ON-bipolar cell gene expression during retinal degeneration: Implications for optogenetic visual restoration

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ARTICLE INFO

Keywords
Bipolar cell
Gene expression
Optogenetics
Inherited retinal degeneration

ABSTRACT

Purpose: Retinal bipolar cells survive even in the later stages of inherited retinal degenerations (IRDs) and so are attractive targets for optogenetic approaches to vision restoration. However, it is not known to what extent the remodelling that these cells undergo during degeneration affects their function. Specifically, it is unclear if they are free from metabolic stress, receptive to adeno-associated viral vectors, suitable for optogenetic tools and able to propagate signals by releasing neurotransmitter.

Methods: Fluorescence activated cell sorting (FACS) was performed to isolate labelled bipolar cells from dissociated retinae of litter-mates with or without the IRD mutation Pde6 b e1rt1 selectively expressing an enhanced yellow fluorescent protein (EYFP) as a marker in ON-bipolar cells. Subsequent mRNA extraction allowed Illumina® microarray comparison of gene expression in bipolar cells from degenerate to those of wildtype retinae. Changes in four candidate genes were further investigated at the protein level using retinal immunohistochemistry over the course of degeneration.

Results: A total of sixty differentially expressed transcripts reached statistical significance: these did not include any genes directly associated with native primary bipolar cell signalling, nor changes consistent with metabolic stress. Four significantly altered genes (Smr2, Slf2, Anxa7 & Cntn1), implicated in synaptic remodelling, neurotransmitter release and viral vector entry had immunohistochemical staining colocalising with ON-bipolar cell markers and varying over the course of degeneration.

Conclusion: Our findings suggest relatively few gene expression changes in the context of degeneration: that despite remodelling, bipolar cells are likely to remain viable targets for optogenetic vision restoration. In addition, several genes where changes were seen could provide a basis for investigations to enhance the efficacy of optogenetic therapies.

Abbreviations

ANOVA Analysis Of Variance
AAV Adeno associated virus
EYFP Enhanced Yellow Fluorescent Protein
FACS Fluorescence Activated Cell Sorting
FDR False detection rate
HSPG Heparin Sulphate Proteoglycan
IHC Immunohistochemistry
IRDs Inherited Retinal Degenerations P40, P90, P120, P150
LTD/P Long term depression/potentiation PKC α - Protein Kinase C - α

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https://doi.org/10.1016/j.exer.2020.108553
Received 19 June 2020; Received in revised form 8 March 2021; Accepted 23 March 2021
0014-4835/© 2021
1. Introduction

Advances in retinal gene therapy delivery methods, such as adeno-associated virus (AAV), have allowed retinal gene replacement to become a reality for patients suffering from certain inherited retinal degenerations (IRDs) (Russell et al., 2017). With this success, attention has turned to expanding the use of these proven vectors with alternative strategies for visual restoration such as optogenetics - the expression of exogenous light sensitive proteins within an excitable cell - which may be applied to a wide range of IRDs, regardless of the causative mutation.

AAV delivered optogenetic tools have been shown to restore electrophysiological and behavioural responses to light in animal models of IRDs (Cehajic-Kapetanovic et al., 2015; De Silva et al., 2017; Doroudchi et al., 2011) by rendering surviving cells in the degenerate retina sensitive to light. This general principle of survivor cell stimulation has also been demonstrated clinically with electronic retinal prostheses already in clinical use for vision restoration (Edwards et al., 2018; Luo and da Cruz, 2016).

While effective, the stimulation of retinal ganglion cells - especially by epiretinal prostheses - bypasses much of the early image processing carried out in the retina. This makes specific stimulation of cells higher in the retinal hierarchy, such as the bipolar cell, conceptually attractive. However, significant retinal remodelling does occur after the death of the photoreceptor (Gilhooly and Acheson, 2017; Jones and Marc, 2005), potentially compromising the suitability of bipolar cells as targets for such stimulation. Indeed, changes in morphology, synaptic connections, electrophysiological responses and receptor expression (Dunn, 2015; Gayet-Primo and Puthussery, 2015; Kalioniatis et al., 2016; Marc and Jones, 2003; Marc et al., 2003, 2007; Varela et al., 2003) have been observed in human and animal studies.

To date, it is not understood how changes specifically within the bipolar cells during degeneration will affect their long-term viability as optogenetic targets. Particularly, if they are free from metabolic stress, receptive to adeno-associated viral vectors, suitable for opsin based optogenetic tools and able to propagate their signal by releasing neuro-
transmitter in response to exogenous optogenetic stimulation. While studies of general gene expression changes in animal models of the degenerate retina exist in the literature (Dorrell et al., 2004; Hackam et al., 2004; Hornan et al., 2007; Michalak et al., 2013; Punzo and Cepko, 2007; Yu et al., 2018), none has considered the retinal bipolar cell in isolation.

Investigation of bipolar cells is particularly apposite for two reasons: first, the development of delivery tools to specifically target dis-

| Protein Function | Comments | Human disease caused defect in gene | Mouse knock outs | Expression at P90 (gene array) | Expected location of protein within cell | Previous IHC of Pde6b<sup>W/W</sup> retina | 2-way ANOVA – difference by genotype (Pde6b<sup>W/W</sup> vs Pde6b<sup>+/+</sup>) | Significant Post Hoc Tests |
|------------------|----------|----------------------------------|------------------|-----------------------------|-----------------------------------------|-----------------------------------|---------------------------------------|----------------------------------|
| Shroom 2 | Regulates actin cytoskeleton and therefore cell shape, axon projection and organelle location (essential for viral transduction, second messenger systems and neurotransmitter release) (Fairbank et al., 2006). | Implicated in retinal degeneration with deafness (Fairbank et al., 2006). | Knock down - failure of retinal lamination, full knock out - no retinal phenotype. Other Shrm family members may compensate (Fairbank et al., 2006). | Up (p = 0.042) | Cell membrane (Bourney et al., 2007) confirmed in cell culture (Fig. S1) & IHC (Fig. 2) | Retinal pigment epithelium, bipolar cell bodies, inner plexiform layer (Bourney et al., 2007) | Yes F (1, 8) = 23.85; p = 0.0012 | Sidak P90 → p = 0.0136; p120 → p = 0.0026 |
| SrM2 | Sulphatase 2 | Extracellular endosulphatase. Removes sulphate residues from cell surface HS PG residues (Morimoto-Tomita et al., 2002) - important in the entry of AAV into cells (Summenford and Samalski, 1998) as well as retinal synaptic plasticity | Not reported | Down (p = 0.024) | Cell surface, extracellular (Morimoto-Tomita et al., 2002) Confirmed in cell culture (Fig. S1) & IHC (Fig. 3) | Bipolar cell bodies, outer plexiform layer photoreceptors synapses (Orlandi et al., 2018) | No F (1, 8) = 3.775; p = 0.0879 | Sidak P40 vs P90 → p = 0.0003; P90 vs P150 → p = 0.0307; P120 vs P90 → p = 0.0157; Pde6b<sup>W/W</sup> vs Pde6b<sup>+/+</sup> vs P120 → p = 0.0126; P120 vs P150 → p = 0.0199 |
| Sulf2 | Annexin a7 | Calcium dependent phospholipid binding protein implicated in synaptic neurotransmitter release and the bipolar cell light response (Cashby and Pollard, 2002; Grewal et al., 2016; Hoque et al., 2014). PKGs (important in activation and termination of bipolar cell light response) phosphorylates annexin a7 promoting membrane fusion and so neurotransmitter release (Haque et al., 2014). PKGs expression was not altered in our gene array comparison. | Not reported | Down (p = 0.049) | | | Yes F (1, 9) = 5.31; p = 0.0467 | Sidak P40 vs P90 → p = 0.0003; P90 vs P150 → p = 0.0307; P120 vs P90 → p = 0.0157; Pde6b<sup>W/W</sup> vs Pde6b<sup>+/+</sup> vs P120 → p = 0.0126; P120 vs P150 → p = 0.0199 |
| Anxa7 | Membrane fusion, neurotransmitter release | | No gross neurological phenotype, retina not examined (Grewal et al., 2016) | | | | Yes F (1, 8) = 3.775; p = 0.0879 | Sidak P40 vs P90 → p = 0.0003; P90 vs P150 → p = 0.0307; P120 vs P90 → p = 0.0157; Pde6b<sup>W/W</sup> vs Pde6b<sup>+/+</sup> vs P120 → p = 0.0126; P120 vs P150 → p = 0.0199 |
| Ctnm1 | Contactin 1 | Neuron projection development, synaptic remodelling | A cell surface glycoprotein implicated in synaptic plasticity (Davisson et al., 2011) | | | | Yes F (1, 8) = 3.775; p = 0.0879 | Sidak P40 vs P90 → p = 0.0003; P90 vs P150 → p = 0.0307; P120 vs P90 → p = 0.0157; Pde6b<sup>W/W</sup> vs Pde6b<sup>+/+</sup> vs P120 → p = 0.0126; P120 vs P150 → p = 0.0199 |

Table 1

Genes differentially expressed between Pde6b<sup>W/W</sup> and Pde6b<sup>+/+</sup> retiniae at P90, grouped by broad function based on gene ontology terms annotated to the gene’s entry on the gene ontology consortium database (two listed for each gene). Δ = change in expression compared to Pde6b<sup>W/W</sup> (i.e. ‘+’ = up regulated; ‘-’ = down regulated) please see Table S2 for corresponding FDR adjusted p-values.

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crete retinal cell populations (such as cell specific promoters and AAV capsid tropism (Cronin et al., 2014; de Leeuw et al., 2014; de Silva et al., 2015; Juttner et al., 2019; Kleine Holthaus et al., 2020; Lu et al., 2016; Scalabrinio et al., 2015)) have made cell-specific delivery a realistic possibility. Secondly, human opsins such as rhodopsin (Cehajic-Kapetanovic et al., 2015; Gau et al., 2015), cone opsin (Berry et al., 2019), melanopsin (De Silva et al., 2017; Lin et al., 2008) and variants (van Wyk et al., 2015) are being described as sensitive optogenetic tools. These are known to couple to endogenous G-protein signalling cascades (Hughes et al., 2016) allowing greater signal amplification compared to microbial opsins, such as channelrhodopsin (Lagali et al., 2008), which lack such coupling. However, this coupling could be affected by changes in levels of constituents of these cascades in bipolar cells during retinal degeneration. Therefore, investigation of retinal bipolar cells specifically in IRD models is paramount in determining if this conceptually attractive strategy of bipolar specific targeting is likely to be feasible for the clinical translation of optogenetics.

The principal objective of this study was to confirm the continued expression of the principal components of both the ON-bipolar light signalling and other second messenger cascades during IRDs. Here we show that, despite remodelling, bipolar cells undergo remarkably limited transcriptomic changes in response to the loss of synaptic inputs from photoreceptors, even in the late stages of the disease in an animal model.

The secondary aim of the study was to identify differentially expressed genes for further characterisation in both Pde6b<sup>wt/wt</sup> and Pde6b<sup>rd/rd</sup> retinas using immunohistochemistry. Together, these findings will be central to guiding investigations to effectively translate bipolar cell targeted optogenetic therapies into clinical use.

2. Methods

2.1. Mouse lines

All experiments involving animals were performed in accordance with the Animals for Scientific Procedures Act 1986, licence no. 30/3371 and approved by the University of Oxford animal welfare and ethical review body and in accordance with the declaration of Helsinki and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A transgenic mouse line ("L7.Cre.EYFP.Pde6b<sup>wt/wt</sup>", supplementary methods) was used which:

1. Expressed Cre recombinase under the control of the ON-bipolar cell specific promoter “L7” (also known in the literature as “Pcp2”)
2. Were homozygous for “floxed” EYFP at the Rosa26 locus and therefore expressed EYFP in L7 (retinal ON-bipolar) cells.
3. Were either Pde6b<sup>wt/wt</sup> or Pde6b<sup>rd/rd</sup> (wildtype or retinal degeneration phenotype)

2.2. Isolation of RNA from retinal bipolar cells and comparison with gene array

Six L7.Cre.EYFP.Pde6b<sup>wt/wt</sup> and Six L7.Cre.EYFP.Pde6b<sup>rd/rd</sup> mice underwent cervical dislocation at P90 to P91, 6 h into their light phase with immediate enucleation. All mice were littermates, three of each group were female. Retinas were dissected with special care to remove retinal pigment epithelium before cell dissociation using a papain dissociation kit (Worthington Biochemical, Lakewood, USA) according to the manufacturer’s instructions (Fig. 1).
The resulting dissociated cells were subjected to fluorescent activated cell sorting (FACS) (Fig. 1) with 97.3 ± 1.8% of cells in the YFP + isolate co-staining for PKCa on ICC. RNA was extracted from cell isolates and processed using standard methods for use on a MouseWG-6 v2 Expression BeadChip (Illumina). Expression levels were compared using Lumi (Du et al., 2008) and Limma (Ritchie et al., 2015) packages for R (Team, 2013) with quintile normalization. Results were corrected for multiple testing using FDR testing (Benjamini, 1995) (supplementary methods).

When isolating dissociated retinal cells in such a way, the large number of rod photoreceptor cells in the wildtype retina as well as their invaginated bipolar cell synapses could potentially lead to rods being carried over with labelled bipolar cells into the isolate. Increasing the length of enzymatic dissociation can minimise rod contamination, yet such approaches must be weighed against the risk of damage to the isolated cells or RNA with extended papain dissociation times. These protocols not only require longer periods for cells in a ‘non-physiological’ state before mRNA extraction, but lead to greater loss of cell processes (dendrites and axons). These are especially relevant to bipolar cells given the dramatic changes to these parts of the cell during degeneration.

Previous studies have attempted to control for rod carry over in different ways - for example, Siegert et al. (2012) normalise all gene expression levels based on expression of known rod specific genes whereas Punzo et al. (Punzo and Cepko, 2007), exclude any known rod specific genes from further analysis while Berg et al. (2019) examine changes in array expression of known photoreceptor genes to quantify levels of contamination.

We specifically interrogated our samples (prior to gene array) for two known rod specific genes (Rho & Cnga1) in a small number of samples using qPCR to confirm relative sample purity (supplementary methods, Fig. 1). Despite this, our gene array data did present differential expression of a small number of genes with either a rod ontological annotation or where previous literature reported low or absent protein expression in bipolar cells compared to photoreceptors (Table S1). To avoid difficulties in interpretation, we excluded these genes from further investigation (Berg et al., 2019; Punzo and Cepko, 2007) but did not systematically alter our data otherwise (Siegert et al., 2012). Like all of the approaches described above, this relies on an (fortunately available) a priori knowledge of gene photoreceptor specificity which should be borne in mind when extrapolating conclusions beyond this model.

### 2.3. Identifying candidate genes for further investigation

As in previous studies, we combined several practical strategies to prioritise those probes found to have significantly different expression for further investigation (Berg et al., 2019; Michalakis et al., 2013; Punzo and Cepko, 2007; Siegert et al., 2012). After removal of genes with rod annotations (Table S1) interaction & pathway prediction software (Fabregat et al., 2016; Warde-Farley et al., 2010) was used to identify shared functions or pathways linking groups of differentially expressed genes. All differentially expressed genes were also searched...
for on the RetNet (Daiger et al., 1998) database of human retinal disease genotype-phenotype relations to identify potentially clinically relevant candidates.

For each candidate, a search was performed on the NCBI® Gene database to extract gene ontology annotations and on the Medline® database allow systematic review of the literature (supplementary methods). Gene ontology terms were used to group the genes into six broad functional groups (Table 1) while review of primary literature allowed prioritisation of an initial candidate in each group for further investigation (supplementary methods) and identification of any potential photoreceptor specific genes (Table S1). Within each of first four groups, one candidate was prioritised (supplementary methods) to be further characterised by IHC staining.

### 2.4. Immunohistochemistry & semiquantitative image analysis

At each of four timepoints (P30, P90, P120 and >P150), Pde6bw/w and Pde6bw/w mice (n = 3 per genotype, unless otherwise indicated) underwent cervical dislocation before immediate enucleation and processing of retina for IHC (supplementary methods). Images were taken at a point three fields of view (at 40 magnification) from the ora serata in three sections from one eye in each animal. Colocalised staining for the proteins of interest and a bipolar cell marker were taken as a semiquantitative index of protein expression levels in bipolar cells and were determined using Costes’ method, described and validated previously (Costes et al., 2004). In brief, this involves normalising the number of pixels demonstrating colocalization above threshold in each image to the number of non-zero pixels in the image to give an index of colocalization that could be compared between images (supplementary methods). A two-way ANOVA with Sidak’s method to account for multiple comparisons between groups and Tukey’s test for comparison with groups over time.

### 3. Results

#### 3.1. Gene array comparison

Retinal ON-bipolar cells were isolated using FACS of dissociated retina from L7.Cre:EEFP transgenic mice which were additionally either wildtype or homozygous for a clinically relevant IRD mutation (Pde6bw/w or Pde6bw/w). cDNA libraries were extracted from the resulting EEFP+ (ON-bipolar) cell isolates were processed and subjected to an Illumina® mouse gene array. To quantify contamination of these isolates with rod photoreceptors, qPCR for two rod specific genes (Cnga1, Rho, chosen a priori) was performed to ensure that neither was detectable at a significant level in any bipolar cell isolate (Cnga1 – undetectable, Rho <1% in YFP + isolate compared to YFP –, Supplementary methods). Gene array analysis revealed sixty-six probes corresponding to sixty genes were shown to have differential expression between the degenerate and non-degenerate samples with a p value of <0.05 (False detection rate (FDR) testing, Fig. 1, Tables 1 and S2, supplementary methods).

#### 3.2. Candidate genes

Following this, a sequence of methods was employed to highlight the most relevant genes, beginning with pathway analysis and database searches to highlight groups of genes with common function. Pro-
tein-protein interaction prediction software (Warde-Farley et al., 2010) highlighted one common function (“transition metal ion binding”) linking five of the differentially expressed genes (Atox1, Clip1, Msrb2, Mt1 & Mt2) involved in preventing and repairing oxidative damage. This was reinforced by use of the Reactome® knowledge base (Fabra et al., 2016), demonstrating two similar overrepresented pathways (“Metallothionein metal binding” p = 0.009 & “Response to metal ions” p = 0.013) involving similar genes (Msrb2, Mt1 & Mt2). Searches of the RetNet database (Daiger et al., 1998) revealed only one differentially expressed gene (Srn2) to be implicated in human retinal disease.

Genes known to be related to the native bipolar cell light signalling pathway (Grm6 +1.054, p = 0.8573; Gnao1 -1.23, p = 0.9835; Gnb5 -1.13, p = 0.9670; Gna13 -1.01, p = 0.7109; Trpm1 +1.19, p = 0.8035 (log2 fold change, adjusted p value, FDR, Table S3, Fig. S3) were specifically queried, and while highly expressed in absolute terms, none were significantly differentially expressed between groups. Similarly queried were genes more generally implicated in cell signalling (Table S3) and remodelling (e.g. glutamate, glycine and GABA receptors, Table S4) with no significantly differentially expressed genes identified.

As these methods revealed only one unifying functional pathway - “metal ion binding” (supplementary results) – this data driven approach was complemented by a manual, systematic, evaluation (including literature and gene ontology consortium annotation review) for each differentially expressed gene. This was used to group the potential candidates by function (Harris et al., 2004) and score them for relevance to our research question (supplementary methods). The highest scoring potential candidate in each group was selected for further investigation at the protein level using IHC: Srn2, coding for a gene involved in cell shape regulation, Sfl2 heparin proteoglycan metabolism, Anxa7, neurotransmitter release and Cntn1 in synaptic remodelling (see Table 1).

3.3. Immunohistochemical staining

Antibody labelling for proteins encoded by each of the selected genes (Srn2, Sfl2, Anxa7, Cntn1) showed co-localisation with classical ON bipolar markers (CHX10 or PKCa) at postnatal day 90 (P90) in our IHC study – although these proteins were typically not expressed exclusively within ON BCs (Figs. 2-5). A semi-quantitative index of staining was recorded for each genotype (Pde6bwt/wt & Pde6bwt/dh) at each timepoint (P40, P90, P120, P150) to give an impression of how protein levels may change over time.

There was a difference between genotypes in this index for all proteins excepting Slf2 (Sulphatase 2) [Srn2 F (1, 8) = 23.85; p = 0.0012, Sfl2 F (1, 8) = 3.775; p = 0.0879, Anxa7 F (1, 9) = 5.31; p = 0.0467; Cntn1 F (1, 13) = 8.272; p = 0.0130]. In addition, differences in staining over time were seen in Srn2 (Shroom 2) & Sfl2 [Srn2 F (3, 8) = 4.865; p = 0.0327, Sfl2 F (3, 8) = 19.9; p = 0.005, Anxa7 F (3, 9) = 0.1829; p = 0.9053, Cntn1 F (3, 13) = 0.168; p = 0.9161]. Whilst an interaction between genotype and time was seen in Srn2 and Cntn1 (Contactin 1) [Srn2 F (3, 8) = 0.09; p = 0.0059, Sfl2 F (3, 8) = 2.718; p = 0.1148, Anxa7 F (3, 9) = 0.6712; p = 0.5909, Cntn1 F (3, 13) = 7.366; p = 0.0039]. (Please see Figs. 2-5 and supplementary material for post hoc analysis.)
Table 2
Details of four differentially expressed genes prioritised for further characterisation with IHC. The bottom four rows refer to semi-quantitative IHC co-localisation seen in Figs. 2–5, p = “adjusted p value”; n.s. = P > 0.05

| Symbol | Δ log2 | Ontology term 1 | Ontology term 2 |
|--------|--------|----------------|----------------|
| A - Actin cytoskeleton, microtubules and intracellular transport<br>Ap3m2 | −1.85 | anterograde synaptic vesicle transport | intracellular protein transport |
| Cng | 3.52 | microtubule cytoskeleton organization | microtubule binding |
| Sept4 | 1.54 | cilium assembly | mitotic cytokinesis/melanosome organization |
| Srn2 | 1.81 | actin cytoskeleton organization | actin cytoskeleton organization regulation of cell migration |
| Tmbb10 | −2.15 | | |
| B - Heparin sulphate proteoglycan metabolism<br>Exil3 | −2.05 | heparan sulphate proteoglycan biosynthesis | protein glycosylation |
| Sulf2 | 1.43 | heparan sulphate proteoglycan metabolic process | arylsulfatase activity |
| C - Cell signalling, calcium homeostasis<br>Anxa7 | −1.56 | calcium ion binding | membrane fusion |
| Gaby | 2.25 | sperm capacitation | calcium-mediated signalling |
| Gabrg2 | −1.56 | gamma-aminobutyric acid (GABA)ergic signalling | synaptic transmission, GABAergic response to calcium ion |
| Pde1c | −1.96 | signal transduction | calcium ion arginine transport |
| Slc7a3 | −2.87 | amino acid transport | SNARE binding |
| Unc13a | −2.11 | neurotransmitter secretion | |
| D - Neural cell growth, survival & remodelling<br>Ctn1 | −1.79 | neuron projection development | nervous system development |
| Enh1 | 2.15 | axon guidance | pre-synapse assembly |
| Hdac9 | −3.69 | DNA repair | chromatin organization |
| Lynx1 | −8.26 | synaptic transmission, cholinergic regulation of cell proliferation | acetylcholine receptor binding |
| Mif | −1.91 | | positive regulation of axon regeneration |
| Nrm | −1.40 | nuclear membrane cell adhesion | membrane recognition |
| Pcdha7 | −1.98 | | histone ubiquitination |
| Phc1 | 1.93 | cellular response to retinoic acid | |
| Ptprr | −3.72 | negative regulation of ERK1 and ERK2 cascade | ERK82 signalling pathway |
| Sfrs1 | 1.72 | mRNA 5′-splice site recognition | regulation of transcription, DNA-templated |
| Soc5 | −1.64 | regulation of growth | JAK-STAT cascade |
| Ya2 | −2.54 | regulation of transcription, DNA-templated | DNA-templated transcription, DNA-templated |
| E - Aerobic and anaerobic respiration, cellular response to stress<br>Atox1 | −1.62 | transition metal ion binding | stress response to oxidative stress |

4. Discussion

4.1. Bipolar cells

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

Targeting bipolar cells assumes that, within a degenerate retina, these cells retain levels of the second messengers required for light signalling. It also assumes such cells survive in a stable state without metabolic stress to allow effective propagation of this signal by synaptic neurotransmitter release and, importantly, are able to be transduced by AAV. Increasing evidence confirms that bipolar cells do undergo significant remodelling in the later stages of retinal degeneration with changes in morphology, synaptic connections, electrophysiological responses and receptor expression (Cuena et al., 2014; Dunn, 2015; Gayet-Primo and Puthussery, 2015; Kalloniatis et al., 2016; Marc and Jones, 2003; Marc et al., 2007; Michalakis et al., 2013; Strettoi et al., 2002).

4.2. Gene expression in context

Here we show that, despite this remodelling, bipolar cells undergo remarkably limited transcriptomic changes in response to the loss of synaptic inputs from photoreceptors, even in the late stages of the disease. While gene array studies of whole retina may not have statistical power to detect very small changes in expression of single genes, our approach of limiting our comparison as far as possible to a single cell type in a clinically relevant disease model, will accentuate those changes that are biologically most relevant.

Given the marked changes seen at the anatomical level over the whole retina during the neural remodelling of degeneration, it is perhaps surprising that we found such a small number of genes were differentially expressed in bipolar cells in this context (66 out of a total of c.20,000 probes). The absence of differential expression of genes re-
lated to the native bipolar cell light signalling cascade, second messag-
ing in general or glutamatergic transmission (Tables S3 and S4) is par-
ticularly interesting given reports of functional loss of sensitivity to glu-
tamate even early in degeneration (Varela et al., 2003). We see no sig-
nificant alteration in expression of genes associated with glutamate re-
ceptor subunits (nor with GABAergic, nor glycnergic receptors, Table S4) which is intriguing given the shift from metabotropic to ionotropic transmission seen at a functional and anatomical level in bipolar cells during degeneration (Dunn, 2015; Marc et al., 2007; Varela et al., 2003).

This finding of such stability at a gene expression level is particu-
larly informative when seen in the light of studies where opsin based
optogenetic tools (Cehajic-Kapatianovic et al., 2015; De Silva et al., 2017; Lin et al., 2008) are targeted to ON-bipolar cells to functionally
restore light responses in degenerate retina. Therefore, despite marked functional and anatomical remodelling, the parts of the bipolar cell sig-
nalling cascade necessary for optogenetic restoration appear to persist
both at a gene expression and functional level during retinal degenera-
tion in the Pde6b<sup>−/−</sup> model. Given that the rd1 mutation causes an IRD
in humans similar in phenotype to that of the model, these findings are
particularly interesting from a translational point of view (if they are
reflected in human bipolar cells). In counterpoint however, the huge va-
riety of causative mutations in human IRDs should still be borne in
mind when extrapolating results.

4.3. Identifying candidate genes

With a relatively small number of differentially expressed genes overall and no obvious candidate genes presented for further investiga-
tion by data driven approaches (supplementary results), a systematic
literature review for each gene could be used to group and prioritise
those most promising for further characterisation at the protein level
(Srm2, SIf2, Anxa7, Cntn1). Immunohistochemical staining at the
timepoint corresponding to the gene array (P90) confirmed expression of
all four proteins in bipolar cells of both Pde6b<sup>−/−</sup> and Pde6b<sup>+/−</sup> retinas. Staining of similar retinas at other timepoints over the course
of degeneration (Figs. 2–5, Table 2) could be additionally analysed in
a semi-quantitative manner to given an impression of likely relative pro-
tein expression over time in order to guide potential future investiga-
tions.

4.4. Further characterisation of selected candidate genes

The pattern of Shroom 2 staining (Fig. 2) that we see in degenera-
tion, with a maxima in mid degeneration (where neuronal modelling is at
its highest), is consistent with its described role in cytoskeleton remodel-
ling, cell shape regulation and membrane blebbing, given the retraction
of bipolar cells axons and change in shape seen in histological studies of
degeneration (Jones and Marc, 2005; Strettoi et al., 2002). A corre-
sponting upturn in staining at P150 in wildtype animals was seen in
multiple replicates and could perhaps be explained by an increase in
neural remodelling in older mice – which would certainly be an interest-
ing target for further investigation. In the broadest terms, this may sug-
gest intervention earlier in the course of degeneration may be beneficial
whilst cytoskeleton and membrane activity (such as AAV entry, pay-
load trafficking and neurotransmitter release) are possibly less dis-
rupted.

The Heparin Sulphate Proteoglycans (HSPG), from which Sul-
phatase 2 removes sulphate residues, are involved in (but not essential
to) the binding of AAV in advance of its entry to the cell (Summerford
and Samulski, 1998). Importantly, AAVs are less able to bind HSPGs
that are less sulphonated (for example due to increased sulphatase ac-
tivity) and indeed HSPGs have been found to be functionally important
in retinal cell transduction efficiency, especially by the intraretinal
route (Boye et al., 2016; Woodard et al., 2016). Interestingly, our semi-
quantitative IHC (Fig. 3), unlike gene array data, suggests no differ-
ence in Sulphatase 2 staining compared to wildtype during degenera-
tion. However, this could represent an increase in protein turnover (and
hence RNA levels), a shift to the secreted, extracellular form of the pro-
tein during degeneration (Morimoto-Tomita et al., 2002) from the cell
surface bound sulphatase 2 or indeed post-transcriptional changes at
the mRNA level and so gene expression levels do not directly reflect
the level of cell staining. Therefore, it may be fruitful to investigate quan-
titatively HSPG sulphonation in various cell types of the degenerate
retina, compared to wildtype. Or indeed, if changes in Sulf2 levels can
manipulate AAV transduction efficiency (for example by investigating
AAV transduction in a Sulf2 knock out retina (Table 2).

Effective retinal optogenetic therapy requires neurotransmitter re-
lease from targeted cells; Annexin a7 is central to this process and inter-
acts with PKCα, an enzyme known to regulate bipolar cells’ light re-
sponse kinetics (Table 2) (Hoque et al., 2014) (supplementary discus-
sion). Annexin 7 IHC staining in our series is just significantly different
from wildtype during degeneration (but with no individual timepoint
identified as significant on post hoc analysis), this could suggest that
this aspect of the bipolar signalling cascade is indeed still functional,
but with a reduced rate of protein turn over when light signalling is lost
during retinal degeneration. A more comprehensive understanding of
the role of Annex 7’s in the wildtype bipolar cell light response will need to
be determined if any downregulation is likely to directly impair bipo-
lar cells ability to act as optogenetic targets, or indeed represent an op-
portunity to manipulate response kinetics.

To act as optogenetic targets, bipolar cells must not only be able to
release neurotransmitter, but to maintain useful synapses to communi-
cate the transduced light signal. Contactin 1 has a role in regulating
synaptic plasticity in the nervous system, so our finding that it was
downregulated at P90 in retinal degeneration – a process defined by
neural remodelling (Jones and Marc, 2005) - was perhaps surprising.
The transient drop in IHC staining (at P90 only) in our series (Fig. 5 and
Table 2) is however congruent with the findings of Haenisch et al. who
also noted an initial marked decrease of neural Cntn1 mRNA expression
followed by an increase back to baseline when investigating nerve
crush (deafferentation) in zebra fish (Haenisch et al., 2005). Which, in
isolation, could perhaps predict a benefit to early optogenetic intervention
in the retina, restoring afferent signal input in an attempt to prevent a
drop in Contactin 1 and any resulting maladaptive synaptic remodel-
ling.

4.5. Limitations

While representing the first description of transcriptomic changes in
bipolar cells in the context of degenerative retina remodelling, there are
limitations to our approach that must be borne in mind when extrapo-
lating results.

Firstly, as alluded to above, we predicted that rod photoreceptors
may be a contaminant of our cell isolates and therefore assessed sample
purity in two ways – by interrogating our microarray samples for ex-
pression of genes known to be specific for rods and by performing RT-
qPCR for a small number of these markers. However we did not quan-
tify markers specific to other retinal cell types to absolutely exclude
contamination from other populations.

Secondly, microarrays incorporate multiple technical controls and
have been shown to faithfully replicate the mRNA quantification re-
sults of other methods (such as qPCR) (Arikawa et al., 2008; Canales
et al., 2006; Morey et al., 2006) and in meta-analysis studies show con-
cordance across array platforms (Brown et al., 2017). Therefore, we
did not employ alternative methods to externally validate our microarray
findings at the mRNA level, but rather, investigated a subset of can-
didate genes by IHC. This approach provided a wealth of additional in-
formation on both the spatial distribution within the retina as well as
whether mRNA changes actually affected protein levels. However,
mRNA modifications, transport and post translational modifications could potentially occur. Indeed, further investigation of such processes will form an important future direction of investigation – especially as they may explain the lack of change in staining for Sulf2 & Anxa7 at P90.

Thirdly, as this is the first study to investigate transcriptional changes in on-bipolar cells during retinal degeneration, a direct positive-control - a gene already known to be up or down regulated in this context – was lacking. Such positive controls provide a valuable technical validation of transcriptomic datasets but were not possible in this context.

5. Conclusion

Here we present the first comparison of gene expression in bipolar cells of degenerate and non-degenerate retinas. Our findings suggest relatively few changes in gene expression with degeneration, including genes essential to effective optogenetic bipolar light signalling. This suggests, that despite remodelling, bipolar cells are likely to remain viable and effective targets for optogenetic vision restoration and we highlight candidate genes where further investigation is likely to improve the translation of this important technique.

Declaration of competing interest

No relevant conflicts of interest.

Acknowledgements

George Nicholson for assistance in producing Fig. S1. Cambridge Genomic Services for performing the Illumina gene microarray.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2021.108553.

Funding

This work was supported by The Wellcome Trust (grant number 205151/Z/16/Z); The Wolf Fischer Trust; The German Research Foundation (DFG) (grant number LI2846/1-1) and The Biological Basic Sciences Research Council (grant number BB/M009998/1).

Financial support

Heidelberg Engineering, Optos, Genentech, Alimera Sciences.

Equity owner

Carl Zeiss Meditech, Fresenius Medical Care.
M.J.G., D.H., S.H., S.N.P., R.E.M. and M.W.H.

Statement

Aspects of this work were presented at the 2018 Association for Research in Vision and Ophthalmology annual meeting at Honolulu, Hawaii, USA.

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