Title: Long term restoration of visual function in end-stage retinal degeneration using subretinal human melanopsin gene therapy

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Abstract:

Optogenetic strategies to restore vision in patients who are blind from end-stage retinal degenerations aim to render remaining retinal cells light sensitive once photoreceptors are lost. Here, we assessed long-term functional outcomes following subretinal delivery of the human melanopsin gene (OPN4) in the *rd1* mouse model of retinal degeneration using an adeno-associated viral vector. Ectopic expression of OPN4 using a ubiquitous promoter resulted in cellular depolarisation and ganglion cell action potential firing. Restoration of the pupil light reflex, behavioural light avoidance and the ability to perform a task requiring basic image recognition were restored up to 13 months following injection. These data suggest that melanopsin gene therapy via a subretinal route may be a viable and stable therapeutic option for the treatment of end-stage retinal degeneration in humans.

Significance statement:

Inherited retinal degenerations may result in blindness due to a progressive loss of photoreceptor cells. We assess subretinal delivery of human melanopsin using an adeno-associated viral vector to remaining retinal cells in a model of end-stage retinal degeneration. Human melanopsin being already present in the eye is unlikely to generate an immune response when introduced via gene therapy. Furthermore, this method of delivery has been proven to be safe in clinical trials and may be more effective at delivering vector in primates than the alternative method of intravitreal injection. We demonstrate long-term vector expression and restoration of visual function indicating this therapy could be stable and efficacious in treatment of patients with end-stage retinal degenerations.

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Introduction

Inherited retinal degenerations such as retinitis pigmentosa (RP) affect 1 in 4,000 people (1), causing significant visual morbidity and blindness due to a progressive loss of photoreceptor cells. Even in end-stage disease, the remaining retinal layers and central visual projections remain structurally intact. Stimulation of these remaining cells is potentially sufficient to mimic visual responses and restore vision and by this means the subretinal electronic implant has shown proof of principle for restoration of vision in patients after severe photoreceptor loss (2).

An alternative gene therapy strategy involves the expression of transgenes encoding photosensitive proteins in remaining retinal cells, making them directly light sensitive in the absence of rods and cones (3-7). A candidate protein for this purpose is melanopsin, the photopigment naturally present in a subset of ganglion cells that are intrinsically photosensitive (ipRGCs) (8). Melanopsin is particularly suited to this purpose since it is native to the human eye (9) and therefore is less likely to be immunogenic. Melanopsin shows greater sensitivity to light than alternative microbial optogenetic tools such as channelrhodopsin-2 (3, 10, 11) or halorhodopsin (4), but has slower kinetics. Furthermore, the melanopsin transduction cascade involves the activation of ubiquitously expressed Gnaq/11 type G-proteins (12) permitting signal amplification in multiple host cell types (13, 14).

Previous work used intravitreal delivery of an adeno-associated viral vector (AAV) to express mouse melanopsin in ganglion cells with restoration of visual responses (5). We investigated whether human melanopsin (OPN4) could be effectively delivered via an alternative subretinal approach, using a ubiquitous (CBA) promoter to drive expression in all remaining outer retinal cells for several reasons. Subretinal vector delivery is well established in human clinical trials

(15, 16), but has not been assessed in combination with a CBA promoter as an optogenetic approach for vision restoration. Transduction of cells in the upstream retina maximises the potential of retaining complex processing of the visual signal. Furthermore, increased availability of chromophore (retinal) in the outer retina may be required for effective photon capture in the absence of specialised outer segment discs. Other studies have used AAV vectors containing a mouse bipolar-cell specific enhancer to target a melanopsin.mGluR6 chimera (17) or rhodopsin (6) to bipolar cells via intravitreal injection. However, there is variation in anatomy between primates and mouse models (18) and this may render the intravitreal approach less effective in humans. Virions delivered via intravitreal injection are diluted more in primates compared to mice due to the larger volume of the vitreous, reducing the concentration of vector reaching retinal cells. The inner limiting membrane on the retinal surface is also thicker in primates than in rodents (19), through which virions must pass to reach target cells. The increased risks of an inflammatory response following intravitreal AAV injection (20) may also limit the translational potential of this route of delivery. We therefore assessed transduction following subretinal delivery of OPN4 and whether this could support long-term restoration of light sensitivity and visual function in a mouse model of end-stage RP.

Results

Long-term expression of human melanopsin in degenerate retina is achieved following subretinal delivery of an AAV vector

In order to model the extensive photoreceptor loss seen in end-stage RP, the *rd1* mouse was used. These mice have a nonsense mutation in the *Pde6b* gene, which leads to rapid degeneration of rod photoreceptors followed by loss of cones (21). Retinal tropism was assessed 4 and 15 months after subretinal delivery of a single capsid mutant AAV vector (rAAV2/8(Y733F) CBA-*OPN4*-IRES-DsRed) in six to eight week old *rd1* mice. This bicistronic vector included a DsRed fluorescent marker to permit visualisation of transduced cells within the retina and ensure vector-driven expression. Retinal flatmounts stained with human OPN4 specific antibody showed widespread retinal transduction in treated eyes, which was sustained up to 15 months post injection (figure 1A,B), but none in age-matched untreated controls (figure 1C,D). Human melanopsin showed appropriate membrane localisation (figure S1, figure S2E), with the DsRed marker confirming vector-driven expression (figure 1E). Histological sections demonstrated robust and widespread OPN4 expression throughout the inner nuclear and inner plexiform layers of the degenerate retina in treated eyes, with immunohistochemistry using horizontal, bipolar (figure 1F,G, figure S1) and Müller cell (figure S2) specific antibodies and cell morphology indicating widespread transduction of these cell types. There was no significant transduction of cell bodies in the ganglion cell layer following subretinal vector delivery, as assessed by cell morphology and ganglion cell specific staining (figure S2).

Human melanopsin expressed in the degenerate retina is able to mediate a functional response to a light-stimulus

Expression of the ‘immediate early’ gene c-Fos, is a marker of cellular depolarisation and has been widely used to monitor melanopsin driven light responses in ipRGCs in the degenerate retina (22, 23). Comparison of light induced c-Fos expression in the inner nuclear layer (INL) of vector treated versus untreated retina showed a 2.5 fold increase in the number of c-Fos positive cells in treated eyes (effect of treatment p=0.0197, 2 way ANOVA, figure S3A-G). High levels of co-localisation were observed for both DsRed and c-Fos (figure S3A-C), and human OPN4 and c-Fos (figure S3O-Q) confirming light induced depolarisation of transduced cells in treated retina. In addition, some adjacent c-Fos positive cells were observed that did not appear to express OPN4 (or DsRed), which might indicate depolarisation of neighbouring cells and cell to cell signalling resulting from ectopic expression of OPN4 in the degenerate retina.

Multi-electrode array recordings from *ex-vivo* retinal explants were performed four months after subretinal vector delivery to assess whether expression of OPN4 in the INL was able to drive action potential firing in retinal ganglion cells, and therefore generate a signal that could be centrally transmitted. The percentage of electrodes showing a light-dependent increase in action potential firing in treated retinae (43.1±5.6% electrodes, N=6 retinae, n=144 electrodes) was more than doubled compared to untreated age-matched controls (18.1±7.3% electrodes, N=9, n=88 electrodes; p=0.028, two-tailed unpaired t-test, figure 2A). Responses observed in untreated retina were consistent with the activation of endogenous ipRGCs, whereas treated retinae include both these responses and those originating from ectopic expression of human OPN4 (figure 2C). In treated but not untreated retina some electrodes showed a reduction in spike firing rate following light stimulation (figure 2B), which may add to the complexity of the visual signal generated following OPN4 expression in inner nuclear cells. Visualisation of DsRed fluorescence using 540nm light (figure 2D) confirmed that the location of responsive

electrodes in treated retinae was highly correlated to areas transduced by the OPN4 vector (figure 2 C-E, S4A), indicative of these being responses from ectopically expressed OPN4.

Irradiance response curves (IRCs) generated for responsive electrodes in retinae following vector delivery (n=51, N=3) versus controls (n=22, N=4) revealed similar overall light sensitivity between groups (figure 2F): with half maximal responses (EC50) observed at 13.4±0.11 log photons/cm^2/s in electrodes from treated retina versus 13.2±0.16 in controls (p=0.26, two-tailed unpaired t-test). However, at sub-saturating levels of light (3.99x10^{14} photons/cm^2/s), the maximal spike firing rate was significantly higher on responsive electrodes from treated retinae (80.0±6.2Hz, n=54) versus untreated controls (44.6±5.4Hz, n=25; p=0.0006, two-tailed unpaired t-test, figure 2G-H). A range of response kinetics was observed for both treated and untreated groups, including transient and sustained changes in action potential firing (figure 2B and figure S4B,C). At sub-saturating levels of light (3.99x10^{14} photons/cm^2/s), the time taken to reach maximal firing rate was significantly lower in OPN4 treated retinae (6.63±0.5s, n=54) compared to untreated controls (16.9±2.9s, n=25; p<0.0001 unpaired t-test, figure 2I), indicating different response kinetics between ganglion cells firing in OPN4 treated retinae versus responses recorded from native ipRGCs in untreated controls.

Visual function restored by human melanopsin expression in the degenerate retina was sustained at 13 months

The pupil-light reflex (PLR) was evaluated to determine if light-dependent signals generated by ectopic OPN4 expression could affect central targets. Mice that received a unilateral subretinal injection of OPN4 vector were compared with age-matched mice that received a sham injection of phosphate buffered saline and also with untreated controls. The consensual PLR was assessed

to avoid any surgical effect on pupil constriction in the treated eye. Two cohorts of mice were evaluated, one at two months after injection (figure S5) and another at thirteen months to determine long term functional improvement. Thirteen months after OPN4 vector delivery, pupil constriction was significantly greater in treated eyes compared to controls at multiple light intensities (overall treatment effect $p=0.013$, repeated measures two way ANOVA, figure 3A,B). Pupil constriction was faster in the OPN4 vector group compared to sham-injected and untreated groups, (interaction between treatment and time on pupil area: $p<0.0001$ repeated measures 2 way ANOVA, figure 3C). Furthermore, pupil constriction was significantly reduced in older sham-injected (effect of age $p=0.0018$, repeated measures two way ANOVA) and untreated ($p=0.0067$) control groups compared to their younger counterparts (figure 3E,F). This effect was not seen in the OPN4 vector treated group ($p=0.217$, figure 3D), indicating a sustained treatment effect in the older cohort following OPN4 vector delivery.

In order to assess whether information generated by ectopic expression of OPN4 in the retina could drive visually guided behaviour, animals were assessed using a behavioural light avoidance assay (24, 25) based on the natural preference of mice to avoid brightly lit environments (figure 3G). Thirteen months after OPN4 vector delivery, there was a significant difference in the percentage of time spent in the brightly lit chamber between groups (treated 37.65%±6.7, sham-injected 64.66%±6.8, untreated 57.65%±7.7, $p=0.03$ one way ANOVA, figure 3H), with the vector treated group showing behaviour closest to wild type mice. This was not due to a difference in general or anxiety-related locomotor activity, since the number of transitions between light and dark chambers was similar between groups ($p=0.88$, one way ANOVA, figure 3I). Functional effects seen were unlikely to be mediated by residual cones in the *rd1* mouse, since these cells were morphologically abnormal and there was no difference in

numbers of remaining cells between treated, sham-injected and untreated groups (figure S6A-D, p=0.123 one way ANOVA). Furthermore, all *rd1* mice selected were homozygous for the *gpr179* mutation (figure S6E,F), excluding any input from residual photoreceptors in mediating ON bipolar cell depolarisation via the mGlur6 cascade in this mouse model (26).

Visual responses requiring image-forming vision are generated following melanopsin gene therapy in the degenerate retina

Pupillometry and behavioural light avoidance indicated that subretinal OPN4 delivery restored or improved light responses; however signalling to the visual cortex is not necessary to mediate such effects. To study cortical responses we examined light-induced changes in visual cortex blood flow using laser speckle contrast imaging (figure 4A). Six months after subretinal delivery of OPN4, both eyes were stimulated by 480nm light of two second duration and cerebral blood flow (CBF) recorded over the visual cortices. Treated mice showed an increase in CBF with an initial peak at 5.4 seconds after light onset, consistent with the peak expected in wild type animals (25) (figure 4B,C,S7A). There was no clear corresponding initial peak in the sham-injected group (figure S7B).

Finally we assessed whether ectopic expression of OPN4 would aid image-forming vision. For this purpose we used the one-trial spontaneous object recognition test (27, 28). In wild type rodents with no retinal degeneration, a change in visual environment disrupts object recognition, indicating that these animals encode and remember the background visual environment in which an object is encountered (27, 28). By contrast, mice with visual deficits are not able to detect the visuospatial context of an object (29), indicating that they cannot encode visual information regarding their environment. Object recognition performance was analysed to determine visual

context recognition: this was determined by the ratio of time spent exploring a novel object relative to a previously encountered object. Two cohorts of mice were evaluated, one at two months (figure S7C) and another at thirteen months after injection (figure 4D). Following OPN4 vector delivery 13 months previously, treated mice showed a significant change in recognition ratio dependent on their visual environment ($p=0.04$ for effect of visual-context on object recognition performance; 2 way ANOVA with Bonferroni post hoc test, figure 4E). Combining younger and older cohorts, the effect of visual context on behaviour was highly significant in the treated group overall ($p=0.003$, split-plot ANOVA, table S1). No significant changes were seen in sham-injected or untreated control groups, suggesting an inability to form and retrieve an association between the object and visual context in these mice. Melanopsin vector treated mice showed a behavioural pattern similar to that observed in wild type mice with functional rods and cones in a test requiring image-forming vision.

Discussion

Data presented here demonstrate that a functional human melanopsin gene (OPN4) can be delivered to remaining retinal cells in a mouse model of end-stage retinal degeneration. This was achieved via subretinal injection of an AAV vector using a ubiquitous promoter, an approach currently validated in AAV gene therapy clinical trials (15, 16). Ectopically expressed OPN4 mediated depolarisation of outer retinal cells and ultimately ganglion cell action potential firing, resulting in long-term restoration of the pupil light reflex and behavioural light avoidance up to at least 13 months following injection. Finally, subretinal OPN4 expression led to light-induced changes in visual cortex blood flow and provided long-term improvements in a visually guided behavioural task that requires image-forming vision. In combination, these results suggest that this approach may be clinically useful in vision restoration in patients with end-stage RP.

In the interpretation of these data, a consideration is the mechanism by which visual responses were restored. We believe that responses detected arose from activation of retinal circuitry involved in image-forming vision rather than augmenting existing ipRGCs for several reasons. Human melanopsin was not detected by immunohistochemistry in ganglion cell membranes in transduced retinae. Similarly, light-induced c-Fos expression in vector treated areas of retina was seen in multiple cells within the inner nuclear layer, whereas this pattern was not seen in the INL of controls. MEA recordings revealed a greater percentage of responsive electrodes in treated retinae compared to untreated controls, along with differences in firing rates and response kinetics, suggesting that a larger number of ganglion cells were generating light-induced action potentials in treated retinae.

To assess functional responses *in vivo* a number of assessments were used including behavioural light avoidance since wild type animals with functional rods and cones show aversion to bright light. Treated mice spent less time in the bright chamber compared to control mice, which showed an apparent preference for the bright chamber. This may be due to an inability of control mice to detect the difference in brightness between the two chambers resulting in exploration being guided primarily by non-visual cues e.g. subtle differences in temperature, auditory or olfactory cues. Interestingly, previous work including rodless/coneless mice has also demonstrated that mice have a preference for the front half of the chamber irrespective of whether the animal was placed there first or the test was performed in complete darkness (24).

Treated mice showed the ability to form and retrieve an association between an object and its visual environment in the visual context recognition task. In contrast, control groups were able to perform the object recognition task using non-visual cues (since recognition ratios in these mice were above chance or 0.5) but performance did not vary according to visual environment. This test has been validated for the assessment of rod/cone-dependent image forming visual responses (29). Previous work has also investigated the effect of changes in background irradiance on performance with wild type mice requiring a substantial change in irradiance (e.g. an increase from 10 to 350 lux) to disrupt object recognition performance (29). Therefore context dependent behaviour in treated mice is likely to be caused by the change in visual environment, rather than any changes in background irradiance caused by the different test arenas.

A potential concern is the effect of retinal remodelling seen in end-stage degeneration on restoration of visual function. Multiple changes within the remaining retina have been described in mouse models and human tissue (30-33) including neuronal morphological changes, cell death, network rewiring and formation of gliosis between the RPE and neural retina. Certain

elements of remodelling such as the glial seal may potentially be overcome by subretinal injection, since the hydrostatic force generated could allow AAV to penetrate areas of gliosis. The process of remodelling does however demonstrate plasticity (30) and since we use a ubiquitous promoter to deliver melanopsin to the degenerate retina, it is possible that some of these abnormal connections from a variety of cells are used to restore visual responses. The clinical phenotype in human retinitis pigmentosa is of a rod-cone dystrophy in the majority of patients, which may also be variable with previous studies demonstrating differing degrees of degeneration even in the presence of the same genetic mutation and level of vision (34). Careful selection of potential patients for OPN4 optogenetic therapy would therefore be required since the ideal candidate would have severely affected vision yet grossly intact inner retinal structure (as visualised by ocular coherence tomography) and some remaining inner retinal function, detected for example using electrical phosphene testing (34, 35).

Human melanopsin as an optogenetic tool has significant advantages in its suitability for translation to patients. Being a native protein, OPN4 is unlikely to induce an immune response and secondly OPN4 shows greater light sensitivity than other optogenetic tools. We detected ganglion cell firing during MEA recordings at the lowest stimulus intensity tested ($1.20x10^{12}$photons/cm^2/s), whereas previous reports document a minimum stimulus of $1x10^{14}$ (36) or $1x10^{15}$photons/cm^2/s (3) for detection of ganglion cell responses following channelrhodopsin gene therapy and $1x10^{16}$ for halorhodopsin (4). We also report restoration of visually guided behaviour in the visual context recognition task at a light intensity of 50lux, corresponding to low level indoor lighting. The brightest light stimulus we used was $2x10^{16}$photons/cm^2/s during pupillometry (however significant differences between groups were seen at lower intensities) or approximately 13000 lux (table S2) equivalent to daylight

conditions. The brighter stimulus intensity used for pupillometry in our experiments compared to previous studies using melanopsin likely reflects the use of different animal models (37) and our use of a 2 second white light stimulus (chosen to be a more useful stimulus for image-forming vision) whereas other studies used monochromatic light (37) and longer stimulus durations more suited for activating ipRGCs (5).

The quality of vision that might be restored by OPN4 gene therapy is likely to be affected by its response kinetics. Although slower than classical (rod/cone) photopigments, the detection of transient light responses following melanopsin stimulation (figure S4B) as previously described (38) may be a useful input for image forming vision. Furthermore, not all ganglion cell spikes are transmitted at the retinogeniculate synapse allowing for modification of the visual signal (39). Melanopsin gene therapy may provide functionally useful vision in a static visual environment such as in the visual context recognition test, which may be useful to patients in terms of aiding navigation. For more dynamic environments, modification of the visual input through devices such as image processing glasses (40) may be required.

The use of human melanopsin delivered via subretinal injection using a CBA promoter for optogenetic restoration of vision has not previously been described. We demonstrate effective transduction of end-stage degenerate retina resulting in sustained restoration of visual function. The effects may be mediated by transduction of bipolar and horizontal cells. This approach has significant potential for translation to patients since although technically more challenging, subretinal delivery has been established as safe in current clinical trials and provides the advantage of delivering a high concentration of vector to residual retinal cells, whereas the alternative method of intravitreal delivery may not be as effective in humans. Targeting the outermost surviving retinal layers will likely allow greater levels of signal processing to be

performed by existing retinal circuitry, potentially resulting in restoration of more complex visual responses.

Materials and Methods

All animal experiments were conducted in accordance with Association for Research in Vision and Ophthalmology statements on care and use of animals in ophthalmic research. C3H/HeNHsd-*Pde6b*^{rd1} (*rd1*) mice were 6 to 8 weeks old at time of intraocular injection. Treated eyes were injected with a dose of $1.5x10^9$ vg per eye of AAV2/8(Y733F) CBA-*OPN4*-IRES-DsRed vector, with an equivalent volume of phosphate buffered saline injected in sham treated eyes and age-matched untreated eyes also used as controls. Further experimental details are described in SI Materials and Methods.

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Figure Legends

Figure 1. Long-term expression of human melanopsin is achieved in the degenerate retina following subretinal delivery of an adeno-associated viral (AAV) vector. Flatmounts of the *rd1* mouse retina assessed following immunolabelling for human OPN4 (green) at 4 months (A) and 15 months (B) after subretinal OPN4 vector delivery reveal widespread transduction. Labelling of human OPN4 was absent from untreated age-matched controls (C,D scale bar 500μm). Appropriate membrane localisation of human melanopsin was evident demonstrating a network of transduced cells, with DsRed fluorescence confirming vector driven OPN4 expression (scale bar 25 μm) (E). Successful transduction of bipolar cells (labelled by PKC alpha, purple, F) and horizontal cells (labelled by calbindin, purple, G) using a ubiquitous promoter was evident following co-labelling with OPN4 (green) and Hoechst nuclear stain (blue) (scale bar 25 μm).

Figure 2. Human melanopsin expressed in the degenerate retina is able to mediate a functional response to a light-stimulus. Multi electrode array (MEA) recordings from ex-vivo *rd1* mouse retinae showed a higher percentage of electrodes demonstrating light-induced increases in action potential firing in treated retinae compared to untreated controls (A) ($p=0.028$, two-tailed unpaired t-test, treated N=6, untreated N=9). Examples of raw data obtained from individual electrodes following 60 second 480nm light pulses are shown (B). Light responses recorded from electrodes from one treated retina are shown (C) (represented as mean spike firing rate (Hz) measured in 1s bins. Data shown are 30s of baseline recording followed by

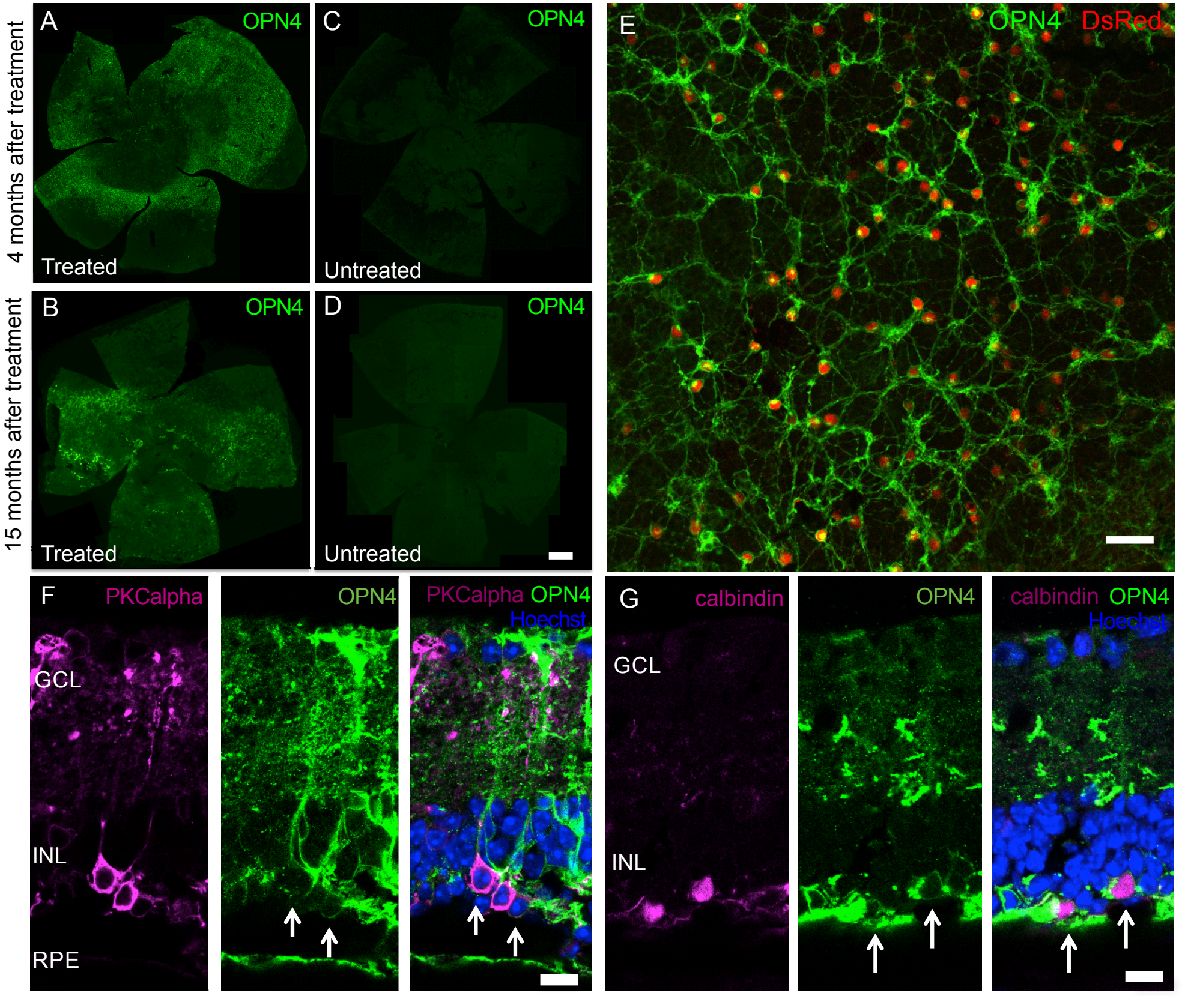
a 60s 480nm light stimulus at 3.99 x 10^{14} photons/cm^2/s; for original image of the retina showing location of DsRed positive cells see Figure S4). Visualisation of DsRed fluorescence (dots) illustrates the area of the retina transduced by the human OPN4 vector relative to the position of MEA recording electrodes (D). A representation of DsRed expression (highlighted in red) is shown to indicate the transduced areas of the treated retina in (C) relative to the positioning of electrodes (E). In combination, this illustrates an increase in light-dependent action potential firing within regions of transduced retina. Irradiance response curves generated from responsive electrodes in treated (n=51, N=3) and untreated (n=22, N=4) retinae shows a similar sensitivity of responses between groups (F), with action potential firing detected at the lowest light intensity assessed (1.20x10^{12} photons/cm^2/s). The mean response of all electrodes to a 3.99 x 10^{14} photons/cm^2/s light stimulus is shown (G). Blue bars indicate duration of light stimuli (B,C,G). There was a greater increase in spike firing rate in response to a light stimulus in treated versus untreated retinae (H); and time to maximal response was shorter in treated retinae compared to untreated controls (I) (*** $p<0.001$, two-tailed unpaired t-test). For further description of response kinetics see Figure S4.

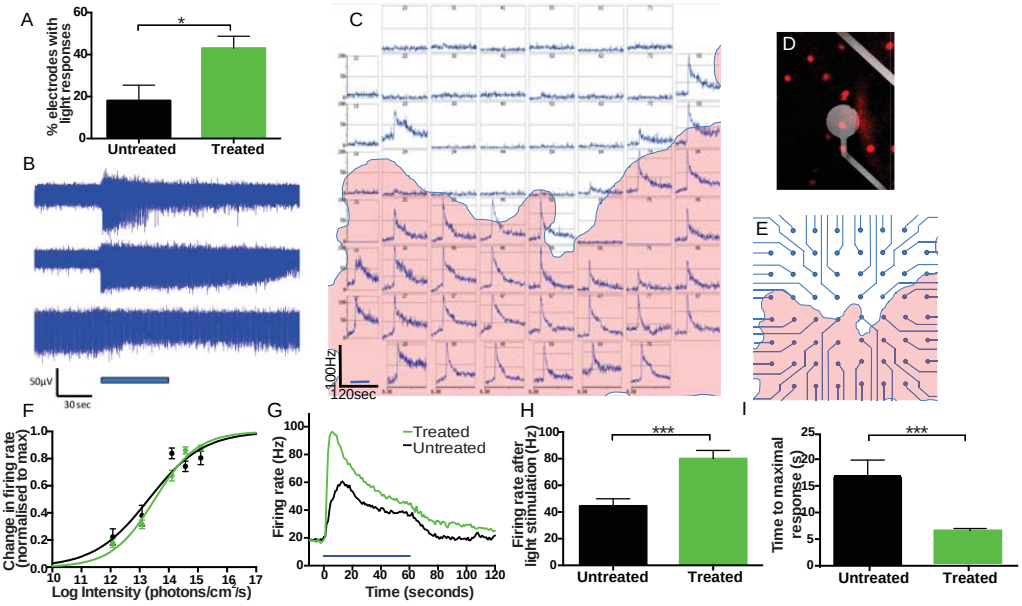
Figure 3. Visual function restored by human melanopsin expression in the degenerate retina was sustained at 13 months. Representative images show levels of pupil constriction observed thirteen months after human OPN4 vector delivery, compared to age-matched sham-injected and untreated controls (A). Significantly more pupil constriction was observed in mice treated with OPN4 vector compared to sham-injected (red*) and untreated controls (black*), at multiple light intensities (B) measured at two seconds after light onset (treated n=5, sham-injected n=8, untreated n=13). Time course of pupil constriction at 2x10^{15} photons/cm^2/s

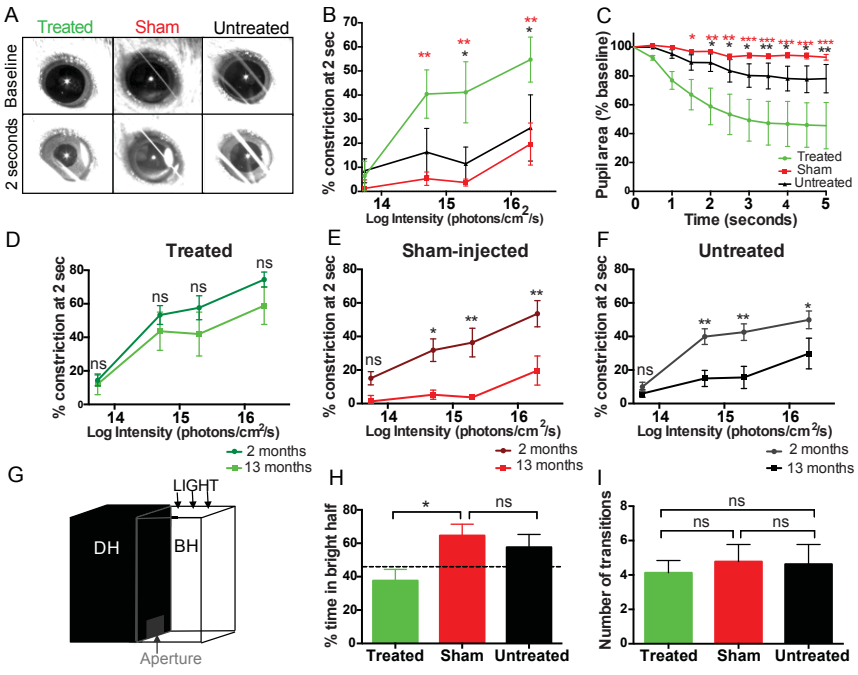
demonstrated a faster response in the treated group (C). Pupil constriction was not significantly different in treated mice (D) at 2 and 13 months, whereas the level of pupil constriction declined with age in sham-injected (E) and untreated mice (F). (2 month cohort: treated n=10, sham-injected n=9, untreated n=12; ns= non significant, *$p<0.05$, **$p<0.01$, repeated measures 2 way ANOVA with Tukey post hoc test). In the behavioural light-dark assay, mice could move freely between the bright half (BH) of the test chamber illuminated by a white light stimulus of 200 lux at ground level and the dark half (DH), (G). Mice treated with OPN4 vector thirteen months previously spent less time in the bright half of the chamber compared to sham-injected controls (H) (treated n=8, sham-injected n=9, untreated n=8; $p=0.03$, one way ANOVA with Tukey post hoc test). The number of transitions between compartments was similar across groups (I).

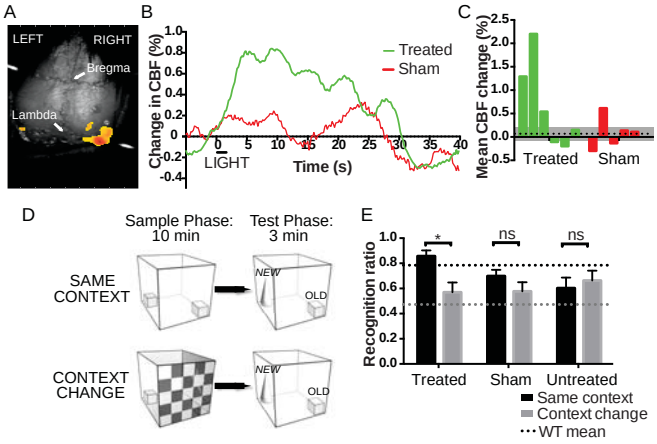
Figure 4. Visual responses requiring image-forming vision are generated following melanopsin gene therapy in the degenerate retina. Laser speckle cortical imaging was used to measure changes in visual cortex blood flow following a 2 second 480nm light stimulus of 2000 lux intensity (A). OPN4 vector treated mice showed an appropriate light-dependent peak in cortical blood flow (CBF) compared to sham-injected controls (B; treated n=5, sham-injected n=4). The mean percentage change in cortical blood flow for each animal in the first ten seconds after light onset is shown (C). The dashed line indicates mean change in sham-injected animals with standard error of the mean (grey). Visual context recognition testing (D): in the ‘same context’ condition the visual environment is identical in both phases. In the ‘context change’ condition there is a change in visual environment between phases but all other factors are constant. The recognition ratio is calculated as n/(n+f), i.e. time spent exploring the novel object (n) as a fraction of the total time spent exploring both the novel and a familiar (f) object. Thirteen

months after OPN4 vector delivery, recognition ratios for the novel object were significantly different in treated mice when the visual environment was the same versus when it was changed indicating a visual environment-dependent change in behaviour (E) ($p=0.04$, two way repeated measures ANOVA with Bonferroni post hoc test; dashed line= wild type mean, $*p<0.05$; treated n=9, sham-injected n=8, untreated n=11).


4 months after treatment
15 months after treatment
A
OPN4
Treated
C
OPN4
Untreated
B
OPN4
Treated
D
OPN4
Untreated
E
OPN4
DsRed
F
PKCalpha
OPN4
PKCalpha OPN4
Hoechst
GCL
INL
RPE
G
calbindin
OPN4
calbindin OPN4
Hoechst
GCL
INL


A
% electrodes with light responses
60
40
20
0
*
Untreated
Treated
B
50µV
30 sec
C
100Hz
120sec
D
E
F
Change in firing rate (normalised to max)
1.0
0.8
0.6
0.4
0.2
0.0
10 11 12 13 14 15 16 17
Log Intensity (photons/cm²/s)
G
Firing rate (Hz)
100
80
60
40
20
0
Treated
Untreated
0 20 40 60 80 100 120
Time (seconds)
H
Firing rate after light stimulation (Hz)
100
80
60
40
20
0
***
Untreated
Treated
I
Time to maximal response (s)
25
20
15
10
5
0
***
Untreated
Treated


A
Treated
Sham
Untreated
Baseline
2 seconds
B
% constriction at 2 sec
Log Intensity (photons/cm2/s)
C
Pupil area (% baseline)
Time (seconds)
Treated
Sham
Untreated
D
Treated
% constriction at 2 sec
Log Intensity (photons/cm2/s)
ns
2 months
13 months
E
Sham-injected
% constriction at 2 sec
Log Intensity (photons/cm2/s)
2 months
13 months
F
Untreated
% constriction at 2 sec
Log Intensity (photons/cm2/s)
2 months
13 months
G
LIGHT
DH
BH
Aperture
H
% time in bright half
Treated
Sham
Untreated
I
Number of transitions
Treated
Sham
Untreated


A
LEFT
RIGHT
Bregma
Lambda
B
Change in CBF (%)
1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
0
5
10
15
20
25
30
35
40
LIGHT
Time (s)
Treated
Sham
C
Mean CBF change (%)
2.5
2.0
1.5
1.0
0.5
0.0
0.5
Treated
Sham
D
Sample Phase:
10 min
Test Phase:
3 min
SAME
CONTEXT
NEW
OLD
CONTEXT
CHANGE
NEW
OLD
E
Recognition ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
ns
ns
Treated
Sham
Untreated
Same context
Context change
WT mean