so that a fresh odour sample could be presented at each test, and the test was performed under dim red light to eliminate any visual cues. Two parameters were calculated for analysis: the recognition ratio for the sample and test phases, and distance travelled in metres, see section 2.7.10 for full experimental details.

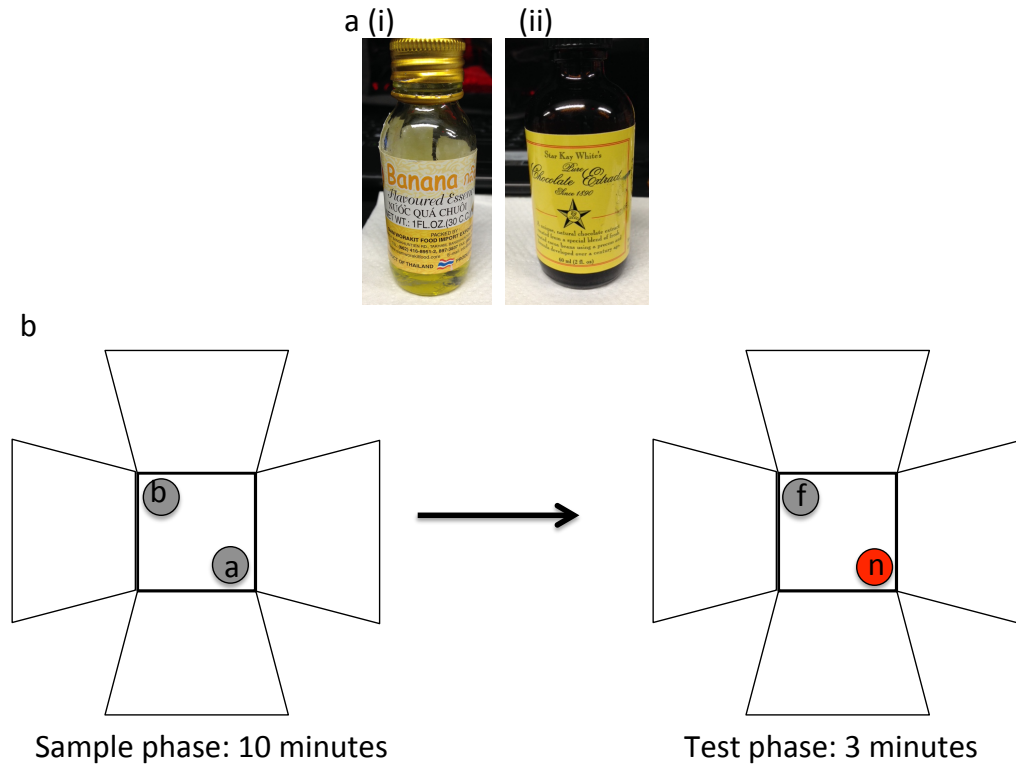


Figure 7.6 Odour discrimination testing.

In the sample phase, a mouse is placed into an open top box with two small glasses each containing 1ml of an identical liquid (e.g. a(i) banana essence) and allowed to explore for 10 minutes. After a short delay, the mouse is placed into a further open top box with one replicate of the odour previously encountered (grey circle, f) and another novel and distinct odour e.g. chocolate essence (a(ii) red circle, n). (b) The recognition ratio is calculated for the test phase as $n/(n+f)$, ie the time spent exploring the novel odour as a fraction of the total time spent exploring both odours. For the sample phase the ratio is calculated as $a/(a+b)$ i.e. using the location at which the novel odour will be placed in the test phase as the numerator. This then accounts for any place preference the mouse may have within the box aside from odour.

7.3 Results

7.3.1 Pupil light reflex

The pupil light reflex (PLR) was assessed in *rd1* mice following subretinal delivery of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector compared to sham-injected and untreated age-matched mice. Wild type mice of the same background strain that did not carry the *rd* mutation (non-*rd* C3H mice) (40) were also assessed using the same apparatus to validate the testing protocol.

7.3.1.1 Assessment in wild type mice

Kinetics of the pupil response of wild type mice (n=5) revealed a rapid constriction in the first 2 to 2.5 seconds after the onset of light and subsequently a sustained pupil constriction after stimulus offset, figure 7.7, the latter being mediated by ipRGCs (258). Since the objective was to evaluate any change in the pupil response driven by melanopsin gene therapy and to distinguish this from the ipRGC mediated response, for further analysis pupil constriction within the first two seconds was used. Irradiance response curves were generated using four different light intensities and marked pupil constriction was seen even at the lowest light intensity ($60.2 \pm 8.2\%$ (mean $\pm$ standard error)) with full pupil constriction at the highest two light intensities ($84.6 \pm 2.4\%$ and $86.4 \pm 2.2\%$), figure 7.8.

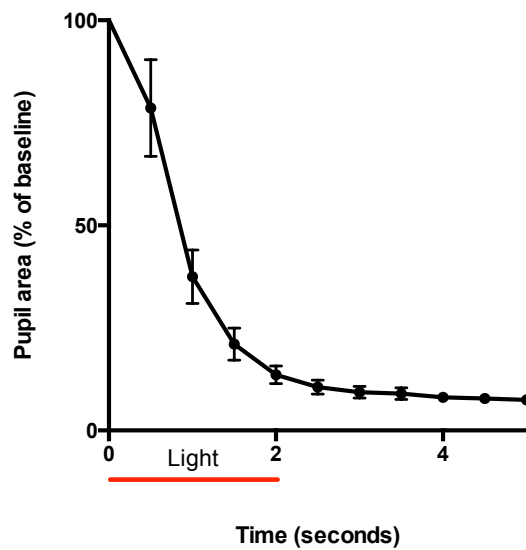


Figure 7.7 Kinetics of the pupil light response in wild type mice.

Pupil area compared to baseline is shown following a 2 second stimulation with white light of intensity 2×10^{16} photons/cm²/s demonstrating a fast constriction within the first two seconds and then prolonged constriction following termination of the light stimulus (n=5).

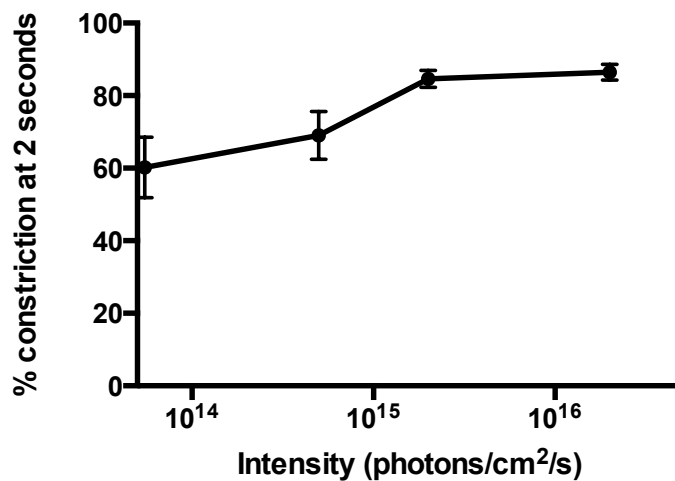


Figure 7.8 Irradiance response curve in wild type mice.

Percentage pupil constriction is shown following a two second white light stimulus, analysed at the two second time point after light onset. There is marked pupil constriction even at the lowest light intensity used, with full pupil constriction seen at the highest two intensities (n=5).

7.3.1.2 Irradiance response curves: two months after treatment

Mice that had been treated unilaterally in the left eye with rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector (the right eye being untreated, n=10) were compared with age-matched mice that had bilateral sham injections of phosphate buffered saline (n=9) and untreated natural history controls (n=12), figure 7.9a. Irradiance response curves showed very little pupil constriction in all groups using the dimmest light intensity. There was a significant effect of treatment ($p=0.04$) and light intensity ($p<0.0001$) on the amount of pupil constriction (repeated measures two way ANOVA; interaction between the two factors did not reach significance ($p=0.07$)). More pupil constriction was evident in treated mice than in sham-injected controls at all but the dimmest light intensity (all $p<0.05$, Tukey's post hoc test) and there was significantly increased pupil constriction in the treated group compared to both control groups at the highest light intensity (treated $73.3 \pm 4.7\%$ constriction versus sham-injected $51.9 \pm 8.5\%$ ($p=0.036$), treated versus untreated $49.9 \pm 5.3\%$ ($p=0.01$)), figure 7.9b.

7.3.1.3 Kinetics of pupil constriction: two months after treatment

Previous work has shown that the pupil response driven by ipRGCs has a longer latency than that in wild type mice (53). Therefore kinetics of pupil constriction in the three groups were compared. Pupil constriction in response to the brightest light stimulus was faster in the treated group compared to sham-injected and control groups, figure 7.9c. Statistical analysis of the first five seconds of this response, showed a significant effect of time ($p<0.0001$, repeated measures two way ANOVA) and a significant interaction between treatment and time ($p=0.0009$) on pupil area as a percentage of baseline. Post hoc analysis revealed a significantly smaller pupil area in the treated group at time points between 1.5 to 2.5 seconds compared to sham-injected mice, and 1.5 to 2 seconds compared to untreated mice (at 1.5 seconds pupil area compared to baseline: treated group $36.8 \pm 3.8\%$ versus sham-injected $58.9 \pm 7.9\%$ ($p=0.008$); versus

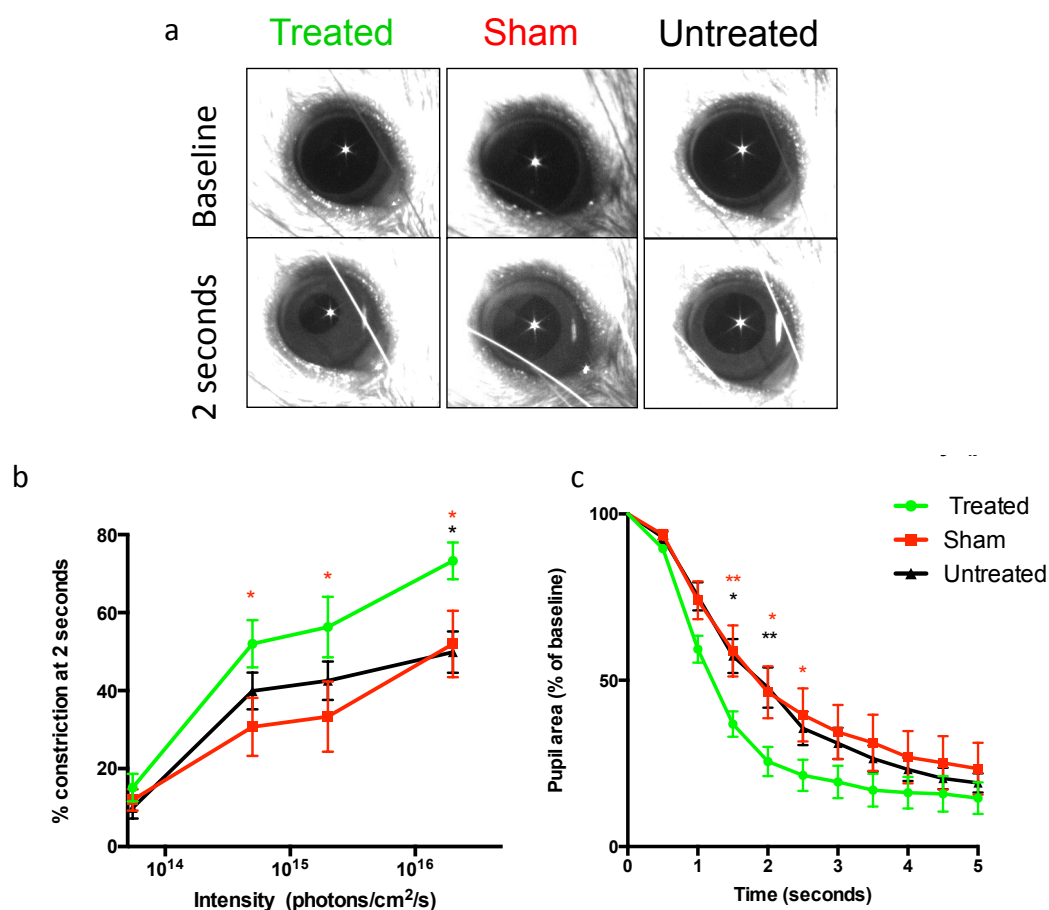


Figure 7.9 Pupillometry in *rd1* mouse model of retinal degeneration 2 months after treatment with human melanopsin.

(a) Representative images showing pupil size at baseline and after 2 seconds in the treated, sham and untreated groups following a 2 second white light stimulus. (b) Irradiance response curves demonstrate that pupil constriction in the treated group (green) is greater than in sham treated animals (red) and untreated controls (black). (c) Time course of pupil constriction at 2×10^{16} photons/cm²/s demonstrates a faster response in the treated group (treated n=10, sham n=9, untreated n=12). * (red) denotes significance between treated and sham, ** between treated and untreated, 2 way ANOVA with Tukey post hoc test.

untreated $57.3 \pm 5.1\%$ ($p=0.01$)), figure 7.9c. Therefore treatment with melanopsin gene therapy also reduced the latency of pupil constriction in these degenerate mice.

7.3.1.4 Treatment with atropine: two months after treatment

As evident from the data above, there was significant pupil constriction within the duration of the two second light stimulus used. This would have reduced the amount of light entering the stimulated eye and may have diminished the size of the treatment effect seen. In previous work, topical atropine drops have been used to dilate the pupil of the stimulated eye to prevent its constriction and maximise the light stimulus (259, 260). Therefore a further experiment was done as per the protocol above but with the application of a drop of 1% atropine to the stimulated (left) eye 30 minutes prior to commencing pupillometry.

Following the use of atropine, the absolute amount of pupil constriction was reduced with only $29.7\% \pm 3.8$ pupil constriction seen at the maximum light intensity in treated mice, possibly indicating systemic absorption of the drug. However, the treatment effect seen was greater ($p=0.0035$, two way repeated measures ANOVA; effect of light intensity $p<0.0001$, no significant interaction between factors). There was more pupil constriction in the treated group compared to the sham-injected group at the three higher light intensities ($p<0.05$, $p<0.05$ and $p<0.01$ respectively, Tukey's post hoc test) and compared to untreated eyes at the two highest light intensities ($p<0.01$ and $p<0.05$), (at the highest intensity: pupil constriction treated group $29.7\% \pm 3.8$ versus sham-injected $16.5 \pm 2.9\%$ ($p=0.002$); versus untreated $19.1 \pm 3.8\%$ ($p=0.01$)), figure 7.10.

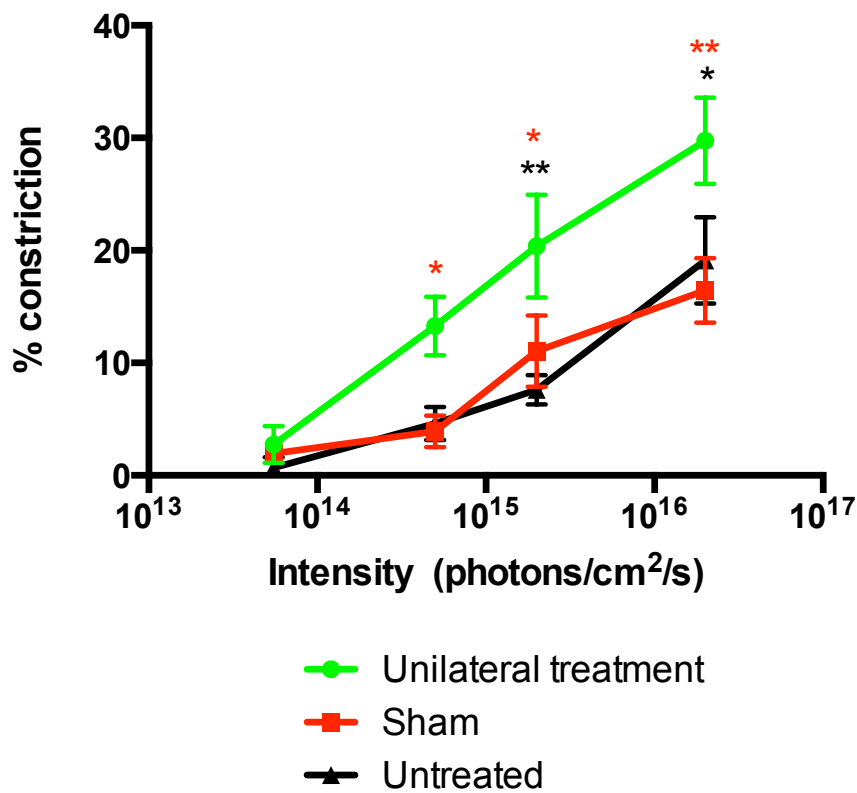


Figure 7.10 Irradiance response curve after treatment with topical atropine to the stimulated eye.

Pupil constriction following a 2 second white light stimulus is greater in the treated group (green) than in sham treated animals (red) and untreated controls (black) analysed at the 2 second time point after light onset (treated n=10, sham n=9, untreated n=12). * (red) denotes significance between treated and sham, ** between treated and untreated, 2 way ANOVA with Tukey post hoc test.

7.3.1.5 Irradiance response curves: thirteen months after treatment

Mice that had been treated unilaterally with rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector in the left eye thirteen months previously (with the right eye being untreated, n=5) were compared with age-matched mice that had bilateral sham injections (n=8) and untreated natural history controls (n=13), figure 7.11a. There was a significant effect of treatment ($p=0.013$) and light intensity ($p<0.0001$) on the amount of pupil constriction (repeated measures two way ANOVA with interaction between the two factors not reaching significance ($p=0.13$)). There was more pupil constriction in treated mice than in sham-injected controls at all but the dimmest light intensity (all $p<0.01$, Tukey's post hoc test) and increased pupil constriction in the treated group compared to the untreated group at the middle two light intensities (both $p<0.05$), (at 2×10^{15} photons/cm²/s intensity: pupil constriction treated group $41.1\% \pm 12.6$ versus sham-injected $3.7 \pm 1.4\%$ ($p=0.005$); versus untreated $15.5 \pm 6.6\%$ ($p=0.04$)) figure 7.11b.

7.3.1.6 Kinetics of pupil constriction: thirteen months after treatment

Examining the second brightest light stimulus (2×10^{15} photons/cm²), there was a significant effect of treatment on the kinetics of pupil constriction ($p=0.008$, repeated measures 2 way ANOVA) with also a significant effect of light intensity ($p<0.0001$) and a significant interaction between treatment and time ($p<0.001$). Pupil constriction was faster in treated eyes than sham-injected eyes from 1.5 seconds onwards after onset of the light stimulus ($p<0.05$ to $p<0.001$), and in untreated eyes after 2 seconds ($p<0.05$ to $p<0.01$), figure 7.11c.

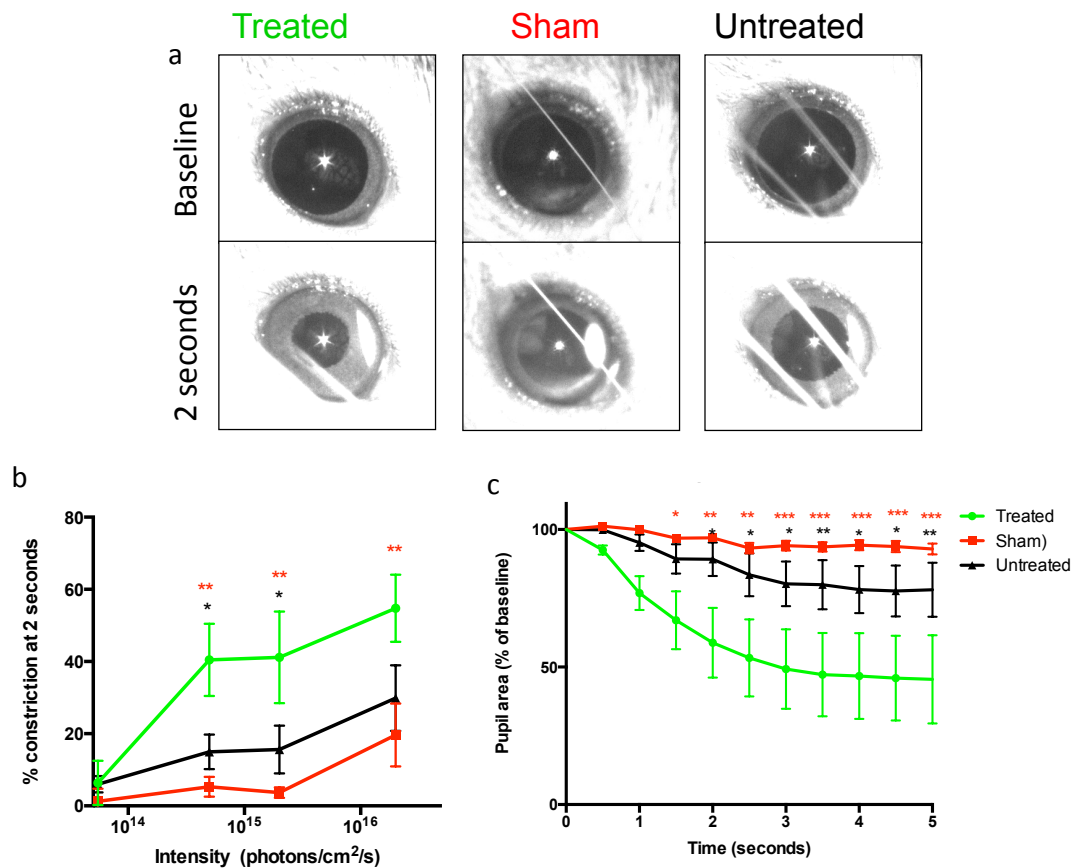


Figure 7.11 Pupillometry in *rd1* mouse model of retinal degeneration 13 months after treatment with human melanopsin.

(a) Representative images showing pupil size at baseline and after 2 seconds in the treated, sham and untreated groups following a 2 second white light stimulus. (b) Irradiance response curves demonstrate that pupil constriction in the treated group (green) is greater than in sham treated animals (red) and untreated controls (black). (c) Time course of pupil constriction at 2×10^{15} photons/cm²/s demonstrates a faster response in the treated group (treated n=5, sham n=8, untreated n=13). * (red) denotes significance between treated and sham, ** between treated and untreated, 2 way ANOVA with Tukey post hoc test.

7.3.1.7 Comparison of pupil constriction between young and old cohorts

Examining kinetics of pupil constriction at the highest light intensity (2×10^{16} photons/cm²), it was evident these were different between the cohort tested 2 months after injection (4 month old mice) versus those tested 13 months after injection (15 month old mice), figure 7.12 a,b. Analysing pupil size at the 5 second time point, there was a significant effect of age ($p < 0.0001$) and treatment ($p < 0.01$). In the sham-injected and untreated mice, pupil area at 5 seconds was smaller in younger mice versus older mice (sham-injected $23.29\% \pm 7.8$ versus 70.65 ± 9.12 , $p < 0.001$; untreated 22.33 ± 3.36 versus 56.436 ± 11.83 , $p < 0.01$), indicating reduced pupil constriction in the older control groups. However, there was no significant difference in pupil area at 5 seconds between the young and old melanopsin treated cohorts ($14.55\% \pm 4.78$ versus 25.93 ± 9.12).

7.3.2 Behavioural light aversion

Behavioural light aversion (BLA) was tested in both cohorts of mice i.e. 2 months or 13 months after treatment, and compared to sham-injected and untreated controls along with wild type mice of the same background strain.

7.3.2.1 Accuracy of ANY-Maze tracking

Videos were analysed using ANY-maze software to track the position of the mouse in the bright half of the test arena. Accuracy of this tracking was confirmed by comparison with manual tracking in a group of untreated *rd1* mice. There was no significant difference between the percentage of time spent in the bright half calculated by manually scoring the videos versus ANY-maze tracking ($59.94\% \pm 8.375$ versus $57.04\% \pm 8.23$, $p = 0.81$ unpaired t-test), figure 7.13. Therefore further analysis of videos was performed using ANY-maze.

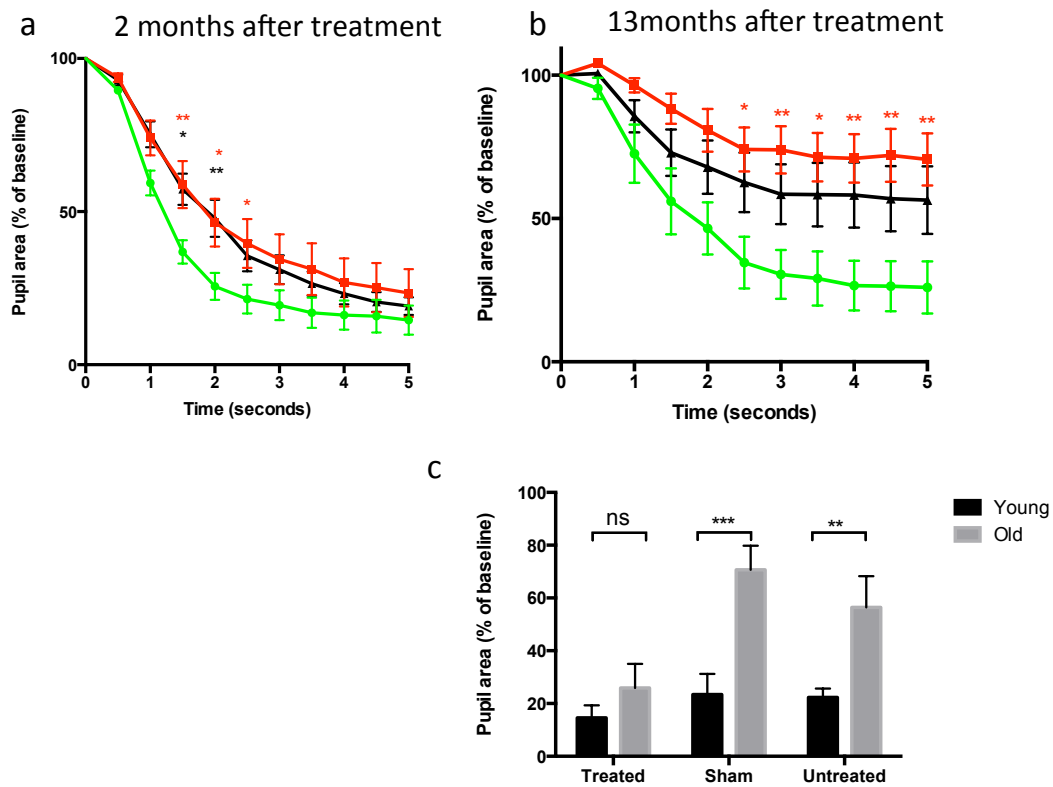


Figure 7.12 Comparison of pupil constriction in mice with age

Pupil constriction was assessed in mice that were (a) 2 months after treatment (i.e. 4 month old mice; treated n=10, sham n=9, untreated n=12) and those (b) 13 months after treatment (i.e. 15 month old mice; treated n=5, sham n=8, untreated n=13) following 2 second stimulation with white light of 2×10^{16} /photons/cm²/s intensity * denotes significance between treated and sham, **between treated and untreated, 2 way ANOVA with Tukey post hoc test. (c) Comparison of pupil area compared to baseline at 5 seconds between young and old mice of each treatment group reveals a significant effect of age in sham-injected and untreated mice, but not in melanopsin treated mice. ** p<0.01 *** p<0.001 one way ANOVA with Tukey post hoc test.

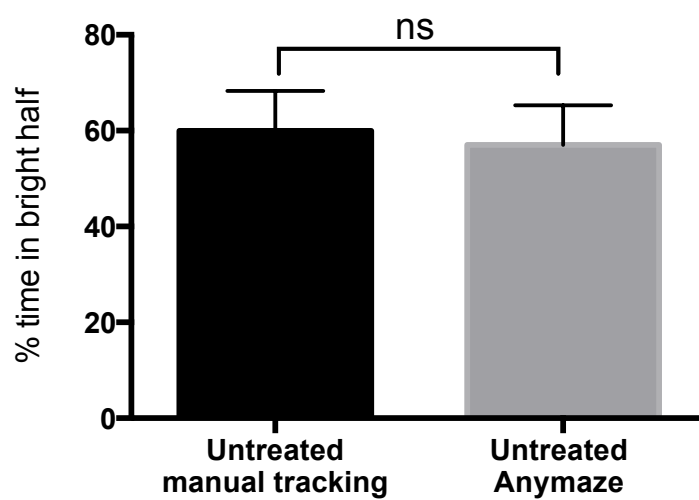


Figure 7.13 Manual versus automated tracking

Comparison of manual scoring of videos of untreated mouse movement during the behavioural light avoidance assay compared to automated tracking of videos with ANY-maze. ns= non- significant, unpaired two-tailed t-test; n=10.

7.3.2.2 Wild type mice

To establish behaviour of normal-sighted mice using the experimental protocol, five month old wild type mice of the same background strain (n=5) were tested. The percentage of time spent in the bright half of the chamber was $48.0 \pm 6.3\%$ (mean $\pm$ standard error), indicated by the dashed line in figure 7.14b.

7.3.2.3 Two months after treatment

Examining behaviour of mice over the time course of the five minute test, there appeared to be no difference between groups, (treated n=8, sham-injected n=10, untreated n=10), figure 7.14a. Further analysis revealed no significant difference in the percentage of time spent in the bright half of the test arena in the first three minutes of the test between groups, (treated $71.87\% \pm 3.0$, sham-injected $66.17\% \pm 7.74$, untreated $57.04\% \pm 8.23$, $p=0.31$ one way ANOVA), figure 7.14b.

The number of transitions between compartments is a measure of anxiety of mice during the test, and this was measured to ensure that a difference in level of anxiety between mice was not a contributing factor. There was a significant difference in the number of transitions between groups (one way ANOVA, $p=0.026$). Post hoc testing revealed a significantly higher number of transitions in the treated group versus the untreated group (treated 7.87 ± 1.95 , untreated 2.87 ± 0.58 , $p=0.03$), figure 7.15. Treated animals also made more transitions than sham-injected animals (sham 3.75 ± 0.88), although this did not reach statistical significance on post hoc testing ($p=0.08$). Therefore, treated animals appear to have higher anxiety levels in the behavioural light avoidance test compared to controls, although the absolute amount of time spent in the bright half of the test arena is similar between groups.

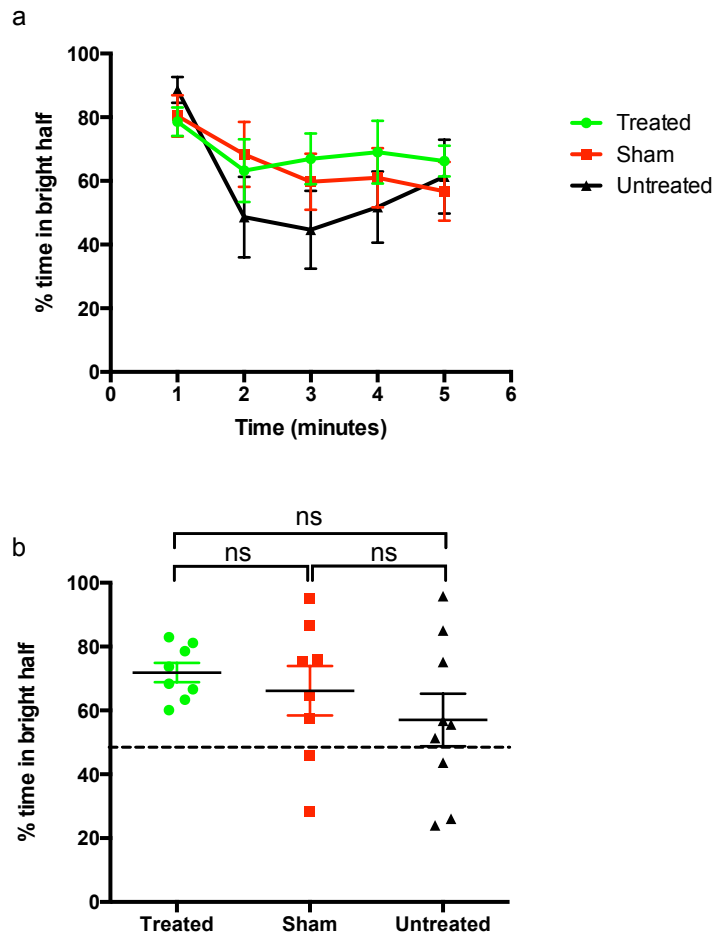


Figure 7.14 Behavioural light avoidance 2 months after treatment.

(a) Time course over the 5 minute test period showing percentage of time spent in the bright half of the test arena per minute (b) Percentage of time spent in the bright half per treatment group in the first 3 minutes; treated $n=8$, sham-injected $n=10$, untreated $n=10$; dashed line indicates mean of wild type (non-rd C3H) mice, $n=5$; ns= non-significant, one way ANOVA with Tukey post hoc test.

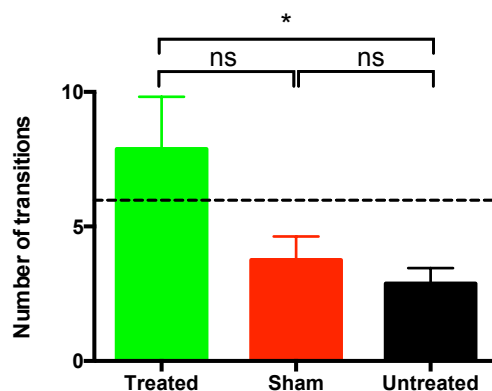


Figure 7.15 Number of transitions made between compartments during the behavioural light avoidance assay 2 months after treatment.

Treated animals made more transitions than sham-injected or untreated controls in the first 3 minutes; dashed line represents wild type mean; treated $n=8$, sham-injected $n=10$, untreated $n=10$; * $p<0.05$, one way ANOVA with Tukey post hoc test.

7.3.2.4 Thirteen months after treatment

Thirteen months after treatment, treated animals tended to spend less time in the bright half of the test arena compared to controls during the earlier part of the five minute test, (treated $n=8$, sham-injected $n=9$, untreated $n=8$) figure 7.16a. Analysing the first three minutes, there was a significant difference in the percentage of time spent in the bright half between groups (treated $37.65\% \pm 6.7$, sham-injected $64.66\% \pm 6.8$, untreated $57.65\% \pm 7.7$, $p=0.03$ one way ANOVA), figure 7.16b. Post hoc analysis revealed a significant difference between treated and sham-injected groups ($p=0.03$, Tukey's post hoc test). The difference between treated and untreated groups did not reach significance ($p=0.14$), and there was no significant difference between sham-injected versus untreated mice ($p=0.76$).

Examining transitions between the bright and dark halves of the arena, there were no differences between groups (treated 4.13 ± 0.72 , sham-injected 4.78 ± 0.99 , untreated 4.63 ± 1.15 , one way ANOVA $p=0.88$), indicating no difference in anxiety levels between the three groups, figure 7.17.

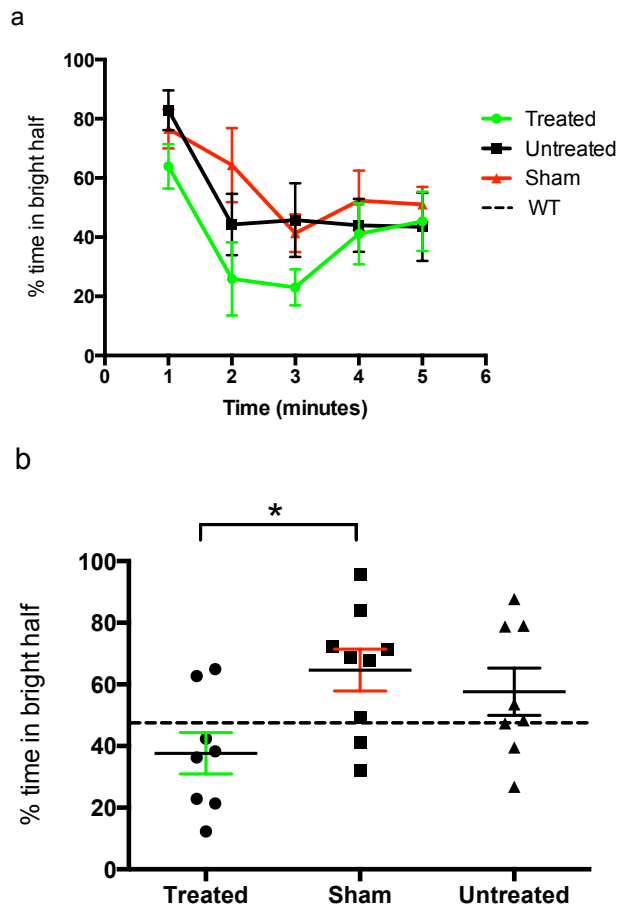


Figure 7.16 Behavioural light avoidance 13 months after treatment.

(a) Time course over the 5 minute test period showing percentage of time spent in the bright half of the test arena per minute (b) Percentage of time spent in the bright half in the first 3 minutes per treatment group, treated n=8, sham-injected n=9, untreated n=8; dashed line indicates mean of wild type (non-rd C3H) mice, n=5; ns= non-significant, one way ANOVA with Tukey post hoc test.

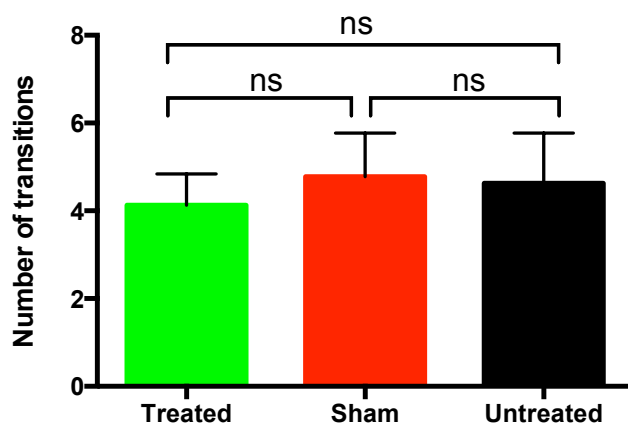


Figure 7.17 Number of transitions made between compartments during the behavioural light avoidance assay 13 months after treatment.

There was no difference between groups regarding the number of transitions made in the first 3 minutes; treated n=8, sham-injected n=9, untreated n=8; ns=non-significant, one way ANOVA with Tukey post hoc test.

7.3.3 Visual context recognition

Behavioural light avoidance suggests that an animal can detect light and that this can drive an expected behaviour. In order to assess whether treatment with melanopsin gene therapy could restore image-forming vision in these mice rather than light-detection alone, a visual environment recognition test was performed.

7.3.3.1 Wild type mice

In wild type animals of the same background strain as the test groups (n=5), recognition ratio was compared between the two visual contexts, i.e. the amount of time spent exploring the novel object divided by the total time exploring objects.

When the visual environment was constant, ('same' context) wild type (i.e. normally sighted) mice spent more time exploring the novel object compared to the previously encountered one (ratio 0.78 ± 0.08 (mean $\pm$ standard error)) suggesting that they recognised the latter object as familiar. When there was a change of environment, wild type mice spent approximately equal amounts of time exploring the novel and familiar objects (ratio 0.47 ± 0.12), suggesting that they no longer recognised the latter in its new visual environment. The difference in ratio between 'same context' and 'change of context' conditions was statistically significant ($p=0.04$, two-tailed paired t-test) figure 7.18a.

Distance travelled was also different in the 'Same' versus 'Change' conditions indicating a visual-context specific increase in exploratory behaviour, (figure 7.18b, $1.93\text{m} \pm 0.41$ in 'Same' context versus 2.53 ± 0.37 with a 'Change' in context) although this did not reach statistical significance on ($p=0.24$ paired t-test).

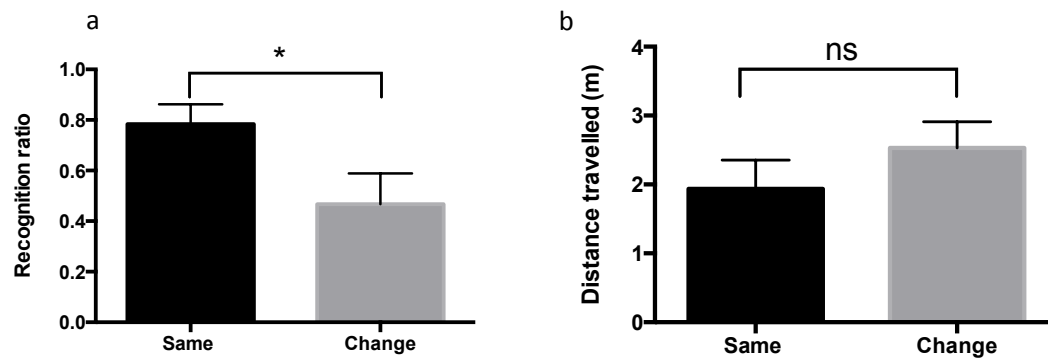


Figure 7.18 Visual context recognition testing in wild type mice

(a) Recognition ratio of the novel object (time spent exploring novel object/total time spent exploring objects) when the visual environment is constant i.e. 'Same' or where there is a change in visual environment. (b) Distance travelled per minute within the test arena during the trials. * $p < 0.05$, two-tailed paired t-test. $n=5$.

7.3.3.2 Two months after treatment

Mice that had been treated with melanopsin gene therapy two months previously, sham-injected mice and untreated controls were tested according to the protocol above (treated $n=12$, sham-injected $n=10$, untreated $n=12$). There was a significant effect of context on distance travelled during the test phase ($p=0.0001$, two way repeated measures ANOVA with treatment and context as factors) and a significant interaction between treatment and context ($p=0.04$) indicating that the degree of behavioural change according to context varied between treatment groups, figure 7.19a. Post hoc analysis revealed a significant difference in distance travelled in the treated group between the 'Same' and 'Change' conditions ($1.35\text{m} \pm 0.2$ versus $2.41\text{m} \pm 0.15$, $p=0.0002$, Bonferroni post hoc test) indicating increased exploratory behaviour when the visual environment was changed. There was no significant difference in exploratory behaviour in different contexts in the sham-injected ($1.0\text{m} \pm 0.18$ versus $1.51\text{m} \pm 0.22$, $p=0.16$) and untreated groups ($1.55\text{m} \pm 0.24$ versus $1.79\text{m} \pm 0.24$, $p=0.94$).

There was a trend for a difference in recognition ratio according to visual context in the treated group (ratio 0.72 ± 0.25 in 'Same' context versus 0.53 ± 0.19 in 'Change' of context), with no such differences seen in the sham-injected or untreated groups (sham: 0.63 ± 0.11 in 'Same' context and 0.62 ± 0.07 in 'Change' of context; untreated: 0.63 ± 0.10 in 'Same' context and 0.68 ± 0.07 in 'Change' of context), figure 17.9b. However, using two way repeated measures ANOVA for analysis with treatment and context as factors, neither factor nor interaction reached statistical significance.

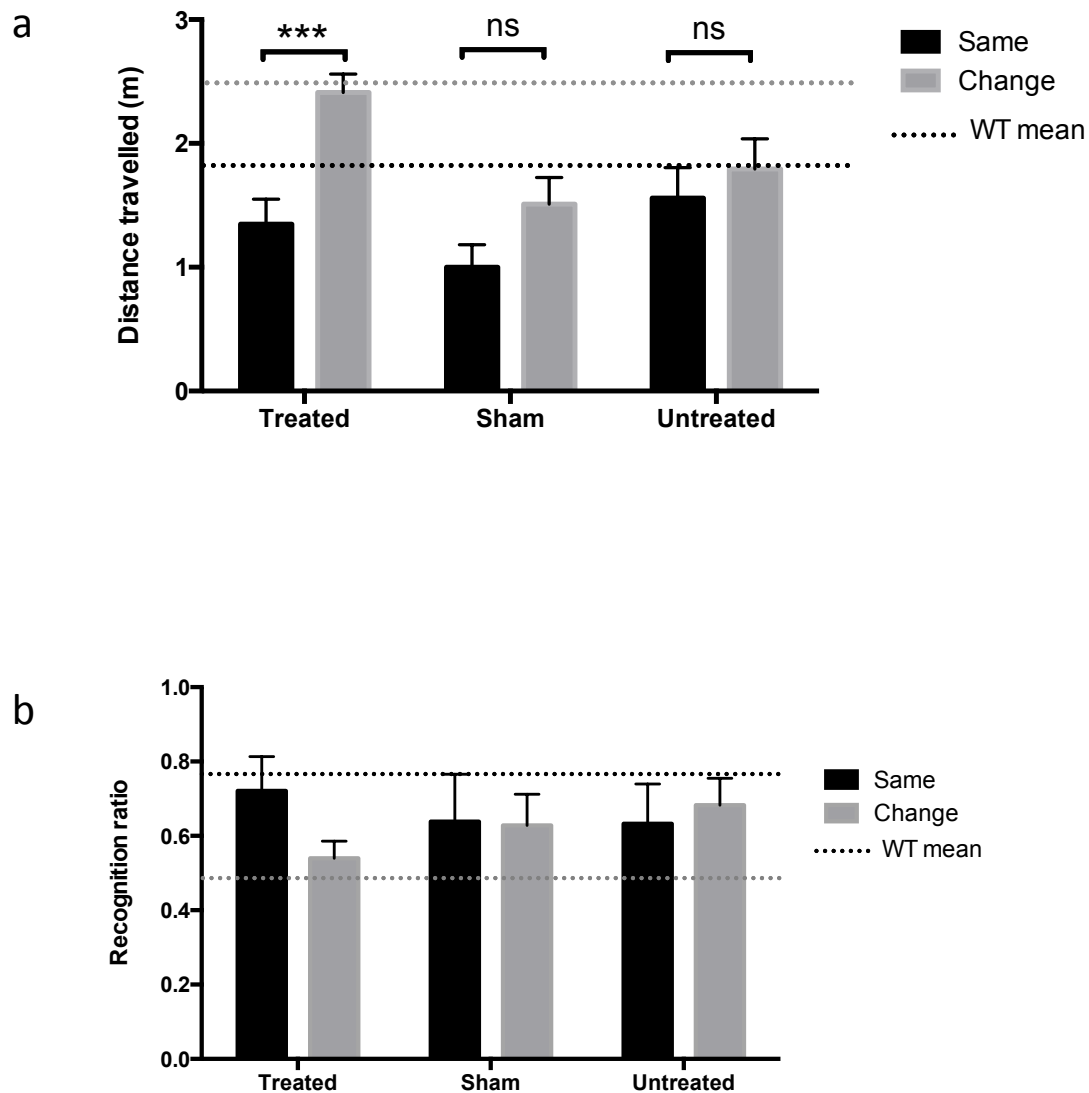


Figure 7.19 Visual context recognition testing 2 months after treatment

(a) Distance travelled in metres during the test phase when visual environment is the same (black bar) or where there is a change in environment (grey bar) for each group demonstrating a significant increase in exploratory behaviour in treated mice when the visual environment is changed. (b) Recognition ratios (time spent exploring the novel object/total time exploring objects) when the visual environment is the same (black bar) or there is a change in environment (grey bar) indicating a trend for visual environment-dependent behaviour in treated mice. Treated $n=12$, sham-injected $n=10$, untreated $n=12$; dashed line= wild type mean, $***p<0.001$, 2 way ANOVA with Bonferroni post hoc test.

7.3.3.3 Thirteen months after treatment

There was a significant effect of visual context on distance travelled in this cohort (treated $n=9$, sham-injected $n=8$, untreated $n=11$; $p=0.0003$ for context, $p=0.72$ for treatment with no significant interaction between factors, two way repeated measures ANOVA), figure 7.20a. Post hoc analysis revealed a significant difference in distance travelled in the treated group between the 'Same' and 'Change' conditions ($1.31\text{m} \pm 0.27$ versus 2.48 ± 0.27 ; $p=0.003$, Bonferroni post hoc test). There was no significant difference in the sham-injected ($1.70\text{m} \pm 0.47$ versus $2.56\text{m} \pm 0.23$, $p=0.06$) and untreated groups ($1.77\text{m} \pm 0.20$ versus $2.04\text{m} \pm 0.20$, $p>0.99$). This indicates a visual context specific change in exploratory behaviour in the treated group only.

Regarding recognition ratio, there was a significant difference in recognition ratio in the treated group between the 'same' and 'change' conditions (ratio 0.85 ± 0.05 versus 0.56 ± 0.07 , $p=0.04$, Bonferroni post hoc test), demonstrating a change in behaviour when the visual environment is changed, figure 7.20b. There was no significant difference in the sham-injected (0.69 ± 0.05 versus 0.58 ± 0.07 , $p=0.91$) and untreated groups (0.60 ± 0.08 versus 0.66 ± 0.08 , $p>0.99$). The treated group showed a behaviour pattern closest to wild type, suggestive of the ability to detect a change in visual context in these animals.

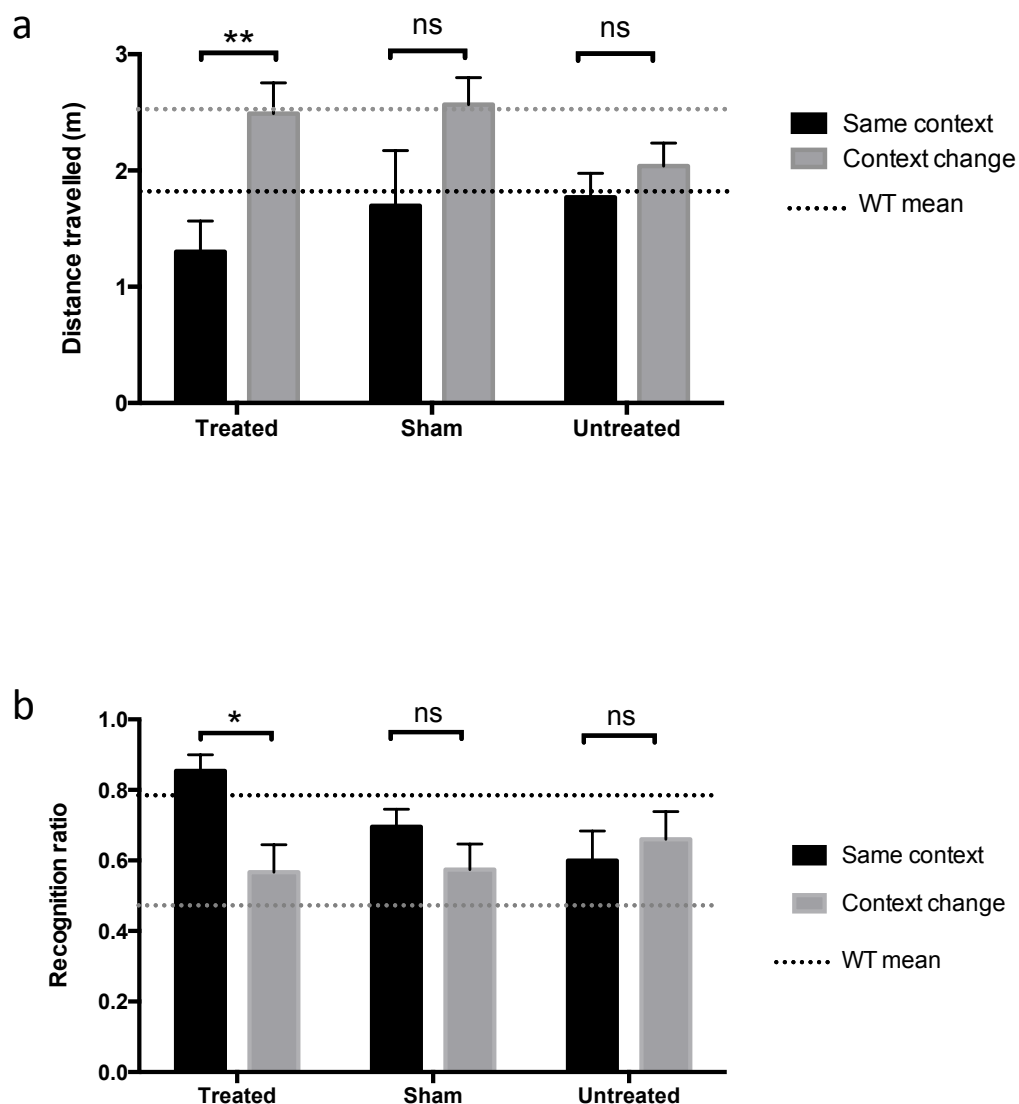


Figure 7.20 Visual context recognition testing 13 months after treatment

(a) Distance travelled in metres during the test phase when visual environment is the same (black bar) or where there is a change in environment (grey bar) for each group demonstrating a significant increase in exploratory behaviour in treated mice when the visual environment is changed. (b) Recognition ratios (time spent exploring the novel object/total time exploring objects) when the visual environment is the same (black bar) or there is a change in environment (grey bar) indicating a visual environment-dependent change in behaviour in treated mice. Treated $n=9$, sham-injected $n=8$, untreated $n=11$; dashed line= wild type mean, * $p<0.05$, ** $p<0.01$, 2 way ANOVA with Bonferroni post hoc test.

7.3.4 Optomotor response

The tests above suggest that treated mice are able to detect the change in visual environment in the visual context recognition test, which is a 4 by 4 cm square pattern in static conditions. The optomotor response was assessed to see if treated mice could respond to a dynamic stimulus and if so what the limits of resolution might be.

The cohort of mice tested two months after treatment was compared to sham-injected controls (treated n=12, sham-injected n=10). Neither group showed head tracking in response to the stimulus (treated: 0.166 ± 0.11 head tracks per minute, sham: 0.2 ± 0.13 head tracks per minute, n=10, p=0.84 unpaired two-tailed t-test), figure 7.21a. The cohort of mice tested 13 months after treatment was compared to sham-injected and untreated controls (treated n=10, sham-injected n=9, untreated n=9). Some treated animals showed head tracks occasionally (0.33 ± 0.13 head tracks per minute). This was not significantly different from sham-injected (0.04 ± 0.03 head tracks per minute) and untreated mice (0.11 ± 0.05 head tracks per minute, p=0.07 one way ANOVA), figure 7.21b. In all cases the degree of head tracking seen in any of the retinally degenerate groups was markedly less than that observed in under the same conditions in WT *non-rd* C3H controls (n=5). These mice were able to track the 0.1 cycles/degree grating with 5.6 ± 0.6 (mean $\pm$ standard error) head tracks per minute.

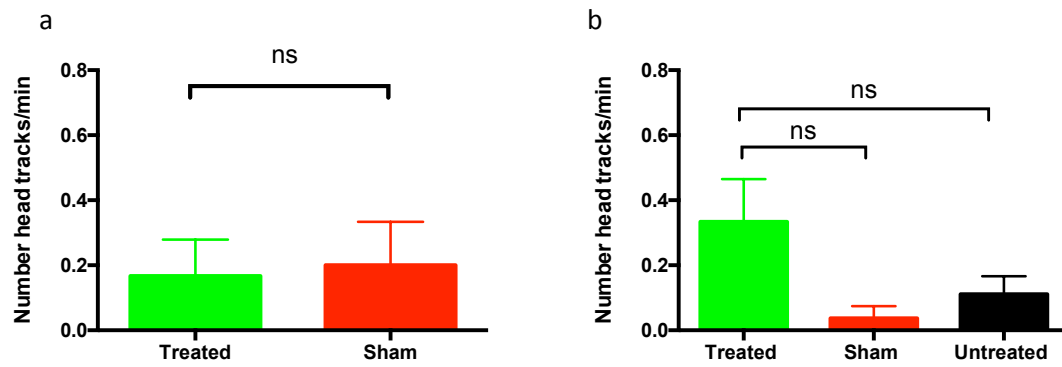


Figure 7.21 Optomotor response following treatment with melanopsin gene therapy.

Number of head tracks per minute in treated mice compared to controls (a) 2 months after treatment (treated n=12, sham-injected n=10; unpaired t-test) and (b) 13 months after treatment (treated n=10, sham-injected n=9, untreated n=9; one way ANOVA). No significant differences were seen between groups. ns=non-significant.

7.3.5 Odour testing

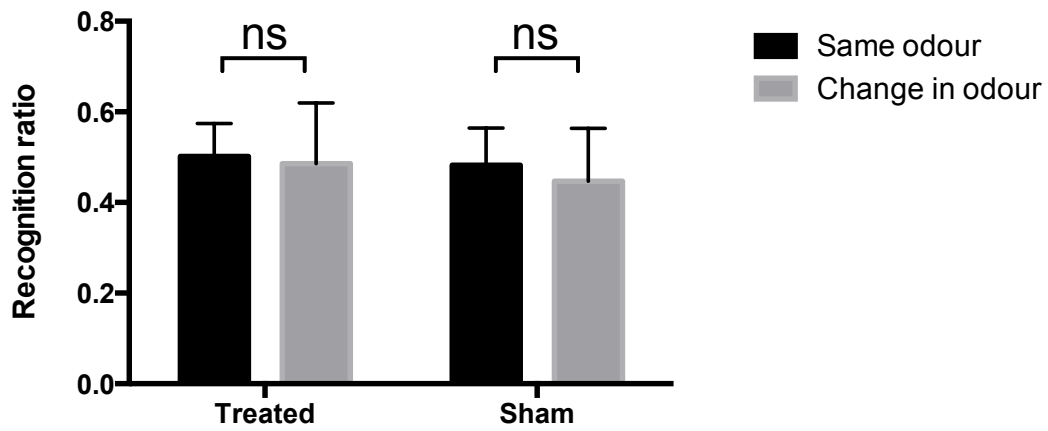
A modification of the object recognition test was used to assess the ability to discriminate odours (254).

7.3.5.1 Two months after treatment

Two groups were compared: mice treated with melanopsin gene therapy and sham-injected mice (both groups $n=9$). In the sample phase, the same odour was placed two corners of the test arena, whereas in the test phase two different odours were placed the test arena. The recognition ratio was calculated as the time spent in the location of the new odour /total time exploring in either phase. In the treated group there was no significant difference in ratio between the odour being the same or changed (ratio 0.501 ± 0.07 sample phase, versus 0.485 ± 0.134 test phase, 2 way repeated measures ANOVA with Bonferroni post hoc test $p>0.99$), figure 7.22a. There was also no difference between the two conditions in the sham-injected mice (ratio 0.483 ± 0.08 sample phase, versus 0.446 ± 0.117 test phase, $p=0.99$) indicating poor performance in this test in both treatment groups.

The distance travelled was significantly higher in the sample phase compared to the test phase in both groups (treated $1.04\text{m} \pm 0.08$ versus 0.40 ± 0.09 ; sham-injected 1.04 ± 0.11 versus 0.43 ± 0.07 , both $p<0.0001$), figure 7.22b. This could be explained by familiarity with the test arena in the second phase and therefore less exploratory behaviour. An alternative explanation could be that the mice could not detect the difference between odours during the test phase and so had no incentive to explore.

a



b

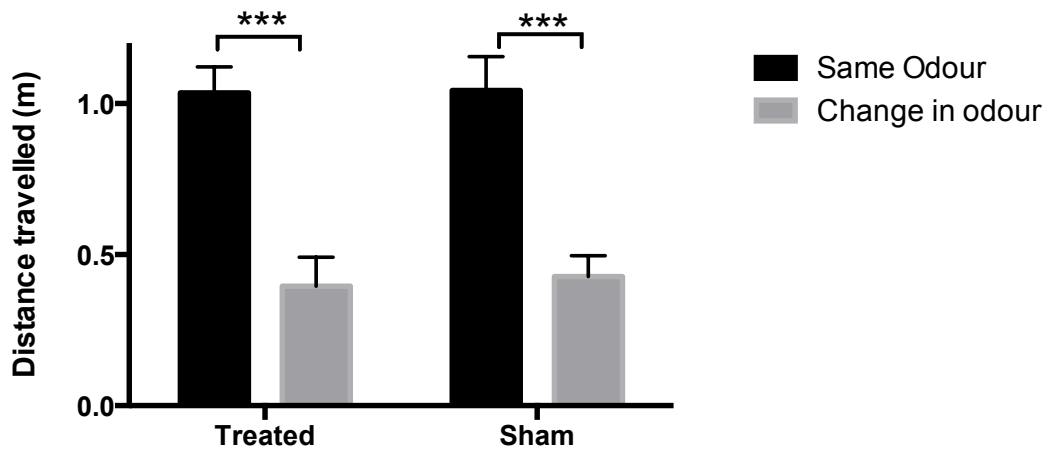


Figure 7.22 Odour discrimination testing 2 months after treatment

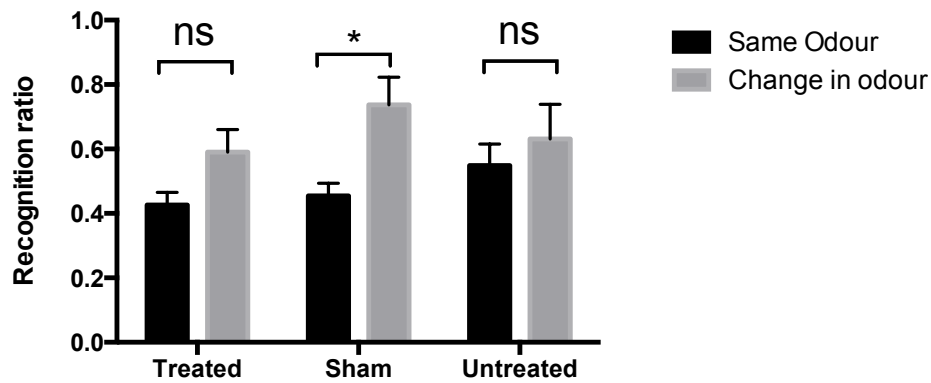
(a) Recognition ratios (time spent exploring the location of the novel odour/ total time exploring) in the sample and the test phase. There was no significant difference between phases or groups, both groups $n=9$. (b) Distance travelled in each phase of the test. Mice moved a greater distance in the sample phase (same odour) versus the test phase (change in odour) in both test groups *** $p<0.001$, 2 way repeated measures ANOVA with Bonferroni post hoc test.

7.3.5.2 Thirteen months after treatment

Three groups were compared: treated, sham-injected and untreated controls (treated n=9, sham n=7 untreated n=8). Recognition ratios were significantly higher in the test phase (change in odour) compared to the sample phase (same odour) in all groups indicating ability to discriminate odours in this task ($p=0.005$ for phase, 2 way repeated measures ANOVA). Post hoc testing revealed this difference to be significant only in the sham-injected group (ratio 0.455 ± 0.04 sample phase, versus 0.737 ± 0.09 test phase, $p=0.03$ Bonferroni post hoc test). The treated and untreated groups followed a similar trend, but post hoc testing did not reach statistical significance (treated ratio 0.426 ± 0.04 sample phase, versus 0.591 ± 0.07 test phase, $p=0.22$; untreated ratio 0.549 ± 0.07 sample phase, versus 0.631 ± 0.11 test phase, $p=0.78$), figure 7.23a.

The distance travelled was significantly higher in the sample phase compared to the test phase in all groups (treated $1.21\text{m} \pm 0.11$ versus 0.61 ± 0.09 ; sham-injected 1.36 ± 0.18 versus 0.75 ± 0.09 ; untreated 1.17 ± 0.10 versus 0.69 ± 0.056 ; all $p<0.01$), figure 7.23b. Therefore this aspect of behaviour was similar in both the young and older cohorts of mice.

a



b

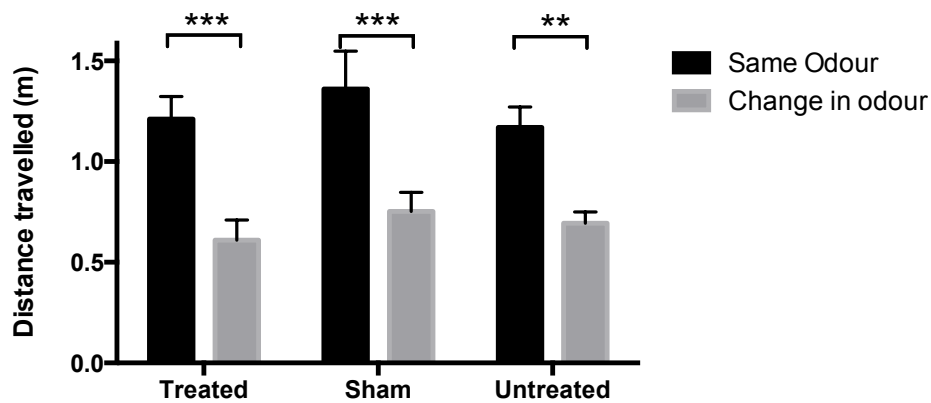


Figure 7.23 Odour discrimination testing 13 months after treatment

(a) Recognition ratios (time spent exploring the location of the novel odour/ total time exploring) in the sample and the test phase. There was a trend for increased time spent exploring the novel odour in all groups, this reached significance in the sham group only. (treated $n=9$, sham $n=7$ untreated $n=8$). (b) Distance travelled in each phase of the test. Mice moved a greater distance in the sample phase (same odour) versus the test phase (change in odour) in both test groups * $p<0.05$, ** $p<0.01$ *** $p<0.001$, 2 way repeated measures ANOVA with Bonferroni post hoc test.

7.4 Discussion

In previous chapters, it has been established that AAV gene therapy was capable of producing long-term expression of human melanopsin in the degenerate retina. Furthermore, the results suggest this ectopic expression leads to an increase in photosensitivity within the retina and some functional increase in signaling to the visual cortex. In this chapter, several physiological and behavioural tests were performed to assess whether this increase in retinal photosensitivity could lead to an improvement in light-driven responses or visual function. Different aspects of the visual pathway were tested: the pupil light reflex assesses whether signals generated by light incident upon the retina can be transmitted to central targets and feed into the appropriate neural pathway to mediate consensual pupil constriction. The behavioural light avoidance assay examines whether the light detected can drive a behavioural response, and the visual context recognition test develops this further to examine whether a change in visual environment, and not just ambient light levels, can be detected and used to drive a change in behaviour. The optomotor response assesses if a dynamic stimulus can be detected and aims to quantify visual acuity. Finally, it was assessed if long-term blindness affects or leads to augmentation of olfactory abilities i.e. cortical remodeling, and whether this is affected by melanopsin gene therapy.

The sensitivity of pupil constriction to light was increased in treated mice compared to controls 2 months after subretinal delivery of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed, with faster time course of constriction also seen in the treated group. This indicates that the additional visual information detected by expression of human melanopsin in the degenerate retina can feed into existing retinal circuitry to drive a consensual pupil response. The treatment effect was not only maintained at thirteen months, confirming longevity of expression, but was even greater than that seen at 2

months. This could indicate that human melanopsin expression was not at its maximal level at 2 months but an alternative explanation may be due to loss of other photoreceptive cells. Native melanopsin cells (ipRGCs) are intact in the *rd1* mouse model and previous work has shown that ipRGCs are significantly reduced in the aged mouse retina at 700 days (227). It is therefore likely that a number of ipRGCs driving pupil constriction in the sham-injected and untreated cohorts 2 months after treatment (4 month old mice) had degenerated in the cohort tested 13 months after treatment (15 month old mice). This is consistent with maximal pupil constriction in the sham-injected and untreated groups being significantly greater in the younger mice compared to older mice. The mice tested in the work presented here were approximately 500 days old, whereas those in the study above were older, but it is still likely that a significant proportion of ipRGCs had degenerated. The enhanced treatment effect of human melanopsin gene therapy in the older cohort is therefore very encouraging since it indicates the ongoing ability of ectopic melanopsin expression to integrate visual signals into retinal circuitry despite potentially an ongoing degenerative process.

In assessing the treatment effect seen following subretinal delivery of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed, it is important to consider whether the increased sensitivity seen is due to augmentation of an existing photoreceptive cell in the degenerate retina. As discussed above, the sustained increase in pupil constriction in treated mice between the younger and older cohorts despite age-related loss of ipRGCs in conjunction with data presented in chapter 4 illustrating that ganglion cells are not directly transduced by the vector, suggests that augmentation of the pupil response in treated animals is not mediated by ipRGCs but also involves other neural pathways within the retina. Another group of photoreceptive cells to consider are residual cones in the *rd1* retina in younger mice. Although these are morphologically abnormal and do not elicit any functional response on MEA testing, as discussed in chapter 6, it cannot be

completely excluded that these cells could drive some of the pupil constriction seen in the cohort at 2 months (4 month old mice). The majority of these cones have degenerated by the 13 month assessment (when the mice are 15 months old)(75) and since the treatment effect of melanopsin gene therapy on pupil constriction is maintained or greater at this time point, this is unlikely to be mediated via stimulation of residual cones.

A further observation in the older cohort is that the pupil constriction in untreated mice is slightly greater than that of sham-injected controls, whereas the difference is small or absent at 2 months. A possible explanation could be that subretinal injection and temporary separation of the neurosensory retina from the RPE has a long-term detrimental effect on the retina. In this case, the maintained effect in the treated group at 13 months is even more encouraging, as any detrimental effect of the subretinal injection has been overcome.

The behavioural light avoidance assay demonstrated that treated mice showed greater behavioural light aversion compared to sham-injected controls in the older cohort, indicative that treated mice could use the photosensitivity induced by ectopic expression of melanopsin in the degenerate retina to drive a behavioural response. In the younger cohort, no significant effect was seen overall between treatment groups comparing the percentage of time spent in the bright half or illuminated chamber. However treated animals made significantly more transitions than the control groups in the cohort tested at 2 months, indicating a greater level of anxiety in these mice. This is likely to be a confounding factor and makes it difficult to draw firm conclusions that there was no difference between groups. In the older cohort, the number of transitions and therefore anxiety levels were similar across all groups, increasing the reliability of the result that treated mice demonstrated greater behavioural light aversion. A

previous study has shown enhanced behavioural light aversion following administration of topical atropine drops to tested mice (259), and therefore future testing using this protocol may increase the treatment effect seen further.

Visual context recognition tests investigate whether treated animals are able to detect a stimulus reliant on image-forming vision and not just a change in ambient light levels. There was a significant increase in exploratory behaviour when the visual environment was changed in melanopsin treated animals at both 2 months and 13 months after treatment, indicating that these animals were able to detect this change. In fact, treated mice tested at 13 months performed better in this task than those tested at 2 months since there was a statistically significant change in recognition ratio according to visual context in the older animals, whereas a similar trend was seen but did not reach statistical significance in younger treated mice. No such effect was seen in controls. The cohorts of mice were independent, with each mouse being naïve when tested, and it may therefore be that over time, mice learned to use the new visual information received from treatment with human melanopsin gene therapy, resulting in better performance in the older treated group.

In order to assess the limits of vision gained in treated mice, the optomotor response was examined. There was no significant head tracking in treated mice or controls in either the younger or older cohorts in response to a 0.1 cycles/degree stimulus rotating at two revolutions per minute. This can be explained by the kinetics of the melanopsin response, which are slow as described in chapter 6, and may not be able to respond quickly enough to the OKN stimulus. Therefore it seems likely that the level of vision restored by melanopsin gene therapy could be that of visual environment recognition, or the ability to see static images rather than dynamic ones at this speed.

Finally odour discrimination was performed to assess whether olfactory sensation was enhanced in aged degenerate mice and if this was affected by melanopsin gene therapy. A number of reports have documented increased odour discrimination in blind humans (252, 253). In blind mice one study describes enhanced function using auditory cues (261), but none has examined odour. In the young cohort, both treated and sham-injected animals performed poorly in the odour task. In the older animals there was increased odour discrimination in all groups but post hoc testing only reached statistical significance in sham-injected animals. Therefore there may be some augmentation of olfactory sensation in blind animals, but no conclusions can be drawn as to whether melanopsin gene therapy has an impact on this effect.

7.6 Conclusions

The data presented demonstrates that treatment with human melanopsin gene therapy augments the pupil light response after two months of treatment and this effect is maintained and even greater at thirteen months. In the older cohort, treated mice showed increased behavioural light aversion compared to sham-injected animals indicating the ability to use the light sensitivity conferred by human melanopsin gene therapy to drive a behavioural response. In both young and old cohorts of mice, behavioural testing showed the ability of treated animals to detect a change in visual environment. Performance was even better in animals after thirteen months treatment suggestive that these animals may have learned to use the new visual information they received. These data indicate that the increased photosensitivity derived from expression of human melanopsin in the degenerate retina can generate functional useful information, which can restore or improve light-driven responses and even enhance performance in a test that relies on image-forming vision in mice with end-stage retinal degeneration.

Chapter 8 Discussion

8.1 Overview

The aim of this thesis project has been to develop a gene therapy strategy to restore visual function in end-stage retinal degeneration using human melanopsin as an optogenetic tool. The melanopsin AAV vector was optimised and subsequently assessed extensively *ex vivo* and *in vivo* for gene expression and function.

With regards to vector optimisation, an AAV2/8 capsid with a single tyrosine to phenylalanine amino acid mutation was identified that could effectively transduce cells of the degenerate retina in the *rd1* mouse. Bipolar cell transduction was seen both in this model and in human retinal explants *ex vivo*, which is the first reported example of human bipolar cell transduction using a capsid mutant AAV vector. Of three promoters assessed, the ubiquitous CMV enhancer/CBA promoter gave by far the highest levels of expression of human melanopsin in the degenerate retina, with the wild type rather than a codon optimised melanopsin sequence resulting in greater protein expression.

Therefore the optimal vector rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed was assessed for further experiments. Using this ubiquitous promoter, successful RPE, horizontal cell, bipolar cell and Müller cell transduction was demonstrated in the degenerate retina. Long term, sustained expression was seen with high levels of human melanopsin being detected in the degenerate retina 15 months after subretinal injection. This was functionally active with evidence of light-induced responses throughout the visual pathway.

Following rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed injection, *cfos* upregulation in response to light stimulation was evident in areas of vector transduction consistent with cell depolarisation. *Cfos* expression was not only seen in human melanopsin expressing cells in the inner nuclear layer, but also in adjacent cells, indicative that ectopic melanopsin mediated depolarisation can drive more widespread responses within the circuitry of the retina. This was supported by MEA recordings, which demonstrated an increased percentage of electrodes showing light-dependent action potential firing in treated retinas compared to controls. The spatial distribution of responses in treated retinæ also correlated to areas of vector transduction. This suggests that melanopsin induced depolarisation of cells could generate a useful signal that could be transmitted centrally.

Confirmation of ability of the ectopic human melanopsin driven signal to feed into appropriate neural circuitry and reach central targets was demonstrated via increased sensitivity of the pupil light response in treated mice compared to controls. Appropriate connections must be made to drive the consensual pupil constriction assessed in these experiments. Functional effects were sustained and even greater at 13 months after subretinal injection of the vector, indicating longevity of functional restoration and these connections.

The greater question to address was whether expression of melanopsin might be useful for restoring image-forming vision, rather than only augmenting light-sensitivity. Laser speckle cortical imaging demonstrated a physiological change in visual cortex blood flow in response to light in treated animals, indicating the visual information signalled by expression of human melanopsin in the degenerate retina can reach this key target. Furthermore visual context recognition testing, which is a task of image-forming vision, demonstrated the ability of treated mice to be able to detect a change in visual

environment, compared to sham-injected and untreated controls. These data are the first evidence that melanopsin gene therapy can restore image-forming visual responses, since the only previous study using melanopsin gene therapy via an intravitreal approach used behavioural light avoidance and two-choice discrimination as functional assays, neither of which necessarily require image-forming vision (88). Interestingly the treated group tested 13 months after delivery of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed performed better than mice tested 2 months after vector injection, and it may be that animals learn to use the new visual information generated by ectopic human melanopsin expression over time. Collectively, these results suggest that expression of human melanopsin in the degenerate retina can restore functional visual responses, and even enhance performance in a test that relies on image-forming vision.

8.2 Ubiquitous versus cell specific targeting strategies

The work presented in this thesis is the first reported example of a ubiquitous promoter being used to deliver an optogenetic tool via a subretinal approach. Previous studies have used a ubiquitous promoter via an intravitreal approach via which the main cells transduced are ganglion cells (88, 155). The alternative approach of using a cell specific promoter has been used to target bipolar cells (87, 111, 159) or ganglion cells (157). The strategy used in this thesis has several advantages: the first is that levels of expression achieved using ubiquitous promoters are generally higher than with the use of cell-specific ones, as illustrated in chapter 4 and also other studies (262). In addition, many cell surface receptors and other proteins are downregulated in the degenerate retina. Therefore expression of protein using a cell specific promoter may also be downregulated with time (190). Data presented here illustrate this advantage since the CMV enhancer/CBA promoter was still able to drive high levels of human melanopsin

expression at 15 months after injection. This is of particular importance in translating such work to patients: a therapy that required frequent re-administration or that became less effective as degeneration progressed would be much less attractive as a treatment option. Due to its long-term efficacy, a single subretinal injection of rAAV2/8 Y733F CMV.CBA-*OPN4* could potentially restore visual responses in the long term or even indefinitely.

A criticism of this ubiquitous approach is alteration of the normal retinal circuitry. In a wild type retina, light-induced hyperpolarisation of photoreceptors leads to depolarisation of ON rod and ON cone bipolar cells. In contrast, OFF cone bipolar cells hyperpolarise. Following delivery of human melanopsin to the degenerate retina using an AAV vector with a ubiquitous promoter, potentially all three classes of bipolar cell maybe transduced. This would introduce light-induced depolarisation into OFF bipolar cells technically abolishing the OFF pathway, which would impact on restoration of completely normal levels of visual function. However, several areas of evidence exist to support that some useful vision could still be restored despite the absence of an OFF channel of information at the bipolar cell level.

The first is the subretinal electronic implant. The stimulating chip in this device is inserted into the subretinal space underlying the residual neuroretina i.e. inner nuclear layer in end-stage retinal degeneration. Individual electrodes within the chip deliver monophasic voltage pulses to adjacent bipolar cells (83), and thus in this set up only an ON signal is delivered. Following implantation of the subretinal implant, patients who previously had no vision were able to localise objects and three out of nine patients were able to read letters (141). Therefore activation of the ON pathway alone in this model restored visual responses.

Further evidence for successful restoration of vision using an ON stimulus only comes from a recent study targeting Channelrhodopsin-2 to ON bipolar cells using AAV containing a Grm6 enhancer/ SV40 promoter and packaged in a 7m8 capsid variant of AAV (159). Despite only ON bipolar cells being transduced by the channelrhodopsin vector, both ON and OFF responses to light were seen in MEA and visual cortex recordings. The mechanism of this OFF response is hypothesised to be the communication between rod ON bipolar cells to AII amacrine cells, which then make a glycinergic synapse to OFF cone bipolar cells. No such OFF responses were seen in MEA recordings of degenerate retina transduced by the rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector, although some electrodes showed an initial light-induced increase in action potential firing followed by inhibition. The lack of classical OFF responses may have been due to kinetics of the melanopsin response, the fact that some cone OFF bipolar cells were also transduced, or that not enough rod ON bipolar cells were transduced to effect this alternative pathway (previous reports targeting channelrhodopsin to ON bipolar cells have not shown OFF responses (87, 111)). However it may be possible that the light-induced suppression of activity seen in some electrodes following retinal transduction by the melanopsin vector presented in this thesis could potentially be centrally processed to obtain some OFF characteristics regarding the light response.

Studies of the electroretinogram (ERG) also provide information regarding visual potential in the absence of an OFF pathway. Hyperpolarising bipolar cells and horizontal cells (which are also inhibitory) can be selectively blocked using two pharmacological agents *cis*-2,3-piperidine dicarboxylic acid (PDA) and kynurenic acid (KYN). Applying these via intravitreal injection in macaques, a depolarising or entirely positive photopic ERG response was seen, i.e. lacking the hyperpolarising contribution of OFF cone bipolar cells (263). Furthermore, several patients with retinal dystrophies have been found to

have this characteristic depolarising ERG phenotype, implying a defect in OFF bipolar cells (264). These patients were clinically diagnosed with cone-rod or rod-cone dystrophy and had Snellen visual acuity ranging from 20/30 to 20/100. These individuals would have had significantly less severe retinal degeneration than those end-stage patients that might be treated with melanopsin gene therapy. However these findings illustrate that abnormalities or absence of the OFF pathway can still generate an ERG signal and could result in some useful vision.

Pursuing an ON bipolar cell specific strategy using melanopsin may ultimately give a retinal signal that is most physiological. However, as presented in this thesis, using a single capsid mutant vector to target bipolar cells using the CHX10 or 4xMg6 promoter was unsuccessful. Therefore use of an AAV capsid that can drive higher levels of expression, such as the 7m8 capsid used to target Channelrhodopsin to mouse ON bipolar cells (159) may be necessary. Previous studies have shown species differences in transduction patterns (112), and therefore further work is required to ascertain whether this capsid would be effective in targeting bipolar cells in the primate retina. In addition, administration of high dose AAV by intravitreal injection has been documented to drive an immune response (133), and this may be exacerbated in the presence of a multiple modifications of the AAV capsid (181).

Therefore, data presented in this thesis regarding longevity of expression and functional restoration using an AAV melanopsin vector with a ubiquitous promoter, and previous safety and efficacy data using ubiquitous CMV enhancer /CBA promoters (79, 100), suggests that this would be a logical first vector to use in clinical trials evaluating melanopsin gene therapy in humans.

8.3 Mechanism of action

A further consideration to address is whether visual responses restored following subretinal delivery of rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed could be attributed to activation of retinal circuitry involved in image-forming vision and not a result of augmenting existing intrinsically photosensitive ganglion cells (ipRGCs).

This question was considered carefully in experimental design. Treated mice were compared to either sham-injected and/or untreated control mice in all experiments, with all groups having an intact ipRGC pathway. Therefore any effect seen in the treated group over and above the control groups should be attributed to ectopic expression of melanopsin by the AAV vector.

The transgene included in the AAV vector was human melanopsin and this could be identified using a human melanopsin specific antibody, allowing areas of melanopsin expression driven by the vector to be accurately identified. Inclusion of DsRed in the construct also confirmed areas of vector transduction. The AAV human melanopsin vector effectively transduced retinal pigment epithelium and cells of the inner nuclear layer, with processes extending into the inner plexiform layer and ganglion cells layers. No direct transduction of cells in the ganglion cell layer was detected, making it unlikely that ipRGCs were transduced and therefore augmented by the vector.

Light induced *cfos* upregulation in vector treated areas of retina was seen in multiple cells in the inner nuclear layer, suggestive that the human melanopsin expressed in these cells was able to mediate cell depolarisation. The pattern of *cfos* expression in controls was different, with fewer *cfos* positive cells overall and the majority of these being in the ganglion cell layer.

Examining responses from MEA recordings, a greater percentage of responsive electrodes were seen in treated retinas compared to untreated controls, suggestive that a larger number of ganglion cells were generating light-induced action potentials in treated retinas i.e. not ipRGCs alone. Furthermore, there was greater variation in responses in treated retinas versus controls, in terms of firing rate, kinetics and sensitivity, which would imply that a number of different ganglion cell types were firing in treated retinas.

Responsive animals in the treated group showed large, early light-induced changes in blood flow in the visual cortex using the technique of laser speckle cortical imaging (LSI), whereas no such response was seen in sham-injected controls. It is difficult to be entirely certain, but is unlikely that this response is channelled by ipRGCs. Previous work has demonstrated projections from ipRGCs to the dorsal lateral geniculate nucleus (LGN), the latter being the origin of cells in the thalamocortical visual pathway (265). Following a 60 second light stimulus in the rodless/coneless (*rdrd/cl*) degenerate mouse, sluggish light responses were detected in the dorsal LGN, and following a twenty second light stimulus responses were detected in some cortical visual centres in the *rdrd/cl* mouse such as the posterior/ medial aspects of V1, V2M and the retrosplenial dysgranular cortex (265). Therefore the experimental protocol used following subretinal injection of AAV melanopsin vector attempted to minimise activation of ipRGCs projecting to the dorsal LGN with input to the visual cortex by using a short two second light stimulus since long sustained light pulses are usually utilised to stimulate ipRGCs (47). The responses detected in mice with retinal degenerations may be age-related since previous work has documented responses in the visual cortex of *rd1* mice using short (0.125Hz) stimuli only in 6 month old but not in 9 month old mice(180). Mice assessed using LSI following melanopsin vector delivery were 8 months old at the

time of testing, and therefore the responses seen are likely to be secondary to ectopic melanopsin expression since no responses were seen in age-matched control mice, which had an intact ipRGC system.

IpRGCs are known to play an important role in driving the pupil light reflex and therefore again a short duration two second light pulse was used to attempt to minimise their stimulation. Melanopsin in ipRGCs is known to mediate the late tonic pupil constriction seen following a light stimulus (258), and so the time point chosen for analysis of pupil size was at two seconds, which in a wild type mouse would be in the phase of the rod/ cone contribution to the pupil light reflex. In addition, in the cohort of mice that were 15 months old at the time of assessment, overall pupil constriction in sham and untreated groups was reduced. This was likely to have been due to age-related loss of ipRGCs. Pupil constriction was similar in young and old treated animals, indicative that the treatment effect was maintained despite ipRGC degeneration.

Treated mice in the older cohort demonstrated behavioural light aversion (BLA) compared to sham-injected controls. Previous studies have shown in rodless/coneless mice i.e. that have ipRGCs as their only functional photoreceptive cell, BLA is seen after twenty minutes in a brightly illuminated test arena (1300lux) (259). Therefore, to assess the effects of the rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector a lower light intensity of 200 lux, and a shorter test time of 5 minutes was used to try to minimise activation of ipRGCs.

Finally in the visual context recognition test, treated animals were able to detect a change in their visual environment whereas sham-injected and untreated animals did not show such behavioural changes. The visual stimulus was a static 4cm x 4cm chequer board pattern and a large white star on a black background. The task required the

presence of image-forming vision to drive a behavioural response. There is only one previous report of ipRGCs being able to mediate navigation of a water maze using visual gratings (60). However these results have been questioned since the sluggish cortical responses mediated by ipRGCs alone would probably not be compatible with a timeframe of visual fixation (265). Secondly the model used had an intact but non-functional retina with mutations in *Gnat1*^{-/-} and *Cnga3*^{-/-}. Other work has illustrated that a residual ERG is still present in the total knockout mouse (*Gnat 1*^{-/-}, *Cnga3*^{-/-} and *Opn4*^{-/-}) driven by *Gnat1* independent signalling in the rod pathway (71). Therefore it is not certain whether the visual responses described to drive navigation of the water maze in the latter study are attributable to ipRGCs. In the visual environment recognition tests, again conditions were tailored as far as possible to avoid ipRGC stimulation. The arena was illuminated by a dim light of 50 lux intensity and the test phase was only 3 minutes. Sham-injected and untreated mice, which had intact ipRGC systems, did not show appropriate behavioural changes in response to a change in environment.

In summary, ipRGCs were not directly transduced by the rAAV2/8 Y733F CMV.CBA-*OPN4*-IRES-DsRed vector, and therefore treatment effects seen are unlikely to be attributed to direct augmentation of melanopsin in these cells. There is a possibility that light responses could have been channelled via ipRGCs to central targets to mediate the treatment effects seen, particularly in pupillometry experiments. However, as far as possible experimental conditions were designed to avoid ipRGC stimulation. Furthermore, *cfos* induction and MEA recordings showed activation of a number of different cell types suggestive that the AAV vector led to activation of retinal circuitry outside the ipRGC system.

8.4 Optogenetic tools

The work presented in this thesis provides a solid foundation for the use of human melanopsin as an optogenetic tool for the restoration of visual responses in the treatment of patients with end-stage retinal degenerations. The major limitation of use of human melanopsin for this purpose is its slow kinetics, particularly in signalling light offset. Therefore, a further avenue of investigation to optimise the use of melanopsin for restoration of vision could be to modify its structure and/or signalling to speed up its response. Alternative options would be to use non-mammalian opsins such as Channelrhodopsin-2 (ChR2) (87, 111), Halorhodopsin (89) or a chemically engineered light-gated ionotropic glutamate receptor (LiGluR) (266). Although these options have faster kinetics than melanopsin, two of which when used as an optogenetic tool were able to restore an optomotor response (87, 89), their activation requires significantly higher levels of light. All functional experiments presented in this thesis were carried out at light intensities within the physiological light range. Use of ChR2 or halorhodopsin may require significant modification of visual input not only to adjust for the intense light levels required, but also due to the comparatively small range of light intensities over which these opsins are able to adapt (171).

The greatest advantage that the use of human melanopsin has over other strategies, is its natural presence in the human eye and therefore it is unlikely to generate a significant immune response (88). ChR2 and halorhodopsin are non-mammalian opsins, and thus ectopic expression could be immunogenic (171). Previous studies in mice have not demonstrated a detectable local or systemic immune response following subretinal delivery of ChR2 using a capsid mutant AAV vector (111), however in systemic gene therapy, testing using animal models has not necessarily been able to predict human immune responses to the AAV capsid (267). As discussed above, more recent work has

focused on intravitreal delivery of ChR2, and aside from the potential immune response to ChR2 itself, high doses of intravitreal AAV vector have been demonstrated to be immunogenic in primates (133). Therefore subretinal delivery of human melanopsin is likely to be the safest and best tolerated optogenetic strategy currently available for restoration of visual function in the human eye.

8.5 Conclusions

In summary, data presented in this thesis has led to the generation of an optimised capsid mutant AAV vector for delivering human melanopsin to the degenerate retina. Following a single subretinal injection of vector in the *rd1* mouse model, sustained expression of human melanopsin was seen at 15 months. In these retinas there was significant light-induced expression of *cfos* and increased light-dependent action potential firing using multi-electrode array recordings indicative of the melanopsin expressed being functional. Furthermore, using an assay of the consensual pupil light reflex, it was demonstrated that the visual signal generated could input into existing neural circuitry. Physiological changes were seen in the visual cortex following light-stimulation and visually guided behaviour was seen in treated mice, including visual context recognition. These data provide evidence that gene therapy using human melanopsin may restore some level of image-forming vision. Therefore with further development, this optogenetic strategy has great potential for treatment of patients with end-stage retinal degeneration, irrespective of their underlying genetic mutation.

Chapter 9 References

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