PHOTORECEPTOR TRANSPLANTATION IN THE DEGENERATING RETINA

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I, Amanda Claire Barber, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
ABSTRACT

Retinal degenerative disorders are the leading cause of blindness in the developed world, resulting in loss of the photoreceptor cells and vision. Few treatments are available and none can reverse the loss of sight. Photoreceptor transplantation offers the potential to restore vision by replacing cells lost in disease. Previous work has demonstrated that, following transplantation, rod-precursor cells can migrate into the retina, differentiate into mature phenotypes and confer increased sensitivity to light in the rod range. However, rigorous assessment of whether transplanted photoreceptors can actually restore vision is required.

In order to do this, we first optimized rod photoreceptor transplantation. To test the functionality of the transplanted rod photoreceptors, we selected a model in which improvements could be assessed unambiguously. The Gnat1<sup>-/-</sup> mouse, a model of stationary night-blindness, lacks rod α-transducin and thus has no rod function. We demonstrate that transplanted cells robustly integrate forming synaptic connections with the recipient. Integrated cells form inner/outer segments that appropriately express phototransduction proteins. Single cell recordings demonstrate that integrated cells are light responsive and intrinsic imaging of the visual cortex shows that visual signals generated by transplanted rods project to higher visual areas. These cells are also capable of restoring optokinetic head-tracking and visually-guided behavior in response to scotopic visual stimuli.

A major question remains as to how amenable the heterogeneous diseases encompassed within retinal degenerative disorders will be to photoreceptor replacement and if treatment of late-stage disease is feasible. We performed a comprehensive assessment of photoreceptor transplantation in 6 murine models of inherited retinal degeneration encompassing different types and stages of degeneration. Transplantation is feasible in all models examined but disease type has a major impact on outcome, as assessed both by the morphology and number of
integrated rod-photoreceptors. Integration can increase, decrease or remain constant with disease progression, depending upon the gene defect, with no correlation with disease severity. Robust integration into late-stage disease is possible in some disease types. We assessed features of the recipient microenvironment known to change during degeneration, namely gliosis, outer nuclear layer cyto-architecture and outer limiting membrane (OLM) integrity. Disruption of glial scarring and OLM integrity significantly increased integration to levels sufficient to restore optokinetic head-tracking responses in a model with an otherwise poor transplantation outcome.

Together, these findings demonstrate the feasibility of photoreceptor transplantation as a strategy for the restoration of vision in retinal disease.
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Oral presentation at the 24th Head Group Meeting (2012) Institute of Child Health, UCL. Retinal Repair in the Degenerating Retina: transplanted photoreceptor efficiency and visual function.

Oral presentation at 5th Pro- RETINA Research-Colloquium (2009) Potsdam, German. Improved protocols for photoreceptor cell transplantation.


CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

Retinal degenerations, including retinitis pigmentosa (RP) and age-related macular degeneration (AMD), are the leading cause of blindness in the developed world. The resulting photoreceptor cell loss is permanent as the mammalian retina lacks the ability to regenerate. Limited treatment options exist for patients with these retinal degenerative disorders. Gene therapy can correct the defective gene; however this relies on the presence of the endogenous photoreceptor cells, offering little hope for patients with extensive photoreceptor cell death in late disease. In addition, for the many retinal degenerative disorders have multi-genetic and multi-factorial underlying causes, and in these the usefulness of gene therapy may be limited. Photoreceptor replacement could be offered as a standalone or complementary treatment with the aim of replacing the cells lost in the degenerative process and restoring visual function.

Regenerative medicine holds significant potential for the treatment of CNS injury and disease. As part of the CNS, the retina is a prime candidate for developing cell therapies to treat CNS degenerative disorders: it is easily accessible for both surgical intervention and in vivo testing; it has an immune privileged status and has a well characterized structure with a relatively small number of neuronal cell types. More specifically, photoreceptor replacement therapy could be considered as a feasible solution to treat retinal degenerative disorders as transplanted cells need only make single short synaptic connections to restore the visual pathway and in the initial stages of retinal degeneration, the inner retinal circuitry remains largely intact.

Proof-of-concept experiments have demonstrated the feasibility of photoreceptor replacement therapy (Bartsch et al., 2008; Kwan et al., 1999; Lakowski et al., 2010;
Maclaren et al., 2006). However, in order for this strategy to be considered as a clinical treatment, there are a number of issues that need to be addressed, some of which will be investigated in this thesis. In both Chapter 3 and 4, methods to improve transplantation efficiency, by optimizing transplantation protocol and manipulating the recipient microenvironment, are examined with the aim of achieving robust integration within the degenerating recipient retina. In Chapter 3, rigorous investigation of the functional capabilities of the transplanted cells is undertaken with the aim of determining whether transplanted cells can integrate into the recipient visual system, respond to light and restore vision in the degenerate retina. In Chapter 4, the breadth of application of photoreceptor replacement therapy is examined to assess how permissive different disease types are to photoreceptor transplantation and whether degeneration state impacts upon transplant outcome.
1.2. The Eye

1.2.1. Basic Structure and Function

The eye has evolved as a highly specialized organ whose principle function is to capture an image and convey it as a neural message to the brain, resulting in visual perception of the external environment. Vision begins when the front optical components of the eye, the cornea and lens, focus the incoming light onto the retina at the back of the eye (Figure 1.1a). The cells responsible for the conversion of light into a neural signal are the photoreceptors and can be found in the outer most layer of the retina (Figure 1.1b). Light must travel through the entire thickness of the retina to reach these outer layers where the photoreceptors are situated (Figure 1.1b). Here, photons are absorbed by photoreceptor visual pigments, triggering a biochemical reaction, known as the phototransduction cascade, which results in an electrical signal being transmitted to ganglion cells in the inner retina, via second order bipolar cells (Figure 1.1b). The ganglion cell axons pass across the inner surface of the retina and exit the eye at the optic disk where they form the optic nerve (Figure 1.1b). The optic nerve becomes the optic tract after crossover at the optic chiasm (Figure 1.1c) and propagates the neural signal to a region of the brain called the lateral geniculate nucleus (LGN). Here the axon synapses with a single neuron which projects to the striate portion of the visual cortex whereby visual information is processed (Figure 1.1c).
FIGURE 1.1. THE HUMAN EYE: BASIC STRUCTURE AND FUNCTION

1.2.2. GROSS ANATOMY AND FUNCTION OF THE EYE

The eyes of all vertebrates share a common structural plan and can be described in three distinct layers: the external, intermediate and inner layer (Figure 1.1a).

The external outer layer is formed by the sclera and cornea, both of which broadly function as a protective outer surface to reduce the risk of trauma and to act as a barrier to environmental pathogens. The opaque sclera is a tough yet flexible tissue, which allows it to resist varying intraocular pressure and maintain the shape of the globe. The cornea forms the transparent anterior part of the eye and together with the lens is responsible for refracting the light entering the eye through the pupil. The cornea is avascular, to maintain transparency, and has a specialised laminar structure consisting of the corneal epithelium, the stroma and the corneal endothelium (Figure 1.1d). Adult limbal epithelial stem cells are found at the limbus of the cornea and allow continuous regeneration of the corneal epithelium. This ensures that the air-tear film interface and thus oxygen supply is efficiently maintained. The corneal stroma functions to maintain transparency of the cornea and consists of highly organized layers of collagen fibers. Finally, the non-regenerative corneal endothelium functions to maintain hydration of the stroma (Figure 1.1d).

The intermediate layer of the eye, called the uveal tract, can be divided into the anterior components, the iris and ciliary body, and the posterior component, the choroid (Figure 1.1a). The choroid consists of pigmented connective tissue, which absorbs light that passes through the retina and reduces light reflection within the eye to improving contrast sensitivity. Often referred to as the vascular layer, the choroid supports a dense meshwork of blood vessels that provide oxygen and nutrients to the outer retina. The iris contains papillary muscles that control pupil diameter and therefore the amount of light entering the eye. The outer anterior edge of the iris, an area known as the ciliary margin, is where the iris attaches to the sclera and the ciliary body (Figure 1.1e). The ciliary body contains the ciliary muscles and it terminates at the ora serata where
the neural retina begins (Figure 1.1e). The ciliary body has three functions: aqueous humour production, accommodation and the production of lens zonules. It is lined by a double layer ciliary epithelium consisting of a non-pigmented and a pigmented layer. It can be divided into two regions, the anterior pars plicata and the posterior pars plana (Figure 1.e). Ciliary processes extend from the par plicata and secrete aqueous humour into the anterior chamber. Components of the vitreous are produced in the pars plana and secreted into the vitreous cavity. The pars plana is a flat region whereby the stroma is continuous with the choroid and where the outer, pigmented ciliary epithelium layer is continuous with the retinal pigmented epithelium (RPE) and the inner transparent epithelial layer is continuous with the neural retina. Zonular fibers, arising from the ciliary body, attach to the lens anchoring it in position and allow the ciliary muscles to fine tune lens light refraction, a process known as accommodation.

Finally, the internal layer, which lines the inner surface of the eye, encompasses the photosensitive neural retina and the RPE (Figure 1.1a). The RPE lies adjacent to the neurosensory retina and is firmly attached to the underlying choroid. It is a layer of single pigmented epithelial cells, homogeneous in size and hexagonal in shape, and connected by a series of tight junctions. The RPE has a series of functions vital for vision such as providing trophic support to photoreceptor, maintaining retinal homeostasis and shielding the retina from excess light.

In the human retina, the optic disk is located towards the centre of the retina and serves as an exit point for the optic nerve and an entry point for several major incoming blood vessels that supply retina. No light sensitive tissue is found in the optic disk, causing a break in the visual field, more commonly known as the blind spot. The macular is specialized for high acuity vision and is a located adjacent to the optic disk in the centre of the human retina. The fovea, located near the centre of the macular, has a high spatial density of cone photoreceptors, which accounts for the high visual acuity and sharp central vision. Although the overall percentage of cones is similar in mouse and human (3%), the mouse retina lacks the macular region and the cones are
evenly distributed across the visual field. The fovea is surrounded by the parafovea and in turn by the perifovea; in these regions rod photoreceptors gradually begin to appear and visual acuity is reduced as reflected by a lower density of cone photoreceptors. The peripheral retina, beyond the macular, is rod photoreceptor dominant and delivers highly compressed, low-resolution information.
1.3. **THE RETINA**

1.3.1. **STRUCTURE AND INTRODUCTION TO CELL TYPES**

The retina consists of two main divisions: the supportive RPE, formed by a single layer of columnar pigmented epithelia cells and the sensory retina, including rod and cone photoreceptor both responsible for phototransduction and the neurons which carry out the first steps in visual processing and propagate the signal to the brain. The retina is a highly laminar structure (Figure 1.2a and 1.2b). In all vertebrate retinas, the same principle lamina structure is conserved: the neural cell bodies are grouped into three distinct nuclear layers separated by two plexiform layers rich in synaptic connections.

**A) NEURAL LAYERS**

The neuronal layers of the retina house the three principle neuronal cells that make up the direct visual pathway: the photoreceptor, bipolar and ganglion cells (Figure 2b). The outer nuclear layer (ONL), containing the cell bodies of the rod and cone photoreceptors, is found adjacent to the RPE. Bipolar cell bodies reside within the inner nuclear layer (INL) and propagate the signal from photoreceptors in the outer retina to ganglion cells in the inner retina. The INL also contains horizontal and amacrine cell bodies, which provide lateral modulation. Müller cell bodies are also located in the INL; these glial cells span the entire breadth of the retina and provide both structural and metabolic support. Finally, the ganglion cell layer (GCL) contains both ganglion and displaced amacrine cell bodies. Ganglion cell axons pass within the nerve fiber layer to the optic disk where they form the fibers of the optic nerve.
FIGURE 1.2. THE STRUCTURE OF THE RETINA

(a) A schematic diagram showing a cross-section of the posterior eye. Adapted from http://dev.ellex.com/2rt (b). Schematic (left) and histological (right) cross-section of the retina illustrating the laminar structure of the retina and the position of each cell type within the distinct layers of the retinal. Note that the retina throughout this thesis is presented with the apical retina orientated at the bottom, in keeping with the clinical approach of orientation.

Abbreviations: RPE, retinal pigmented epithelium; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cells layer; NFL, nerve fiber layer).
B) PLEXIFORM LAYERS

The outer plexiform layer (OPL), located between the ONL and INL, is where photoreceptor cells synapse with bipolar cell dendrites (Figure 1.2b) Here too, laterally extending horizontal cell processes can be found forming synapses with photoreceptors and bipolar cells (Figure 1.2b). The inner plexiform layer (IPL) functions as a relay station for the bipolar cells to synapse with the ganglions cells. In addition, synaptic connection with amacrine cells input into the neuronal network here.

C) RPE

The apical membranes of the RPE cells contain processes that extend into the interphotoreceptor matrix (IPM), a region between the RPE and ONL that contains the photoreceptor segments (Figure 1.2b). The basolateral membrane of RPE cells contacts Bruch’s membrane, which maintains the retinal-blood barrier and the immune privileged status of the retina (Figure 1.2a). The pigmented melanin granules within the RPE cells function to absorb light not captured by the adjacent photoreceptors, which reduces backscatter and so improves image resolution. The RPE plays major role in the visual cycle and is the principal site for 11-cis-retinal regeneration. In addition, the RPE provides trophic and metabolic support essential for the normal function and survival of photoreceptor cells. The RPE is also involved in the renewal process of photoreceptor outer segments, which are constantly renewed due to photo-oxidative damage. The tips of the outer segments are shed into the IPM and phagacytosed by the RPE, preventing debris build up. The function of the photoreceptors heavily relies on interaction with RPE cells; death of either cell type can result in secondary degeneration of the other.
D) OTHER IMPORTANT STRUCTURES AND CELL TYPES IN THE RETINA

**Outer limiting membrane**

The outer limiting membrane (OLM) is formed between the end-feet of the Müllner glia and the plasma membrane of the photoreceptor inner segment at the outer edge of the retina. It comprises of a series of adherens junctions, or zonula adherens, which are formed from cadherin-catenin transmembrane protein complexes that maintain cell-to-cell adhesion. These junctions are linked to the actin cytoskeleton within the cell cytoplasm, via essential adapter proteins including ZO-1 and CRB1. The OLM functions as a barrier between the photoreceptor segment region and the neural retina, preventing the diffusion of phototransduction cascade molecules. Müllner cells also form the inner limiting membrane, which functions as a diffusion barrier between the neural retina and vitreous humour at the inner surface of the retina.

**Interphotoreceptor matrix**

The adult IPM surrounds the rods and cones and is rich in chondroitin-6-sulphate proteoglycans (Hollyfield et al., 1999). Briefly, proteoglycans are a group of proteins that covalently bind large carbohydrates called glycosaminoglycans and on the basis of this can be classified into 4 main groups depending on the content of the bound glycosaminoglycan side chain. These are chondroitin sulphate (CS) proteoglycans, dermatan proteoglycans, keratan proteoglycans and heparin sulphate proteoglycans (Inatani and Tanihara, 2002; Tanihara et al., 2002). Traditionally, proteoglycans in the IPM were thought to function simply as a structural link between the RPE and neural retina (Kuehn and Hageman, 1999). However, their role is more multifunctional and they are involved in many regulatory functions in processes including cell adhesion, cell migration and differentiation through their ability to bind many other ECM proteins and growth factors (Ruoslahti, 1989).
**Retinal Glial Cells**

Müller cells are the principle glial cells of the retina and provide structural support, spanning from the inner limiting membrane (ILM) at the vitreous surface to the outer limiting membrane (OLM) of the ONL (Figure 1.3a) with their cell bodies located in the INL (Figure 1.3a). Müller glia have a variety of roles in maintaining a healthy retinal environment. They provide metabolic support to neurons and are rich with glycogen granules, mitochondria and oxidative enzymes and they absorb extracellular potassium ions to maintain neuronal homeostasis and protect neurons. In addition, they have high concentrations of glutamine synthase to recycle glutamate and protect neurons from excess exposure to glutamine. Müller cells also act as optic fibers, guiding light along their processes towards the photoreceptors (Figure 1.3b) (Franze et al., 2007).

Other glial cells of the retina include retinal astrocytes and microglia. Astrocyte cell bodies and their processes are restricted to the inner retina where they act as axonal and vascular glial sheaths. Microglia are found throughout the retina and exist in two forms: resident microglia and infiltrating microglia. Both are thought to be involved in phagocytosis of retinal neurons following trauma.
FIGURE 1.3. MÜLLER GLIAL CELLS

(a) Schematic diagram of a Müller cell (orange) illustrating its location within the retina, stained with H&E. (b) Illustration demonstrating the role of the Müller cell as a natural optical fibers. Light is guided through the Müller cell processes towards the photoreceptor (Franze et al., 2007). (c) Schematic diagram demonstrating the structure of the adherens junctions that form the outer limiting membrane between the Müller glia (orange) and photoreceptors (green). Enlargement illustrates the interaction between ZO-1 (red) and the actin cytoskeleton (black lines), a defining feature of these adherens junctions (West et al., 2008).
1.3.2. THE PHOTORECEPTOR: STRUCTURE AND FUNCTION

The photoreceptor is the principal light-sensitive cell in the retina. The mammalian retina contains two types of photoreceptors: rods and cones (Figure 1.4a). Primates and humans posses one type of rod photoreceptor and three types of cone photoreceptor, called L, M and S cones, each with a different spectral sensitivity (Figure 1.4b). The murine retina contains a single rod and two cone types, S and M/L. All photoreceptors share a common general morphology consisting of four subcellular compartments: the outer segment, the inner segment, the cell body residing in the ONL and a synaptic terminal located in the OPL (Figure 1.4a). The outer segment is the light sensitive part of the cell and, together with the inner segment, can be found distal to the outer limiting membrane and projecting into the IMP located between the RPE and ONL with the tip of the outer segment buried in the apical processes of the RPE (Figure 1.2b).

A) OUTER SEGMENT

The outer segment contains all of the components needed to absorb photons and convert photons into an electrical signal, a process known as phototransduction. The outer segment can be considered as a modified cilia and is connected to the inner segment by the ciliary stalk, which contains connecting tubules that extend part way into the outer segment providing structural scaffolding and allowing for the transport of molecules between the inner and outer segment (Figure 1.4a).

Electron microscopy reveals layered membrane structures, called disks, horizontally stacked throughout the length of the outer segment (Figure 1.4a). These disks are derived from in-foldings of the plasma membrane, however in rod photoreceptors, although also derived from the plasma membrane, the disks are not continuous with
the membrane surface and exist as independent structures. In contrast, cone outer segments disks are composed of, and continuous with, surface membrane. In addition, cone outer segments are much shorter in length than rod outer segments and in some areas outside of the foveal region, appear tapered in appearance. The visual pigment is incorporated within the disk membranes existing as a transmembrane protein. Visual pigment contains a protein portion, variations in which determine its spectral sensitivity, and a light-capturing portion called the chromophore. Usually only one type of visual pigment is expressed in each type of photoreceptor: rhodopsin, S (blue) cone opsin, L (red) cone opsin or M (green) cone opsin.

B) INNER SEGMENT

The inner segment contains all of the components needed for the high metabolic demands of the photoreceptor cell generated by phototransduction. The inner segment is packed full of mitochondria and manufactures ATP to power the sodium-potassium pump necessary for stabilizing the resting potential. Visual pigment is also synthesized in the inner segment region and transported to the outer segment via the ciliary stalk. The inner segment diameter appears wider in cones than in rods.

C) NUCLEUS

Cone nuclei are situated at the outer apical edge of the ONL near the OLM, whereas the rod nuclei lie basal to those of the cones in the inner regions of the ONL. In addition, cone cell nuclei have an irregular shaped clumps heterochromatin, which rods have a single large central clump of heterochromatin. In general, fibers projecting away from the cell nucleus both to the synapse and inner segment are thicker in cones compared to rods.


**D) SYNAPSE**

Both rods and cones release glutamate at the synaptic terminal located in the OPL. The rod synaptic terminal, called a spherule, appears smaller in size than cone synapse terminals; it contains a single invagination with a single ribbon synapse (Figure 1.4a). Rod terminals are generally found at the outer regions of the OPL and lie basal to cone terminals. Processes from two horizontal cells and two to five rod bipolar cells can be found within a rod synaptic cleft. Conversely, the cone synapse terminal, termed a pedicle, has multiple ribbon synapses and several synaptic cleft invaginations, each containing processes from two horizontal cells and multiple bipolar cells (Figure 1.4a). Bipolar cell processes can also be located near to the invaginations or close to the base of the synapse terminal.
FIGURE 1.4. ROD AND CONE PHOTORECEPTOR: STRUCTURE AND SPECTRAL SENSITIVITY

(a) Illustration demonstrating the different structure of rod and cone photoreceptors cells, including inner and outer segments and the synaptic terminal. Adapted from http://alexandria.healthlibrary.ca/documents/notes/bom/unit_6/Lec%2042_ribdasil_hise.xml and http://www.nature.com/nrn/journal/v5/n10/fig_tab/nrn1497_F1.html (b) Graphical illustration (top) and graph (bottom) showing the spectral sensitivity of human rods, S-cones (blue), M-cones (green) and L-cones (red). Adapted from http://www.planetary.org/rrgtm/emspectrum.html.
**E) RODS AND CONES: SPATIAL DENSITY AND SENSITIVITY**

Rod photoreceptors are extremely sensitive and pool visual information allowing highly sensitive vision in dim scotopic lighting conditions. Rod photoreceptors express the visual pigment rod rhodopsin with a peak of sensitivity around 500nm (Figure 1.4b). Rod photoreceptors far outnumber cone photoreceptors in the mouse and human retina; cones make up only 3% of total photoreceptors population in both species. More specifically, there are approximately 100 million rod photoreceptors compared to 6.4 million cone photoreceptor in the human retina. Despite this discrepancy, rods are absent in the fovea, which is solely comprised of cone photoreceptors. Instead, rods densely populate the peripheral retina allowing highly sensitive but low acuity peripheral vision. In the rodent, where the fovea is absent, cones are distributed evenly throughout the retina.

Cone photoreceptors provide high acuity vision, are adapted for colour vision and operate in bright lighting conditions. Each type of cone photoreceptor expresses a different cone opsins visual pigment, which detects specific wavelengths of light. Human vision is trichromatic, mediated by three cone subtypes: L-cones that detect long wavelengths of (red) light with a peak sensitivity at 564nm, M-cones that detect medium wavelengths of (green) light with a peak at 533nm and S-cones that detect short wavelengths of (blue) light with a peak at 437nm (Figure 1.4b). As mentioned previously, L and M cones are densely packed in the fovea region of the human retina. S-cones account for only 10% of the overall cone population in the human retina and are absent in the fovea. The murine retina has di-chromatic vision, as it possess just two subtypes of cone photoreceptors: S-cone and L/M-cone subtype. In the rodent retina, L/M-cone distribution is mostly limited to the superior retina and S-cone distribution in the inferior retina.
1.3.3. **The Phototransduction Cascade**

The phototransduction cascade refers to the biochemical process that converts a photon of light into an electrical signal. Three cells in the retina are able to do this: rod cells, cone cells and melanopsin containing photosensitive ganglion cells. All mammalian visual pigments are called rhodopsins and contain the chromophore 11-cis-retinal, which is covalently embedded within the protein portion of the rhodopsin molecule, called the opsin (Figure 1.5a). The visual pigments can be found within the outer segment disk membranes.

**A) Dark Current**

In the dark, voltage-gated cation channels in the outer segment remain open: the intracellular environment is negatively charged compared to the extracellular environment so an inward electrical current of sodium and calcium ions is maintained. To maintain a negatively charged intracellular environment, K+ selective channel in the inner segment pump out K cations. The gated cation channels in the outer segment plasma membrane are kept open when bound with guanosin 3’,5’ cyclic monophosphate (cGMP) which diffuses freely throughout the cytoplasm and levels are high in the dark. The overall result is that photoreceptors remain depolarized in the dark, keeping voltage gated calcium channel in the synapse terminal open. Calcium causes synapse vesicles to fuse with the cell membrane and release the neurotransmitter glutamate. Glutamate is thus continuously released in the dark.
**B) Activation of the Phototransduction Cascade**

**Photoactivation**

11-cis retinal consists of a head region linked to a hinged tail (Figure 1.5a). When 11-cis retinal is struck by a photon, the energy is absorbed and the chromophore photoisomerises whereby it changes shape: the tails straightens converting it into the all-trans retinal isomer. This change forces the rhodopsin molecule (R) to expand resulting in activation (R*) (Figure 1.5a and b).

**G protein activation**

In turn, the R* molecule activates a G-protein on the disk membrane surface (Figure 1.5b). G-proteins (G) consist of three subunits which bind the nucleotide: α subunit, β subunit and γ subunit. The α-subunit binds GDP nucleotide when inactive. When an inactive G-protein encounter and activated rhodopsin (R*) molecule, the α-subunit aligns with R* and GDP is replaced with GTP (Figure 1.5b). This interaction results in activation of the G-protein α subunit (G*) causing it to separate from the G-protein and rhodopsin molecule (Figure 1.5b). The R* molecule can activate several hundred G-proteins.

**PDE Activation**

G* binds to the γ subunit of cG phosphodiesterase (PDE; this exposes the catalytic site of either the α- or β-subunits and thus activates PDE (PDE*). PDE* hydrolys cGMP producing GMP, which is inert and unable to bind to the cGMP-gated channels. The intracellular concentration of cGMP declines resulting in a closure of the cG-gated cation channels (Figure 1.5b).
**Photovoltage**

The closure of the cGMP-gated cation channels results in a reduction of the inward movement of calcium and sodium ions. However, the efflux of K+ ions in the inner segment is maintained. The overall result is a net loss of positively charged ions in the photoreceptor and the transmembrane potential hyperpolarizes, this change in transmembrane potential in response to photon absorption is called photovoltage.
FIGURE 1.5. ACTIVATION OF THE PHOTOTRANSDUCTION CASCADE

(a) Illustration showing the location of photoreceptor outer segment disks membrane and the positioning of rhodopsin in the disk membrane (left). The molecular structure of rhodopsin (right), consisting of opsin and the chromophore 11-cis-retinal. Upon light activation, 11-cis-retinal photoisomerises converting into the all-trans-retinal isomer (Adapted from (Purves et al., 2001). (b) Illustration of molecular steps involved in photoactivation (modified from (Leskov et al., 2000). Step 1: Photon (hv) activates a rhodopsin to R*. Step 2: R* activation G. G releases bound GDP in exchange for GTP, α-subunits (G*) dissociates. Step 3: G* binds to and activates phosphodiesterase (PDE) activating. Step 4: PDE* hydrolyzes cGMP. Step 5: Reduced levels of cytosolic cGMP cause cyclic nucleotide gated channels to close, preventing further influx of Na+ and Ca²⁺.
C) Deactivation of Phototransduction Cascade

Termination of the phototransduction cascade requires a number of processes, illustrated in Figure 1.6. Firstly, cGMP levels must be restored to allow re-opening of the cGMP gated cation channels; secondly activated rhodopsin(R*) must be deactivated and finally the chromophore must be recycled.

**Restoration of cGMP levels**

By restoring the cell's cGMP levels, through a reduction in cGMP degradation (Figure 6a) and an increase in synthesis (Figure 1.6b), the cGMP-gated cation channels re-open.

**Reduced cGMP degradation**

GTPase Activating Protein (GAP) binds to the Gα* subunit which stimulate Gα* to hydrolyse the bound GTP converting it into GDP (Figure 1.6a). This hydrolysis causes the Gα* subunit to break away from the PDE* thereby deactivating it and halting the degradation of cGMP to GMP.

**Increased cGMP synthesis**

Fundamentally, the intracellular levels of calcium control cGMP synthesis. Guanylate Cyclase Activating Protein (GCAP) binds calcium (Figure 1.6b). When intracellular levels of calcium decrease, GCAP dissociates from it's bound calcium and activates guanylate cyclase (GC). GC is a membrane-associated enzyme called guanylate cyclase (GC) that once activated by GCAP increases the rate of synthesis of cGMP from GTP (Figure 1.6b). GCAP is associated with GC and controls the rate of cGMP synthesis.
Deactivation of R*

Rhodopsin kinase (RK) is an enzyme present in the disk membrane that binds to R* (Figure 1.6c) and is itself regulated by recoverin (Rec), which is a calcium binding protein. When intracellular calcium levels fall following phototransduction, calcium dissociated from recoverin releasing rhodopsin kinase (Figure 1.6c). Using ATP, rhodopsin kinase phosphorylates R* allowing a cytoplasmic protein, arrestin (Arr), to recognize and bind to the rhodopsin molecule replacing the rhodopsin kinase. Arrestin remains bound to the rhodopsin molecule until the all-trans chromophore is removed, following which, the phosphorylated opsin remains in the disk membrane waiting to receive a new 11-cis retinal chromophore.

Chromophore recycling

Once released from the inactive phosphorylated rhodopsin molecule, all-trans retinal is converted all-trans retinol by the enzyme retinal dehydrogenase. All-trans retinol is enveloped by an all-trans retinol binding protein and transported to the RPE. Retinyl-ester isomerase is an enzyme present within RPE cells, which converts all-trans retinol to 11-cis retinol. A second enzyme, 11-cis retinol dehydrogenase, converts 11-cis retinol to 11-cis retinal. This is then transport across the interphotoreceptor matrix enveloped by 11-cis retinal binding protein to the photoreceptor outer segment. Here, phosphatase dephosphorylates the deactivated rhodopsin molecule allowing 11-cis retinal to re-enters the opsin thus reforming the inactive visual pigment.
FIGURE 1.6. DEACTIVATION OF THE PHOTOTRANSDUCTION CASCADE

(a) Diagram illustrating inactivation of PDE*. GTPase-activating proteins (GAP) enhances hydrolysis of Gα*-PDE6*. The Gα* subunit dissociated, deactivating PDE* and halting the degradation of cGMP. (Adapted from Pugh and Lamb, 2000). (b) Diagram illustrating restoration of cGMP, which is partly achieved by increasing cGMP production. Guanylate cyclase (GC) activity is enhanced by guanylate cyclase activating protein (GCAP). When bound to calcium, GCAP is inactivated. When calcium levels fall following photoactivation, calcium dissociates from GCAP, allowing it to increase GC activity and cGMP synthesis (Adapted from Stockman et al., 2007; Stockman et al., 2008). (c) Diagram illustrating inactivation of activated rhodopsin (R*). Rhodopsin kinase (RK) forms a complex with calcium bound recoverin (Rec). As calcium levels fall, RK is released which in turn phosphorylates R* which is then quenched by arrestin (Arr). (Adapted from Pugh and Lamb, 2000).
1.4. RETINAL CIRCUITRY AND CORTICAL PROCESSING

1.4.1. RETINAL CELL TYPES AND CIRCUITRY

The retina contains five major neuronal cell types; each can be further divided into distinct subtypes resulting in a total over 70 different types of neuronal cells found within the retina. This vast array of neurons results in the formation of multiple pathways and circuits to channel the visual signal; this allows the neural retina to carry out the first steps in processing the visual information prior to the signal being transmitted to the brain. Here, each major neural cell type is discussed with the view to describing some of the common underlying retinal circuitry. Figure 1.7 illustrates the basic circuitry of the retina. Understanding of retinal circuitry is paramount if we are to accurately assess how our transplanted cells integrate into the recipient retinal environment.

A) THE PRIMARY ROD PATHWAY

Photoreceptors hyperpolarize in response to light resulting in a reduction of glutamate release at the photoreceptor synaptic terminal. The electrical signal is propagated to second order neurons: horizontal cells and a variety of bipolar cell subtype. There is also some evidence to suggest rod/cone gap junction coupling, whereby the rod photoreceptors can signal via the cone pathway at the level of the photoreceptor (Smith et al., 1986). However, in the classic primary rod pathway, rod photoreceptors project to rod ON-bipolar cells, which in turn innervate All amacrine cells that converge with the cone visual pathway at the level of the cone bipolar cell (Figure 1.7a).
FIGURE 1.7. BASIC CIRCUITRY OF THE RETINA

(a) Schematic diagram illustrating the classic rod primary pathways. Rod (red) signals are conveyed to AII amacrine cells (AII) via the rod ON-bipolar cell (ON-RBP). The rod signal converges with the cone OFF pathway when AII amacrine signals synapse with cone OFF bipolar cells (OFF-CBP) and OFF ganglion cells (OFF-GC). The rod signal converges with the cone ON pathway when AII amacrine electrically signal to cone ON bipolar cells (ON-CBP) via connexin36 channels (Cx36). Cone ON bipolar cells synapse with ON ganglion cells (ON-GC), which project to the brain. (b) Schematic diagram illustrating an alternatively rod signaling pathway. Rod signals can converge with both the ON and OFF cone pathway at the level of the photoreceptor which is facilitated by electrical coupling between rod and cone photoreceptors. Adapted from (Seeliger et al., 2011).
B) Bipolar Cells

Bipolar cells are excitatory glutaminergic interneurons, whose cell bodies reside in the INL, and are responsible for conducting the visual signal to ganglions located in the innermost part of the retina. Bipolar cells dendrites can receive input from either rod or cone photoreceptors but not from both and are named accordingly as rod bipolar cells and cone bipolar cells. There is only one type of rod bipolar cell, the rod ON-bipolar cell and 13 recorded distinct types of ON or OFF cone bipolar cells.

Bipolar cells are subdivided as ON or OFF bipolar cell types depending on whether they hyperpolarize or depolarize in response to glutamate. In the dark, photoreceptor cells are constantly depolarized and thus, glutamate is continuously released. Glutamate activates mGluR6 receptors found on ON-bipolar cells resulting in closure of cation channels and a hyperpolarizing, inhibiting response. Conversely glutamate depolarizes the OFF-bipolar cell served by ionotropic glutamate receptors. Following light exposure, the photoreceptor hyperpolarizes resulting in a reduction in glutamate release, thus removing the inhibitory effect upon the ON-bipolar cell. As a consequence, the ON-bipolar cell depolarizes and becomes active. The converse effect occurs in the OFF-bipolar cells, whereby the reduction in photoreceptor glutamate release, results in hyperpolarisation and therefore silencing of the OFF-bipolar cell. ON-bipolar cells descend deeper into the IPL where they synapse with ON ganglion cells, whereas OFF-bipolar cells ramify at the outer IPL where they synapse with OFF-ganglion cells.

There is only one type of rod bipolar cell, which is categorised as an ON-bipolar cell as it depolarizes in response to light stimulation. The dendrites of rod bipolar cells branch profusely and receive input from as many as 120 rods allowing high sensitivity but low acuity. Each rod synapses with between two and five different rod bipolar cells. It is unusual for a rod bipolar to connect directly with a ganglion cell; instead 90% of rod bipolar cells output to AII amacrine cells.
C) Horizontal Cells

In addition to connecting with bipolar cells, rod and cone photoreceptors also synapse with horizontal cells, whose cell bodies reside at the apical side of the INL. Horizontal cell are involved in a lateral inhibition circuit with the photoreceptors and help to modulate multiple photoreceptor input to bipolar cells and control the subsequent magnitude of bipolar activation. Following a light stimulus, the photoreceptor hyperpolarizes and glutamate release is inhibited. This has a hyperpolarizing effect on the adjoining horizontal cell, which subsequently reduces GABA release. The reduction of GABA has an anti-inhibitory effect on the photoreceptor, i.e. the photoreceptor then depolarizes and increases glutamate release. Each horizontal cell collates the signal from many neighboring photoreceptors and the greater the stimulation of the horizontal cell terminals by photoreceptors, the larger the inhibition feedback and the smaller the effect each individual photoreceptor has on its connecting bipolar cell. Thus, lateral inhibition acts to reduce the effect of the overall levels of light intensity on the bipolar cells and as a result enhances spatial differences in light intensity allowing the eye to adjust vision under bright and dim light conditions.

Horizontal cell can be subdivided into HI (Type B) horizontal cells with axons and HII (Type A) horizontal cells without axons. There are three types of lateral inhibition: one exclusive to rod and rod bipolar which is mediated by the axon terminals of HI cells, the second is mediated by the dendritic terminals of HI cells serving L and M cones and the third is mediated HII dendritic terminals which serves all cone types.
**D) AMACRINE CELLS**

Amacrine cells are mostly laterally projecting inhibitory interneurons found within the INL and in the GCL, where they are aptly named displaced amacrine cells, and are responsible for modulating bipolar cell signal input to ganglion cells. Amacrine cells exist in many diverse subtypes, such as dopaminergic, starburst and AII cell, which all signal using a variety of neurotransmitters; however, the vast majority use either GABA or glycine. Although the exact functions of many amacrine cells remain unknown, two functions seem to be commonly repeated. Firstly, amacrine GABA-mediated lateral feedback, exists in all bipolar cell terminals in the IPL and secondly, many ganglion cells are coupled to amacrine cells by gap junctions forming a lateral inhibition feed-forward circuit. The most common type of amacrine is the AII amacrine cell, known as the rod amacrine cell. All amacrine cells play an essential role in the primary rod visual pathway, which ultimately converges and ‘piggy-backs’ the cone pathway in the IPL. Glutaminergic rod bipolar axon terminals synapse with (AII) amacrine cell processes, which contain post-synaptic AMPA glutamate receptors. All amacrine cells are glycinergic neurons and in turn make inhibitory synapse with the axon terminal of OFF cone bipolar cells. In addition, AII amacrines also make connections with ON cone bipolar cells using electrical synapses or gap junctions. This duel connection to the cone pathway allows both ON and OFF signals to be generated in dark lighting conditions.
**E) Ganglion Cells**

Ganglion cells receive input from bipolar and amacrine cells and are responsible for transmitting visual information from the retina to the brain. Ganglion cells have long axons that run across the vitreal surface of the retina and form the optic nerve, which projects to several different regions of the brain.

Ganglion cells spontaneously fire action potentials and the visual information is transmitted to the brain in the form of the rate at which these action potentials are generated. In general, excitation of ganglion cells by bipolar cells increases the rate of firing. Conversely, amacrine cell inhibition decreases the rate of firing, although there are some exceptions to this rule. Ganglion cells use the excitatory neurotransmitter glutamate.

There are many ganglion cell subtypes, each thought to transmit specific channels of visual information. Ganglion cell subtypes are categorised based on their projections, morphology and functions and can be grouped into five main classes. The first three classes of ganglion cell, Midget, parasol and bistratified ganglion cells, project to different regions of the LGN. The fourth class of ganglion cells included photosensitive ganglion cells, which contain the photopigment, melanopsin, allowing them to respond directly to light in the absence of rod and cone photoreceptors. These ganglion cells project to the suprachiasmatic nucleus (SCN) and are responsible for maintaining circadian rhythms. These ganglion cells also project to the Edinger-Westphal nucleus via the LGN and contribute to the pupil light reflex. The fifth group of ganglion cells project to the superior colliculus and accessory optical system. These neural pathways function to direct the eyes to regions of interest and helps maintain the direction of the gaze in the presence of head movement.
1.4.2. Cortical processing and visual perception

Visual perception is the process of interpreting visual information gathered from light in the environment activating sensory photoreceptors in the eye. As previously mentioned, different types of ganglion cells project to different locations within the brain, however most ganglion cell axons terminate in specific regions of the LGN in the thalamus. The LGN exists in both hemispheres of the brain and acts as a relay centre. Neurons exiting the LGN project via the optic radiations pathway to the primary visual cortex in the occipital lobe of the brain, also known as V1 (Figure 1.8).

A) Visual cortex

The visual cortex is responsible for processing visual information and consists of several sub regions the most studied being the primary visual cortex, also known as V1. Additional regions include the extrastriate visual cortical areas V2, V3, V4 and V5 (Figure 1.8). The visual cortex exists in both hemispheres of the brain, each hemisphere receives visual information from the ipsilateral visual field.

V1 receives visual input from the LGN and outputs via two pathways. Firstly, the dorsal stream goes through V2 down to V5 finishing in the posterior parietal cortex. This pathway is associated with coordinating movement of the arms and eyes, motion and location of objects. Secondly, the ventral stream travels through V2, then V4 and terminated in the inferior temporal cortex. This pathway is associated with long-term memory and object recognition. V1 has a well-defined map of spatial information, also referred to as retinotopic mapping.
FIGURE 1.8. VISUAL PATHWAYS TO THE VISUAL CORTEX

Illustration showing the projections from the retina to the brain. Projections relay via the lateral geniculate nucleus (LGN) and from there the signals are passed on to the visual cortex. The visual cortex is mainly divided into five areas as V1, V2, V3, V4 and V5. Area V1, which is known as striate cortex or the primary cortex, is the most important and one of the most intensively studied areas of the brain.

B) Retinotopy

Retinotopy refers to the spatial organization of visual areas of the brain. Many of the brain structures receiving visual input do so in a way whereby positions within the brain can be mapped to visual fields in the retina. The discovery of visual field maps arose when a neurologist observed a correlation between areas of trauma in the visual cortex with loss of specific visual fields (Fishman, 1997). The organization of the visual pathways allows this retinotopic map and there is some degree of visual field overlap. Retinotopic mapping is apparent in many brain structures receiving visual input, including the visual cortex, the superior colliculus in the brainstem, and the LGN in the thalamus. The retinotopic maps in V1, in which the initial steps of visual processing are performed, are very precise: adjacent neurons map to neighboring visual fields in the retina. In other cortical areas, the retinotopic maps are more complex.

C) Optokinetic Reflexes

The optomotor response refers to the innate optokinetic head-tracking reflex where the head moves to track a whole-field moving visual stimulus (Prusky et al., 2000; Prusky et al., 2004). This pathway has not been studied extensively but investigation into neural processing in the zebrafish showed that retinal ganglion cells project to a small region of neurons in the hindbrain which connect to afferent motor neurons in the spinal cord (Ireland, 1979; (Portugues and Engert, 2009).
1.5. DEVELOPMENT OF THE EYE

The eye is derived from three embryonic tissues, each developing into different structures of the eye. The retina is derived from neuroectoderm and is considered part of the central nervous system (Figure 1.9a-e). The mesoderm develops into the cornea, sclera and uveal tract and the lens arises from the surface ectoderm (Figure 1.9f).

1.5.1. DEVELOPMENT OF THE EYE CUP

A) NEUROECTODERM

The neural plate develops very early in the embryo from the ectoderm. An inward groove in the neural plate develops, which deepens forming the neural fold (Figure 1.9b). Ultimately the neural folds meet and the neural tube is formed (Figure 1.9c-e). Different regions of the neural tube go on to develop distinct regions of the CNS governed by dorsal-ventral patterning of secreted signaling molecules. Optic vesicles are formed when the dorsal neural tube begins to fold early in development, appearing as hollow structure that protrude from both sides of the forebrain (Figure 1.9d and e). As the vesicles grow they form a narrow stalk-like connection to the developing brain, called the optic stalk, which develops into the optic nerve. The optic vesicles begin to invaginate, forming an optic pit (Figure 1.9f). As the optic cup forms, two distinct layers are apparent: the inner layer of the cup, which undergoes repeated rounds of division in order to form the laminated neural retina, and the outer layer, which remains as a single monolayer that differentiates to form the RPE (Figure 1.9f). The retina develops in an inside-out order, with the ganglion cells forming first in the inner retinal layers and the photoreceptor cells in the outer retina maturing last (Figure 1.9).
**B) SURFACE ECTODERM & MESODERM**

The region of surface ectoderm located above the optic pit is induced by the optic vesicle and begins to thicken forming the lens placode (Figure 1.9f). The formation of the lens placode is thought to act as an inducer and triggers the infolding of the optic pit to form the optic cup. The lens placode goes on to differentiate and also invaginates eventually separating from the ectoderm to form the lens vesicles (Figure 1.9f). Again this acts as an inducer transforming the surface ectoderm into the cornea and eyelid.

The mesoderm surrounding the optic cup eventually differentiates into the corneoscleral tunics. In addition, during the invagination process, some mesenchyme migrates into the optic cup and is essential for the development of the iris, ciliary body and blood vessel development as well as acting as an inducer for optic nerve and RPE development. Retinoic acid plays an essential role in guiding mesoderm differentiation (Duester, 2008).
FIGURE 1.9. DEVELOPMENT OF THE EYE CUP

Illustrations demonstrating development of the eye-cup from the neural plate. (a) Eye cup development begins with the neural plate. (b) Neural folds form as the neural plate begins to fold upwards. (c) Optic grooves are formed when the neural plate invaginates. (d) The neural folds to meet each other and the optic vesicles are formed. (e) The neural tube seals off: the forebrain grows upwards and the optic vesicles grow outwards. (f) At this point the optic vesicles contact the surface ectoderm and development of the lens placode is induced. The optic vesicle and surface ectoderm invaginate together forming the optic cup. The inner layer of the bilayered optic cup forms the neural retina; the outer most layer forms the RPE.

Adapted from: (Lamb et al., 2007).
1.5.2. DEVELOPMENT OF THE RETINA

Development of the neural retina begins when the optic vesicles are formed. Neurogenesis occurs in an inside to outside manner, starting around embryonic day (E) 11 in the mouse. Initially, retinal progenitor cells (RPCs) undergo rapid symmetric proliferation in early development to expand the progenitor pool. The RPC cell bodies follow a clearly defined pattern of interkinetic nuclear translocation during the cell cycle: mitosis occurs in the ventricular zone, at the outside edge of the retina, RPCs then migrate to the vitreal surface in G₁ and undergo DNA replication associated with S-phase at the vitreal surface before migrating back to the ventral zone in G₂ (Figure 1.10a) (Dyer and Cepko, 2001; Norden et al., 2009; Pearson et al., 2005). As development progresses and the RPC pool is sufficiently expanded, there is a shift to asymmetric division, allowing a subset of RPCs to exit the cell cycle and become post-mitotic precursors, which can go on to differentiate into a specific cell type, which is strictly governed by extrinsic and intrinsic cues. Later in development, RPCs are thought shift to symmetrical division where two post-mitotic daughter cells are produced. RPCs are multipotent and have the potential to give rise to all 7 cell types of the retina; however differentiation occurs in a highly conserved temporal fashion and as development progresses RPC competence to produced different cell types becomes restricted (Turner and Cepko, 1987; Wetts and Fraser, 1988). There is a strict order in which the cell types exit the cell cycle and it occurs in overlapping waves (Figure 1.10b). The first wave of cell genesis includes ganglion cells, horizontal, cone photoreceptors and amacrine cells. The second wave gives rise to rod photoreceptors, bipolar cells and Müller glia cells (Lamb et al., 2007). Differentiated astrocytes develop outside of the neural retina and migrate along the optic nerve. A large array of transcription factors, mostly basic helix-lool-helix (bHLH) and homeodomain factors, expressed by subsets of RPCs or their post-mitotic progeny, together with extrinsic factors from the environment induce and regulate the differentiation of the specific cell types within the neural retina (Ohsawa and Kageyama, 2008). Homeodomain factors
regulate retinal layer specificity and bHLH factors are important for determining cell fates within each layer.
a

Outer (ventricular) surface

b

Ganglion cells
Horizontal cells
GABAergic amacrine cells
Cones
Other amacrine cells
Müller cells
Rods
Bipolar cells
Synaptic layers form:


cecal time (weeks)
Post-natal time (weeks)

-3 -2 -1 0 1 2


c At birth

d 2 weeks

e 4 weeks

OLM
IPL
ILM
FIGURE 1.10. DEVELOPMENT OF THE RETINA AND CIRCUITRY

(a) Illustration showing the stages of the cell cycle in the developing vertebrate retina. Proliferating cells undergo DNA replication (S phase) at the vitreal surface, before migrating to the outer (ventricular) surface during G2 of the cell cycle. Upon reaching the ventricular zone, the cell enters mitosis (M) and the resulting daughter cells either return to the vitreal surface during G1 ready to re-enter the cell cycle once more or terminally differentiate (G0). (b) Graphical illustration of cell birth timing for each cell type in the vertebrate retina. (c) Illustrations showing the development of retinal circuitry. (c) Immature rod (R) and cone (C) photoreceptors send transient processes to the inner plexiform layer (IPL). (d) Bipolar cells (B) enter into the pathway when the photoreceptors retract their processes, forming synapses with photoreceptors in the outer plexiform layer (OPL). (e) Photoreceptors mature, developing inner segments (IS) and outer segments (OS). Abbreviations: A, amacrine cell; G, ganglion cell; H, horizontal cell; ILM, inner limiting membrane; OLM, outer limiting membrane.

Adapted from: (Lamb et al., 2007).
1.5.3. DEVELOPMENT OF PHOTORECEPTORS AND RETINAL CIRCUITRY

As mentioned above, cone photoreceptors commit to the photoreceptor lineage prior to rods, between E11.5 to E18.5 in the mouse. Conversely, rods are born from E12.5, with the peak of rod birth around postnatal day (P) 0 and rod genesis is complete by ~P5 (Carter-Dawson and LaVail, 1979; Young, 1985). Although committed to the photoreceptor cell fate, both rods and cones undergo several more phases of development until developing a mature phenotype (Figure 1.10c-d) (Bruhn and Cepko, 1996; Watanabe and Raff, 1990). During this period several transcription factors regulate the photoreceptor differentiation process.

*Otx2* is expressed in several regions in early development, including the eye field. It is essential for the correct development of the RPE and is expressed in post-mitotic neuroblast cells that later form ganglion cells, bipolar cells and photoreceptors (Baas et al., 2000; Bovolenta et al., 1997). In addition to its essential role in the development of many retinal cell types, *Otx2* promotes photoreceptor cell fate determination: the peak of Otx2 expression coincides with photoreceptor cell birth, declining as photoreceptors maturation occurs. *Otx2* enhances Cone-rod homeobox protein, *Crx*, promoter activity in photoreceptor precursor cells. *Crx*, is the principle transcription factor that directs rod and cone photoreceptor cell differentiation and together with *Otx2*, activates the transcription of many photoreceptor genes (Furukawa et al., 1997). *Rorβ* is downstream of *Crx* and through its downstream target *Nrl*, acts as a switch directing photoreceptors to the rod cell fate (Jia et al., 2009).

*Nrl* is a transcription factor expressed only in rod photoreceptors in the retina and is essential for rod development (Akimoto et al., 2006; Mears et al., 2001; Swain et al., 2001). Coupled with the actions of *Nrl*, photoreceptor nuclear receptor, *Nr2e3*, is also expressed specifically by rod photoreceptors and acts as a dual transcription regulator. Together with *Crx*, it activates transcription of rod specific genes while repressing cone
specific genes. Many other transcription factors are involved rod survival and maturation including, *NeuroD* and *bHLH*.

Synaptogenesis between retinal neurons occurs in distinct phases (Figure 10c-d) and there is a progressive restructuring throughout the development process (see review (Reese, 2011). The lateral retinal circuits are formed first, starting with connections between ganglion and amacrine cells in the IPL followed by connections between photoreceptors in the OPL. Bipolar cells are last to differentiate and so the vertical retinal networks are formed much later in the developmental process.
1.6. RETINAL DEGENERATION

Retinal degenerations are the leading cause of blindness in the developed world (World Health Organisation, 2010). Many retinal degenerative disorders share a common fate: the death of retinal neurons and the subsequent loss of vision. The loss of photoreceptor cells, as a result of age-related, genetic or environmental factors, occurs in diseases such as age-related macular degeneration (AMD), retinitis pigmentosa (RP) and diabetic retinopathy (DR) and accounts for over 50% of all cases of blindness (Congdon et al., 2004).

1.6.1. COMMON RETINAL DISEASES

A) RETINITIS PIGMENTOSA

RP is the most common form of inherited retinal degeneration and can be inherited in an autosomal dominant, autosomal recessive or X-linked fashion. RP encompasses an extraordinary range of heterogenic disorders, affecting 1 in 4000 individuals worldwide (Resnikoff et al., 2004; Shintani et al., 2009; Sohocki et al., 2001). To date more than 240 gene mutations have been associated with RP (Retinal Information Network), causing defects in a variety of cellular components involved in structure, function and maintenance primarily within the rod photoreceptor cell but also within the underlying RPE cells. Many RP diseases result from mutations affecting the rod rhodopsin gene, accounting for approximately 25% of autosomal dominant inherited disease forms (Hartong et al., 2006). Photoreceptor cell death in many forms of RP follows a two-stage process. Rod photoreceptors degenerate first, as indicated by the early clinical symptoms of night-blindness and loss of peripheral vision. As the disease progresses, the fundus develops a pigmented appearance and the second wave of cell death occurs and affects the cone photoreceptor cells, as a result of which, central vision, visual acuity and colour vision begins to deteriorate. Traditionally, the mechanism of
photoreceptor cell death was attributed to apoptosis. However, current investigations reveal a more complex pathway involving non-apoptotic processes. Several groups have implicated that necrotic cell death mechanisms may also be involved as indicated by deregulation of cyclic nucleotide metabolism, increased activation of calpain and PARP (see review (Shintani et al., 2009).

**RP: Current and future treatment options**

To date there are no routinely available treatment options for patients with RP to reverse the photoreceptor cell loss. Current management of the disease aims to slow down the disease progression, including vitamin supplementation and light avoidance therapies. Daily supplementation with vitamin A can reduce disease progression in some patients postponing blindness by up to 10 years (Berson, 2007). Future treatments can be divided into three strategic approaches, each categorised by the stage of disease it is suited to treat (Jacobson and Cideciyan, 2010; Kalloniatis and Fletcher, 2004; Sahni et al., 2011). Firstly, in the early stages of disease prior to photoreceptor cell death, it may be possible to halt degeneration using gene therapy, which aims to correct the underlying genetic abnormality. This strategy involves the use of viral or non-viral vectors to either replace or silence the expression of a mutated gene. In recent years, some exciting advances in treatment Leber’s congential amaurosis (LCA) have been reported. LCA is an autosomal recessive X-linked RP disease. One of the genes frequently affected is RPE65, whereby mutations in RPE65 lead to loss of function. RPE65 is expressed in RPE cells and is involved in the regeneration of visual pigment. Three human phase II clinical trials using AAV-2 to replace the mutant RPE65 gene with the wildtype version are underway (Bainbridge and Ali, 2008; Hauswirth et al., 2008; Maguire et al., 2008). Sustained improvements were reported in some patients and importantly no major side effects have been found to date. These studies are now moving onto phase III. Future challenges for gene therapy include treating autosomal dominant conditions, where siRNA and ribozymes
could be employed to silence the abnormal gain of function gene mutation prior to gene replacement with the wildtype corrected version. In addition, only 60% of the causative gene mutations causing RP are known, consequently, gene therapy relies on identification of the gene mutation and application of such a therapy is therefore restricted (Hartong et al., 2006). Finally, this condition can involve multiple genetic changes and mechanisms: gene replacement of a single mutated gene may not completely halt disease progression.

Neuroprotective strategies have been examined by administration of neurotrophic growth factors or agents that inhibit pro-apoptotic pathways to prolong photoreceptor viability. Although some promise has been shown using ciliary neurotrophic factor (CNTF) and brain derived growth factor (bFGF), such studies were also associated with significant adverse effects with variable treatment outcomes (Emerich and Thanos, 2008; Uteza et al., 1999).

Electrical implants and retinal transplants have been proposed as a potential future therapy to treat patients with advanced degenerative retinal disease following major neuronal cell death and loss of laminar structure as a means to restore vision. Different types of electrical implants have been developed including cortical visual prosthesis, supra-choroidal implant, epiretinal implant and subretinal implants. Several studies have demonstrated tolerance and some functional improvements in human patients receiving epiretinal and subretinal implants (Ahuja et al., 2011; Chader et al., 2009; Gerding et al., 2007; Rizzo, III et al., 2003; Zrenner et al., 2011). Although these devices are effective at generating electrical impulses following light stimulation (Humayun et al., 2003), the connection of electronic hardware to the remaining recipient biological tissue is likely to be complex and the degree of rescue of visual acuity is unknown.

An alternative and potentially complementary approach the strategies described above is the replacement of photoreceptors by transplantation. Indeed this may be a more
feasible and practical option as cells have an innate ability to make synaptic connections with secondary neurons (Maclaren and Pearson, 2007). Photoreceptor replacement therapy will be discussed in detail in section 1.6.

B) AGE-RELATED MACULAR DEGENERATION

AMD is associated with both genetic and environmental risk factors and is a disorder affecting 10-20% of people aged over 65 years old, with 14 million suffers worldwide (Enzmann et al., 2009; Klein et al., 1992; Penfold et al., 2001). With the aging population likely to increase significantly over the coming years, the socio-economic impact of age-related conditions is set to rise. Four key risk factors have been identified: age, genetic, high body mass index and smoking. In particular, mutations in the genes encoding proteins of the complement immune system, such as factor H (CFH), have been identified as potential risk factors for the development of AMD.

AMD is primarily a disease of the RPE around the macular region. The secondary degeneration of the neurosensory retina results in loss of central vision and thus AMD has a dramatic impact on patient quality of life, often rendering them unable to live independently. It occurs in the nonexudative form, affecting 90% of AMD sufferers (Curcio et al., 1996; Singh and Maclaren, 2011), and the more severe yet treatable exudative form. Clinical signs of nonexudative AMD include pigmented disruption of the macular and the appearance of extracellular deposits called drusen residing between the RPE and Bruch’s membrane. The resulting geographic atrophy of the RPE, photoreceptors and underlying choriocapillaris is thought to be associated with widespread oxidative damage (Shen et al., 2007). Activation of the complement immune system has also been attributed to the pathogenesis of AMD: it is suggested that accumulation of extracellular deposits activates a local inflammatory response accelerating the development of drusen formation (Anderson et al., 2002; Donoso et al., 2006; Enzmann et al., 2009; Tezel et al., 2004). Approximately 10-15% of patients with nonexudative AMD progress to the exudative form of AMD, which involves
abnormal growth of blood vessels into the retina (Sunness, 1999). Exudative AMD is characterized by choroidal neovascularization (CNV) whereby choroidal blood vessels break through Bruch’s membrane and extend into the retina. These blood vessels are often very leaky leading to accumulation of blood below the macular causing secondary damage to the photoreceptor cells.

**AMD: Current and future treatment options**

Currently there are no treatment options for nonexudative AMD however some therapies do exist for exudative AMD. The most successful treatment option to date has been achieved with frequent vitreal injections of anti-VEGF agent, including ranibizumba (Lucentis) and bevacizumba (Avastin). These anti-angiogenic agents cause a regression of CNV blood vessels and visual improvement in 25-40% of patients (Brown et al., 2006; Enzmann et al., 2009; Rosenfeld et al., 2006). Several studies investigated the role of dietary supplementation of antioxidants to slow progression of nonexudative AMD and the use of laser therapy to reduce CNV, both with limited success.

As a consequence of the lack of treatment options for nonexudative AMD patients, current research in stem cell therapy has intensified as it offers the potential to replace both the degenerate RPE and photoreceptor cells. Central macular RPE replacement by translocating autologous RPE tissue from the periphery can prevent loss of central visual in some patients with AMD (Joussen et al., 2006). This has lead several groups to investigating the potential a cell-based transplantation therapy as a strategy that will avoid destruction of peripheral RPE tissue. Various cell types have been investigated, however, human ES-derived RPE cell generated *in vitro* for transplantation purposes have shown the most promising results so far. Some preservation of retinal function has been demonstrated in the RCS rat after transplantation of hES-derived RPE cells (Carr et al., 2009; Idelson et al., 2009; Lund et al., 2006; Vugler et al., 2008). In
addition, some promising results using iPS-derived RPE cells in the RCS rat have been demonstrated (Carr et al., 2009). Phase I/II safety trials using hES-derive RPE cells to treat patients with Stargardt’s disease, a juvenile onset macular degeneration, are now underway and have been shown to be well tolerated with no signs of tumorigenicity or immune rejection (Schwartz et al., 2012). However, despite these recent advances, the restoration of vision in patients with late stage disease will also require replacement of photoreceptors.
1.6.2. General Pathology of Retinal Degeneration

A) Photoreceptor Degeneration

The stress pathways and cell death mechanisms involved in many retinal degenerative diseases are an area of intense investigation and are likely to be very different in each disease subtype. However, in both rod-cone and cone-rod degenerations, a number of typical changes occur in the degeneration process. During the initial stress phase of degeneration, photoreceptor neurites sprout and extend towards the inner retina. Prior to death, these neurites and the pre-synaptic terminals are retracted. As the photoreceptors begin to die, the interphotoreceptor matrix becomes compressed and the remaining photoreceptor outer segments become compromised resulting in remodeling of the inner segment followed eventually by retraction of the remaining photoreceptor axons and cell death (see reviews (Jones et al., 2005; Jones et al., 2012; Jones and Marc, 2005).

B) Inner Retinal Re-modeling

The retina comprises of many specific neural circuits, as previously described. The death of the photoreceptors leaves the remaining neural retina de-afferented. In the final stages of photoreceptor loss, the cone photoreceptors die acting as a key trigger for negative global remodeling of the remaining neural retina (Jones and Marc, 2005). Both rod and cone bipolar cells, located in the INL, retract their dendrites from the OPL, and some undergo cell death at this point. In addition, rod horizontal cells retract their dendrites whereas cone horizontal cells undergo hypertrophy and elaborate new neurites towards the IPL. Neurons in the IPL begin to extend new neurites into the inner nuclear layer towards the remnant OPL. This remodeling process is accompanied by global neuronal cell death, migration and rewiring by new synaptic connections (Jones and Marc, 2005).
C) GLIAL RESPONSE

The Müller cell plays a pivotal role in the degenerative process and become activated in response to injury or disease. Initially, Müller glial cells may promote neuron survival by producing a variety of neuroprotective growth factors and antioxidants (Frasson et al., 1999). However, as degeneration progresses activated Müller cells undergo reactive gliosis and form a glial scar. Retinal gliosis functions to protect the retina following injury. The development of the glial scar functions to stabilize the fragile retinal tissue after injury and cell death (Jones and Redpath, 1998) and to protect the healthy part of the retina from the damaged area (Bush et al., 1999). In advanced glial scar formation, where extensive photoreceptor cell death ensues, a fibrous seals forms between the retina and RPE to allow re-modeling of the inner retinal neurons (Jones and Marc, 2005).

Typically, gliosis is characterized by a dramatic increase in intermediate filament expression and a pronounced hypertrophy of Müller cells (Figure 1.11b). This occurs throughout the retina and is the first step in the formation of the gliotic scar (Bignami and Dahl, 1979; Bringmann et al., 2000; Bringmann and Reichenbach, 2001; Eisenfeld et al., 1984; Fan et al., 1996; Landiev et al., 2006; Jones and Marc, 2005; Sethi et al., 2005). Indeed, upregulation of the intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin is considered the hallmark of gliosis. Intermediate filaments are highly stable structural components of the cellular cytoskeleton. However, little is known of their exact function in the stress response. Studies in the brain suggest that intermediate filaments play a major structural role to allow the process of glial hypertrophy and stabilization of the fragile tissue after injury (Eng and Ghirnikar, 1994). Upregulation of GFAP and vimentin originates in the end foot region of the Müller cell at the vitreous side of the retina (Sarthy et al., 1989). As the reactive gliosis process continues, the intermediate filaments extend outward into the Müller cell body and up into the apical processes forming lateral branches (Figure 1.11b) (Lewis and Fisher, 2003). The main process and lateral branches undergo further hypertrophy and
processes can extend into the subretinal space forming a subretinal gliotic scar (Lewis and Fisher, 2003). The gliotic scar refers to the lateral GFAP+ve processes that extend along the outer edge of the ONL and into the subretinal space.

Much of our understanding of retinal gliosis was pioneered in the CNS and later investigated in the retina. In the quiescent retina, GFAP expression is restricted to the retinal astrocytes whose cell bodies reside at the inner margin of the retina in the ganglion cell layer and the end foot domain of the Müller cells with very few filaments extending deeper than the border between the GCL and inner plexiform layer (Figure 1.11a). One exception is at the very periphery of the retina, where GFAP is expressed throughout the Müller cells. In addition, a gradual increase in GFAP expression has been shown to occur with age (Eng et al., 1998).

There is still much to be investigated with regard to what stimulates reactive gliosis and intermediate filament expression and how expression is regulated. The accumulation of GFAP in Müller cells is probably not a direct effect of neuronal cell death. Indeed, GFAP accumulation can either precede photoreceptor cell death, as it does in the Prph2rd/rd mouse, or it can occur after cell death, as it does in the PDE6βrd1/rd1 mouse (Ekstrom et al., 1988). GFAP expression is modulated by a wide variety of factors. Lewis et al. and other groups reported that intravitreal injections of basic fibroblast growth factor (bFGF) or CNTF resulted in an increase in the expression of both GFAP and vimentin and that CNTF mediates this response via the JAK/STAT signaling pathway (Lewis et al., 1999; Wang et al., 2002). Other reports have suggested that glial activation is mediated by the resident microglia in the retina (Harada et al., 2002).
(a) In the quiescent retina, intermediate filament, such as GFAP, expression is restricted to the retinal astrocytes in the inner retina. (b) GFAP upregulation begins in the end foot region of the Müller cell at the vitreous side of the retina and extends throughout the retina. At the outer edge of the ONL lateral branches form and processes extend into the subretinal space forming a lateral gliotic scar. Adapted from (Franze et al., 2007).
In addition to the changes in intermediate filament expression and Müller cell morphological changes, the gliotic scar also includes changes in the composition of the surrounding ECM and in the case of the retina, the IPM.

Many groups have shown that chondroitin sulphate proteoglycans (CSPGs) are upregulated in response to CNS injury (Davies et al., 1997; Fitch and Silver, 1997; Gates et al., 1996; Levine, 1994; Lips et al., 1995; McKeon et al., 1991; Pindzola et al., 1993). It has also been reported that CSPGs are upregulated in the retina in response to injury and disease (Escher et al., 2008; Inatani et al., 2000; Kennan et al., 2002; Landers et al., 1994). A number of studies indicate that many different cell types secrete CSPGs. *In vitro* studies in which the neural retina was cultured as explants demonstrated that CSPGs were secreted into the media, suggesting that it the neural retinal cells are responsible for the secretion of proteoglycans in injury (Murillo-Lopez et al., 1991). Inatani et al. (2000) reported that GFAP was co-localised with the CSPG neurocan in the ischemic retina suggesting that Müller cells may be responsible for the secretion of CSPGs. This has also been observed in the retina of *Rd1* mice and in the RCS rat (Zhang et al., 2003; Zhang et al., 2004). However, other studies have indicated that some CSPGs are also produced by RPE cells and exist as both soluble and membrane bound forms (Iwasaki et al., 1992).

It is thought that CSPGs act as guidance cues for neurite growth in both development and injury. Much of the early work examining the role of CSPG was pioneered in the CNS. *In vitro* studies by several groups have demonstrated the inhibitory role of CSPGs in the developing CNS where CSPGs act as boundary cues for neurite outgrowths (Dou and Levine, 1994; Emerling and Lander, 1996; Fawcett et al., 1989; Friedlander et al., 1994; Smith-Thomas et al., 1994). In addition, the role of CSPGs in CNS injury has been thoroughly investigated and several groups have demonstrated inhibition of neurite outgrowths in the region of the glial scar *in vivo* (Davies et al., 1999;
Kerschensteiner et al., 2005; McKeon et al., 1991). Furthermore, the removal of CSPG in the spinal cord enhanced neurite outgrowth *in vivo* (Bradbury et al., 2002). Brittis *et al.* (1992) showed that CSPGs also play a role in retinal development; this *in vitro* study demonstrated that CSPGs also act to negatively regulate axon growth of ganglion cells in the developing retina and that removal of CSPG *in vitro* enhanced ganglion cell axonal growth (Brittis et al., 1992).
1.7. REGENERATIVE MEDICINE

The regenerative capacity of mammalian photoreceptors is limited and treatment options are available for only a minority of patients suffering from a subset of the diseases mentioned previously. Thus there is a very real need for the development of new therapies (Shintani et al., 2009).

The field of regenerative medicine focuses on tissue/organ regeneration or replacement when the body’s own repair mechanisms fail in patients with severe injuries or chronic diseases. Many of these conditions, including Alzheimer’s, Parkinson’s, spinal cord injury, congenital heart-failure and retinal degenerative disorders have limited treatment options and regenerative medicine offers hope to replace the lost tissue and ultimately restore physiological function.

Several concurrent approaches have been pursued to promote successful tissue regeneration. These include the stimulation of endogenous regeneration mechanisms to regenerate damaged tissue, the restoration of function by transplanting bioengineered artificial organs and finally, cell replacement therapy, which aims to replace the cells lost in disease and injury. Investigation into cell therapy has included the use of many donor cell populations. However, recently much of the focus has been on the use of stem cells because of their self-renewal properties and ability to give rise to a variety of specialized cell types.
1.7.1. PHOTORECEPTOR REPLACEMENT THERAPY

As described in the previous section, some photoreceptor preservation can be achieved using therapeutic strategies, such as gene replacement therapy or neuroprotection therapies. Photoreceptor transplantation offers a complementary approach by replacing the cells lost in the degenerative process. There has been a long-standing interest in using cells to repair the degenerate retina. Over a number of years, several transplantation strategies using immature donor tissue, either as whole retinal sheets or cell aggregates, have shown morphological integration into recipient rodent models. Success of either transplantation strategy will be reliant on the ability of the donor cell/tissue to form segment structures capable of capturing light and the ability to integrate into the visual system by forming synaptic connections with the recipient retina.

A) RETINAL SHEET TRANSPLANTATION

Many studies have attempted transplantation of whole retinal sheets, from either embryonic or neonatal sources, into the subretinal space of the degenerating recipient (Arai et al., 2004; Seiler and Aramant, 2005). The rationale of this approach lies in the highly organized laminar structure of the retina; a retinal sheet has a fully formed structure and may be a feasible option for the treatment of late stage disease when the ONL is completely degenerate and the foetal sheets could replace large sections of retina. In order for the transplanted sheets to connect to the recipient retina, either the retinal ganglion cells of the donor sheet must extend processes through the degenerating host retina or the recipient inner retinal cells must elaborate dendrites into the graft. Morphological host-recipient integration has been reported on the basis of donor neurite extension and synaptic tracer experiments, although the number of contacts was extremely low (Ehinger et al., 1991; Seiler et al., 2005). These studies
demonstrated survival, lack of immune rejection and some differentiation of the grafted tissue.

Several reports have reported functional improvements following neuroblastic progenitor sheet transplantation in the rat (Arai et al., 2004; Seiler et al., 2010; Seiler and Aramant, 2005). The precise explanation for the improvements observed in these studies remains unclear, although is likely that growth factors released by the foetal tissue improved the survival of the endogenous photoreceptor cells via a neurotrophic effect (Liljekvist-Soltic et al., 2008). Further studies would be required to prove the specificity of the visual improvements reported. Clinical studies in humans using this approach showed some subjective improvements in vision (Radtke et al., 2008), but as these patients also received intraocular lens implants, it is difficult to assess the source of the improvement.
**B) CELL SUSPENSIONS**

Other strategies have investigated the potential of transplanting single cell suspensions. Successful transplantation of photoreceptors in this manner requires a donor cell that is capable of migrating into the outer nuclear layer of the recipient retina; differentiating into the mature photoreceptor phenotype, forming segment structures capable of capturing light, and successful integration into the visual system by forming synaptic connections with second order neurons. The quest to find a donor cell capable of all of these feats has been a long-standing investigation.

**Neural progenitor cells**

Brain-derived and retinal-derived neural precursor cells share a common origin, both originating from the neuroectoderm of the neural plate in development (Chow and Lang, 2001). It was thought that using neural progenitor cells, rather than more mature neural cells, would be optimal because immature neurons retained their intrinsic capacity to migrate and differentiate. Several studies examine the use of brain-derived neural stem (NS) cells and neural progenitor (NP) cells as a donor cell source (Conover and Allen, 2002; Klassen et al., 2007; Mellough et al., 2007; Mizumoto et al., 2003; Sakaguchi et al., 2003; Takahashi et al., 1998). Although early studies demonstrated that brain-derived neural precursor cells were able to robustly integrate into the immature recipient retina (Chacko et al., 2000; Sakaguchi et al., 2003; Van Hoffelen et al., 2003), further studies yielded disappointing results when transplants using neural progenitor cells were performed into adult recipients, as cell migration and integration into the adult recipient retina was limited (Sakaguchi et al., 2005; Young et al., 2000). It was concluded that the age of the host played a key role in determining transplantation outcome. Furthermore, although these cells were capable of migrating and integrating into all layers of an immature host retina, the cells did not differentiate into mature retinal phenotypes (Marquardt and Gruss, 2002). While some cells
developed retinal neuronal morphologies, they did not express retinal specific markers (Sakaguchi et al., 2005; Sam et al., 2006; Takahashi et al., 1998; Young et al., 2000).

**Retinal progenitor cells**

The lineage restriction of brain-derived NS and NP cells and their subsequent inability to differentiate into mature retinal phenotypes led groups to investigate immature retinal neural cells as a potential donor source for photoreceptor transplantation. These cells are clearly already programmed to become retinal cells and were therefore thought to be better able to form retinal phenotypes after transplantation.

Retinal progenitor cells isolated from E17 rat retina and expanded in culture prior to transplantation into S334ter recipient rats showed the ability to differentiate into photoreceptor phenotypes expressing photoreceptor markers such as recoverin and rhodopsin. However, although donor neurite extensions were observed when transplanting RPC into the immature recipient retina very limited integration was observed in the adult retina (Qiu et al., 2005).

**Post-mitotic retinal precursor cells**

It was assumed that the adult retina was not permissive to donor cell integration as it lacked the environmental development extrinsic cues needed to promote RPC migration in to the adult host retina. These studies had assumed that only immature progenitor cells retained the ability to migrate and integrate into the recipient retina, however, two studies indicated that an older donor cells may also retain this ability.

Kwan et al. (1999) demonstrated that donor cells isolated from postnatal day 7-9 mice transplanted into adult Rd1 recipients were able to survive in the subretinal space and extend neurites towards the host retina. In addition, further studies using cultured P1 GFP positive retinal cells, demonstrated some integration in the Rho<sup>−</sup> recipients.
(Klassen et al., 2004). However, despite expression of mature photoreceptor markers in the latter study, the integrated cells shown in both reports lacked mature photoreceptor morphologies.

The breakthrough came in 2006 by MacLaren and Pearson et al., later confirmed by Bartsch et al. 2008, when it was demonstrated that robust integration and differentiation can be achieved in the adult retina if the donor cells that are transplanted are at the correct ontogenic stage in development. Post-mitotic precursor cells, as opposed to stem/progenitor cells or more mature retinal cells, were identified as the most efficient donor cell population to integrate in the adult retina. Furthermore, it was demonstrated that these cells could form synapses in the correct location, respond to glutamate stimulation and restore a basic visual response, the pupillary light reflex, in the Rho−/− mouse model of retinal degeneration (Maclaren et al., 2006). This study demonstrated proof-of-concept for rod photoreceptor transplantation, showing robust migration, integration and some basic functional connectivity in both the adult and degenerate retina.
1.7.2. LONGEVITY AND IMMUNOLOGICAL CONSIDERATION

Depending on the nature of the donor cell source, immune rejection may present a major problem for photoreceptor replacement therapy. Traditionally the eye has been described as an ‘immune-privileged’ site that will allow survival of grafted tissue. However, it has been demonstrated that despite survival of integrated cells up on 12 months post transplant, there is a marked decline in the number of integrated cells from 4 months post transplant, an effect associated with activation of an adaptive immune response (West et al., 2010). Although autologous donor cells are likely to be optimal for long-term survival of transplanted cells, immune suppression maybe effective to ensure survival of successfully transplanted cells from non-autologous donor sources. Continuous immune suppression using cyclosporine A enhanced long-term survival of transplanted cells in wildtype recipients (West et al., 2010). In addition, an acute inflammatory innate immune response was demonstrated 1 month post transplantation in some wildtype recipients, suggesting that immune modulation of both the innate and adaptive immune response maybe required. It must also be considered that long-term survival of transplanted photoreceptor cells in the degenerating recipients retina maybe far more compromised than in the wildtype recipient as a result of the pro-inflammatory microenvironment. A donor cell source that could circumnavigate the potential immunological complications would be a significant advantage.
1.7.3. **Generation of appropriate donor cell sources**

When considering photoreceptor replacement therapy as a treatment for human retinal disease, a fundamental problem arises in that an appropriate source of donor cells is required. There are a number of considerations that need to be addressed in the search for a suitable donor cell source: ethical suitability, ease of availability, and immunological compatibility. The MacLaren and Pearson et al. (2006) study demonstrated that the precise developmental stage of the donor cell is a key requirement for successful photoreceptor transplantation. Harvesting human photoreceptor precursor cells at the correct ontogenic stage would require donor tissue from the developing retinae of foetuses between 10-16 weeks gestation, a strategy that raises both ethical and practical concerns because of the limited availability of such tissue. Stem cells may offer an attractive alternative because of their capacity generate an abundant supply of cells and the potential to avoid any immunological complications. The future challenge is to generate an equivalent post-mitotic precursor phenotype *in vitro* to provide an integration-competent alternative donor cell source.

The fundamental properties of a true stem cell are that they are pluripotent and able to differentiate into all cell types from all three germ layers; that they are able to proliferate indefinitely; and finally that they are able to self-renew by asymmetric division producing both a replacement stem cell and a daughter cell. Many research groups are investigating the potential of stem cells, including adult retinal stem-like cell (RS), Müller stem-like cells (MS), embryonic stem cells (ES) and induced pluripotent stem cells (iPS) as a future donor cell source for photoreceptor replacement therapy. Their potential as donor source is discussed below.
A) MÜLLER STEM-LIKE CELLS (MS)

Müller glia cells from the mammalian adult retina have been reported to have some stem-like properties when cultured in vitro; they can grow as neurospheres, express some neural stem cell markers and following differentiation promoting culturing conditions express mature retinal cell markers such as recoverin, S-opsin and peripherin (Das et al., 2006; Lawrence et al., 2007; Nickerson et al., 2008; West et al., 2009). However, their functional capabilities have yet to be characterized and their ability to integrate into the host retina following transplantation appears limited (Bull et al., 2008; Lawrence et al., 2007; Singhal et al., 2008).

B) ADULT RETINAL STEM-LIKE CELLS (RS)

Adult derived-RS cells harvested from the ciliary body, iris and, in some circumstances retinal tissue, can be grown as neurospheres in culture (Ahmad et al., 2000; Carter et al., 2007; Gu et al., 2007; Gualdoni et al., 2010; Haruta et al., 2001; Kokkinopoulos et al., 2008; Macneil et al., 2007; Mayer et al., 2005; Tropepe et al., 2000). Furthermore these cells can proliferate and differentiate into glial and neuronal cell types. Although several studies have suggested to have successfully induced differentiation into photoreceptor phenotypes whereby a number of mature photoreceptor markers, such as rhodopsin, recoverin and transducin are expressed (as reviewed in (West et al., 2009) differentiation efficiency remain low and recent reports question the extent of true photoreceptor differentiation (Gualdoni et al., 2010). Furthermore, following transplantation the integration efficiency of these cell types remains poor (Akagi, 2005; Canola et al., 2007; Chacko et al., 2000) and the functional capabilities of these cells remains unknown as they have not been demonstrated to be light-sensitive (Jomary and Jones, 2008). The usefulness of adult derived RS cells at present remains limited and ES and iPS cells may offer a more promising alternative (Boucherie et al., 2011; West et al., 2009).
C) Embryonic stem cells (ES)

ES cells proliferate and differentiate to produce all of the cells of the developing embryo. ES cells isolated from the inner cell mass of the mouse, monkey and human blastocyst have shown the ability to differentiate into retinal cell types, including photoreceptors (Boucherie et al., 2011; Ikeda et al., 2005; Lamba et al., 2006; Lamba et al., 2009; Osakada et al., 2008). Differentiation efficiency into photoreceptor cell fates remains relatively low, with ~17% expressing rod markers such as rhodopsin and recoverin (Osakada et al., 2008). Interestingly, differentiation efficiency of human ES towards retinal cell fates appears to be lower than mouse, with only ~8.5% of cells expressing rhodopsin (Osakada et al., 2008). More recently, it was discovered that mouse ES cells grown in 3D Matrigel cultures generated a synthetic retina with a self-organised laminar structure producing all the major classes of retinal neurons, including photoreceptors (Eiraku et al., 2011). If this 3D culturing system could be used to grown human ES, it may provide a method for more efficient production and identification of photoreceptor precursors at the correct ontogenetic stage for transplantation (Ali and Sowden, 2011; Eiraku et al., 2011). Human ES cells have been shown to integrate into the mouse retina and express mature rod photoreceptor markers such as recoverin and rhodopsin (Lamba et al., 2009). A major challenge surrounding the use of ES cell for regenerative medicine is the ethical issues concerned with the use and destruction of human embryos to isolate ES cells. In addition, ES derived donor cells do not address the immunological complications and tissue matching would still be a technical challenge.
D) INDUCED PLURIPOTENT STEM CELLS (iPS)

In 2006, it was discovered that adult somatic cells can be reprogrammed to produce pluripotent stem cells by inducing forced retroviral expression of four transcription factors: Oct4, Sox2, c-Myc and Klf4 (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Although very similar to ES cells in their molecular, morphological and development profile, the true extent of their reprogramming is still being assessed and some epigenetic abnormalities exist (Stadtfeld et al., 2010). Efficiency of iPS cell differentiation in vitro towards photoreceptor cell fates remains low, with only 6-8% of cells expressing rhodopsin in vitro (Osakada et al., 2009). It is also emerging that specific iPS cell lines retain epigenetic memory restricting retinal differentiation (Hirami et al., 2009; Lamba et al., 2010; Osakada et al., 2009). Transplantation of iPS derived retinal cells has, to date, largely been unsuccessful. There are reports of some integration into the recipient retina, but convincing examples of photoreceptor morphologies are lacking (Lamba et al., 2010; Parameswaran et al., 2010). This may be due to the donor cells not being at the correct development stage required for robust cell integration (Maclaren et al., 2006). An additional challenge of using photoreceptor donor cells derived from iPS cells for transplantation is the safety issues surrounding teratoma formation. The transcription factors used to reprogram somatic cells are known oncogenes that could be reactivated during the differentiation process thus posing a safety risk for their use in humans (Nakagawa et al., 2008). Several groups are working to restrict the use of these transcription factors and using non-integrating viruses to circumvent this problem (Brambrink et al., 2008; Kiskinis and Eggan, 2010). In addition, if the underlying cause of disease was genetic, correction of the mutated gene in the autologous iPS derived cells using gene therapy will be required.

Provided that the safety issues are be addressed, the differentiation efficiency is improved and the integration efficiency is robust, the use of iPS generated photoreceptors for transplantation could offer a number of advantages compared with other donor cell sources, namely the immunological benefit of transplanting autologous
cells and avoidance of the ethical issues surrounding use of ES cells derived from embryos.

1.8. FUTURE CHALLENGES FOR PHOTORECEPTOR REPLACEMENT THERAPY

In order for photoreceptor transplantation to be considered as a future therapy to treat retinal degenerative disorders, a number of key issues need to be addressed. Transplantation efficiency needs to be enhanced, as far greater numbers of integrated cells are likely to be required than previously reported to restore higher visual functions (Bartsch et al., 2008; Lakowski et al., 2010; Maclaren et al., 2006; West et al., 2008; West et al., 2010; Yao et al., 2010). Furthermore, the functionality of the transplanted cells needs to be assessed, as does the breadth of application of cell transplantation therapy. Robust integration in the degenerating retina must be demonstrated and the ability to restore higher visual functions must be achieved before photoreceptor replacement therapy can be seriously considered as a future treatment for retinal degenerative disorders.
1.8.1. DIFFICULTIES IN INTERPRETATION OF CELL INTEGRATION

Before addressing the key issue highlighted in the previous paragraph, it is worth noting that a common problem in the field has been the terminology used to describe successful transplantation. Indeed, the term ‘integrated’ has been used to describe transplanted cells showing full morphological integration through to geographic proximity to the host ONL.

Figure 1.12 illustrates some of the different interpretations of cell integration and successful transplantation. The images present in Figure 1.12a were taken from several reports where the authors suggested that transplanted donor cells were correctly integrated into the host retina (Klassen et al., 2004; Lamba et al., 2009; Ma et al., 2011; Tucker et al., 2011). Some of these studies suggested that these cells presented processes extending into the plexiform layer (Klassen et al., 2004; Ma et al., 2011), formed functional synapses (Lamba et al., 2009; Tucker et al., 2011) and developed segments structures that co-stained with phototransduction cascade markers (Tucker et al., 2011), statements that are not clearly represented in the images presented in the reports. The morphology of the transplanted cells is ambiguous and their location within the retina is disorganized (Figure 1.12a). Indeed, many of the cells described as integrated lacked clear mature photoreceptor morphologies, including synapse and segment structures, and co-staining with mature photoreceptor marker is not clearly demonstrated (Figure 1.12a i, ii, iv, v, vi). In addition, in the studies performed by Lamba et al (2009) and Tucker et al (2011), identification of the donor cell within the recipient retina is compromised. The former study relies on staining for dsRED, which in the images presented appears weak and possibly non-specific, as the entire ONL shows positive staining (Figure 1.12a iv; (Tucker et al., 2011)). The latter study used an adenovirus to drive GFP expression in the human-ES-derived donor cells prior to transplantation (Figure 1.12a iv, v and vi). The use of viruses to label
donor cells must be carefully controlled as any virus particles left in the donor cell media can transduce the endogenous recipient photoreceptors creating false positives, a phenomenon that has been observed in our lab (pers com Emma West). Furthermore, in many of the images it is difficult to distinguish the host ONL (Figure 1.12a ii and iii) and several of the images are not co-stained with DAPI to facilitate identification of the retinal laminar structure (Figure 1.12a iv, I and vi). In contrast, images presented in Figure 1.12b demonstrate transplanted cells with defined mature rod morphologies consisting of an easily identifiable cell body clearly located within the recipient ONL, synaptic-bouton like structures correctly located in the OPL and elongated segment structures extending into the subretinal space (Bartsch et al., 2008; Jiang et al., 2010; Maclaren et al., 2006; West et al., 2010; Yao et al., 2010).

Another potential pitfall in the interpretation of integration efficiency data is the methodology used to quantify the number of integrated cells. Several groups count the total number of integrated cells in alternate serial sections, a method that will account for the uneven distribution of the cell mass and integration across the retina (Jiang et al., 2010; Maclaren et al., 2006; West et al., 2010; Yao et al., 2010). However, other groups either failed to mention their method of quantification (Gust and Reh, 2011; Lamba et al., 2009) or assessed the number of integration by counting the number of cells located in the ONL within one microscopic field of view (Ma et al., 2011; Tucker et al., 2011). This method is very limited as it does not account for the very uneven distribution of integration and may easily introduce unintentional bias. Of equal importance is the exclusion criteria adopted when removing data values from a data set. In this report, exclusion criteria was based on where the cell mass was present in the subretinal space or whether any there was evidence of a severe immunological response (for further information of exclusion criteria, see Chapter 2.3.4.1). However, in one study, data was excluded if no integration was observed, despite the presence of a good cell mass and no clear evidence of donor cell mass or an acute recipient
immunological response (Gust and Reh, 2011). This may falsely increase the average integration efficiency.
(a) Examples of ambiguous donor cell integration where donor cell body location is not clearly located in the recipient ONL and integrated cell morphology is poor. (i) Transplantation of donor P1 cultured RPCs isolated from the cba.GFP^ve mouse into Rho^−/− recipient mice. Donor RPCs (green) were described to have migrated into all retinal layers and extended cellular processes within the recipient retina. The ONL was visualized by recoverin-positive staining (red). Adapted from Klassen et al., 2004. (ii), (iii) Transplantation of P1 cultured RPC isolated from the cba.GFP^ve mouse
into Rho<sup>−/−</sup> recipient mice. Scale bar: 20 μm. (ii) Integrated cells (green) were described as co-localising with recoverin (red) as indicated by the white arrow. Adapted from Ma et al., 2011. (iv) Immunocytochemical analysis performed on Rho<sup>−/−</sup> retinal degenerative eyes 21 days after receiving subretinal injections of mouse iPS derived rod precursor donor cells. Cells were generated from the dsRED mouse and identification of donor cells in the recipient retina relied on dsRED staining (red). Integrated cells were described to co-localise with recoverin (green). Scale bar 50μm. Adapted from Tucker BA, et al 2010. (v) and (vi) Examples of transplanted hES-derived rod precursor cells, described to have integrated into the adult Crx<sup>−/−</sup> recipient retina. To allow identification of donor cell post transplantation, a GFP-expressing virus was added to the culturing media. Adapted from Lamba, et al., 2009. (v) Integrated cell were described to co-localise with rhodopsin (red). (vi) Integrated cell were described to co-localise with human Nrl (red).

(b) Examples of clearly defined donor cell integration: donor cell body is clearly located in the recipient ONL and integrated cells adopt a mature rod morphology with well defined synaptic boutons in the OPL and segment structures in the IPM. (i) Examples of integrated Nrl.GFP<sup>+</sup> rod precursor donor cells transplanted into the Rd9 recipient mouse. Adapted from Yao, et al., 2010. (ii) Examples of integrated P1 RPC donor cells, cultured from the cba.GFP donor mouse, and transplanted into wildtype recipients. Integrated RPCs co-label with rhodopsin (red), and recoverin (blue). Adapted from Jiang, et al., 2010. (iii) P4 cba.GFP donor cell integrated into the wildtype recipient mouse demonstrating clearly defined donor cell bodies (full arrows) located in the ONL (data not shown) and synaptic bouton structure (small arrow heads) located in the OPL. Adapted from Bartsch et al., 2008. (iv) Post-mitotic Nrl.GFP rod precursor donor cells integrated into the wildtype ONL following subretinal transplantation. Adapted from West, et al., 2010. Scale bar 50μm. (v) P1 donor cells from the cba.GFP donor mouse integrated in the immature recipient wildtype retina. Adapted from MacLaren, et al., 2006.
1.8.2. IMPROVING TRANSPLANTATION EFFICIENCY

The proof-of-concept of photoreceptor transplantation has been demonstrated. However, to date the efficiency of photoreceptor transplantation remains low with only a few hundred to a few thousand cells integrating into the recipient (Lakowski et al., 2010; Lakowski et al., 2011; Maclaren et al., 2006; Pearson et al., 2010; West et al., 2008; West et al., 2010). Far greater numbers of successfully integrated cells are likely to be required in order to restore meaningful vision. There are a number of ways to maximize integration efficiency. Firstly, purifying the optimal donor cell population using fluorescent activated cell sorting (FACS) and optimizing transplantation techniques, such as cell density and exploring multiple transplantation sites, could improve efficiency. Secondly, transplant outcome is likely to be enhanced by modifying the recipient environment by targeting disruption physical barriers present in the host retina, such as the outer limiting membrane (West et al., 2008). In addition, the degenerating retinal host environment is likely to present some additional challenges for successful transplant outcome. Glial scaring, a hallmark of degenerative disorders, has been show to be inhibitory to neurite extension and regeneration in the CNS (Bradbury et al., 2002; Fawcett et al., 1989; Moon et al., 2001; Morgenstern et al., 2002; Smith-Thomas et al., 1994). A similar glial response in the retina can also be observed and it’s exact role in transplant outcome is yet to be clarified (Kinouchi et al., 2003; Zhang et al., 2003; Zhang et al., 2004) and destruction of the retinal cytoarchitecture of the host. In addition, the use of growth factors is likely to improve the migratory capacity of the donor cell population. Some of these issues will be further addresses in Chapter 3.
1.8.3. ASSESSING FUNCTIONALITY OF TRANSPLANTED CELLS

Considering the low integration efficiency reported in many studies, some impressive and perhaps questionable improvements in visual function has been reported. Currently, there is a lack experimental cohesion in the published reports, making it difficult to cross-reference reported findings from different research groups. Studies use different donor cell types and different recipient at a variety of ages, factors which are likely to affect transplantation and functional output efficiency. Many studies choose to test only one visual output (Klassen et al., 2004; Kwan et al., 1999; Lamba et al., 2009; Maclaren et al., 2006; Tucker et al., 2011) but a thorough assessment of transplanted photoreceptor function, using one type of donor cell and recipient, is required, starting from the level of the photoreceptor up to cortical responses in the brain and finally to demonstrate visual perception by assessing visually guided behavior. In addition, validation of the visual functional assays needs to be affirmed to address the question of how many cells are needed to generate a functional response; this will allow some comparison between research groups using different strategies. Chapter 3 aims to assess the functionality of transplanted photoreceptor cells along with correlating the number of cells required to generate responses in some of the tests used to measure visual output.
1.8.4. ASSESSING BREADTH OF APPLICATION

A fundamental question remains as to how broad an application photoreceptor transplantation therapy will have for the very heterogeneous degenerations encompassed within AMD and Retinitis Pigmentosa (RP). Until now, much of the work concerning photoreceptor transplantation as a strategy for retinal repair has used the wildtype retina (Bartsch et al., 2008; Chacko et al., 2000; Eberle et al., 2011; Gust and Reh, 2011; Kwan et al., 1999; Mizumoto et al., 2003; Pearson et al., 2010; Suzuki et al., 2007; Takahashi et al., 1998; West et al., 2008; West et al., 2009; West et al., 2010) or isolated models of degeneration as a recipient (Lakowski et al., 2011; Lamba et al., 2009; Maclaren et al., 2006; Mellough et al., 2007; Pearson et al., 2010; Singhal et al., 2008; Suzuki et al., 2007; Takahashi et al., 1998; Yao et al., 2010). In order for cell therapy to be established as a feasible approach to treat human retinal disease, photoreceptor transplantation must be assessed in different models of retinal degeneration. The degenerating retinal environment is very different from the wildtype retina and potentially hostile to transplanted cells (Vugler et al., 2007; West et al., 2009), which have to migrate from the site of transplantation, typically the subretinal space, across the inter-photoreceptor matrix and outer limiting membrane (OLM) and into the recipient outer nuclear layer (ONL) which undergoes extensive glial scaring. In addition, global cell death of endogenous photoreceptors results in changes to the remaining retinal cytoarchitecture and creation of a pro-inflammatory host environment. Different disease types are likely to present with very different micro-environments.

Moreover, photoreceptor replacement been proposed as a complementary therapy to treat late-stage disease, however, it is not known to what extent this is possible. Studies in the opossum demonstrated that transplanted neural progenitor cell integration efficiency declines as the recipient matures suggesting that the age of the recipient host may inhibit successfully cell migration (Sakaguchi et al., 2003; Sakaguchi
et al., 2005). Several studies to date have shown that integration into the adult wildtype retina can be achieved when using donor rod photoreceptor precursor cells (Maclaren et al., 2006), however, the impact of the age and degenerative status on transplant outcome has not adequately examined. Photoreceptor transplantation must be assessed at different stages of the degenerative process to address both the feasibility of application and identify any therapeutic time windows.
EXPERIMENTAL AIMS

While considerable progress has been made in recent years concerning the prospects for photoreceptor transplantation as a treatment for retinal degeneration, a number of very significant challenges and questions remain. This thesis addresses two of these:

1) Are transplanted photoreceptors capable of restoring visual function in the dysfunctional recipient retina?

2) Is photoreceptor transplantation efficiency equivalent across different retinal degenerations?

Question 1 is addressed in Chapter 3, with the following specific experimental aims:

(1) To optimise transplantation protocols and techniques in order to improve transplanted rod-precursor cell integration efficiency firstly into wildtype recipient retinae and then into a model of stationary night-blindness, the Gnat1−/− mouse. This will be done by assessing the impact of (i) donor cell injection concentration; (ii) multiple injection sites; (iii) recipient retinal injury, induced by a scleral stick wound and subretinal pre-detachment on transplantation efficiency; and (iv) purification of the donor cell population to include only Nrl.GFP+v+ve-rod precursor cells, using FACS.

(2) To assess the functional capabilities of integrated rod precursor donor cells in the Gnat1−/− mouse model of stationary night-blindness. This includes assessing the ability of transplanted cells to integrate and assume mature rod cell morphology, express appropriate components of the phototransduction cascade and respond to light in an appropriate manner.

(3) To determine whether integrated cells form functional synaptic connections to second order neurons within the recipient retina and whether visual signals generated
by integrated cells are propagated to higher visual areas and are able to drive visually-guided behavior.

Chapter 4 addresses the breadth of application of photoreceptor replacement therapy to ask whether disease type and degeneration state impact upon transplanted cell integration efficiency. To do this I will:

(1) Assess transplantation efficiency across 6 murine models of retinal degeneration at different stages of the disease process, both in terms of the total number of cells integrated into the recipient retina and the morphology of integrated cells.

(2) Assess key factors of the recipient microenvironment known to change during degeneration, namely ONL composition, glial scarring and OLM integrity, at all degeneration stages, to determine whether these correlate with transplantation outcome.

(3) Manipulate glial scarring and OLM integrity using enzymatic and molecular strategies, respectively, to determine their role in transplantation outcome and ask whether their disruption improves integration efficiency.
LIST OF ABBREVIATIONS

RP – Retinitis pigmentosa

AMD - Age-related macular degeneration

RPE - Retinal pigmented epithelium

ONL – Outer nuclear layer

INL – Inner nuclear layer

GCL – Ganglion cell layer

OPL – Outer plexiform layer

IPL – Inner plexiform layer

IPM – Interphotoreceptor matrix

RPC - Retinal progenitor cells

E - Embryonic day

P – Postnatal day

ECM – Extracellular matrix

CSPG - Chondroitin sulphate proteoglycans

CNS – Central nervous system

NS – neural stem cells

NP – neural progenitor cells

ES – embryonic stem cells

hES – human embryonic stem cells

iPS – induced pluripotent stem cells
CHAPTER 2

METHODS

2.1. ANIMALS

C57Bl/6J (Harlan, UK), Nrl.gfp+/+ (A. Swaroop, University of Michigan, USA (Akimoto, 2006)), Crb1<sup>rd/rd</sup> (Jackson Laboratory, (Mehalow et al., 2003)), Gnat<sup>+</sup>−<sup>−</sup> (J. Lem, Tufts University School of Medicine, USA, (Calvert et al., 2000)), Prph2<sup>rd2/rd2</sup> (G. Travis, UCLA (Connell et al., 1991; Travis et al., 1989)), Prph2<sup>Δ307</sup> (J. Farrar, Trinity College Dublin, Ireland (McNally et al., 2002)) PDE6β<sup>rd1/rd1</sup> (Harlan, Wyton, UK) and Rho<sup>−/−</sup> (P. Humphries, Trinity College Dublin, Ireland, (Humphries et al., 1997)) mice were maintained in the animal facility at University College London. All experiments have been conducted in accordance with the Policies on the Use of Animals and Humans in Neuroscience Research, revised and approved by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (Rockville, MD). Animals were maintained under standard cyclic lighting conditions (12 hour light-dark). Adult mice (male and female) were aged 6-8 weeks, unless otherwise stated. C57Bl/6J mice were used as wildtype controls. Nrl.gfp<sup>+/−</sup> were used as the donor cell source for transplantation.
2.2 TRANSPLANTATION

The transplant procedure is illustrated in the methods schematic presented in Figure 2.1.

2.2.1. DISSOCIATION OF RETINAL CELLS

Cells were prepared from postnatal day (P) 4-8 Nrl.gfp+/+ littermates. The mice were sacrificed by cervical dislocation and the neural retina was dissected. Cells were dissociated using a papain-based kit (Worthington Biochemical, Lorne Laboratories UK). Unless otherwise stated, flow cytometry (FACs) was used to isolate the GFP-positive rod precursor cell population. Dissociated cells were re-suspended at a concentration of 20,000,000 cells/mL in oxygenated EBSS containing 1% fetal calf serum (FCS) and stored on ice prior to FACs sorting. Cells were sorted under sterile conditions using the MoFLO DXP cell sorter (Beckman Coulter, High Wycombe UK). The sort uses sterile PBS and a sample pressure of 60psi and a sheath pressure of 60.8psi using a 70μm nozzle. Viability of both the original sample and FACS sorted sample was assessed at the end of the sorting session using Propidium Iodide (PI) to label dead and dying cells. Sorted cells were collected in EBSS containing 20% FCS and stored on ice before being spun at 150G for 12 minutes before re-suspending in re-suspension buffer (EBSS containing 0.005% DNAse) prior to transplantation. Cells were stored on ice until transplantation. Unless otherwise stated, cells were re-suspended at a concentration of 200,000 live cells/μl using a re-suspension buffer (EBSS containing 0.005% DNAse). Cell viability counts were performed using trypan blue and the Sceptre handheld cell counter (Millipore, Watford, UK).
i. Dissect central neural retina

ii. Papain dissociation

iii. Subretinal transplants
200,000 live cell/μl

iv. Terminate 3 weeks post transplant

v. Quantify the number of integrated cells
Donor cells are harvested from the central neural retina of P4-8 Nrl.GFP\textsuperscript{+/+} pups (i) and dissociated using papain (ii). Either FACS-sorted purified GFP\textsuperscript{+/+} rod precursor cells or an unsorted mixed population of dissociated retinal cells are transplanted subretinally at a density of 200,000 live cells per µl in recipient mice (iii). Recipient animals are sacrificed 3 weeks post transplant (iv) and the number of Nrl.GFP\textsuperscript{+/+} donor cell correctly integrated in the ONL of the recipient retina is quantified as illustrated in (v) whereby a schematic of integrated cells are shown on the right panels and a representative confocal image is shown on the left.
2.2.2. TRANSPLANTATION PROCEDURE

Surgery was performed under direct ophthalmoscopy through an operating microscope, as previously described (Maclaren et al., 2006). Briefly, recipient mice were anaesthetised using a reversible anaesthetic regime. A single intra-peritoneal injection of 0.2 ml of a mixture of Dormitor (1 mg/ml medetomidine hydrochloride, Pfizer Pharmaceuticals, Kent UK), ketamine (100 mg/ml, Fort Dodge Animal Health, Southampton, UK) and sterile water for injections in the ratio of 5:3:42 for adult mice. Recipient mice were reversed using antisedan.

The eye was dilated using tropicamide (Bausch&Lomb, Surrey UK) and kept moist with viscotears (Dr Winzer Pharma GmbH, Berlin Germany). Where stated, a scleral puncture wound was performed at the level of the anterior chamber using a sterile 8 mm 34-gauge hypodermic needle (Hamilton, Switzerland). Using the same hypodermic needle, cells were transplanted via a single subretinal injection (1 µl, unless otherwise stated), made at an oblique angle through the superior sclera into the subretinal space, and injected slowly to produce a standard and reproducible retinal detachment in the superior hemisphere. The needle was slowly withdrawn, leading to a self-sealing of the wound tunnel (Figure 2.1, iii)

Chloramfenicol (Martindale Pharmaceuticals, Romford UK) was used as post-operative care for the eye and animals were kept warm during the procedure to minimize the development of cataract. Animals were sacrificed by cervical dislocation 3 weeks post cell transplantation, unless otherwise stated.
2.2.3. INTERVENTIONS

2.2.3.1. siRNA ZO-1
Targeting siRNA against ZO-1 was generated using the sequence 5’ AAGATAGTTTGGCAGCAAGAG 3’ (Invitrogen, UK) as reported previously (Sourisseau et al., 2006). Sterile buffer containing oligofectamine was used to re-suspend the siRNA to the appropriate concentration using RNAase-free plasticware. A proven non-targeting siRNA (AllStars negative control siRNA; cat. no. 1027281; Qiagen, UK), or vehicle (buffer + oligofectamine) was used for control experiments at the equivalent concentration and volume. siRNAs were introduced by subretinal injection (1.5µl) to the superior retina 48 hrs prior to cell transplantation.

2.2.3.2. Chondroitinase ABC
ChABC was prepared and administered according to previous reports (Singhal et al., 2008; Suzuki et al., 2007). Briefly, ChABC was made up in TBS according to manufacturer’s preparation instructions (Sigma-Aldrich, USA). ChABC was reconstituted in 0.01% bovine serum albumin (BSA) aqueous solution. Immediately prior to use, subsequent dilutions were made with buffer (50nM Tris, 0.02% BSA), containing 60mM sodium acetate to activate the ChABC. The buffer solution was adjusted to the optimal pH (8.0) for ChABC activity: ChABC was added to the cell suspension medium at a final concentration of 0.025U/µL, containing 0.005% DNAse.
2.2.4. SLO IMAGING

A HRA2 scanning laser ophthalmoscope (SLO (Heidelberg engineering, Heidelberg, Germany)) with a 55° angle lens was used to perform fluorescence imaging, a method previously described (Luhmann et al., 2009). Briefly, a 488 nm laser was used to scan the retina. The optic disc was positioned at the center of the image and the image focused on the outer retina. Projection images of 30 frames per fundus were taken and evaluated for the appearance of fluorescence.
2.3. Histology, Microscopy & Western Blot

2.3.1. Immunohistochemistry and Histology

2.3.1.1. Immunohistochemistry

For counting, eyes were enucleated and the cornea and lens removed before fixation with paraformaldehyde (PFA). For immunohistochemistry, a corneal burn was used to identify the superior retina before fixation. See Table 1 for specific fixation regimes. The eyecup was cryoprotected before embedding in optimal cutting temperature (OCT) embedding medium (TissueTek, Thermo Fisher Scientific UK) using isopentane and liquid nitrogen.

Serial coronal sections were cut at 20µm thick for counting and sagittal sections were cut at 15µm thick for immunohistochemistry and affixed to super-frosted poly-lysine coated slides (Thermo Scientific, UK). Retinal cryosections were air-dried for 15-30 mins and frozen at -20°C. For immunohistochemistry, retinal cryosections were thawed at room temperature for 30 mins prior to staining. Some antibody protocols required post-fixation and in these cases sections were incubated with PFA at this point.

Sections were washes with Tris-buffered saline (TBS) and incubated in blocking solution (see Table 1 for specific blocking buffers) for 2 hours at room temperature and then incubated with primary antibody at 4 °C overnight. Sections were washed 5x 5 mins with TBS and incubated with secondary antibody for 2 hours at room temperature (RT). Again sections were washes 5x 5mins with TBS before counter-staining with Hoechst 33342 (stock solution 5mg/mL diluted 1:1000 with TBS for working dilution). Negative controls were treated identically and in parallel except the primary antibody was omitted. The following antibodies and staining protocols were used:
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fixation</th>
<th>Blocking solution with TBS</th>
<th>Primary Ab concentration</th>
<th>Secondary Ab concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal anti-GFAP</td>
<td>4% PFA, 1hr</td>
<td>2% NGS, 1% BSA, 0.05% Triton-X</td>
<td>1:500</td>
<td>Gt-anti-rabbit, Alexa 546, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in rabbit DAKO Z0334</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal anti-Vimentin</td>
<td>No fixation, 1% PFA post fix 5 mins</td>
<td>5% NGS, 2.5% BSA, 0.5% Triton-X</td>
<td>1:200</td>
<td>Gt-anti-Mouse, Alexa 488, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in mouse SIGMA V5255</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal anti-CS56 (CSPGs)</td>
<td>No fixation.</td>
<td>1% NGS, 5% Milk, 0.05% Triton-X</td>
<td>1:200</td>
<td>Gt-anti-Mouse, Alexa 488, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in mouse SIGMA C8035</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Polyclonal anti-ZO-1</td>
<td>1% PFA, 30min</td>
<td>5% NGS, 2% BSA, 0.05% Triton-X</td>
<td>1:250</td>
<td>Gt-anti-rabbit, Alexa 546, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in rabbit ZYMED</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal anti-CtB2 (ribeye)</td>
<td>4% PFA, 15min.</td>
<td>1% NGS, 1% BSA, 0.1% Triton-X</td>
<td>1:500</td>
<td>Gt-anti-Mouse, Alexa 546, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in mouse BD Bioscience 612044</td>
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<td>Polyclonal anti-Rod Transducin (gnat1)</td>
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<td>1:1000</td>
<td>Gt-anti-rabbit, Alexa 546, 1:500 Molecular Probes</td>
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<tr>
<td>Monoclonal anti-Rho4D2</td>
<td>4% PFA, 1hr</td>
<td>1% NGS, 1% BSA, 0.1% Triton-X</td>
<td>1:250</td>
<td>Gt-anti-Mouse, Alexa 546, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in mouse Gift from Professor Robert Molday</td>
<td></td>
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</tr>
<tr>
<td>Antibody</td>
<td>Fixation</td>
<td>Blocking solution with TBS</td>
<td>Primary Ab concentration</td>
<td>Secondary Ab concentration</td>
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<td>------------------------------</td>
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</tr>
<tr>
<td>Polyclonal anti-Peripherin2</td>
<td>4% PFA, 15 min</td>
<td>1% NGS, 1% BSA, 0.1% Triton-X</td>
<td>1:1000</td>
<td>Gt-anti-rabbit Alexa 546, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in rabbit Gift from Professor Gabriel Travis</td>
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<td>2% NGS, 1% BSA, 0.05% Triton-X</td>
<td>1:2000</td>
<td>Gt-anti-Mouse Alexa 546, 1:500 Molecular Probes</td>
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<tr>
<td>Produced in mouse Stressgen VAM-PS003</td>
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<tr>
<td>Polyclonal anti-Arrestin</td>
<td>1% PFA, 30 min</td>
<td>2% NGS, 1% BSA, 0.05% Triton-X</td>
<td>1:500</td>
<td>Gt-anti-rabbit Alexa 546, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in rabbit ThermoScientific PAI-731</td>
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<tr>
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<td>1% PFA, 15 min</td>
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<td>1:1000</td>
<td>Gt-anti-rabbit Alexa 546, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in rabbit Gift from Jan Wijnholds</td>
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<td></td>
<td></td>
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<tr>
<td>Polyclonal anti-B-catenin</td>
<td>4% PFA, 1 hr</td>
<td>5% NGS, 2% BSA, 0.1% Triton-X</td>
<td>1:2000</td>
<td>Gt-anti-rabbit Alexa 546, 1:500 Molecular Probes</td>
</tr>
<tr>
<td>Produced in rabbit ABCAM Ab6302</td>
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</tr>
</tbody>
</table>
**TABLE 2.1. ANTIBODY PROTOCOLS**

NGS: Normal goat serum (Abd serotec, Oxford UK). BSA: Bovine Serum Albumin (Sigma Aldrich, Dorset UK).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fixation</th>
<th>Blocking solution with TBS</th>
<th>Primary Ab concentration</th>
<th>Secondary Ab concentration</th>
</tr>
</thead>
<tbody>
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<td>5% NGS</td>
<td>1:500</td>
<td>Gt-anti-rabbit Alexa 546,</td>
</tr>
<tr>
<td>Produced in Rabbit SIGMA C3678</td>
<td>1 hr</td>
<td>2% BSA</td>
<td></td>
<td>1:500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1% Triton-X</td>
<td></td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Polyclonal anti-rabbit PKC-α</td>
<td>4% PFA</td>
<td>2% NGS, 1% BSA</td>
<td>1:500</td>
<td>Gt-anti-rabbit Alexa 633,</td>
</tr>
<tr>
<td>Produced in Rabbit ABCAM</td>
<td>1 hr</td>
<td>0.05% Triton-X</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>
2.3.1.2. TUNEL staining for apoptotic cell bodies

TUNEL staining using an Apoptosis Detection kit according was performed according to the manufacturer’s instructions (Chemicon Apoptosis Detection Kit, S7165, Millipore, USA). Briefly, retinal cryosections were fixed with 1% PFA for 10 mins at RT. After washing with PBS, tissue was post-fixed in pre-cooled ethanol:acetic acid (2:1) for 5 mins. Following a series of washes, equilibration buffer* was applied for 10 seconds at RT prior to incubation with TdT enzyme* at working strength for 1 hour in a humidified chamber. Sections were then submerged in stop/wash buffer* for 10 min. After washing, sections were incubated with anti-digoxigenin conjugate* in a humidified chamber for 30 mins before further washing and counter staining with Hoechst 33342.

* reagents supplied in kit.

2.3.1.3. H&E staining

For histological assessment, eyes were enucleated while leaving the superior rectus muscle in place to provide a landmark for the superior retina. Tissue was fixed in buffered formalin overnight at 4°C. Retinal sections were prepared by step-wise dehydration in isoproponal prior to paraffin embedding (Histocentre). Sections (5 µm thick) were cut in a nasal-temporal direction and affixed to glass slides. Staining using standard haematoxylin and eosin protocols was performed.
2.3.2. ELECTRON MICROSCOPY

2.3.2.1 EM for transplanted cell synapse analysis

For ultrastructural analyses, trans-cardiac perfusions were performed on anesthetized animals via the ascending aorta. Animals were perfused with: (1) 10 ml of heparin saline (1,000 units/ml), (2) 20 ml of 4% PFA/3.75% acrolein (Polysciences, Warrington, PA) in 0.1 M phosphate buffer (pH 7.4), (3) 60 ml of 4% PFA in 0.1 M phosphate buffer. Eyes were enucleated and the eye-cups were fixed with 4% PFA/0.1% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) overnight. Eye-cups were transported to our collaborators at Cornell University, USA for further preparation, imaging and analysis by Sung C.H. and Chuang J.Z. The methods used are described briefly below.

Eye cups were embedded in agarose embedding and sectioned using a vibratome. The free-floating method described by Chuang et al. (2001, 2007) was used for peroxidase immunolabeling. GFP antibody was used to label Nrl.GFP\textsuperscript{\textpm} transplanted cells and a biotinylated D-anti-rabbit secondary was used and visualised with ABC/DAB. All immunolabelled sections were examined by a Leica TCS SP2 microscope (Nussloch, Germany). Immunoreactivity appears as dark precipitation. For ultrastructural analysis, retinal sections were permeabilized by the “freeze-thaw” method, immunolabelled with GFP antibody and post-fixed with 2% glutaraldehyde for 10 mins after incubation with a biotinylated D-anti-rabbit secondary antibody incubation. The immunolabelled sections were visualised using ABC/DAB. Finally, the sections were then flat embedded in Epon resin, and ultrathin sections (70nm thick) were cut. The most superficial sections were counterstained and examined on a Philips CM10 electron microscope. Negative controls were performed in parallel where application of the primary antibodies was omitted.
2.3.2.2. EM analysis of the OLM

Mice were sacrificed and the eyes were fixed (3 % glutaraldehyde / 1 % PFA buffered to pH 7.4 with 0.08 M sodium cacodylate-HCl) for a minimum of 12 hours at 4ºC. Eye cup dissections were performed by removing the cornea and lens and a nasal stitch was made to orientate the eye cups. Following a 15 minute washing step with 2.5% glutaraldehyde buffered to pH 7.4 with 0.1 M cacodylate, specimens were processed as previously described (Tschernutter et al., 2005; West et al., 2008). Briefly, eye-cups were post fixed in the dark for 2.5 hours with 1% aqueous solution of osmium tetroxide. Specimens were then dehydrated using a series of ascending ethanol solutions (from 50-100%) in 10 minute steps with rotation, followed by 2 x 10 minute changes of 100% ethanol and 3 x 10 minutes submersion in 1,2-epoxypropane. Specimens were maintained in a 50:50 mixture of 1,2-epoxypropane and araldite resin for a minimum of 3 hours with rotation, then the submersion solution was changed to fresh araldite resin and maintained for 5 hours with rotation. Eye-cups were embedded and cured for 48hr at 60ºC. Semi-thin sections (0.7µm) and ultrathin sections (0.07µm) were cut using an appropriate diamond knife fitted on a Leica ultracut S microtome (Diatome histoknife Jumbo and Diatome Ultrathin, respectively). Ultrathin sections were collected on copper grids (100 mesh, Agar Scientific), contrast-stained with 1 % uranyl acetate and lead citrate and analysed using a JEOL 1010 Transmission Electron Microscope (80 kV), fitted with a digital camera for image capture. 1% toluidine blue was used to stain semithin sections and imaged using a Leitz Diaplan microscope fitted with a digital camera (DC 500).
2.3.3. Confocal Microscopy

Retinal sections were imaged on a confocal microscope (Leica TCS SPE, Leica microsystems, Milton Keynes, UK). Single confocal sections or merged projection images of a xyz confocal stack are shown. Confocal stacks were approximately 10µm thick with a step size of approximately 1µm. Hoechst 33342 was excited by the 405nm laser, Alexa-488 fluorophore using the 488nm laser and the Alexa-546 fluorophore using the 543nm laser. Images were taken at a resolution of 1024x1024. All images examining glial scaring, OLM integrity, ONL thickness and density presented in Chapter 4 were taken from a standardized location superior to the optic nerve. Where possible, all images showing GFAP, vimentin, CS-56 and ZO-1 presented in Chapter 4 were taken using the same laser intensity, gain and offset settings.

2.3.4. Cell Counts

Counts of integrated cells were taken 3 weeks after transplantation using a fluorescence microscope (Observer Z.1, Zeiss, UK), unless otherwise stated. The use of the Nrl.gfp+/ mouse as a source of donor cells provides a genetic marker for the identification of rod photoreceptors (Akimoto et al., 2006). The average number of integrated cells per eye was determined by counting all the integrated GFP-positive cells in alternate serial sections through each eye. This was doubled to give an estimate of the mean number of integrated cells per eye. Please note that this methodology of counting may result in an overestimation of the total cell number owing to the possibility of double counting one cell in separate sections. Cells were considered to be integrated if the whole cell body was correctly located within the outer nuclear layer, and at least one of the following was visible; spherule synapse, inner/outer processes, inner/outer segments.
2.3.4.1. Exclusion criteria

Animals were omitted from quantification analysis only if there was clear evidence of (1) a transplant occurring intravitreally, rather than subretinally, or (2) if no cell mass (<200 cells) was evident in the subretinal space at the time of counting, an indication that complete reflux of the donor cell suspension had occurred at the time of injection or (3) if eyes demonstrated evidence of significant inflammation or acute immune rejection, as described previously (West et al., 2010, Figure 2.2). Briefly, significant inflammation or immune rejection is indicated by a large influx of auto-fluorescent macrophages within the subretinal space and host retina; this is often accompanied by decreased Nrl.GFP expression in the donor cell mass and the presence of autofluorescent cell debris, indicative of cell death, and morphological disturbances in the recipient retina, including retinal folding and thinning (Figure 2.2, iii). Approximately 22% of wildtype recipients exhibit this morphological appearance, which was accompanied by a significant decline in integration efficiency (West et al., 2010).
This grading scheme was described by West E.L., et al., 2010. Activated tissue macrophages are stained with CD68 (red). Few subretinal macrophages are demonstrated in Grade 1 (i), indicating a lack of immunological response. Several subretinal macrophages are demonstrated in Grade 2 (ii). Grade 3 indicates acute immune rejection at 1 month post transplant and transplanted animals with this appearance would be excluded from analysis. Grade 3 includes accumulation of subretinal and retina macrophages, a reduction in GFP expression in the donor cell mass and disturbances in host retinal morphology.
2.3.4.2. Apoptotic cell counts

TUNEL-staining was used to quantify the number of apoptotic cells counting all TUNEL-positive profiles in each layer of the retina in alternate serial sections. Only sections that encompassed the site of subretinal injection were used, and are thus not representative of apoptosis in the whole eye.

2.3.4.3. Cell count statistics

All data was presented and analysed in keeping with previous reports (Lakowski et al., 2011; Maclaren et al., 2006; West et al., 2008; West et al., 2010). All means are stated ± the standard error of the mean (SEM), unless otherwise stated. N= number of eyes examined, where appropriate. For assessment of integration efficiency, statistical analysis is based on at least two independent transplantation sessions (encompassing cell preparation, FACs sorting and transplantation). Statistical significance was assessed using Graphpad Prism 5 software (Graphpad Inc, La Jolla, USA). P values are presented as P<0.05 = *, p<0.01 = ** and p<0.001 = ***. All data was normally distributed, as assessed using the d'Agostino and Pearson omnibus normality test. Appropriate parametric tests were applied including student t-test when comparing 2 groups; ANOVA with Dunnett’s (when comparing treatment to control) or Tukey’s (inter-group comparison) correction for multiple comparisons, and Pearson’s correlation where appropriate.
2.3.5. **HISTOLOGY ASSESSMENTS: ONL, SEGMENTS & SYNAPSES**

2.3.5.1. **Outer nuclear layer assessment: thickness & density**

ONL thickness measurements presented in Chapter 3 were recorded from image taken at x40 magnification on a Leica fluorescence microscope captured by ImagePro software. Images were taken from 5 regions around the injection site, as indicated by the cell mass. Control images were taken from the same eye but at a site away from the injection site on the opposite hemisphere. ONL thickness was measured at three points in each image for each site. These values were pooled to give an average thickness, which was normalized to the average ONL thickness at the control region. Similarly, ONL measurements were taken from uninjected wildtype mice and normalized in the same fashion.

The ONL thickness measurements presented in Chapter 4 were assessed using ImageJ software from single section confocal images taken at x80 magnification. Measurements were taken from 3 regions of the superior central retina, giving an average ONL thickness. Average ONL measurements were obtained from three independent animals, giving an overall average ONL thickness for that model at a given age. The rate of degeneration was calculated using the overall average ONL thickness measurements: the total loss of ONL thickness between early and late stage degeneration was divided by the number of days over which the degeneration had taken place. ONL density analysis was performed using single section confocal images taken at x80 magnification from one field of view of the superior central retina for three independent animals. ONL density was calculated by counting the number of cell nuclei within the ONL, normalised to the area of the ONL as measured by ImageJ, and expressed as the number of nuclei per 100µm². Measurements were taken using ImageJ software.
Statistics

All means are stated ± the standard error of the mean (SEM), unless otherwise stated. N= number of eyes examined, where appropriate. Statistical significance was assessed using an AVONA with Dunnett’s (comparing against wildtype) or Tukey’s (inter-group comparison) correction for multiple comparisons using Graphpad Prism 5 software (Graphpad Inc, La Jolla, USA). P values are presented as P<0.05 = *, p<0.01 = ** and p<0.001 = ***

2.3.5.2. Outer segment and synapse assessment

In Chapter 4, an estimate of the number of segments and synapses formed by integrated Nrl.GFP⁺⁺-rod-photoreceptors was achieved by counting 100 integrated cells randomly selected from regions across the whole integration site, from 3 independent recipient retinæ. The percentage of integrated cells that had developed segments and synapses was calculated. Note that this is likely to be an underestimate because only a proportion of integrated cells will be in perfect alignment with the plane of sectioning.

Statistics

All means are stated ± the standard error of the mean (SEM), unless otherwise stated. N= number of eyes examined, where appropriate. Statistical significance was assessed using AVONA with Dunnett’s (comparing against wildtype) correction for multiple comparisons using Graphpad Prism 5 software (Graphpad Inc, La Jolla, USA). P values are presented as P<0.05 = *, p<0.01 = ** and p<0.001 = ***
2.3.6. Western Blot Assessment

Neural retinæ were dissected from 4 mice per time point and snap frozen in liquid nitrogen. Retinal tissue was lysed in RIPA buffer (Sigma Aldrich, Gillingham, UK), then cell membranes were disrupted using a sonicator with micro-tip (Soniprep 150, MSE London, UK). Equal amounts of protein (20 µg in Laemmli’s loading buffer, unless otherwise stated) were run on a 12% sodium dodecylsulfate–polyacrylamide gel. The separated proteins were electrotransfered to PVDF membranes (Millipore Watford, UK). Membranes were incubated with blocking solution for 1 hour at room temperature and incubated with primary antibody overnight at 4 °C, diluted in blocking solution. After washing in PBST, membranes were incubated with secondary antibody for 2 hours. Chemiluminescence detection was performed using a Fujifilm LAS-1000 Luminescence Image Analyser after incubation with enhanced luminescence reagent (ECL plus GE Healthcare UK Ltd. Amersham, UK).

2.3.6.1. GFAP

Western blotting for GFAP was performed by Dr Claire Hippert. For assaying GFAP expression from the different mice models, neural retinas from 4 mice per time point were harvested. Samples were run on a 12% sodium dodecylsulfate–polyacrylamide gel. Blocking solution: 5% (w/v) non fat milk, 1% (w/v) BSA in PBS 0.05% Tween, Primary antibody: 1:2500 goat-anti-rabbit GFAP (Dako, Cambridge, UK) diluted with GFAP blocking solution and 1:5000 mouse-anti-mouse Histone H2B (Biolabs, Wilbury, UK) diluted in 5% (w/v) BSA in 0.05% PBST. Secondary antibody: 1:5000 – 1:10000 (Pierce Immunopure goat-anti-rabbit and anti-mouse IgG (H+L) HRP conjugated, Perbio Science UK Ltd., Northumberland, UK). Band intensities were quantified using Image J software and normalized to H2B levels.
2.3.6.1. CSPG

For assaying CSPG expression, neural retinas from at least 3 Rho<sup>−/−</sup> mice were harvested. Due to the nature of CSPG, a non-reducing loading buffer was used (Kinouchi et al., 2003). Samples were run on a 5% sodium dodecylsulfate–polyacrylamide gel. Blocking solution: 5% (w/v) non-fat milk, in PBS 0.05% Tween. Primary antibody: 1:1000 goat-anti-mouse CS-56 (Sigma, UK) diluted with blocking solution and 1:5000 goat anti mouse β-actin (Sigma, UK) diluted blocking solution.
2.4. FUNCTIONAL ASSESSMENTS

Assessment of visual function was performed within the first 8 hours of the light cycle. In order to test rod photoreceptor function, scotopic lighting conditions were employed based on previous reports assessing murine vision, which indicated that light intensities below 0.01 cd.s/m² are considered within the scotopic range (Cachafeiro et al., 2010; Toda et al., 1999). Where appropriate, all procedures carried out prior to testing were performed under dim red light, which is reported to be below the spectral sensitivity of murine detection (Govardovskii et al., 2000). In some of the experiments presented (watermaze testing, optomotor, intrinsic imaging), light intensity was adjusted using neutral density filters where appropriate and stimulus intensities were confirmed by measuring light levels with a IL700 photometer. Under all circumstances, the light intensity was below 0.007 cd.s/m², well within the scotopic range. Untreated Gnat1−/− control mice were tested at these same intensities to confirm an absence of visual responses.
2.4.1. SINGLE CELL RECORDINGS

Animal were transplanted here in the UK and transported to our collaborators at the Johns Hopkins University School of Medicine, Baltimore USA for single cell recording. These experiments were performed by Xue T. and Yau K.W., and the methods they used are described below.

Mice were dark-adapted overnight, anesthetized by Avertin, and enucleated before euthanasia. Each eye was hemisected and the retina removed. The retina, with 3-4 radial cuts around the periphery, was flat-mounted photoreceptor-side up on a GSTF Millipore filter paper and placed in bicarbonate-buffered Ames medium (Sigma-Aldrich) before being chopped into 100-µm slices with a custom-built tissue chopper. The retinal slices together with the underlying filter paper were transferred and mounted sideways in the recording chamber. All procedures were performed in infrared or dim-red light.

Recordings were carried out at physiological temperature, ~35-37 °C, on a Zeiss upright microscope with infrared DIC optics and imaging. The bath solution (bicarbonate-buffered Ames medium equilibrated with 5% CO2/95% O2) was temperature-controlled and run at ~5 ml/min through the 1-ml experimental chamber. Temperature was monitored by a thermistor in the chamber. Integrated Nrl.GFP+ve-rods were identified by their GFP-fluorescence. Data presented is from Nrl.GFP+ve-rod-photoreceptors correctly located within the ONL with an intact inner segment, cell body and synaptic terminal. A total of 9 Nrl.GFP+ve cells were recorded. The number of cells examined for each group was not always because not all parameters were obtainable for each cell. In order to minimize rhodopsin bleaching during fluorescence-based cell-finding, excitation light intensity (450-490 nm) was kept low and the GFP signal was visualized with a highly-sensitive EM-CCD camera (Lucas, ANDOR). The image sampling of the camera was also synchronized with the exciting flash (30 msec). The infrared DIC image of the retinal slice was overlaid with the GFP-fluorescence image to
guide the recording pipette. Despite these measures, the Nrl.GFP	extsuperscript{+ve}-rods were still desensitized by the GFP-excitation light (63.2 µW 450-490nm, ~1.5 sec in total). Accordingly, for all of the experiments reported here, 100-µM 9-cis-retinal (a readily available analog of 11-cis-retinal; Sigma) in 1% BSA-Ames solution was run through the chamber for 1 hour in complete darkness before light stimulation of the recorded cell began.

The recording configuration was modified from a previously-described procedure (Fu et al., 2008; Nikonov et al., 2006). The Nrl.GFP	extsuperscript{+ve}-rod soma was drawn into a snug-fitting glass pipette with a 3-4 µm opening at its tip and containing HEPES-buffered Ames solution, which was made from Ames stock (without NaHCO3, Sigma-Aldrich) with additional 10mM HEPES and 15mM NaCl, pH 7.4 by NaOH. The osmolarity was adjusted to 283 mOsm/L by glucose to match the bicarbonate-buffered Ames. The recorded rod was stimulated with an LED light (λ\textsubscript{max} at 505 nm, with 30-nm bandwidth) delivered through a light guide into the microscope epifluorescence port. The light intensity and duration (10 msec) were controlled by custom circuitry and flashes were delivered at time zero. The intensity of the light source was periodically calibrated with a radiometer. For flash response family recordings (Chapter 3, Figure 3.5A), the flash intensity was calculated retrospectively to adjust for the difference in spectral and response sensitivity as described below. The flash intensities were: 1.1, 8.8, 57, 280, 1403 and 4962 photons µm\textsuperscript{-2} for dark-adapted wildtype; 50, 244, 1,221, 4,318 and 15,973 photons µm\textsuperscript{-2} for bleached/regenerated wildtype rod and 222, 854, 2,675, 9,257, 26,862, 75,701 and 229,766 photons µm\textsuperscript{-2} for bleached/regenerated Nrl.GFP	extsuperscript{+ve}-rod.

Membrane current was measured with a current-to-voltage amplifier (Axopatch 200B, Axon Instruments). All signals were low-pass filtered at 20 Hz (8-pole Bessel) and sampled at 500 Hz. The response–intensity relationship at the transient peak of the response was fit with the Michaelis equation, \( R = R_{\text{max}} \left( I / (I + \sigma) \right) \), where \( R \) is transient-peak response amplitude, \( R_{\text{max}} \) is saturated peak-response amplitude, \( I \) is flash
intensity, and $\sigma$ is the half-saturating flash intensity. Rod sensitivity is inversely proportional to $\sigma$. The following parameters were measured: Time-to-peak (the time lapse from flash to transient peak of dim-flash response); the integration time, $t_i$, of the dim-flash response (a measure of its overall duration, and is given by $t_i = \int f(t)dt/f_p$, where $f(t)$ is the response profile and $f_p$ is its transient-peak amplitude); half-saturating flash intensity (i.e., intensity that elicited a half-maximum response); and $\sigma$, which is inversely proportional to sensitivity. The LED light source was converted to “equivalent” monochromatic light with the $\lambda_{\text{max}}$ of the respective visual pigment for data analysis and display. For rods without bleaching, the LED light was converted to equivalent 498nm light, the $\lambda_{\text{max}}$ of 11-cis-rhodopsin. The GFP epifluorescence-excitation light (see above) bleached over 99.9% rhodopsin (calculated by equation in (Luo and Yau, 2005)). So for bleached/regenerated rods, the light was converted to equivalent 485nm light, the $\lambda_{\text{max}}$ of 9-cis-rhodopsin. The conversion was done by using the normalized action spectrum of rhodopsin, $f(\lambda)$ with $\lambda_{\text{max}}$ of 498nm and 485nm, respectively, and the power-scaled spectrum of the LED light, $L(\lambda)$. The conversion ratio is:

$$Power_{\text{equivalent } \lambda_{\text{max}} \text{ light}} = Power_{\text{ratio}} \times Power_{\text{light with } L(\lambda) \text{ spectrum}}$$

where

$$Power_{\text{ratio}} = \frac{hc}{\lambda_{\text{max}}} \frac{\int L(\lambda) \times f(\lambda) \, d\lambda}{\int L(\lambda) \, d\lambda} = \frac{\int (\lambda \times L(\lambda) \times f(\lambda)) \, d\lambda}{\lambda_{\text{max}} \int L(\lambda) \, d\lambda}$$

Statistics

Data is presented as mean ± SEM, except the sensitivity ($\sigma$) data which was presented as median + range. N= number of cells examined as indicated in parentheses. Statistical significance was assessed using the ANOVA test, or Kruskal Wallis test for the sensitivity ($\sigma$) data, analysed using Origin (Origin Lab Corp.) P values are presented as $P<0.05 = \ast$, $P<0.01 = \ast\ast$ and $P<0.001 = \ast\ast\ast$
2.4.2 ELECTRORETINOGRAM RECORDING

Electroretinogram (ERG) recordings utilize extracellular currents that are generated when a retina responds to light stimulation. Electrical currents generated by the retina spread along the extracellular matrix and can be measured at the cornea. Full field flash ERGs record the mass electrical response generated in the retina. Flashes of light typically elicit a biphasic waveform as recorded by electrodes at the corneal surface. This is comprised of two major components: the negative a-wave, and the positive b-wave (Figure 2.3). The a-wave directly reflects photoreceptor function, whereas the b-wave reflects the function of inner retinal cells including ON bipolar cells. Measuring the amplitude of the wave peaks (Figure 2.3) provides a quantitative assessment of function.
FIGURE 2.3. ELECTRORETINOGRAM ORIGINS AND WAVEFORMS

(a) Schematic of the retina indicating that the a-wave is generated by photoreceptors, whereas the B-wave is generated by inner retinal cells, including ON-bipolar and Müller cells. (b) Example ERG traces illustrating the characteristic biphasic appearance of ERG recording (top) and how the amplitudes are measured (bottom). The negative A-wave amplitude is measured above baseline, while the positive B-wave amplitude is measured below baseline.

Adapted from: [http://webvision.med.utah.edu](http://webvision.med.utah.edu).
Animals were dark-adapted overnight and all subsequent procedures were performed under dim red light. Following anesthesia (using the Ketamine/Dormitor regime described in section 2.2.2), the pupils were dilated using Tropicamide 1%. Viscotears were placed on each cornea to keep it moistened after corneal contact electrodes and midline subdermal reference and ground electrodes were placed. Animals were left for a further 10 mins in complete darkness prior to recording. The use of a thermostatic heat mat and the effect of increasing anesthesia dosage by 10 % of the dose stated in section 2.2.2 was examined in Chapter 3.

ERGs were recorded using an Espion E2 system with ColourDome stimulator (Diagnosys LLC, Lowell, MA). Ganzfeld ERGs were obtained simultaneously from both eyes to provide an internal control. Single flash recordings were obtained at light intensities of 0.003, 0.007, 0.03, 0.5 and 3 cd.s/m$^2$ presented in a ganzfeld dome using a sampling frequency of 5 kHz, a flash duration of 4 ms, and a frequency stimulus of 0.5 Hz. Data were recorded from 10 ms before stimulus onset to 400 ms post-stimulus. The bandpass filter was set between 0 and 1 kHz. Ten responses per intensity level were averaged with an interstimulus interval of 10 s (0.003, 0.007, and 0.03 cd.s/m$^2$) or a 20 s interstimulus interval (0.5 and 3 cd.s/m$^2$). For analysis, the a-wave and b-wave amplitudes (measured from the a-wave trough to b-wave peak) of the treated eyes were paired with the contralateral control eyes.

ERGs were recorded prior to and at 3, 4, 5 & 6 weeks after transplantation. Test eyes received dual (superior and inferior) subretinal injections of 200, 000 FACS Nrl.GFP$^{+ve}$ rod-precursors. The contralateral control eye received an identical injection of sham cells from an age-matched Gnat1$^{-/-}$ donors, or remained untreated. A masked protocol was employed such that the person performing the ERGs and analysis did not know which eye received sham and which eye received Nrl.GFP$^{+ve}$-rod-precursor-cells.
Statistics
The data was analysed in keeping with previous reports (Carvalho et al., 2011; Mihelec et al., 2011). All means are stated ± standard deviation (SD). N= number of animals examined, where appropriate. Statistical significance was assessed using the student t-test or ANOVA with Dunnett’s correction for multiple comparison. Graphpad Prism 5 software (Graphpad Inc, La Jolla, USA). P values are presented as P<0.05 = *, p<0.01 = ** and p<0.001 = ***

2.4.2.1. AAV2/8 Rho.Gnat1

Plasmid construction and production of recombinant AAV2/8
Preparation of AAV2/8 Rho.Gnat1 was performed by Dr Claire Hippert and is briefly described here. Murine Gnat1 cDNA followed by a polyadenylation site was enzymatically digested and extracted from the CMV-Gnat1.SPORT6 plasmid (Source Bioscience, Cambridge UK). This cassette was cloned into pD10 containing the rhodopsin promoter and AAV-2 inverted terminal repeat (ITR) sequences to form the construct pD10/Rho-Gnat1 (total length 8230 bp). The construct was verified by sequencing. Recombinant AAV2/8 serotype particles were produced through a tripartite transfection method into HEK293T cells. AAV8 packaging, helper and pD10/Rho-Gnat1 plasmids were combined with polyethylenimine (PEI, Polysciences, Inc., Eppelheim, Germany) and left to form complexes for 10 minutes. The mixture was added to HEK293T cells and left for 24 hours. The cells were harvested and concentrated 2 days after transfection and lysed using repeated freeze/thaw cycles to release the viral particles. HEK293T cell nucleic acid components were removed by Benzonase (Sigma Aldrich, Dorset, UK) treatment and virus preparation was cleared of cellular debris by multiple centrifugation steps, followed by purification using ion exchange chromatography. The virus preparation was concentrated using a Vivaspin 4 concentrator (10 kDa, Sartorius Stedim Biotech, Fisher Scientific, Loughborough, UK), to
100 – 150 μl. Viral particle titres were determined using dot-blot analysis of purified virus DNA and plasmid controls of known concentrations.

FIGURE 2.4. SCHEMATIC DIAGRAM OF PD10/RHO-GNAT1 CONSTRUCT

Murine *Gnat1* cDNA was obtained from the CMV-*Gnat1*.SPORT6 plasmid and cloned into pD10 plasmid containing the rhodopsin promoter and AAV-2 inverted terminal repeat (ITR). Total length 8230 base pairs.
**ERG recordings**

ERGs were recorded prior to and at 3, 4, 5 & 6 weeks post injection of AAV2/8 Rho.Gnat1 vector. The test eyes received dual (superior and inferior) 2 µl subretinal injections of AAV2/8 Rho.Gnat1 at $10^9$, $10^7$ or $10^5$ viral particles per µL. The contralateral control eye was left untreated. Again, a masked protocol was employed whereby the assessor was blinded to which eye remained untreated and which was treated.

**Cell Counts**

In order to assess the number of cells transduced with the AAV2/8 Rho.Gnat1 vector, prior to termination, mice were light-adapted for 30 mins to ensure translocation of rod α-transducin into the cell body (Elias et al., 2004). Serial sections, 15 µm thick, were cut and stained for rod α-transducin (as described previously in section 2.3.1). The number of rod α-transducin$^{+ve}$ cells was counted in every 3rd section, this number was multiplied by three to give an estimate of the total number of rod α-transducin$^{+ve}$ cells per eye. Please note that this methodology of counting may result in an overestimation of the total cell number owing to the possibility of double counting one cell in separate sections.
2.4.3. INTRINSIC IMAGING OF THE VISUAL CORTEX

Intrinsic imaging of the mouse visual cortex was performed using a method pioneered by Grinvald et al. (1986), to measure the haemodynamic responses in V1. The experiments carried out here were performed with Dr Matteo Rizzi under the guidance of Dr Andrea Benucci and Professor Matteo Carandidi (Institute of Ophthalmology, UCL).

2.4.3.1. Lighting conditions

All experiments were performed under scotopic lighting conditions to test rod-mediated responses (<0.007 cd.s/m²). This was achieved by using neutral density filters to cover the computer screens. Procedures were performed under dim red light, below the spectral sensitivity of murine detection (Govardovskii et al., 2000). Scotopic conditions were confirmed by measuring light intensity with a IL700 photometer and by testing unprocedured Gnat1<sup>−/−</sup> mice to confirm the absence of rod mediated responses.

2.4.3.2. Surgery, alignment & visualisation

Following overnight dark-adaptation, anaesthetized mice (using the Ketamine/Dormitor regime reported in section 2.2.2) were mounted in a stereotactic frame and maintained on a thermostatic heat mat. Pupils were dilated using Tropicamide 1% and viscotears are continually re-applied to the cornea throughout the duration of the experiment to keep it moistened and to prevent cataract formation. Surgery to expose the cortex involved resection of the contralateral scalp region covering the occipital cortex. A extra-cranial chamber was formed by securing a circular metal head plate to the skull using Lambda and Bregma as reference point, with a glass coverslip placed within the metal ring. A large cortical area (5mm x 5mm) was visible through the extra-cranial imaging chamber, which included V1 (see Figure 2.5A).

Following the surgical procedure, mice were positioned relative to the stimulus screen such that the eye contralateral to the exposed cortex was facing the stimulus screen. The screen was approximately 30cm away of the animal. The eye ipsilateral to the
exposed cortex was covered throughout testing. Alignment of the mouse is noted on each figure whereby an arrow *(black)* indicates the anterior-posterior axis of the brain.

The exposed cortex and intrinsic signal images were visualised using a CMOS camera (MV-D1024E-160-CL, Photonfocus AG) running at 10Hz and illuminated with 660-nm light. Two 50 mm Nikkor lenses were used in a macroscope configuration with 1:1 magnification.

### 2.4.3.3. Visual Stimuli

Figure 2.5B illustrates the visual stimuli used. These were presented on two LCD monitors (60 Hz refresh rate) and consisted of striped black and white bars enclosed within one of four rectangular windows. Visual stimuli were designed under the guidance of Dr Andrea Benucci. The striped bar stimuli inside the windows were vertical square-wave gratings (0.03 cycles/°, 100% contrast) whose contrast reversed sinusoidally (2 Hz frequency, 3 s duration). The rectangular windows were aligned either in the horizontal (to the left or right of the visual field) or vertical plane (to the top or bottom of the visual field). The two vertical rectangular windows (50 deg width, 60 deg height) partially-overlapped and covered 90 deg of azimuth (0 to 90 deg). The two horizontal rectangular windows (90 deg width, 30 deg height) covered 60 deg of elevation (-10 to +50 deg). The rectangular windows were partially overlapping to allow maximal coverage stimulation of the visual field. In addition, a blank stimulus (0% contrast, i.e. grey screen) was also presented. The four tessellating rectangular window stimuli and blank were presented 15-20 times in a random order.
A. Setup Schematic

B. Stimulus design

Blank            Stimulus 1        Stimulus 2        Stimulus 3        Stimulus 4        Combined

C. Retinotopy colour combined maps

Stimulus 1     Stimulus 3    Overlap Colour

Stimulus 2     Stimulus 3    Overlap Colour

Stimulus 1     Stimulus 4    Overlap Colour

Stimulus 2     Stimulus 4    Overlap Colour
(A) The cortex is surgically exposed and V1 is visible through the extra-cranial imaging chamber secured to the skull. The contralateral eye is aligned with the stimulus screen. Hemodynamic responses are illuminated with a 660nm LED light source and captured with a CMSO camera. (B) Illustrated the stimulus design where 4 rectangular windows and blank screen are presented randomly. Stimuli 1-4 consist of overlapping windows containing striped black and white bars whose contrast reverses sinusoidally. (C) Combined colour-coded maps of retinotopy were obtained, whereby each quadrant was assigned a colour. Abbreviations: Au, auditory cortex; S1, somatosensory cortex.
2.4.3.4. Imaging and analysis

Images were captured for 3 seconds post-stimulus offset, and maps of the haemodynamic response were obtained for each stimulus presentation.

First, averaged maps were obtained by taking the mean over time and over stimulus repetitions. Each map was then divided by its grand-average (over space and time) thus to obtain relative intensity values (ΔI/I). Finally, correction for uneven illumination and reflectance was made by subtracting the response to the blank stimulus (a grey screen). Unless otherwise stated, the resulting maps were blurred in space with a 2D Gaussian filter (σ = 52μ) to remove pixel noise. These intensity maps were multiplied by a wide 2-D Gaussian window with unit amplitude (sigma = 100 pixels, 1.4 mm) to reduce artefacts at the borders of the window.

Combined colour-coded maps of retinotopy were obtained and illustrated in Figure 2.5C. The four stimuli covered to the top (stimulus 1), bottom (stimulus 2), right (stimulus 3) and left (stimulus 4) of the screen. Each pixel of the response image, was assigned a colour hue depending on the preferred stimulus quadrant: the top left stimulus response was assigned a blue hue; the top right stimulus was assigned a green hue; the bottom left stimulus response was assigned a red hue and the bottom right was assigned a yellow hue. The strength of the response is indicated by the colour saturation. Each colour quadrant represents the average response to two stimuli covering the preferred position; i.e. the left red quadrant refers to the stimulus 2 and 4 in the area where they overlap.
2.4.4. OPTOMOTOR TESTING

Optokinetic head-tracking reflexes can be used as a method to measure contrast sensitivity and visual acuities of mice treated with Nrl.GFP*ve rod precursor cells. In the presence of rotating vertical gratings (OptoMotryTM and (Alexander et al., 2007; Prusky et al., 2004; Umino et al., 2008)), mice will involuntarily move their head in the direction of the rotation, as driven by the optomotor reflex pathway. This system allows one to independently test left and right eye function (Figure 2.6A). Clockwise pattern rotation results in clockwise head tracking, a response that is driven by the left eye (Figure 2.6A, white arrow). Conversely, anti-clockwise pattern rotations result in anti-clockwise head tracking and are driven by the right eye (Figure 2.6A, black arrow). It must be noted, that while this protocol allows independent assessment of left and right eye sensitivities, the contralateral eye is not ‘blind’ to the stimulus and some ‘cross talk’ may be evident. A double-blinded testing protocol was adopted whereby the assessor was masked to both the direction of the rotation and to which eye received transplants of Nrl.GFP*ve rod-precurso cells. Figure 2.6A illustrates the optomotor testing set up, whereby the mouse was placed on a central pedestal surrounded by four inwardly facing LCD computer monitor screens. The mouse was observed by an infrared video camera located above the pedestal. The OptoMotry TM software presents random 7s trials where a sinusoidal striped pattern is rotated either clockwise or anti-clockwise across the computer screens. The assessor is required to select the direction of the pattern rotation based on the animal’s involuntary head tracking optomotor response. In between trials the computer screen returns to 50 % grey. Contrast sensitivity refers to the ability to distinguish between increments of light versus dark, whereas visual acuity is a measure of special resolution and is assessed by changing the width of the striped bars (Figure 2.6B). The program uses a staircase paradigm to titrate the visual acuity and contrast sensitivity thresholds, as determined by ≥ 70 % correct observer responses.
FIGURE 2.6. OPTOMOTOR™ SCHEMATIC SET-UP

(A) The mouse is placed in a centrally located pedestal surrounded by four inwardly facing computer screens and viewed above by an infra-red camera. Sinusoidal rotation of the striped pattern results in small head tracking movements. Integrative function of each eye can be assessed independently as rotation clockwise is dominated by the right eye and rotation anti-clockwise is dominated by the left eye. (B) Contrast sensitivity refers to the ability to discriminate between increments of light and dark and is assessed by changing the shades of the striped bar pattern (top). Visual acuity is a measure of special resolution and is assessed by changing the width of the striped bars (bottom).
Testing of both visual acuity and contrast sensitivity were performed under scotopic conditions lighting condition (<0.007 cd/s/m²) achieved by using neutral density filters to cover the computer screens. Scotopic conditions were confirmed by measuring light intensity with an IL700 photometer and by testing unprocedured Gnat1−/− mice to demonstrate the absence of rod mediated responses. Prior to testing, animals were dark-adapted over night. Testing was performed at least three times before and after (4-6 weeks) cell transplantation on independent days within the first 6 hours of the light cycle.

Contrast sensitivity was measured at the reported optimal special and temporal acuity frequencies whereby wildtype mice respond maximally in scotopic lighting conditions: a spatial frequency of 0.064 cycles/° and at a speed of rotation of 0.75 Hz (Umino et al., 2008). Visual acuity was assessed at 100 % contrast whereby the grating appeared as black and white.

Testing under photopic lighting conditions was also performed, both before and after transplantation, as a positive control and to assess any damage that occurred during surgery. Following a 30 min period of light adaptation (60 cd/s/m²) visual acuity and contrast sensitivity were tested under photopic lighting conditions (60 cd/s/m²). In order to test visual acuity, contrast was fixed at 100 %, whereas to test photopic contrast sensitivity, the spatial frequency was fixed at 0.128 cycles/degree.

Following optometry assessment, animals were sacrificed and the number of correctly integrated cells was quantified (see section 2.3.4.).
Statistics

All data are presented as mean ± standard error of the mean (S.E.M). N = number of animals examined. Statistical significance was assessed using Graphpad Prism 5 software, and applying ANOVA with Tukey’s or Dunnett’s correction for multiple comparisons, or a Fisher’s exact test (2-tailed), where appropriate. P values are presented as P<0.05 = *, p<0.01 = ** and p<0.001 = ***.
2.4.5. VISUALLY GUIDED WATER MAZE TESTING

The water maze task provides a method to measure visual ability in rodents (Prusky et al., 2000).

*Stimulus & experimental set up*

Mice were trained to discriminate between a neutral stimulus (in this case a 50% grey screen) and a grated pattern stimulus to swim to a hidden platform associated with latter visual cue. The visual stimuli were presented on 2 computer monitors (Mitsubishi Diamond Plus 7458, 17’’); each placed side by side facing inwardly into a Y-shaped, water filled tank. The grated visual stimulus consisted of striped black and white bars set at 0.086 cycles/°. All spatial frequencies are calculated with respect to the decision point. Mice were placed in the release shoot and the end of the mid-line divider was considered the decision point (Figure 2.7, red line), whereby once the mouse swims beyond this line the decision was scored as either pass or fail accordingly, irrespective of whether the mouse reached the platform. In addition, the swim-time latency was measured from the time the mouse swam beyond the release shoot until the time is reached the platform. Each session consisted of 10 trials presented in a pseudo-random pattern, LRLRLRLR (L-Left monitor, R- Right monitor). Each mouse was tested over at least three sessions on separate days.
A. Experimental setup

![Diagram](image)

**FIGURE 2.7. SCHEMATIC OF WATER-MAZE SETUP**

Animals are released into a Y-shaped, water-filled tank at the release shoot and trained under photopic lighting condition to swim to a hidden platform was associated with a striped grating visual cue presented on a computer monitor. The decision point (red) demarks whether the animal made a correct or incorrect choice. Animals were considered to have been successfully ‘trained’ when they made > 70% correct choices in 3 sessions (10 trials per session). Testing was performed under scotopic lighting conditions. Adapted from (Wong and Brown, 2007).
Lighting conditions, training & testing

Monitor brightness was calibrated so both screens were equal in luminance and that both the uniform grey stimulus and grating stimulus had an equal level of average luminance. All animals were successfully trained over a 3 week period under photopic lighting conditions (30 cd.s/m²) and were considered ‘trained’ once they had achieved a pass rate of 70% or greater over the previous 30 trials. Prior to photopic testing, animals were light adapted (>60 cd.s/m²) for 30 minutes to bleach any potential rod responses. To test rod-mediated vision, all animals were dark-adapted over-night and trials were performed in scotopic lighting conditions using dim red lighting to view the maze and an infra-red camera to record trials.

Experimental cohorts & post-transplant testing

Once successfully trained, animals were divided into four testing cohorts: 1) Gnat1⁻/⁻ mice receiving dual injections of Nrl.GFP⁺ve-rod-precursors to both eyes (N=9); 2) Gnat1⁻/⁻ mice receiving dual injections of sham age-matched Gnat1⁻/⁻ cells to both eyes (N=6), and 3) uninjected Gnat1⁻/⁻ (N=6) and 4) age-matched C57Bl/6J wildtype control mice (N=4). Animals were transplanted with the appropriate treatment and water-maze testing was performed 3-6 weeks post-transplant at scotopic lighting levels to test rod-mediated vision. Animals continued to receive photopic maintenance training throughout the 3 week interval between transplantation and testing (regardless of having received cells or not) and in between scotopic test sessions to ensure good task recall. The assessor was blinded to which treatment each independent animal had received. Control photopic recordings were taken in between and two days after the final scotopic testing as a positive control.
**Statistics**

All data are presented as mean ± standard error of the mean (S.E.M). \( N = \) number of animals examined. Statistical significance was assessed using Graphpad Prism 5 software, and applying ANOVA with Tukey’s or Dunnett’s correction for multiple comparisons, or a Fisher’s exact test (2-tailed), where appropriate. \( P \) values are presented as \( P<0.05 = \ast, \ p<0.01 = \ast\ast \) and \( p<0.001 = \ast\ast\ast \).
CHAPTER 3

OPTIMISATION & FUNCTIONAL ASSESSMENT OF PHOTORECEPTOR TRANSPLANTATION

3.1. INTRODUCTION

Work in this lab and others, has established proof-of-concept for photoreceptor transplantation (Bartsch et al., 2008; Lakowski et al., 2010; Lakowski et al., 2011; Maclaren et al., 2006; West et al., 2008; West et al., 2010). The fundamental question remains, however, as to whether and to what extent these transplanted photoreceptor cells can restore vision.

Many previous reports have attempted to demonstrate restoration of visual function following the transplantation of a variety of sources of donor cells in different models of retinal degeneration (Klassen et al., 2004; Kwan et al., 1999; Lamba et al., 2009; Maclaren et al., 2006; Tucker et al., 2011). Despite reports of very low numbers of transplanted cell integration, there have been some potentially surprising claims regarding restoration of vision. Restoration of vision is usually reported following assessment of one, or at best two, visual parameters. Rigorous functional testing of transplanted photoreceptor function, both in terms of the potential to integrate within the visual pathway and the ability to contribution to higher visual function, is lacking in current research. The aim of this chapter is to assess the functionality of transplanted photoreceptors from the level of the individual photoreceptor through to higher visual areas.
In the first instance, the ability of the transplanted cells to respond to light must be assessed. To examine whether the transplanted cells have the potential to respond to light most studies thus far have focused on the histological assessment demonstrating photoreceptor segment formation and expression of phototransduction cascade markers (Bartsch et al., 2008; Jiang et al., 2010; Kwan et al., 1999; Lakowski et al., 2010; Lakowski et al., 2011; Maclaren et al., 2006; West et al., 2008; West et al., 2010; Yao et al., 2010). However, the ability of these cells to hyperpolarize in response to a light stimulus has not been yet been adequately assessed.

The functional connectivity of the transplanted cells to the recipient second order neurons must be examined both by demonstrating the formation of new synapses and by demonstrating that the electrical signal generated by the transplanted cell can be effectively propagated to second and third order neurons within the recipient retina. Using electron microscopy, Gouras et al. demonstrated some segment and synapse formation of transplanted P1/P2 retinal cells in both the wildtype and Rd1 recipient (Gouras et al., 1994). However, this study relied on a LacZ reporter and the enzyme β-galactosidase to identify their transplanted cells population. This technique can be inaccurate and difficult to interpret as the product of β-galactosidase activity can spread outside of the LacZ positive cell. The development of the Nrl.gfp+/- donor mouse has allowed a more accurate tool to examine transplanted cell synapse formation within the recipient retina. Several groups have used immunohistochemistry to confirm the morphological appearance of synaptic boutons that co-express with synapse marker (Gouras et al., 1991; Gouras et al., 1994; Kwan et al., 1999; Maclaren et al., 2006; West et al., 2010). However, a closer inspection of transplanted rod photoreceptor cell synapses is required at the ultra structural level to confirm whether these cells form typical rod triad synaptic structures. Progress has been made to demonstrate the functional propagation of the neural signal to second order neurons. Following extracellular field recordings of the ganglion cell layer, threshold responses in scotopic lighting conditions in Rho-/- recipients receiving P1 retinal donor cells were reported,
compared to sham injected and uninjected control animals (Maclaren et al., 2006). In addition, a recent study reported some improvements in ERG b-wave amplitudes following transplantation of human ES derived retinal cells into adult Crx\(^{-}\) recipient mice, despite a very low integration efficiency and poor transplanted cell morphology (Lamba et al., 2009). In contrast, other groups have failed to report improvement in ERG despite successful graft-host connectivity (Seiler et al., 2009), suggesting there are some inconsistencies using ERG recordings as a readout for retinal function. It must be noted that full field flash ERG recording, which measures the current associated with the phototransduction cascade and the subsequent downstream activation of the second order neurons by recording the changes in electrical current at the cornea, offers a global assessment of retinal function. Although this technique can demonstrate the ability of photoreceptors to respond to light (by measuring the a-wave) and whether they are capable of propagating the electrical signal to second and third order neurons (demonstrated by the b-wave), it is likely that many fully functional cells are needed to generate a large enough signal to be detected by an electrode located distal to the retina at the corneal surface.

Finally, propagation of the neural signal to the visual centers in the brain must be examined and the ability to integrate visual input from transplanted photoreceptor cells must be assessed. Visual reflex tests offer a method to assess whether visual information is propagated to lower visual centers in the brain and integrated into an appropriate reflex response. For example, in 2006 it was demonstrated that the basic subcortical light response, the pupillary light reflex, could be restored in a Rho\(^{-}\) model of blindness, following transplantation of P4-5 rod photoreceptor precursors (Maclaren et al., 2006). Optomotor head tracking reflex testing offers an elegant alternative to pupillometry testing and can be used to measure both visual acuity and contrast sensitivity (Alexander et al., 2007; Prusky et al., 2004; Umino et al., 2008).
In order to address whether the electrical signal generated by engrafted cells can be propagated to the visual cortex, imaging the haemodynamic response (intrinsic signals) \textit{in vivo} in the visual cortex in response to visual stimuli can be employed. This method was first developed by Grinvald \textit{et al.} (1986) in the rat and cat cortex as an alternative to using extrinsic dye-signals. Visually mediated behavioural tests can be used to demonstrate that the higher visual centers receive and integrate the visual information generated by grafted donor tissue. Several groups have attempted to demonstrate functional rescue of vision using behavioural testing (Klassen \textit{et al.}, 2004; Kwan \textit{et al.}, 1999), however, many have done so in the absence of any other measure of function. It is therefore difficult to attribute any improvements in behavioural testing specifically to the integration of the transplanted photoreceptors into the visual circuitry. Kwan \textit{et al.} transplanted P7-9 retinal cells into \textit{Rd1} recipients aged 6-8 weeks and reported an improvement in a simple light/dark discrimination test 2 weeks post transplantation compared to untreated control animal (Kwan \textit{et al.}, 1999). However, in that study, they did not perform sham cell injected controls so they could not dismiss the possibility that this behavioural response is mediated by enhancing the survival or activity of the remaining endogenous cones. In 2004, Klassen \textit{et al} transplanted GFP\textsuperscript{+ve} multipotent P1 retinal progenitor cells into \textit{Rho}\textsuperscript{-} recipients and reported an improvement in light mediated behaviour using the exercise-wheel test (Klassen \textit{et al.}, 2004). In this study they demonstrated that when a light stimulus was presented during the dark phase of the diurnal cycle, wheel running was suppressed in the treated aged \textit{Rho}\textsuperscript{-} mice, whereas untreated and fibroblast sham treated animals stopped running only at the brightest light stimulus, suggesting that some rod-mediated vision was restored.

Many of the techniques mentioned above will be employed in this chapter to comprehensively assess transplanted rod-mediated visual function. Histological and electronmicroscopy will be used to analyse expression of phototransduction cascade markers and the morphology of synaptic structures of the transplanted photoreceptor. Electrophysiological techniques, ERG and single cell recordings, will be used to assess
the ability of the transplanted cells to respond to a light stimulus. Haemodynamic imaging of the visual cortex, the visually-guided watermaze test and optokinetic head tracking behavior will be employed to assess whether the responses generated by transplanted cells are propagated to the high visual areas and can contribute to higher visual functions. In order to assess functional rescue, the Gnat1−/− mouse was used as the recipient. After an initial 10% loss of ONL thickness, the degeneration rate in Gnat1−/− mouse remains stationary and the gross morphology of the retina is maintained until 12 months, thus providing a stable cytoarchitectural structure to maximize integration of transplanted cells. Endogenous rod outer segment development in the Gnat1−/− mouse is normal, indicating that the recipient retina is able to provide the necessary structural support of development of transplanted cell outer segments. Rod photoreceptors are non-functional in the Gnat1−/− mouse (Calvert et al., 2000), resulting in a flat baseline to test any improvement in scotopic rod-mediated vision. Finally, cone photoreceptors are reported to function as normal in this model, indicating that the inner retinal circuitry maintains afferent input (Calvert et al., 2000). All of these factors make this model an ideal candidate to test the function of the transplanted photoreceptors.

In order to restore meaningful vision, it is likely that a far greater number of successful transplanted cells than previously reported will be required (Bartsch et al., 2008; Lakowski et al., 2010; Maclaren et al., 2006; West et al., 2008; West et al., 2010; Yao et al., 2010). In this chapter, transplantation techniques are optimized to maximize integration efficiency prior to functional assessment. We assess the optimal donor cell density for maximal integration efficiency. In previous experiments, transplantation of 400,000 P3-5 retinal cells in 1µl volume administered to the superior retina via subretinal injection resulted in several hundred cells successfully integrating into the wildtype recipient retina (Maclaren et al., 2006). However, only 0.25% of the injected cells correctly migrated and integrated into the recipient ONL and the vast majority of cells remained in the subretinal space. Prolonged retinal detachment is known cause
ONL degeneration (Fisher et al., 2005; Mervin et al., 1999) and a study in 2010 demonstrated that transplantation of 400,000 cells results in prolonged periods of retinal detachment and significant host ONL loss (West et al., 2010). Therefore, it is essential to minimize further host retinal injury and ONL damage by maximizing cell integration and reducing cell mass volume.

In previous studies, transplantations were performed by a single injection to the superior retina (Bartsch et al., 2008; Lakowski et al., 2010; Lakowski et al., 2011; Maclaren et al., 2006; West et al., 2008; West et al., 2010). However, it is possible to transplant cells into both the superior and inferior retina and this possibility is explored in this chapter. Administering dual transplants to the superior and inferior retina is likely to increase both the total number of integrated cells and the distribution across which the integration occurs. From a therapeutic perspective, this approach would potentially rescue larger areas of the visual field. Another method that might improve transplantation efficiency is the intentional induction of retinal injury (Guo et al., 2003; Kurimoto et al., 2001; Nishida et al., 2000). Despite a lack of mature retinal phenotypic differentiation, reports using adult hippocampal-derived neural stem cells demonstrated that donor cell migration in the adult recipient was only possible following either mechanical or ischemic injury, (Guo et al., 2003; Kurimoto et al., 2001; Nishida et al., 2000). Similarly, Jiang et al. demonstrated enhanced integration efficiency of cultured retinal progenitor donor cells following photocoagulation laser treatment prior to transplant compared to untreated control (Jiang et al., 2010). Here we assess the impact of two injury techniques on transplantation outcome: the scleral stick wound and subretinal detachment. The scleral stick wound strategy has been previously reported to protect photoreceptor survival in rat models of degeneration (Sakai et al., 1999) and the subretinal detachment model initiates a well characterized, reproducible and robust acute retinal injury response (Fisher et al., 2005; Lewis and Fisher, 2003; Sethi et al., 2005).
Many previous studies have transplanted freshly isolated tissue from the early postnatal retina (Bartsch et al., 2008; Klassen et al., 2004; Kwan et al., 1999; Lakowski et al., 2010; Lakowski et al., 2011; Maclaren et al., 2006; West et al., 2008; West et al., 2010) which contains a mixed population of retinal cells, many of which are inappropriate cell types or are not at the ideal ontogenic stage for successful integration (Maclaren et al., 2006). The \textit{Nrl.GFP}^{+/+} donor mouse contains a genetic marker that enables the identification of the donor cell population that is able to integrate into the recipient retina: indeed, onset of \textit{Nrl} expression correlates with the integration ability of transplanted rod precursor cells (Maclaren et al., 2006). \textit{GFP} expression in the \textit{Nrl.GFP}^{+/+} donor mouse is driven by the \textit{Nrl} promoter, which is activated immediately post terminal differentiation resulting in \textit{GFP} expression in the newly born post-mitotic rod precursor cells. This donor cell population can be purified using Florescence Activated Cell Sorting (FACS) to isolate the \textit{GFP}^{+ve} rod precursors from postnatal \textit{Nrl.GFP}^{+/+} retinas and this technique is explored with in this chapter.
3.2. RESULTS

3.2.1. OPTIMISING TRANSPLANTATION TECHNIQUES

3.2.1.1. Impact of donor cell density on transplanted photoreceptor integration

Here we assessed the impact of donor cell density both on transplanted photoreceptor integration efficiency and recipient ONL integrity. Wildtype adult recipients received a single superior 1µl subretinal injection of 40,000, 100,000, 200,000, 400,000 or 800,000 live unsorted postnatal retinal cells from P3-5 Nrl.GFP+/+ donors. Animals were sacrificed 3 weeks post transplant and the number of cells correctly integrated into the recipient ONL was quantified. The results are summarised in Figure 3.1a. Reducing donor cell density to 200,000 cells / µl (N = 4) did not result in a significant reduction in integration efficiency compared to the standard injection (N = 9) of 400,000 cells / µl (939 ± 57 compared to 952 ± 206 integrated cells, P > 0.05, ANOVA with Dunnett’s correction). Interestingly, increasing donor cell density to 800,000 cells / µl did not significantly increase donor cell integration efficiency (981 ± 163 cells / eye; P > 0.05, ANOVA with Dunnett’s correction; N = 6). A small, but not significant reduction in integration efficiency was observed in those animals transplanted with a donor cell concentration of 100,000 cells / µl (677 ± 106 integrated cells; P > 0.05, ANOVA with Dunnett’s correction, N = 6). However, a significant reduction in integration efficiency was present using a donor cell density to 40,000 cells / µl compared to the standard 400,000 cell / µl transplant (388 ± 100 cells / eye; P < 0.05, ANOVA with Dunnett’s correction; N = 7).

Thus, changing the number of cells transplanted in 1µl from 100,000 to 800,000 cells / µl had little impact on integration efficiency. This means that significant numbers of
cells are likely to remain in the recipient subretinal space. To assess the effect of donor cell density on survival of the underlying recipient ONL, ONL thickness was measured at the site of transplantation (white bars) and normalized to control measurements taken inferiorly in the same coronal section of the same eye. Control measurements from uninjected wildtype animals were also recorded as a reference guide (black bars). As shown in Figure 3.1b (black bars), ONL thickness of uninjected regions of the recipient retina remains comparable to uninjected wildtype ONL thickness. Recipient ONL thickness around the site of injection remained comparable to the adjacent uninjected regions in recipients receiving 40,000 and 100,000 cells/µl (P > 0.05, paired t-test, N = 7 and N = 6, respectively). Recipients receiving 200,000 cells / µl demonstrated a small reduction in ONL thickness although this was not statistically significant (P > 0.05, paired t-test, N = 4). In keeping with previous findings (West et al., 2010), a significant reduction in ONL thickness was observed in those recipients receiving 400,000 cells / µl (P < 0.05 paired t-test, N = 9, Figure 3.1b and 3.1c).

Together, these findings indicate that a density of 200,000 cells per µL is the optimal donor cell density both in terms of maximizing donor cell integration but also for minimising the ONL thinning associated with the presence of the remaining donor cell mass in the subretinal space.
FIGURE 3. IMPACT OF DONOR CELL DENSITY ON TRANSPPLANTED PHOTORECEPTOR CELL INTEGRATION AND RECIPIENT ONL HEALTH

(a) The number of Nrl.GFP\textsuperscript{+ve} cells integrated within the wildtype recipient retina following subretinal injection of different densities of P4-6 donor cells (expressed as cells/µl; mean ± SEM; ANOVA with Dunnett’s correction for multiple comparisons). (b) The ONL thickness of the recipient retina following transplant of different densities of P4-6 donor cells. Measurements are obtained from around the injection site and expressed as a percentage of control-a region adjacent to the injection site (mean ± SEM; paired t-test). (c) Confocal image demonstrating recipient ONL thinning around the injected cell mass 3 weeks post a superior subretinal transplant of 400,000 donor cells. Scale bar 200 µm.

Abbreviations: NS, not significant; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; HOE, Hoechst 33342.
3.2.1.2. Impact of injury on transplanted photoreceptor integration

Previous studies using neural donor stem cells demonstrated that donor cell migration into the adult recipient retina was only possible following mechanical or ischemic injury (Guo et al., 2003; Kurimoto et al., 2001; Nishida et al., 2000). Enhanced RPC integration efficiency has also been reported following photocoagulation laser treatment immediately prior to transplant to induce a localized injury in the retina (Jiang et al., 2010). Here, we examined two methods introducing injury to improve transplantation efficiency that may be more amenable to a clinical setting and less destructive than laser therapy. These approaches include a sclera puncture at the time of transplantation and a subretinal detachment 48 hours prior to the transplantation procedure.

a) Scleral Puncture

Figure 3.2a shows the impact of a minimal surgical injury intervention, using a trans-sclera needle stick puncture was assessed. A scleral puncture was performed in one eye (grey bars) prior to cell transplantation, leaving the contralateral eye as an untreated control (black bars). Immediately after scleral puncture, both control and injury eyes received a single superior subretinal injection of 200,000 unsorted cells in 1uL. Introducing a scleral puncture had no effect on integration efficiency (1329 ± 192 versus 1130 ± 389 in the contralateral control eye; P > 0.05, paired t-test, N = 6). However, although not quite statistically significant, a reduction in the variation of the data was observed (P = 0.06, variance ratio test [F test], N = 6, Figure 3.2b).
b) Subretinal detachment

An acute retinal injury can be introduced by a subretinal detachment. The retinal detachment model is well characterized and the injury response is robust and reproducible (Fisher et al., 2005; Fisher and Lewis, 2003; Sethi et al., 2005).

The superior retina of the treated eye (grey bars) was pre-detached using 1uL EBSS 48 hours prior to transplant, whereas the contralateral control eye (black bars) remained untreated. Donor cells were transplanted into the subretinal space as a single superior 1uL injection of 200,000 unsorted donor cells in both control and injured eyes. Following termination 3 weeks post transplant, a significant 2.4 fold increase in cell integration efficiency was observed in those eyes receiving pre-detachment surgery compared to contralateral control eyes (2547 ± 261 versus 1044 ± 369 integrated cells; P < 0.05, paired t-test; N = 5, Figure 3.2 a).
3.2.1.3. Impact of multiple injection sites on transplanted photoreceptor integration

Here we examined the effects of injecting both the superior and inferior retina (grey bars) with 1uL of cell suspension containing 200,000 unsorted cells per injection site, compared to contralateral control eyes receiving a single superior 1uL injection of the same cell suspension (black bars). Dual injections significantly increased cell integration to 2689 ± 390 cells/eye compared to contralateral control eyes where 904 ± 219 cells were observed to integrate into the recipient retina (Figure 3.2a, P < 0.05, paired t-test, N = 4). As might be expected, integrated cells were observed across a much larger region of the retina following dual transplants.

3.2.1.4. Purifying donor cell population using FACS

Many previous studies have transplanted tissue isolated from the early postnatal retina containing a mixed population of retinal cells, many of which are inappropriate cell types or are not at the correct ontogenic stage for successful integration (Maclaren et al., 2006). Fluorescence Activated Cell Sorting (FACS) was used to purify GFP⁺ve rod precursors population from postnatal donor Nrl.GFP⁺/⁻ retina (P4 - P6).

First, the effect of FACS on cell viability was examined (Figure 3.2c, grey bars). Viability of the total cell sample and GFP⁺ve FACS sample was assessed using propidium iodide (PI). Viability of the mixed cell population following retinal dissociation was 88 ± 3 % (N = 6), of which 38 ± 3 % were GFP⁺ve (Figure 3.2c, grey bars and black bars, respectively). GFP⁺ve cells were collected in EBSS media containing a high concentration of FCS. PI was added to purified GFP⁺ve cells and re-sorted to determine the viability, revealing a 93 ± 2 % viability, of which 90 ± 2 % were GFP⁺ve (N = 6). To conclude, GFP⁺ve donor cell population purity was significantly boosted from 38 % to 90 % following FACs purification (Figure 3.2c, black bars, P < 0.0001, paired t-test, N = 6).
Transplants were performed using a single 1uL superior injection of 200,000 FACS cells, while the contralateral control eyes received a mixed population of retinal cells that had been processed through the MoFlow FACS analyser but were not sorted based on fluorescence to control for any viability issues caused by the physical stress of being forced through the FACS machine: these cells were termed ‘ungated’. In both cases, donor cell suspensions were adjusted in order to transplant 200,000 live cells/µl. Integration in wildtype recipients receiving a FACS purified donor cell population was significantly higher than contralateral control eyes (4359 ± 711 cells versus 438 ± 85 cells; paired T-TEST) P<0.001; N=9, Figure 3.2a). It should be noted that integration efficiency using the ungated donor cell population was lower than when using the unsorted population (438 ± 85 cells versus 953 ± 206 cells, Figure 3.2a, compare to white bar), presumably due to the shearing stress caused by being passed through the FACS machine.

In addition to integrating more efficiently, transplanted FACS purified donor cells (grey bar) integrated over a wider area following transplantation compared to ungated controls (Figure 3.2d, black bar, P < 0.05, paired t-test; N = 5): qualitatively, the region over which cells integrated appeared to extend beyond the injection site and cell mass (Figure 3.2eii). Similarly, the number of cells remaining in the cell mass appeared reduced (Compare Figure 3.2e, i and ii).

To conclude, transplantation of FACS purified rod precursor cells significantly increases integration efficiency and widens the area over which integrated cells are found within the recipient retina.
FIGURE 3.2. TECHNIQUES TO IMPROVED CELL INTEGRATION EFFICIENCY

(a) Cell integration following various treatments to increase integration efficiency (mean ± SEM, paired t-test) (b) Scatter graph showing the variation in cell integration between eyes receiving trans-scleral puncture wound (variance ratio test [F test], N = 6). (c) Histogram showing cells viability assessed using PI viability dye (grey bars) and purity assessed by GFP (black bars) of the initial dissociated mixed population sample and the post-sorted sample (mean ± SEM, paired t-test, N = 6). (d) Histogram showing the degree of spread of integrated cells over histological sections following FACS sorting compared to un-gated controls (mean ± SEM, paired t-test, N = 6). (e) Confocal section highlighting integration and cell mass using (i) unsorted P4 retina and (ii) FACS purified Nrl.GFP⁺ve donor cells.
3.2.1.5. Combined approach

Next we used techniques described above and combined them to assess whether their effects were additive. Wildtype recipients received subretinal pre-detachment surgery 48 hours prior to cell transplantation. Nrl.GFP<sup>+/ve</sup> donor cells were purified using FACS and 200,000 live cells in 1 uL were transplanted subretinally both in the superior and inferior retina. Interestingly, this combined approach did not result in large increases in cell integration, as anticipated, with only 2294 ± 604 cell found to integrate into the recipient ONL (Figure 3.3a, N = 6). In addition, a high exclusion rate was noted: 21 out of 27 eyes analysed were excluded based on our exclusion criteria (see Methods section 2.3.4.1). 13 of the 21 eyes excluded were so due to evidence of an acute immunological response. The remaining 8 were excluded because of an absence of cell mass within the subretinal space. To address whether the surgical trauma of dual pre-detachment surgery followed by dual transplant surgery accounts for the unexpectedly poor integration efficiency and high exclusion rate, the combined approach was repeated omitting pre-detachment surgery. Recipient animals received 1 uL superior and inferior injections each containing 200,000 live FACS purified Nrl.GFP<sup>+/ve</sup> donor rod precursor cells. Three weeks post transplantation, a significant improvement in cell integration efficiency was observed, with an average of 16,759 ± 1705 cells integrated (N = 9) in the recipient retina (Figure 3.3a, P < 0.0001, unpaired t-test). The maximum number of integrated cells found within the wildtype recipient ONL post transplantation was 26,602 integrated cells.
3.2.1.6. Integration in the \textit{Gnat1}^{-} recipient retina

In order to assess the function of transplanted rods, the \textit{Gnat1}^{-} model of stationary night blindness was selected as the recipient model. We first assessed integration in the \textit{Gnat1}^{-} recipient using the optimized transplantation protocols identified above. FACS purified Nrl.GFP^{+ve} rod precursor were injected both to the superior and inferior retina at a density of 200,000 live cells in 1 uL, using the scleral puncture technique to minimize cell suspension reflux. Three weeks post transplant animals were sacrificed and the number of integrated cells was assessed. Integration was very similar to wildtype (18,196 ± 1477 (N = 29), compared with 16,759 ± 1705 (N = 9), respectively, \( P > 0.05 \), unpaired t-test, Figure 3.3a). The maximum number of integrated cells observed to integrate in to the \textit{Gnat1}^{-} recipient ONL was 32,015 Nrl.GFP^{+ve} cells. Figure 3.3b shows representative \textit{in vivo} SLO images of both the superior and inferior cell mass prior to termination, demonstrating that the cell mass covers a large proportion of the retina. Figure 3.3c demonstrates that integration of Nrl.GFP^{+ve} rod-precursor cells occurs primarily around the regions of the two injection sites.

Nrl.GFP^{+ve} rod cells integrated within the \textit{Gnat1}^{-} recipient appear similar in morphology to those that integrate into the wildtype retina (Figure 3.3d, compare i and ii). In both instances, integrated cells were appropriately located within the ONL and presented with typical rod photoreceptor morphologies, with long segment regions and processes extending into the OPL forming synaptic bouton-like structures (Figure 3.3c and d).
FIGURE 3.3. COMBINED TRANSPLANT APPROACH AND INTEGRATION IN Gnat1−/− RECIPIENTS

(a) Cell integration in the wildtype and Gnat1−/− recipient retina using the combined optimized transplant method (mean ± SEM, unpaired t-test). (b) In vivo SLO images of Gnat1−/− recipients 1.5 weeks post transplant. (c) Confocal montage of Gnat1−/− recipient 4-6 weeks post transplant, with a clear integration region observed in both the superior and inferior retina. Scale bar 200µm. Inset, high power confocal image of (i) superior and (ii) inferior injection site, scale bar 50µm. (d) High power confocal image of integrated cell morphologies in (i) Gnat1−/− and (ii) wildtype recipients. Scale bar 25µm.
3.2.2. DO INTEGRATED CELLS RESPOND TO LIGHT AND IS THE SIGNAL PROPAGATED TO SECOND ORDER NEURONS?

3.2.2.1. Histological analysis of phototransduction and synapse proteins

Previous work in this lab has demonstrated that rod precursor cells isolated from the early postnatal retina integrate within the wildtype recipient retina and are morphologically identical to endogenous rods (Lakowski et al., 2010; Maclaren et al., 2006). Here we sought to determine whether the cells integrating within the Gnat1\textsuperscript{-/-} recipient retina were comparable. We therefore performed a closer analysis of integrated rod cells to determine whether these cells appropriately express proteins necessary for phototransduction. We also sought to address the important question of whether these cells form correct synaptic connections with second order neurons.

a) Integrated cells functional expression mature phototransduction markers

As shown in Figures 3.4a and 3.4b, rod \( \alpha \)-transducin, which is absent in endogenous Gnat1\textsuperscript{-/-} rods, and rod arrestin, which is responsible for quenching photoactivated rhodopsin, are both expressed appropriately in the segment region of the transplanted photoreceptor. These phototransduction markers are known to translocate between the segment region and cell body in a light-dependent manner (Elias et al., 2004). Rod \( \alpha \)-transducin translocates to the cell body and synapse upon light stimulation, whereas rod arrestin translocates from the inner to the outer segment region upon light stimulation. To test whether these markers correctly translocate in response to light in the transplanted rod photoreceptors, animals receiving donor cell grafts were either light adapted for 2 hours or dark-adapted over night prior to termination. Eyes were processed for immunohistochemical staining and the location of rod \( \alpha \)-transducin and
rod arrestin in both light and dark conditions was analysed. Figures 3.4a and 3.4b illustrate that rod α-transducin was located in the outer segment regions of integrated Nrl.GFP<sup>+</sup> cells in animals culled after dark-adaptation (Figure 3.4ai). Moreover, staining of retinal sections of animals culled after light adaptation demonstrated that rod α-transducin expressed in GFP<sup>+</sup>-integrated rod cells correctly translocated to the cell body and synapse (Figure 3.4a ii). Conversely, rod arrestin was located in the cell body region of integrated Nrl.GFP<sup>+</sup> transplanted cell in recipient animals culled after dark adaptation (Figure 3.4b i), as reported in the wildtype (Philp et al., 1987). Similarly, in those recipient animals culled after light adaptation, rod arrestin in integrated Nrl.GFP<sup>+</sup> cells had successfully translocated to the outer segment region (Figure 3.4b ii).

**b) Integrated cells develop classic triad synapse structures**

The ability of the transplanted cells to integrate into the host visual circuitry is dependent on their ability to form functional synapses with second order neurons. Histological analysis demonstrated that integrated Nrl.GFP<sup>+</sup>-rods formed spherule-like structures, typical of normal rod synapse morphology (Okuda, 1969), in the innermost layer of the OPL (Figure 3.4c, i-iii). These synaptic structures appropriately contained dystrophin, bassoon and/or ribeye (Figure 3.4c, i-iii insets, respectively), which are major components of the photoreceptor ribbon synapse (Garner et al., 2000). Sections co-staining with antibodies raised against PKC-α, a rod bipolar cell marker showed that these synaptic structures appeared to terminate immediately adjacent to bipolar cell dendritic processes (Figure 3.4c, iii).

We next sought to examine the synapses formed by integrated Nrl.GFP<sup>+</sup> rod photoreceptor cell at the ultrastructural level. This work was carried out in collaboration with Sung C.H and Chuang J.Z., at Cornell University. Figure 3.4d shows electron micrographs, kindly supplied by Sung C.H. and Chuang J.Z., of representative synaptic
terminals of two transplanted Nrl.GFP+ve rod photoreceptor cells, tT1 and tT2 (Figure 3.4d, inset). Figure 3.4 demonstrates that tT1 clearly adopts the classic rod triad innervated synapse structure (Figure 3.4e), with a central bipolar dendritic terminal (pseudo-coloured brown) and lateral horizontal cell axon terminals (pseudo-coloured green). In addition, several classic features of a synapse were observed in tT1: the rod ribbon synapse is easily identifiable (arrow), as are the synaptic vesicles and synaptic cleft (Figure 3.4d).
FIGURE 3.4. INTEGRATED CELL MORPHOLOGY IN Gnat1<sup>−/−</sup> RECIPIENTS

Confocal images of integrated Nrl.GFP<sup>+</sup> rod cells in adult Gnat1<sup>−/−</sup> recipients demonstrating expression of (a) rod α-transducin and (b) rod arrestin following (i) dark adaptation or (ii) light adaptation, scale bar 50µm. (c) Confocal images of integrated Nrl.GFP<sup>+</sup> rod cells in Gnat1<sup>−/−</sup> recipient, demonstrating the presence of synapse structures and expression of the rod synapse markers (i) dystrophin, (ii) bassoon and (iii) ribeye, scale bar 25µm. *Inserts*: high power projection images of defined areas. (d) High power electronmicrograph of DAB<sup>−/−</sup> Nrl.GFP<sup>+</sup> integrated rod cell terminal. *Inset* shows low-power micrograph of two DAB<sup>−/−</sup> Nrl.GFP<sup>+</sup>-integrated rod cell terminals (tT1 and tT2, pseudo-coloured *red*) surrounded by endogenous DAB<sup>−/−</sup> rod-cell synapse terminals (nT1, nT2, nT3: pseudo-coloured *blue*). Scale bar 500nm. (e) Schematic diagram illustrating classic triad rod synapse morphology, adapted from (Kolb, 1970). (f) Electronmicrographs of four DAB<sup>−/−</sup> Nrl.GFP<sup>+</sup> integrated rod cell terminals (i-vi). (g) Electronmicrographs of four DAB<sup>−/−</sup> unlabelled endogenous rod cell terminals (i-vi). Abbreviations: *mit*, mitochondria; *H*, horizontal cell axon terminal; *B*, bipolar cell terminal; tT1 & tT2, transplanted rod cell synapse terminals; nT1, nT2, nT3, endogenous rod cell synapse terminal. Bipolar cell processes are pseudo-coloured *brown*, horizontal cell axon terminals are pseudo-coloured *green*. Arrow indicates rod ribbon synapse structure.
3.2.2.2. Electrophysiology: single cells recordings

In order to assess whether integrated rod photoreceptors can respond to light, suction pipette recordings were performed on integrated Nrl.GFP+ve rod photoreceptor cells by our collaborators Xue T. and Yau K.W. at the Johns Hopkins University School of Medicine, Baltimore USA (Fu and Yau, 2007; Nikonov et al., 2006). Retinal slices were obtained from Gnat1−/− recipients 4-6 weeks post transplant. Correctly integrated Nrl.GFP+ve rods were identified using GFP-fluorescence and the Nrl.GFP+ve cell body was drawn into a glass pipette. The cell searching process resulted in bleaching of the rhodopsin pigment and desensitization of the Nrl.GFP+ve rods. In order to enable for regeneration of the rhodopsin pigment in the absence of the RPE, retinal slices were maintained in the recording chamber for 1 hour in darkness in a bath solution containing 9-cis-retinal. Wildtype (C57BL/6J) uninjected control retinas were subjected to the same excitation-light/pigment-regeneration regime prior to recording to allow comparison between transplanted and normal rods.

Figure 3.5a, kindly supplied by Xue T. and Yau K.W., shows flash-response families of single dark-adapted rods stimuli of increasing light intensities and demonstrate that transplanted GFP+ve rod cells (blue) can respond in a similar fashion to wildtype naive and regenerated rod cells (black and red, respectively). As light intensity increases, the photocurrents also increase in all three cohorts (Figure 3.5a). Figure 3.5bi demonstrates that the maximal amplitude response, as measured by the saturated photocurrent, of Nrl.GFP+ve transplanted rods, although slightly smaller (4.6 ± 0.5 pA, N = 9; blue), is not significantly different to the maximal amplitude responses recorded from naive wildtype rods or wildtype rods subjected to the same excitation/regeneration regime (6.1 ± 0.8 pA, N = 4, black; 6.4 ± 0.7 pA, N = 8, red, respectively; ANOVA, P > 0.05). Figure 3.5bii and 3.5biii demonstrate the dim flash response kinetics. In Figure 3.5bii, Nrl.GFP+ve rod cells are shown to respond significantly slower (175 ± 10 ms, N = 6, blue), as measure by the time-to-peak, compared to untreated wildtype controls (143 ± 6 ms, N = 8, black; ANOVA; P < 0.05). Whether this effect is a consequence of
pigment regeneration by the 9-cis-retinal treatment regime or an intrinsic feature of the Nrl.GFP^+ve transplanted rods remains unclear; 9-cis treated wildtype controls (168 ±4 ms, N = 4, red) were not statistically different to either untreated wildtype rods (black) or Nrl.GFP^+ve transplanted rod (blue) (ANOVA, P > 0.05). The significant technical challenges involved in obtaining these recordings prevented the inclusion of further n numbers. However, it seems probable that these differences are a consequence of the regeneration treatment regime as the overall duration of the response/integration time is significantly longer in Nrl.GFP^+ve rod (292 ± 28 ms, N = 6, blue) and wildtype controls treated with 9-cis-retinal (267 ± 21 ms, N = 4, red) compared to untreated wildtype controls (163 ± 11 ms, N=8, black), suggesting that the treatment regime alone slows the dim flash response kinetic (Figure 3.5B iii; ANOVA, both P < 0.05). Figure 3.5b iv shows the amount of light needed to elicit a half maximum response, σ, which is inversely proportional to sensitivity. Nrl.GFP^+ve transplanted rods were less sensitive than untreated wildtype controls, requiring significantly more light to produce a half maximal response (1.9E4 ± 1.3E4 µm^2 versus 76 ± 14 µm^2; N = 4, blue and N = 8, black, respectively; Kruskal Wallis, P < 0.05). Again, it is unclear whether this drop in sensitivity is an intrinsic issue of the Nrl.GFP^+ve transplanted rod or a consequence of the 9-cis-retinal regeneration treatment regime, as wildtype 9-cis-retinal treated controls (713 ± 130 µm^2, N=4, red) were not significantly different to either untreated wildtype rods (black) or Nrl.GFP^+ve transplanted rods (blue) (Kruskal Wallis, both P > 0.05). It is possible that recorded signals were generated by neighbouring cone cells whereby the electrical signal is conducted to the rods via electrically coupled gap junctions. Recordings were therefore taken from adjacent GFP^+ve cells. On no occasion was a light mediated response observed (Figure 3.5 c).
(a) Flash-response families from single dark-adapted wildtype (black), 9-cis-retinal treated wildtype (red) and 9-cis-retinal treated Nrl.GFP<sup>+</sup> rod cells (blue). (b) Saturated response amplitudes (i) and dim-flash-responses (ii) and (iii) recorded from single dark-adapted wildtype (black), 9-cis-retinal treated wildtype (red) and 9-cis-retinal treat Nrl.GFP<sup>+</sup> rod cells (blue). Mean ± SEM, ANOVA. Individual cell responses are shown (open circles). (iv) Rod sensitivity as measure by the half-saturating flash intensity (σ) recorded from single dark-adapted wildtype (black), 9-cis-retinal treated wildtype (red) and 9-cis-retinal treated Nrl.GFP<sup>+</sup> rod cells (blue). Median ± range, Kruskal Wallis. (c) Representative retinal slice demonstrating a light-sensitive Nrl-GFP rod (red circle) and its associated response trace, and several surrounding non-responsive GFP negative rods (white circles) which demonstrated no response.
3.2.2.3. Electrophysiology: Electroretinogram recordings

To assess whether transplanted rod photoreceptors collectively generate electrical signals in response to light and propagate the signal to the inner retina, full field electroretinogram (ERG) recordings were performed. Full field ERG recordings offer a global measure of the electrical response of the retina to light stimulation and by measuring the amplitude of the wave peaks, the function of the retina can be quantitatively assessed.

a) Designing the ERG scotopic testing protocol

i) Verifying cone function

Little is known about the response threshold and the recovery kinetics of the transplanted rod photoreceptor. Although outer segment formation of the transplanted cells in the Gnat1−/− recipients was good (see Figure 3.3c), even a small reduction in disc formation and rhodopsin expression can result in a reduction in light sensitivity (Machida et al., 2000; Reuter and Sanyal, 1984). For this reason, we planned to have as bright a stimulus as possible to test rod function, in order to maximize the probability of a photon activating a rhodopsin molecule. Although rod photoreceptor function is absent in the Gnat1−/− mouse, retention of normal cone function has been reported (Calvert et al., 2000). To verify this finding, full flash photopic ERG recordings were performed after a 10-min light adaptation on a background light intensity of 20 cd.s/m² and compared to wildtype controls. Figures 3.6a and 3.6b show that some differences in average Gnat1−/− (blue) photopic b-wave and a-wave amplitudes were observed, compared to wildtype controls (black, N = 6). Photopic b-wave amplitudes in Gnat1−/− mice were significantly higher at photopic bright light intensities 10 and 20 cd.s/m², with a recorded amplitude of 141 ± 14 µV and 170 ± 7 µV respectively, compared to wildtype control at the same light intensities, with a recorded b-wave amplitude of 82 ± 18 µV at 10 cd.s/m² and 84 ± 15 µV at 20 cd.s/m² (Figure3.6a ii, unpaired t-test, P < 0.01 and 0.001 respectively, N = 6). At a light intensity of 10 cd.s/m² the average
Gnat1−/− b-wave amplitude is 126 ± 13 % of the average wildtype b-wave amplitude (Figure 3.6b; unpaired t-test, P <0 .01, N = 6). Conversely, there was a small but significant reduction in Gnat1−/− photopic a-wave amplitudes at 1, 2, 3, 5, 10 and 20 cd.s/m², compared to wildtype controls (Figure 3.6a i, unpaired t-test, N = 6). The average Gnat1−/− a-wave amplitude was 67 ± 23 % of the average wildtype a-wave amplitude at 10 cd.s/m² (Figure 3.6b, unpaired t-test, P < 0.05, N = 6). A representative ERG full-flash response is illustrated in Figure 3.6c, showing the higher b-wave and less defined a-wave in Gnat1−/− recipients compared to wildtype controls. In addition, qualitative pronounced differences in photopic flicker recordings can be observed; the size of the oscillatory potential in Gnat1−/− mice appears reduced in size compared to wildtype controls (Figure 3.6d, N = 4). Also at the higher frequencies the amplitudes are much reduced in Gnat1−/− mice compared to wildtype controls (Figure 3.6d, N = 4).
FIGURE 3.6. PHOTOPIC ERG RESPONSES RECORDED FROM Gnat1<sup>−/−</sup> AND WILDTYPE MICE

Photopic ERG responses recorded from light-adapted Gnat1<sup>−/−</sup> mice is shown in blue and recordings from wildtype mice are shown in black. (a) Full flash photopic a-wave (i) and b-wave (ii) ERG responses at each light intensity examined. Mean ± SD, unpaired t-test. Dashed boxes indicate light intensity examine in b. (b) A- and a-wave full flash photopic ERG responses recorded at 10 cd.s/m<sup>2</sup> expressed as percentage of wildtype (i). Mean ± SD, unpaired t-test. (c) Representative photopic ERG traces recorded at 10 cd.s/m<sup>2</sup>. (d) Representative photopic flicker ERG responses recorded from Gnat1<sup>−/−</sup> (left) and wildtype (right) mice. *, P<0.05; **, P<0.01; ***, P<0.001.
ii) Determining scotopic cone response threshold

To design an ERG protocol suitable for detecting signals from transplanted rod photoreceptors, recordings must be performed following dark adaptation in scotopic lighting conditions. The response threshold of cone light detection must be considered as cone signals may mask any signal from transplanted rod photoreceptor cells. Although rods are traditionally thought to respond in dim scotopic light and cones in bright photopic light, there is a significant degree of overlap in the light intensity range in which both rod and cones can detect. This is called the mesopic range and usually spans between 0.01 to 3 cd.s/m² in humans (Toda et al., 1999). With this consideration in mind, the cone response threshold in the Gnat1⁻/⁻ mice was determined following over-night dark adaptation. Full flash ERGs were performed on dark-adapted Gnat1⁻/⁻ mice (Figure 3.7a, N = 6), where light intensity was incrementally increased until a robust cone response was detected. A small b-wave response of 16 ± 4 µV was consistently detected at 0.03 cd.s/m² in Gnat1⁻/⁻ mice (blue) compared with 332 ± 13 µV in the wildtype (black) (Figure 3.7a ii and b ii, P <0.001, unpaired t-test, N = 6); the average Gnat1⁻/⁻ b-wave amplitude (blue) at this light intensity was 5 ± 3 % of the average wildtype b-wave (black) (Figure 3.7b i, P <0.001, unpaired t-test, N = 6). Below this light intensity stimulus, visual responses were not robustly detected in the Gnat1⁻/⁻ model reflecting the absence of rod function and loss of cone ERG responses. At 0.003 and 0.007 cd.s/m² the average b-wave amplitudes were -6.3 ± 6 µV and 1.3 ± 9 µV, respectively (Figure 3.7). At these light intensities where there was no detectable waveform, the placement of a-wave and b-wave measurement cursors were positioned according to the 0.03 cd.s/m² waveform. Note that the standard error was higher at the 0.003 and 0.007 cd.s/m² light intensities than 0.03 cd.s/m² light intensity (± 6 µV and ± 9 µV versus ± 4 µV). This is mostly likely because at the 0.03 cd.s/m² light intensity there is a robust waveform to measure and less noise.

The b-wave proved a more sensitive measure of scotopic retina function in keeping in published data (Lamba et al., 2009). A-wave signals were barely detectable in Gnat1⁻/⁻
mice even at bright mesopic light intensities: at 3 cd.s/m$^2$, an a-wave of just -8 ± 2 µV was detected in the *Gnat1* mouse compared with -241 ± 11 µV in wildtype controls (Figure 3.7a i, P < 0.001, unpaired t-test, N = 6).

A testing protocol was designed that reflected the above considerations comprising of the following light intensities: 0.003, 0.007, 0.03, 0.5 and 3 cd.s/m$^2$ (Figure 3.7a). The first two intensities (0.003 and 0.007 cd.s/m$^2$) were chosen to represent two ‘flat’ baseline light levels well below the *Gnat1* cone response threshold. Any responses at these intensities can be attributed solely to rod function from the transplanted rod photoreceptors. The third intensity, 0.03 cd.s/m$^2$, was chosen as it is at the very bottom of the *Gnat1* mesopic range and provides us with a reliable wave-form to measure. The cone response seen at this light level was 16 ± 4 µV (Figure 3.7aii and bii) and any small improvements by the transplanted rods may be detectable over and above this signal. At 0.03 cd.s/m$^2$ both the light intensity is brighter and the signal to noise ratio is more favorable to detect small ERG signal compared to the 0.007 and 0.003 cd.s/m$^2$ light intensities. Another two brighter intensities (0.5 and 3 cd.s/m$^2$) were chosen as positive controls of cone and retinal function. At a light intensity of 0.5 cd.s/m$^2$ the average *Gnat1* b-wave amplitude was 28 ±1 % of the average wildtype b-wave response (Figure 3.7bi).
Scotopic ERG responses recorded from dark-adapted *Gnat1*−/− mice are shown in *blue* and recordings from wildtype mice are shown in *black*. (a) Full flash scotopic a-wave (i) and b-wave (ii) ERG responses at each light intensity examined. Mean ± SD, unpaired t-test. Dashed boxes indicate light intensity examined in b. (b) A- and b-wave full flash scotopic ERG responses at three light intensities expressed as percentage of wildtype (i). Mean ± SD, unpaired t-test. (ii) Representative scotopic ERG traces recorded at three light intensities. *, P<0.05; **, P<0.01; ***, P<0.001.
iii) Protocol optimisation: anaesthetic regime & temperature control

In order to minimize the signal to noise ratio, responses at each stimulus intensity were averaged over 15 flashes. An interflash delay of 10 seconds was set between each flash to allow full recovery of the rod photoreceptors.

Again, to minimize the signal to noise ratio, the animal must be anaesthetized in such a way as to ensure minimal movement. However, too deep an anaesthesia may cause a reduction in signal amplitude (Weymouth and Vingrys, 2008). Most importantly, the animal must be anaesthetized to the same depth of anaesthesia when recording on different days. Here, the dose of anaesthesia was 10% higher than reported in Chapter 2 (section 2.2.2.) as mice from the Gnat1<sup>−/−</sup> strain proved to have a higher anaesthetic threshold compared to wildtype animals. The additional anaesthesia reduced animal movement making it easier to place the corneal electrodes. Figure 3.8a shows that the increase in anaesthetic dose did not reduce the signal with Gnat1<sup>−/−</sup> animals receiving the standard anaesthesia volume compared to those receiving an addition 10% (18 ± 3 µV versus 19 ± 2 µV b-wave amplitude, respectively unpaired t-test, P > 0.05, N = 6).

Temperature homeostasis is often compromised in small rodents during anaesthesia and a drop in temperature can result in significant decreases in the ERG response (Weymouth and Vingrys, 2008). Therefore, it is essential to regulate the temperature of the mice during the ERG recording procedure. Temperature homeostasis was not routinely used in the existing set up in the lab, so tests using a temperature controlled heat mat were performed to assess any benefits and to check there was no electrical interference with the recording equipment. Figure 3.8a shows that standardizing the temperature of the mice resulted in a small but significant improvement in the amplitude of the signal (24 ± 0.8 µV compared to 18 ± 3 µV; P<0.001, unpaired t-test, N = 6). In addition, 6 animals were recorded on 2 separate days. Figure 3.8b demonstrated that when the variation between the 2 days was calculated for each protocol, although not significant, less variation between the 2 days was observed.
using the fully optimized protocol (the average standard deviation was 2.7 ± 1.7 for the optimized protocol compared to 6.4 ± 3.7 for the original un-optimised protocol, P>0.05, unpaired t-test, N = 6). Further, there was less variation between the individual animals, as indicated by the error bars (Figure 3.8b).
b) Testing transplanted Gnat1−/− recipients

i) Assessing impact of transplant surgical trauma on ERG responses

It is known that prolonged subretinal detachment causes a reduction in ERG amplitudes (Kim et al., 2001; Nour et al., 2003). To determine the impact of surgical trauma on the recovery of ERG responses in Gnat1−/− mice, sham transplants were performed using donor P4-8 cell from Gnat1−/− mice and compared to contralateral uninjected control eyes. ERG recordings were taken each week for 7 weeks post transplantation following dark adaptation. The amplitude of the b-wave ERG response in sham-transplanted eyes were reduced at most time points compared to uninjected contralateral control eyes (Figure 3.8c, paired t-test, N = 6). This reduction was maximal at 1 week post transplant, but improved and remained largely stable and marginally suppressed after 2 weeks post transplant. This is in keeping with previous reports demonstrating a prolonged reduction of b-wave amplitudes following retinal detachment (Nour et al., 2003).
FIGURE 3.8. OPTIMIZATION OF ERG PROTOCOL AND ASSESSING THE LONG TERM IMPACT OF TRANSPLANTATION SURGERY ON ERG RESPONSES

(a) Scotopic full flash ERG response recorded at 0.03 cd.s/m² from dark-adapted Gnat1⁻/⁻ mice following increased anaesthesia and use of a heat mat. Mean ± SD, unpaired t-test. (b) Histogram demonstrated the mean Standard Deviation following scotopic full flash ERG response recorded at 0.03 cd.s/m² from dark-adapted Gnat1⁻/⁻ mice recorded on two separate days. Mean ± SD, unpaired t-test. (c) Scotopic full flash ERG response recorded at 0.03 cd.s/m² from dark-adapted Gnat1⁻/⁻ mice transplanted with Gnat1⁻/⁻ P4-8 cells (sham) to assess the long term surgical impact of the transplant procedure on scotopic b-wave responses. Mean ± SD, paired t-test. *, P<0.05; **, P<0.01; ***, P<0.001.
ii) Testing Gnat1−/− recipients transplanted with Nrl.GFP+ve rod photoreceptors

Having optimized the scotopic recording setup, transplantations of P4-8 Nrl.GFP+ve donor cells were performed and compared with Gnat1−/− sham transplanted contralateral control eyes (N = 6) to control for the impact of the surgery and presence of cells in the subretinal space. ERG recordings were first taken at 3 weeks post injection to allow the retina time to recover from the initial surgical trauma and to permit maturation of any integrated rod photoreceptor cells. Recordings continued until 5 weeks post injection to obtain repeat recordings. As any response is likely to be small, repeat recording are essential to show whether any treatment effects are robust and reproducible.

No improvements in average scotopic b-wave amplitudes recorded at 0.007 cd.s/m² were similar for either Nrl.GFP+ve and sham transplanted eyes compared to uninjected control animals (Figure 3.9a, P > 0.05, unpaired t-test, N = 6). The small b-wave responses observed at this light intensity can be considered as noise; no defined amplitude waveforms were observed on any of the ERG traces. Similarly, at the brighter mesopic intensity of 0.03 cd.s/m², no improvements in average b-wave amplitude compared to sham injected eyes were observed over the time course examined (Figure 3.9b, P > 0.05, paired t-test, N = 6). Indeed, no consistent improvements in average b-wave amplitude in Nrl.GFP+ve transplanted eye were observed or at any of the tested light intensity compared to sham injected eyes (Figure 3.9c, paired t-test, P > 0.05, N = 6), despite an average of 17,539 ± 3426 integrated cells / eye (range 7135-26,616 integrated cells, Figure 3.9b).

Further analysis of the recordings taken from each independent animal showed that none of the individual b-wave responses from Nrl.GFP+ve rod photoreceptor transplanted eyes demonstrated a sustained improvement over the time frame examined compared with contralateral sham treated controls at any of the light intensities examined. Figure 3.9d shows the individual b-wave amplitudes recorded at
0.03 cd.s/m² at each time point for the animal with the highest integration efficiency (26,616 cells integrated in the ONL, Figure 3.9e).

It must be noted that any negative factors associated with surgical trauma were no worse in Nrl.GFP⁺ve rod-precursor transplanted eyes compared to sham transplanted eyes: b-wave amplitudes of Nrl.GFP⁺ve transplanted and sham transplanted eyes were not statistically different at any time point examined (Figure 3.9b, P > 0.05, paired t-test, N = 6). At all time points examined post injection, b-wave amplitudes from both Nrl.GFP⁺ve rod precursor and sham transplanted eyes remained significantly lower than those from uninjected control eyes (Figure 3.9b; for individual p values see graph, unpaired t-test, N = 6).

To summarise, the data demonstrate that following subretinal transplantation of cell suspension, there is a small but significant reduction in b-wave ERG responses, indicative of some sustained surgical damage, in keeping with previous reports (Nour et al., 2003). Moreover, no repeatable improvements in ERG response were observed following transplantation Nrl.GFP⁺ve donor cells despite robust cell integration.
FIGURE 3. FULL FLASH ERG RESPONSES RECORDED FROM TRANSPLANTED Gnat1⁻/⁻ MICE

Full flash ERG responses recorded from dark-adapted uninjected (blue), Nrl.GFP⁺ve rod precursor transplanted (green) or sham transplanted (red) Gnat1⁻/⁻ mice. (a) Average scotopic b-wave amplitudes recorded from dark-adapted mice at 0.007 cd.s/m². (b) Average b-wave amplitudes recorded from dark-adapted mice at 0.03 cd.s/m². (c) Average b-wave responses recorded from dark-adapted mice at each tested light intensity 5 weeks post transplant. (d) Individual b-wave amplitudes recorded 0.03 cd.s/m² from dark-adapted mice at over 5 weeks for the animal with the highest integration efficiency, with 26,616 cells integrated. (e) Confocal image demonstrating robust integration in those mice treated with Nrl.GFP⁺ve rod precursor cells. This is an example where integration of ~ 25,000 cells was observed. Scale bar is 50µm.

Mean ± SD, paired t-test to compare Nrl.GFP⁺ve rod precursor and sham transplanted eyes and unpaired t-test when comparing to uninjected controls. Abbreviations: NS, not significant.

The single cell recording experiments described in section 2.4.1 demonstrated that transplanted rod photoreceptors are able to hyperpolarize in response to scotopic light stimuli in ex-vivo slices. That this response does not lead to a recordable ERG signal can be explained by two hypotheses. Firstly, either the transplanted rod photoreceptors are not able to propagate these signals to second order neurons. Alternatively, as full field ERG responses are a massed response from the entire retina, the number of functionally integrated cells may be below the threshold for detectable ERG responses. An adeno-associated viral vector carrying the Gnat1 transgene was employed to determine the minimum number of functioning rod photoreceptor cells required to generate an ERG response.


Subretinal injections of high titre AAV2/8.Rho.Gnat1 vector (10^9 viral particles per µl) were administered. Repeated full flash ERG recordings were performed every week from 3 to 6 weeks post injection on fully dark-adapted mice to assess scotopic responses and the degree of rescue achievable by AAV2/8.Rho.Gnat1 mediated gene transfer. Representative scotopic ERG traces recorded at 0.03 cd.s/m^2 are presented in Figure 3.10a demonstrating a rescue of a-wave and b-wave in AAV2/8.Rho.Gnat1 treated mice compare to untreated controls. As shown in Figure 3.10bi and ii, near complete rescue of the ERG b-wave amplitude was observed at 0.03 cs.s/m^2 following treatment of AAV2/8.Rho.Gnat1 vector (dark blue): at all intensities examined, there was no significant difference between treated (dark blue) and wildtype (black) b-wave amplitudes (Figure 3.10a i, P > 0.05, unpaired t-test, N = 4). At a light intensity of 0.03 cd.s/m^2, an average b-wave amplitude of 290 ± 79 µV was achieved in AAV2/8.Rho.Gnat1 treated eyes (dark blue), representing 86 ± 19 % of average wildtype responses (337 ±41 µV, black; Figure 3.10a ii, p>0.05, unpaired t-test, N = 4).
Untreated contralateral control eyes (*light blue*) produced a 21 ± 6 μV average b-wave response (6 ± 1% of wildtype), which was significantly lower than both wildtype (*black*) and contralateral AAV2/8.Rho.Gnat1 (*dark blue*) treated eyes (Figure 3.10b ii, P < 0.001, unpaired t-test and P < 0.01, paired t-test respectively, N = 4). In addition, significant improvements in a-wave amplitudes were also observed: the average a-wave amplitude of untreated *Gnat1*−/− mice at 0.03 cd.s/m² was -3 ± 4 μV (7 ± 2% of wildtype), which was significantly lower compared to AAV2/8.Rho.Gnat1 treated *Gnat1*−/− mice (-23 ± 9 μV or 55 ± 18% of wildtype) (Figure 3.10c i and ii, *light blue* and *dark blue*, respectively; P<0.05, paired t-test, N = 4). Full restoration of the a-wave was not achieved: the wildtype a-wave amplitude (-42 ± 7 μV) remained statistically higher than AAV2/8.Rho.Gnat1 treated *Gnat1*−/− mice (-23 ± 9 μV) (Figure 3.10c ii; P < 0.01, unpaired t-test, N = 4).

Full flash photopic ERG recordings were obtained at 10 cd.s/m² following 30-min light adaptation to suppress rod responses. Figure 3.10d demonstrates that there were no significant differences in the average photopic b-wave or a-wave amplitudes from AAV2/8.Rho.Gnat1 treated (*dark blue*) compared to uninjected contralateral control eyes (*light blue*) following stimulus at a light intensity of 10 cd.s/m². This suggests that any improvements in full flash ERGs following dark-adaptation can be attributed to restoration of rod function (Figure 3.10d, P > 0.05, paired t-test, N = 4). A small and non-significant reduction in both the a- and b-wave amplitudes was observed for untreated (*light blue*) and AAV2/8.Rho.Gnat1 treated (*dark blue*) *Gnat1*−/− mice compared to wildtype controls (*black*). This is consistent with the suppression of ERG responses caused by surgical trauma associated with a subretinal injection (Figure 3.10d, P > 0.05, unpaired t-test, N = 4). Following injection of AAV2/8.Rho.Gnat1 vector, subretinal detachments are likely to resolve within 48hr (*pers com Professor Jim Bainbridge*) as there is no cell mass remaining within the subretinal space to prevent re-attachment. This is likely to explain the reduced impact of surgical trauma on ERG responses, compared to the cell transplant experiments. Using
immunohistochemistry and fluorescence microscopy, we assessed rod- α-transducin+ve (Gnat1) expression in the eyes receiving AAV2/8.Rho.Gnat1 vector (10^9 viral particles per µl). The vast majority (>80%) of the rod photoreceptor cells in the ONL express rod-α-transducin suggesting that the transduction efficiency was high (Figure 3.10e). With this degree of transduction, quantification of the number of cells expressing the Gnat1 protein was not possible. However, it was estimated that 90-100% of the rods within the ONL expressed Gnat1, equivalent to >5 million cells.

(a) Representative ERG traces recorded at 0.03 cd.s/m² from dark-adapted wildtype (bottom), AAV2/8.Rho.Gnat1 high titre (10⁹ viral particles per µl) treated (middle) and contralateral untreated control eyes (top). Average full flash ERG (b) b-wave responses and (c) a-wave responses recorded at (i) different light intensities from dark-adapted wildtype (black), AAV2/8.Rho.Gnat1 high titre treated (dark blue) and contralateral untreated control eyes (light blue). Striped bars indicated points used for analysis in ii.

(ii) Average full flash ERG responses recorded at 0.03 cd.s/m² from dark-adapted mice expressed as percentage of wildtype. (d) Average photopic flash ERG a-wave and b-wave responses recorded at 10 cd.s/m² from light adapted wildtype (black), AAV2/8.Rho.Gnat1 high titre treated (dark blue) and contralateral untreated control eyes (light blue). (e) Confocal image demonstrating rod α-transducin staining in eyes treated with high titre AAV2/8.Rho.Gnat1. Scale bar 50µm.

Mean ± SD, paired t-test to compare Nrl.GFP⁺ve vector treated and untreated animals and unpaired t-test when comparing to wildtype controls.

In order to titrate the number of functioning rod cells required to reach the ERG response threshold, various dilutions of the AAV2/8.Rho.Gnat1 vector were injected subretinally in Gnat1<sup>−/−</sup> mice, varying from $10^5$ to $10^7$ viral particles per µl. Repeated ERG recordings were performed at 3-6 weeks post injection and the average responses of these recordings are presented (Figure 3.11). Animals were sacrificed following 30-min light adaptation to allow translocation of the Gnat1 (rod α-transducin) protein into the cell body. Using immunohistochemistry and fluorescence microscopy, the number of rod α-transducin<sup>+</sup> photoreceptor cells was quantified.

Eyes of AAV2/8.Rho.Gnat1 treated Gnat1<sup>−/−</sup> mice (dark blue) with >127,000 Gnat1<sup>+</sup> cells (range 127,500 - 155,000 Gnat1<sup>+</sup> cells, N = 3) had clear b-wave ERG responses even at the lowest scotopic intensity stimuli. Following dark-adapted ERG recording, significant improvement in the average b-wave amplitude was demonstrated compared to the contralateral untreated control eyes (light blue) at 0.03 cd.s/m² (Figure 3.11a and b, pink box, P < 0.05, paired t-test), and at every other light intensity tested including the lowest scotopic light stimuli of 0.007 and 0.003 cd.s/m². Figure 3.11c i shows the averaged scotopic b-wave amplitudes at various light intensities for an individual Gnat1<sup>−/−</sup> mouse treated with AAV2/8.Rho.Gnat1 (10<sup>7</sup> viral particles per µl) in one eye (dark blue) compared to the contralateral untreated control eye (light blue). At 0.007 cd.s/m² the average b-wave amplitude was 42 ± 12 µV and at 0.03 cd.s/m² the average b-wave amplitude was 62 ± 19 µV. Significant improvements at all light intensities tested were observed compared to the respective uninjected control eyes (Figure 3.11c i, P < 0.05, paired t-test).

Conversely, expression of rod α-transducin in ~60,000 cells or less (N = 8) following AAV2/8.Rho.Gnat1 treatment, did not result in any significant improvements in average b-wave amplitudes recorded from dark-adapted mice at 0.03 cd.s/m² or at any other light intensity tested (Figure 3.11 a and b, P > 0.05, paired t-test). Figure 3.11di shows the averaged b-wave amplitude at various light intensities for an individual Gnat1<sup>−/−</sup>
mouse treated with same vector titre \((10^7\) viral particles per µl), where only \(~59,000\) cells were Gnat1\(^{\text{ve}}\) (Figure 3.11c ii). The averaged b-wave amplitudes for AAV2/8.Rho.Gnat1 treated eyes \((\text{dark blue})\) were not significantly different to those from contralateral uninjected control eyes \((\text{light blue})\) at 0.03 cd.s/m\(^2\) (Figure 3.11a: treated 26 ± 8 µV versus untreated 23 ± 7 µV, \(P > 0.05\), paired t-test) or at any of the light intensities tested (Figure 3.11c i, \(P > 0.05\), paired t-test).

Therefore, it can be concluded that the ERG sensitivity threshold following gene therapy treatment with the AAV2/8.Rho.Gnat1 vector, lies between \(~60,000\) and \(~127,000\) functional rod photoreceptor cells. These data suggests that the number of functionally integrated cells following transplantation of Nrl.GFP\(^{\text{ve}}\) rod precursor cells (max 26,600) is currently insufficient to generate potentials detectable by ERG recordings.
Individual animal responses & cell counts

0.03 cd/s/m²

Amplitude [μV]

Number of cells expressing Gnat1

Improvement over un.injected control

Number of cells expressing Gnat1

C. Individual Animal Scotopic Intensity Series

155,058 Gnat1+ cells

Amplitude [μV]

Light intensity (cd/s/m²)

Gnat1+ (AAV8.gnat1 treated)

rod α-transducin

ONL

~150,000 rod α-transducin** cells

D. Individual Animal Scotopic Intensity Series

59,289 Gnat1+ cells

Amplitude [μV]

Light intensity (cd/s/m²)

Gnat1+ (AAV8.gnat1 treated)

rod α-transducin

ONL

~60,000 rod α-transducin** cells
FIGURE 3.11. TITRATING ERG RESPONSES USING DILUTIONS OF AAV2/8.RHO.GNAT1 VIRAL VECTOR

(a) Correlation between individual animal (Gnat1−/−) average b-wave responses recorded at 0.03 cd.s/m² from dark-adapted AAV2/8.Rho.Gnat1 treated eyes (dark blue) plotted against the number of the of rod α-transducin⁺ve cells. The corresponding contralateral untreated control eyes’ average b-wave amplitude values are also plotted (light blue). (b) ERG b-wave responses recorded from dark-adapted AAV2/8.Rho.Gnat1 treated Gnat1−/− mice: graph shows improvement in individual animal average b-wave responses from treated eyes compared to the contralateral untreated control eyes’ responses recorded at 0.03 cd.s/m² plotted against the number of the of rod α-transducin⁺ve cells. Pink box denotes those animals that demonstrated both a significant improvement compared to the contralateral untreated control eye and >127,000 rod α-transducin⁺ve cells. Dashed boxes indicate individual animals that are analysed in c and d. (c) Complete light intensity series ERG responses recorded after dark-adaptation demonstrating average b-wave amplitudes for an individual animal with ~155,000 rod α-transducin⁺ve cells, represented by the confocal image (ii), below. (d) (i) Complete light intensity series ERG responses recorded after dark-adaptation demonstrating average b-wave amplitudes for an individual animal with ~59,000 rod α-transducin⁺ve cells, represented by the confocal image (ii), below. Scale bar 50µm.

Mean ± SD, paired t-test. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer.
3.2.2.4. Intrinsic imaging of the visual cortex

Retinal signals are amplified along the visual pathway and it is therefore possible that despite that the signals generated by integrated Nrl.GFP\(^{+}\)ve rods are not sufficient to detect a global ERG response, the signals may be transmitted to the higher visual centers and detectable in the brain. To test this, optical intrinsic imaging of the primary visual cortex (Grinvald et al., 1986) was performed. Optical intrinsic imaging provides a measure of cortical intrinsic haemodynamic responses over large regions of the primary visual cortex (V1). Activity in neurons in V1 leads to changes in blood flow and oxygen levels. Following a period of activation, deoxygenated blood can be detected around the region of neuronal activity and this results in changes of reflected light as deoxygenated blood has different absorption properties to oxygenated blood. In V1 areas of the retinal visual field are topically mapped (Maldonado et al., 1997) so changes in the position of a visual stimulus will result in a change of the cortical haemodynamic response locus. Four partially overlapping visual stimuli were used, which covered the majority of the visual field of one eye (Figure 3.12a). The stimuli consisted of flickering black and white bars presented on a grey background (Figure 3.12a). The exposed visual cortex was illuminated with a 660-nm light and imaged using a CMOS photodetector camera as illustrated in Figure 3.12a.

As shown in Figure 3.12bi, photopic stimuli induce a strong intrinsic haemodynamic cortical responses in both Gnat\(^1\)/\(^{-}\) and wildtype mice (N = 5). In addition, retinotopy was observed in both strains when the position of the visual stimulus was changed, concurrent changes in the locus of the cortical response were also observed (Figure 3.12b i). The focus of these studies was to assess rod-mediated vision and here we present for the first time an assessment of rod photoreceptor derived intrinsic responses in the visual cortex under scotopic lighting conditions. Animals were dark-adapted over night prior to imaging, surgery was performed under dim-red light conditions and the visual stimulus intensity was within the scotopic range (0.007 cd.s/m\(^2\)). Figure 3.12bii demonstrates that under scotopic conditions, cortical
responses were only observed in wildtype mice. Again, these were observed in distinct regions of V1, according to the position of the visual stimulus (N = 5). No responses were detected in Gnat1−/− mice tested under scotopic lighting conditions (N = 7), reflecting a lack of rod-derived signaling.
a. Setup Schematic

b. Optical intrinsic imaging in scotopic and photopic conditions
(a) Schematic diagram illustrating experimental setup to image intrinsic haemodynamic responses in the visual cortex. Four overlapping stimuli were presented on a monitor. (b) Intrinsic imaging of wildtype and Gnat1−/− mice under (i) photopic and (ii) scotopic lighting conditions using different stimuli. Black arrows indicate the approximate location of the mouse (posterior-to-anterior axis). Scale bars=1mm; ΔI/I=relative intensity values

Abbreviations: Au, auditory cortex; S1, somatosensory cortex; V1, primary visual cortex
Next, \textit{Gnat1}^{-}\textit{/-}} recipient animals were transplanted with either P4-8 Nrl.GFP^{+ve} rod precursor cells or age-matched sham (\textit{Gnat1}^{-}\textit{/-}) cells. At 3-5 weeks post transplantation, animals were imaged under scotopic conditions to test rod-driven intrinsic cortical responses in V1 of the visual cortex. Animals receiving Nrl.GFP^{+ve} donor cell transplants all demonstrated robust scotopic stimulus driven intrinsic cortical responses (Figure 3.13a, \(N = 5\)). Furthermore, partial restoration of retinotopy was achieved: overlapping visual stimuli resulted in responses in overlapping regions of V1 as indicated by the colour maps in Figure3.13a. It must be noted that not all stimuli resulted in a cortical response in all animals: for example no responses were observed in animal 5 when using stimulus 2 and 3 (Figure 3.13a). This is most likely explained by the fact that transplanted cells integrate primarily around the injection sites and integration occurs patches across of the retinal area, but not the entire visual field. Cortical responses, tested under scotopic lighting conditions, was absent in mice transplanted with sham donor cells (Figure 3.13b; \(N = 3\)), as was seen with untreated \textit{Gnat1}^{-}\textit{/-}} control animals (Figure 3.14; \(N = 7\)). Histological analysis of the transplanted retinae post testing confirmed robust integration in all Nrl.GFP^{+ve} rod precursor transplanted eyes (10,782 - 31,075 integrated cells).
a. SCOTOPIC: Gnat1/- Nrl.GFP transplanted

b. SCOTOPIC: Gnat1/- sham transplanted
FIGURE 3.13. INDIVIDUAL ANIMAL SCOTOPIC HAEMODYNAMIC INTRINSIC IMAGES OF Gnat1−/− MICE

Scotopic haemodynamic intrinsic images of Gnat1−/− mice post transplant of (a) Nrl.GFP+ve rod precursor cells or (b) Gnat1−/− sham cells. Black arrows indicate the approximate location of the mouse (posterior-to-anterior axis). Imaging artefacts created by uneven bone surfaces are indicated by asterisks (red). Scale bars = 1mm; ΔI/I=relative intensity values.
SCOTOPIC: Gnat1-/- Untreated

**FIGURE 3.14. INDIVIDUAL ANIMAL SCOTOPIC HAEMODYNAMIC INTRINSIC IMAGES OF UNTREATED CONTROL Gnat1+/- MICE**

Black arrows indicate the approximate location of the mouse (posterior-to-anterior axis). Scale Imaging artefacts are indicated by asterisks (*red*). Scale bars = 1mm; $\Delta I/I =$ relative intensity values.
3.2.2.5. Behavioural testing of Nrl.GFP*ve rod precursor transplanted Gnat1+/− recipients

In order to assess whether transplantation of rod photoreceptor precursor cells can contribute to and improve higher measures of visual function, two behavioural tests were employed: optomotor head-tracking reflex testing and the visually guided water-maze test. Both require input of visual information, which is integrated by visual centers in the brain resulting in motor responses. The optomotor head-tracking response reflects functional integration of the visual stimulus orchestrated by the sub-cortical brainstem. Conversely, the visually-guided water maze test requires cognitive processing of visual information in higher brain regions including the visual cortex.

a) Optomotor head tracking results

Optomotor head tracking responses to a rotating pattern can be assessed to measure contrast sensitivity and visual acuity (Alexander et al., 2007; Prusky et al., 2004; Thomas et al., 2004b; Umino et al., 2008). A full description of the methods used to test optomotor head tracking response was previously described in Chapter 2, section 2.4.4. Optomotor testing and analysis was performed by Dr Rachael Pearson, who kindly supplied the data presented in Figure 3.15.

Optomotor head tracking responses in wildtype and untreated Gnat1+/− mice were characterized under scotopic and photopic lighting conditions, to confirm the absence of rod mediated vision yet retention of cone mediated vision in Gnat1+/− mice. Under scotopic lighting conditions, head tracking responses testing both contrast sensitivity and visual acuity were retained in wildtype mice yet, as anticipated absent in untreated Gnat1+/− mice (Figure 3.15, ai and aii, N = 4 and N = 12, respectively). In contrast, under photopic lighting conditions, optomotor head tracking responses in wildtype and Gnat1+/− mice were similar (Figure 3.15, a i and ii, both N = 12). Previous reports have demonstrated that murine visual acuity is worse in scotopic condition than in photopic
conditions (Umino et al., 2008): indeed in wildtype mice the visual acuity threshold was $0.488 \pm 0.003$ cycles / degree in photopic lighting conditions and $0.245 \pm 0.01$ cycles / degree in scotopic lighting conditions.

$Gnat1^{-/-}$ recipient mice were transplanted with FACS sorted Nrl.GFP$^{+ve}$-rod precursor cells by both superior and inferior subretinal injections and the contralateral control eye received sham cells (age-matched $Gnat1^{-/-}$ cells). Photopic optomotor head tracking responses were recording pre- and post-transplant as a positive control, and demonstrated no change in either visual acuity or contrast sensitivity for sham injected or Nrl.GFP$^{+ve}$ injected eyes demonstrating that cone function was neither improved nor compromised as a result of the surgery or cell transplant. Animals were dark-adapted overnight and tested in scotopic lighting conditions to assess rod-mediated vision (Figure 3.15, c i and d i). Optomotor responses were observed in mice treated with Nrl.GFP$^{+ve}$-rod precursor cells in rod mediated scotopic lighting conditions: the mean contrast sensitivity threshold was $1.3 \pm 0.1$ compared to $15 \pm 0.6$ in wildtype animals (Figure 3.15, c i) and the mean visual acuity threshold was $0.097 \pm 0.007$ cycles / degree compared to $0.245 \pm 0.01$ cycles / degree in wildtype animals.

A small optomotor response was observed in the contralateral sham injected eyes. To examine whether effect is a consequence of trophic effects generated by the presence of the sham cell mass or surgery or whether this response is can be attributed to ‘cross-talk’ between the visual fields driving the head tracking response, two further experiments were performed. In the first instance, a cohort was transplanted with P1 unsorted cells (known to have poor integration efficiency) to control for any trophic effects of the immature cell mass. No optomotor response was observed in scotopic lighting conditions when testing for either visual acuity or contrast sensitivity. Secondly, a cohort was injected with sham age-matched $Gnat1^{-/-}$ cells in both eyes (Figure 3.15, c and d). Similarly, no optomotor responses were recorded when testing contrast sensitivity or visual acuity in scotopic lighting conditions. This indicates that there is some degree of ‘cross-talk’ between each visual field in driving optomotor head
tracking responses and that small responses can be detected by the non-dominant eye, (i.e. during clockwise rotation, the left eye dominates the response but not exclusively, and vice versa).

Animals were scarified following optomotor testing and the number of Nrl.GFP\textsuperscript{+ve} cells successfully integrated within the recipient ONL was quantified. Robust integration following transplant was demonstrated; more over a significant positive correlation between the number of integrated cells and the degree of rescue achieved for both contrast sensitivity and visual acuity was demonstrated (Figure 3.15, c ii and iii).
a. Pre-testing wildtype and Gnat1⁻/⁻ responses

Scotopic | Photopic
---|---
Wildtype | n = 4
Gnat1⁻/⁻ | n = 12

Scotopic | Photopic
---|---
Wildtype | n = 4
Gnat1⁻/⁻ | n = 12

b. Photopic responses

Scotopic | Photopic
---|---
Pre-Post | Pre-Post
Gnat1⁻/⁻ (Nl:GFP eyes) | (sham eyes)
---|---
N = 12 | N = 12

Scotopic: Contrast sensitivity

Scotopic: Visual acuity

R² = 0.5978
P = 0.003

R² = 0.5317
P = 0.007
FIGURE 3.15. OPTOMOTOR HEAD TRACKING RESPONSES

(a) Contrast sensitivity and visual acuity response thresholds recorded from Gnat1<sup>−/−</sup> and wildtype mice in cone-mediated photopic light conditions and rod-mediated scotopic lighting conditions. (b) Contrast sensitivity and visual acuity response thresholds recorded in photopic lighting conditions from Gnat1<sup>−/−</sup> mice transplanted with Nrl.GFP<sup>+</sup>ve cells and sham (Gnat1<sup>−/−</sup>) cells in the contralateral control eye. Contrast sensitivity (c i) and visual acuity (d i) response thresholds recorded in scotopic lighting conditions. Head-tracking response threshold correlation with the number of integrated cells for contrast sensitivity (c ii) and visual acuity (d ii).
b) Watermaze testing

To assess whether photoreceptor transplantation can contribute to higher visual function, the visually-guided water-maze test, which requires cognitive processing of visual information, was employed. The water-maze is Y-shaped and the mice are trained to swim to a hidden platform that is associated with a visual cue; a pattern grating appearing on a computer monitor (Figure 3.16a). Water-maze training was performed by Dr Claire Hippert and Yanai Duran prior to transplantation. Testing and data analysis was performed by Dr Rachael Pearson, who kindly supplied the data presented in Figure 3.16a.

Watermaze training was performed under photopic cone-mediated lighting conditions. Figure 3.16a demonstrates that Gnat1<sup>-/-</sup> mice (N = 21) were able to learn and complete the task as well as wildtype animals (N = 4), indicated by a ≥ 70 % correct response rate. Testing under scotopic lighting conditions, revealed the Gnat1<sup>-/-</sup> mice (N = 21) performed no better than chance, as anticipated, compared to wildtype (N = 4) animals that were able to complete the task with a ≥ 70 % correct response rate (Figure 3.16a).

Of the 21 Gnat1<sup>-/-</sup> mice trained, three testing cohorts were formed. Gnat1<sup>-/-</sup> mice received either dual transplants of Nrl.GFP<sup>+/+</sup>-rod precursor cells in both eyes (N = 9); dual transplants of sham age-matched Gnat1<sup>-/-</sup> cells in both eyes (N = 6) or remained untreated (N = 6). Trained wildtype untreated control animals were test alongside the Gnat1<sup>-/-</sup> cohorts. All animals were re-tested under scotopic lighting conditions 4-6 weeks post transplant in order to assess rod-mediated visual function. Figure 3.16c demonstrates that 4 out of the 9 Gnat1<sup>-/-</sup> recipients receiving Nrl.GFP<sup>+/+</sup>-rod precursor cell transplants and all of the wildtype control animals (N = 4), were able to solve the task making ≥ 70 % correct responses. None of the sham injected or untreated Gnat1<sup>-/-</sup> mice were able to solve the task (Figure 3.16c), and the average response of these cohorts was no better than 50 % chance (Figure 3.16d). The average response of the Nrl.GFP<sup>+/+</sup> treated Gnat1<sup>-/-</sup> recipients was significantly greater than the untreated and sham treated control groups (Figure 3.16d). Furthermore, figure 3.16e demonstrates
that the Gnat1−/− Nrl.GFP+ve treated recipient cohort that were able to complete the task (performers only), did so with an average swim-time of 15.1 ± 0.8 sec to reach the hidden platform, compared with 7.1 ± 1.4 sec for wildtype mice. This was significantly faster compared with 26.4 ± 1.1 and 26.0 ± 1.2 for sham-injected Gnat1−/− and untreated Gnat1−/− animals, respectively (Figure 3.16e). Furthermore, the ability to perform the water-maze under cone-mediated photopic lighting condition post transplant remained at ≥ 70 % throughout the experiment (Figure 3.16f), however, a small temporary drop in photopic pass rate during maintenance training was observed 2 weeks immediately after injections (data not shown).

Following water-maze testing the animals were sacrificed and the number of Nrl.GFP+ve cells correctly integrated into the recipient Gnat1−/− ONL was quantified. Robust integration was observed (Figure 3.16g), more over a significant positive correlation between the number of integrated Nrl.GFP+ve rod cell and the ability to solve the watermaze task was demonstrated (Figure 3.16g and compare h i and ii).
FIGURE 3.16. WATER-MAZE TESTING RESULTS

(a) Schematic of water-maze setup (Adapted from (Wong and Brown, 2007)). Animals are trained to swim to a hidden platform associated with a striped grating visual cue. (b) Average pass rate for wildtype (black bars) and Gnat1-/- animals (grey bars) in rod-mediated scotopic and cone-mediated photopic lighting conditions. (c) Individual pass-rates of Nrl.GFP-treated, sham (black), sham-injected (dark grey) and uninjected (mid grey) Gnat1-/- mice and wildtype uninjected (light grey). (d) Average pass-rate for each cohort. (e) Average swim-time to platform latencies for each cohort, including all (light grey) and correct choice only (dark grey). (f) Average photopic pass-rate for Gnat1-/- receiving Nrl.GFP-transplants (light grey bars) or sham-transplants (dark grey bars) both before and after transplant. (g) Graph demonstrating correlation between the number of integrated Nrl.GFP rod cells and the ability to solve the water-maze task. Red circle highlight individual animals presented in (h). (h) Examples of integration in animal No 6 (i) that successfully completed the watermaze task and animal number 5 (ii) that did not.
3.3. DISCUSSION

Transplantation of photoreceptor cells offers a potential future strategy for the treatment of retinal degenerative disorders that culminate in impaired vision and ultimately blindness. Over the last decade, proof-of-concept for photoreceptor transplantation has been investigated. Donor cells, if at the correct ontogenic stages (Lakowski et al., 2010; Lakowski et al., 2011; Maclaren et al., 2006), are capable of migrating into and integrating within the recipient retina. These cells form typical photoreceptor morphologies and express mature photoreceptor markers (Bartsch et al., 2008; Jiang et al., 2010; Klassen et al., 2004; Kwan et al., 1999; Lakowski et al., 2010; Lakowski et al., 2011; Maclaren et al., 2006; West et al., 2008; West et al., 2010). Some limited improvements in visual function have been reported (Klassen et al., 2004; Kwan et al., 1999; Maclaren et al., 2006). However, for robust repair of visual function, integration efficiency required significant improvement on the low numbers previously reported to date. In the first part of this chapter, methodological approaches to improve transplant procedure and ultimately increase integration efficiency were assessed. Together, these led to a 17-fold improvement, compared to our earlier reports (Maclaren et al., 2006; West et al., 2008; West et al., 2010), which was achieved through a combined transplant approach, using a purified donor cell population and multiple transplant injection sites.
3.3.1. IMPROVING TRANSPLANTATION EFFICIENCY

Some reports have indicated that the presence of non-integrated graft cells may be beneficial to the endogenous retina by providing neurotrophic support, through the release of pro-survival growth factors (Keegan et al., 2003; Lawrence et al., 2000). However, potential surgical damage to the surviving recipient retina must be considered if photoreceptor transplantation is to be proposed as a therapy to treat mid-stage disease. Previous reports have indicated that photoreceptor transplantation can lead to significant thinning of the recipient ONL (West et al., 2010), an observation likely to arise from the low integration rate leading to the presence of large numbers of non-integrated cells remaining in the subretinal space. Prolonged detachment of the retina is known to result in apoptotic cell death of retinal photoreceptors (Cook et al., 1995; Stone et al., 1999). Survival of the graft cells in the subretinal space may prevent functional re-attachment of the retina after surgery and may act as a barrier separating the endogenous recipient retina from the overlying RPE, which plays an essential role in maintaining photoreceptor health. With this in mind, photoreceptor transplantation replacement therapy may be more appropriate for the treatment of mid- to end-stage disease, when the recipient retina ONL has significantly degenerated. Perhaps in this case, larger number of donor cells could be transplanted with less risk of damaging the remaining host inner retina.

However, here we sought to minimize host retinal damage by reducing the concentration of cells injected, to reduce the remaining donor cell mass size, while retaining the injection volume so keeping the size of the detachment area with the aim of maximizing the spread of cell integration. Recipient retinal damage was minimal when reducing the donor cell density to 200,000 cells / µl, from the previously reported 400,000 cells / µl. Importantly, donor cell integration efficiency was maintained. The preservation of integration efficiency may be part explained by the availability of integration competent donor cells; if these integration competent cells are in
abundance, availability will not be a limiting factor until much lower donor cell numbers are used. In addition, injection of fewer donor cells may reduce their retention within the cell mass and thus promote donor cell migration away from the transplanted cell mass into the recipient ONL. Donor cell migration and integration may be inhibited by local cell-to-cell interactions within the dense cell mass which may provide enough trophic support for donor cell survival. Donor cells that are relatively isolated may migrate into the retina in order to seek such interactions and survival cues.

In these cell density experiments, a mixed population of postnatal retinal cells was transplanted, including Müller glial cells. GFAP is a marker of glial cell activation and reactive gliosis, a process known to inhibit neuronal regeneration (Bradbury et al., 2002; Bradbury and Carter, 2010; Fawcett and Asher, 1999). Examination of the donor cell mass revealed significant expression of GFAP, most likely originating from the donor Müller glia, which appeared to encase the Nrl.GFP+ve photoreceptor cell mass. The presence of these Müller glia may present a barrier to donor rod precursor cells that would otherwise be capable of migrating away from the donor mass.

Previous studies have demonstrated that successful rod transplantation requires donor cells at the correct ontogenic stage in development, a process that coincides with the onset of expression of Nrl in rod precursor cells (Maclaren et al., 2006). Therefore, here FACS was employed as a method to isolate and purify this population from Nrl.GFP+/− donors. In addition, it also offered a method to circumvent the potentially negative effects of transplanting an unsorted donor population that (1) may hinder integration and (2) remain in the subretinal space after transplantation prolonging retinal detachment. Using FACS to purify the P4-P6 donor cell population, the donor cell purity could be significantly increased to 90% Nrl.GFP+ve (a 2.3-fold improvement on unsorted populations, which had 38% of cells Nrl.GFP+ve) with little effect on overall cell viability.

Following transplantation of FACS purified Nrl.GFP+ve rod precursor donor cells, integration was significantly increased (4.7-fold) compared to unsorted controls.
Interestingly, the degree of improvement was greater than anticipated: by purifying the population 2.3-fold integration increased 4.7-fold, suggesting that some other factors, such as those discussed above, may be involved and act to impede cell integration when using unsorted donor cells. The improvement in cell integration was accompanied by an increase in the distribution of the integration and a qualitative reduction in the size the donor cell mass compared to unsorted controls. The latter observation is unlikely to be explained by increased integration alone as only 10% of transplanted cells go on to integrated within the recipient retina. It may be that FACS sorted donor cell masses lack the survival cues associated with an unsorted donor cell mass, resulting in increased integration but reduced longevity of those cells that do not integrate. To investigate this more thoroughly, the number of cells residing in the subretinal space would need to be quantified using a ubiquitous cell marker. Donor cells from offspring of cross between the Nrl.GFP+/+ mouse and a ds-RED mouse could be used in this instance. In addition it may also be useful to assess cell death within the subretinal cell mass cell death using a cell death detection assays such as TUNEL staining.

Reduced integration efficiency was observed when using unpurified donor cells that where processed through the FACS machine, termed ‘ungated’ cells compared to unsorted unprocessed donor cells. A small non-insignificant reduction in cell viability was observed when assessed immediately post FAC sorting, prior to transplant. FACS uses pressure differentials to sort cells, a process that is likely to induce some shearing stress responses (Lakowski J., pers com), and a decline in cell viability is not surprising. However, it is possible that the true extent of cell loss was underestimated, as cell death is a process that occurs over several hours and may not be detected within the time frame examined here. Further investigations would be required to fully assess the impact of FACS sorting on cell viability and integration. Early termination, with 24 hours, of animals transplanted with ‘ungated’ FACS-processed cba.GFP+/+ donor cells could be used as a method to assess the true extent of cell viability loss.
The number of dead cells within the GFP\textsuperscript{+ve} cell mass could be quantified using cell death detection markers.

Despite significant improvements in cell integration efficiency following purification of the donor cell population using Nrl.GFP as a marker, only 8.5% of the total number of injected cells integrated into the recipient retina. Previous reports indicate that cells that are too immature or mature fail to integrate efficiency and fail to develop into mature photoreceptors (Maclaren et al., 2006). This suggests that there is a very specific developmental time window during which donor cells successfully integrate. The rod photoreceptor population exhibits a long maturation period and although the peak of Nrl expression is around P4 (Akimoto et al., 2006), many of the Nrl.GFP\textsuperscript{+ve} cells in a P4 population will be at different stages of maturation. Furthermore, Nrl expression begins when the immediately after terminal differentiation and the beginning of the maturation process; however, it continues to be expressed once maturation is complete. Identification of a second marker at the end of the developmental time window and using it to exclude those Nrl.GFP\textsuperscript{+ve} cells that are already too mature, would allow heightened purification of the optimal donor cell source and may further increase the proportion of transplanted cells that can integrate into the recipient retina. Moreover, while Nrl certainly identifies a population of integration-competent cells, there may be other markers that better define this population (Lakowski et al., 2011). For example, preliminary evidence suggests that FACS purified P8 Nrl.GFP\textsuperscript{+ve} cells are better than FACS purified P4 and P10 Nrl.GFP\textsuperscript{+ve} cells, suggesting that the most integration-competent rod precursor cells may not be immediately post mitotic as initially thought (data not shown). In addition, the use of a fluorescent genetic marker to identify the appropriate donor population is not desirable for a human therapy. Identification of appropriate cell surface markers to isolate integration-competent donor cells from differentiated stem cell cultures will be required. Several groups have began to identify cell surface markers that could be used to both enhance the isolation of the most integration competent donor cell population and to purify the donor population in a
method conducive with a human therapy (Eberle et al., 2011; Lakowski et al., 2011). CD24 and CD73 have been identified as cell surface markers that can isolate a highly integration competent donor cell population, more so than using the expression of a single genetic marker, Nrl.GFP (Lakowski et al., 2011).

Several previous reports have demonstrated that injury can have a positive effect on photoreceptor survival (Sakai et al., 1999) and transplantation outcome (Guo et al., 2003; Jiang et al., 2010; Kurimoto et al., 2001; Nishida et al., 2000). Retinal injury initiates a number of changes in the retina, including the release of several growth factors that may have neurotrophic effects and promote transplanted cell integration (West et al., 2012b). Kwan et al. 1999, had adopted the use of the scleral puncture technique and had hypothesized that this technique may reduce transplant reflux by reducing intra-ocular pressure prior to injection of the cell suspension (Kwan et al., 1999). Here we demonstrate that performing the minimally invasive scleral puncture surgery immediately prior to transplant had no effect on integration efficiency. However, we did find that scleral puncture surgery immediately prior to transplant results in a reduction in integration variation, a phenomenon that could be attributed to a reduction in intra-ocular pressure and the subsequent reflux of the cell suspension.

Using a more invasive technique to initiate an acute local retinal injury, we found that a single subretinal detachment of the retina 48-hours prior to a single subretinal transplant led to a 2.4 fold increase in integration efficiency. Neural retina reattachment following a subretinal detachment is known to induce outer-segment re-growth and outgrowth of rod axons into the inner retina (Fisher and Lewis, 2003): processes could be beneficial to promote transplanted cell integration. The improvement in cell integration demonstrated here is similar to improvements reported by Jiang et al. 2010 who demonstrated an increase in cell integration of cultured RPC following photocoagulation laser treatment prior to transplant (Jiang et al., 2010). Several studies have attributed the beneficial effects of injury on transplant outcome to the release of trophic growth factors (Guo et al., 2003; Kurimoto et al., 2001; Nishida et al., 2000;
Sakai et al., 1999) and changes within the composition of the extracellular matrix (Jiang et al., 2010). Indeed, subretinal detachment is known to initiate a glial response and an array of growth factors and cytokines are upregulated within hours of the detachment (Nakazawa et al., 2006), some of which could provide neurotrophic support that may promote donor cell survival or act as chemoattractants to promote donor cell migration. For example, mRNA levels for fibroblast growth factor (bFGF) are known to be upregulated in the ONL at 72 hours following subretinal detachment (Nakazawa et al., 2006) and bFGF suppresses apoptosis, thus promoting neuronal survival (Cardone et al., 1998). Indeed, ectopic expression of the neurotrophic growth factor insulin-like growth factor was shown to increase integration efficiency in the wildtype retina (West et al., 2012). Other reports examining transplants into an pre-injured retina have demonstrated that N-cadherin, thought to promote neurite outgrowth (Neugebauer et al., 1988), is upregulated at the OLM around the detachment site (Chen and Ma, 2007) and may promote transplanted cell migration. In addition, the mechanical trauma of the subretinal detachment to the recipient retina may cause a localized transient disrupt to the OLM, a proven barrier to cell integration (West et al., 2008). Manipulation of these factors by other more targeted and less traumatic means may prove a useful strategy to further improve cell integration efficiency (see Chapter 4). In addition, several of these factors may be altered in the degenerating retina. For example, as cell death ensues, OLM integrity may become compromised, which is likely to promote donor cell integration. These studies indicate that successful integration may be achieved in the degenerating retina, in which a similar injury response may be activated. The impact of disease type, disease progression and the subsequent changes in the degenerative recipient microenvironment on transplantation outcome will be examined in greater detail in Chapter 4.

Despite moderate improvements in donor cell integration reported in the Jiang study, laser treatment resulted in considerable damage and cell death to the recipient retina (Jiang et al., 2010) and subretinal detachment is known to initiate death of retinal cells
(Cook et al., 1995; Stone et al., 1999). Using retinal injury as an adjunctive strategy to enhance transplanted photoreceptor integration efficiency in a clinical setting needs to be carefully balanced against any negative effects to the surviving recipient retina. We found that excessive injury following dual pre-detachment surgery prior to dual transplant surgery can lead to marked reductions in integration efficiency and poor recipient cytoarchitecture. Widespread detachment may damage host vasculature (Mervin et al., 1999), suggesting the metabolic demands of the newly grafted donor cell mass and recipient retina may not be met and cell death ensues. Although the retina is considered to be immune privileged, injury, for example as a result of widespread subretinal detachment, may compromise the choroid and blood-retinal-barrier functions, which may facilitate an acute immune response. Evidence of immune rejection was indeed observed in several eyes transplanted using the combined approach, more so than observed with single injections. The increased incidence of intravitreal injection observed in this cohort may arise as a consequence of damage to the recipient retina caused by the dual pre-detachment surgery, leading to structural weakness and increasing the likelihood of unintended intra-vitreal injection.
3.3.2. HISTOLOGICAL AND FUNCTIONAL ASSESSMENT OF INTEGRATED ROD PRECURSOR DONOR CELLS

Integration and maturation of transplanted rod precursor cells into the wildtype and degenerating retina has now been robustly demonstrated by a number of groups. Firstly, qualitative assessment of transplanted cells shows that they do develop mature rod morphologies with slender segment structures projecting into the IPM (Bartsch et al., 2008; Maclaren et al., 2006). Here we demonstrate that integration in the Gnat1−/− recipient is comparable to that achieved in wildtype recipients, both in the total number of cells integrating into the recipient ONL and in the development of mature rod morphologies. Segment formation of rod precursor cells transplanted into Gnat1−/− recipients appears similar to rod transplanted into the wildtype recipient. Further studies as assessed from histological fluorescence sections at the ultrastructure level are required to confirm the structural similarity of transplanted cell outer segment development with that of endogenous wildtype rod outer segments: measuring segment length and quantification of the number of outer segment disks would provide quantitative assessment of this. Secondly, expression of several mature photoreceptor markers has been reported, including expression of rhodopsin and recoverin (Bartsch et al., 2008; Jiang et al., 2010; Kwan et al., 1999; Lakowski et al., 2010; Lakowski et al., 2011; Maclaren et al., 2006; West et al., 2008; West et al., 2010; Yao et al., 2010). In this chapter, we demonstrate functional expression of two mature rod markers in transplanted rod cells: rod-α-transducin and arrestin. These proteins undergo opposing light-induced translocation. When the retina is exposed to light arrestin is rapidly translocated to the outer segment region in rod photoreceptors. This is mirrored by a rapid translocation of rod-α-transducin to the inner segment region (Elias et al., 2004). The rapidity of this movement suggests that the primary function of this translocation is to terminate the phototransduction cascade (mediated by the role of arrestin) to protect rod photoreceptors against light damage. In darkness, translocation of both proteins
occurs in the appropriate opposing direction and does so in a slower manner and is thought to maximize rod sensitivity in low lighting conditions (Elias et al., 2004). Thus, not only do transplanted rod photoreceptor cells integrated within the recipient retina express markers of the phototransduction cascade, they also undergo appropriate light-mediated translocation of some of these proteins.

Single cell recordings demonstrated that integrated Nrl.GFP^+ve cells respond to light in a manner similar to wildtype rods. We demonstrated that the transplanted cells hyperpolarize and that the extent of the response is determined by the intensity of light used. Experiments to determine the maximal photocurrent demonstrate similarities between Nrl.GFP^+ve integrated rod and wildtype rod cells in the saturated photocurrent amplitude. In order to test rod function, recordings were performed in the dark. However, GFP-fluorescence was used to identify and locate the integrate Nrl.GFP^+ve cells. This process also resulted in bleaching of the rhodopsin pigment and subsequent desensitization. As a consequence, recipient retinal ex-vivo slices were maintained in a bath of 9-cis-retinal for one hour after cell searching to allow regeneration of the rhodopsin pigment. 9-cis-retinal is an analogue of the native 11-cis-retinal chromophore and can be used exogenously to substitute the native 11-cis-retinal, which is usually regenerated by the RPE. 9-cis-retinal behaves in a very similar manner to the native 11-cis-retinal chromophore, but has a shorter \( \lambda_{\text{max}} \) and the photoisomerisation reaction is known to be slower and less efficient (Peteanu et al., 1993; Sekharan et al., 2006). Chromophore substitution is the most likely explanation for the differences observed in the dim-flash response kinetic of the Nrl.GFP^+ve transplanted cells compared to untreated naïve wildtype controls and wildtype rods cell treated with the regeneration regime that had similarly reduced kinetics. The results demonstrating an overall reduction in sensitivity are less clear. GFP^+ve rods show a significant reduction in sensitivity compared to untreated wildtype rods, whereas 9-cis-retinal treated wildtype rods were not statistically different to either cohort. This raises the question as to whether the reduction in rod sensitivity observed in Nrl.GFP^+ve rods is due to the 9-cis-
retinal treatment regime or is an intrinsic property of the transplanted cells. Either scenario is possible, or even a combination of the two. In the former scenario, incomplete regeneration/reconstitution of the native rhodopsin with 9-cis-retinal chromophore would result in less visual pigment and a reduction in rod sensitivity. Similarly, any differences in segment development of the transplanted cells may result in smaller outer segments, leading to fewer disks and consequently a reduction in rod cell sensitivity. To further investigate this in discrepancy further recording from many more cells would be required. This was not possible due to the considerable technical challenges involved in recording from these cells.

The development of a functional synapse is critical for a light-mediated electrical signal generated in the rod photoreceptor cells to be propagated to the second order neurons and on to the brain. Here we present for the first time conclusive evidence for the classic rod triad synaptic structure formed by the transplanted rod photoreceptor cells and the endogenous second order neurons. EM analysis showed that at the ultrastructural level synapses formed by Nrl.GFP+ve transplanted cells are remarkable similar in structure to wildtype rod synapses, with the rod ribbon synapse and classical triad structure being very apparent. We also demonstrated that the connectivity of transplanted cells to the recipient retinal circuitry is similar to wildtype connectivity; we showed the presence of both horizontal and bipolar cells dendrites in the transplanted cell synaptic cleft. Immunohistochemistry revealed that Nrl.GFP+ve spherule synapses co-localise with several rod synapse markers. Other studies have also demonstrated the expression of synapse markers in transplanted photoreceptor using immunohistochemistry (Gouras et al., 1991; Gouras et al., 1994; Kwan et al., 1999; Maclaren et al., 2006; West et al., 2010), and our findings support the notion that correct synapse formation is conserved when rod precursor cells are transplanted into Gnat1−/− recipients. Furthermore, confirmation of the ultra-structure of transplanted rod cell synapse presented in this chapter complement earlier, less conclusive studies using EM to suggest transplanted cell synapse formation (Gouras et al., 1991).
ERG recordings are the standard method of assessing visual function (Carvalho et al., 2011; Lamba et al., 2009; Mihelec et al., 2011; Tucker et al., 2011). We also used this technique to address whether scotopic responses from transplanted rod photoreceptors could be detected and to ask whether signals generated by transplanted photoreceptors can be propagated to second order neurons, as measured by the b-wave. However, ERG responses were not detectable in Gnat1−/− animals transplanted with Nrl.GFP+ve rod precursor cells, despite robust integration of transplanted cells. It must be noted here that other groups have reported rescue of ERG responses following the integration of very few transplanted cells (Lamba et al., 2009; Tucker et al., 2011). Lamba et al. (2009) demonstrated a 22 µV improvement in b-wave amplitude following full field flash scotopic ERG recordings at bright mesopic light intensities (exact light intensity not reported) in Crx−/− mice, which lack both cone and rod responses, transplanted with human embryonic stem cell-derived retinal cells. In these animals an average of ~3000 cells was reported to integrate into the host retina of which only 36 % expressed recoverin, and outer segment formation was entirely absent. Tucker et al. 2011, reported a 95 µV b-wave amplitude following transplantation of adult mouse derived iPS cells photoreceptor differentiated toward rod precursor fate in Rho−/− recipients. In this study, total cell integration counts were not performed and integrated cell morphology also appeared poor. In addition, this report failed to mention whether the improvements in ERG recordings were robust and it is unclear whether the data presented was obtained by repeat recordings on separate days. Other groups have failed to detect a recordable ERG. Seiler et al (2009) reported good integration of feline fetal retinal sheets into the dystrophic feline recipient retina, but were not able to demonstrate ERG responses.

Given the discrepancy between our findings and those described in the reports above, we sought to examine the number of cells required to generate a robust and reproducible ERG response. Using AAV2/8.Rho.Gnat1 viral vector, we were able to restore scotopic b-wave amplitudes in Gnat1−/− mice to near wildtype levels. The a-
wave was also restored but not to wildtype levels. Incomplete rescue of a-wave function to wildtype levels in Gnat1−/− mice is not surprising as this model undergoes a degree of retinal degeneration (Calvert et al., 2000) and even minimal degeneration and shortening of the outer segment is likely to reduce a-wave amplitude. Previous reports, using gene therapy to rescue achromatopsia by AAV2/8.CNGB3, also reported a full rescue of b-wave amplitude but not a-wave amplitude (Carvalho et al., 2011).

Importantly, by titrating the amount of virus administered we were able to demonstrate that only those animals with rod-α-transducin expression in >125,000 cells demonstrated a measureable and reproducible restoration of the ERG b-wave. It is important to note that gene supplementation, as used here, rescues the function of endogenous rod photoreceptors already wired into the host visual system. In contrast, transplanted cells must migrate into and functionally integrate within the recipient retinal circuitry. None-the-less, this figure is far higher than the number of integrated cells observed using our optimized transplant procedure. It must be noted that these data offers only an indication of the number of transplanted cells likely to be needs to drive an ERG response and one must remain cautious when comparing gene therapy to cell transplantation. Gene therapy may not be able to restore the diseased endogenous photoreceptor cell to wildtype levels, especially if the outer segments have undergone any degeneration. On the other hand, the degree of rescue using cell transplantation would depend on how efficiently the transplanted cells integrate into the visual system and whether outer segment and disk formation is equivalent to endogenous rods. A slight outer segment shortening of the endogenous photoreceptors has been reported in the Gnat1−/− mouse (Calvert et al., 2000), a finding that was reflected in the degree of rescue of ERG responses to near wildtype levels. It is likely that at least the same number, if not more, of integrated transplanted cells will be required to produce the same degree of rescue demonstrated using the gene therapy approach. Use of multi-focal ERG may provide an alternative method to full flash ERG and will allow assessment of smaller local responses in the retina (Seeliger
et al., 2003), however practical considerations have thus far limited its use in small rodents

Although a popular and easy technique to recorded retinal function full field flash ERG recording can only offer a global assessment of retinal function. Here we have demonstrated that integrated transplanted Nrl.GFP+ve cells robustly integrate and differentiate, and can respond to light and appear to form typical synaptic structures. However, the number of cells integrated remained below the number required to generate a robust ERG response. It is likely that many more fully functional transplanted cells will be required in order to generate a signal large enough to be detected by an electrode located distal to the retina at the corneal surface.

In this study we present for the first time intrinsic imaging of the visual cortex in scotopic lighting conditions mediated by the rod photoreceptors. We demonstrate cortical responses similar to wildtype in the visual cortex of Gnat1−/− mice at bright photopic lighting condition, reflecting the maintenance of the cone visual system. Imaging of V1 following dark-adaptation and light stimulation under scotopic lighting conditions demonstrated an absent of intrinsic signal in Gnat1−/− mice where responses were maintained in wildtype mice. This confirms the lack of rod photoreceptor function and subsequently rod derived cortical responses in the Gnat1−/− mouse. Imaging of intrinsic haemodynamic signals in V1 following dark-adaptation of Gnat1−/− recipients transplanted with rod precursor cells demonstrated that visual signals generated in response to scotopic visual stimuli by integrated rod precursor donor cells were detected in the visual cortex. This demonstrated that successful transplantation of rod precursor donor cells was sufficient to restore rod derived cortical activity; furthermore partial restoration of retinotopy in V1 was also demonstrated. It must be noted that not all stimuli presented in the intrinsic imaging experiments resulted in responses for all Nrl.GFP+ve treated animals. This is not surprising. Firstly transplanted were made only to the superior and inferior retina, which resulted in integration across only some areas of the visual field. Furthermore, within the transplantation sites integration is not
uniform. In some instances, this can result in focal patches of cell integration. Together, these likely result in only partial restoration of the visual field.

Other studies have gone some way to show that visual signals generated by transplanted donor material can be successfully propagated to subcortical areas of the brain and indeed some restoration of responses in the superior colliculus has been reported following transplantation of E19.5 rat fetal retinal sheets into S332ter recipient rats (Thomas et al., 2004a). Similarly, another report by the same group demonstrated positive responses to a flash stimulus in the superior colliculus in 1 month old PDE6β<sup>rd1/rd1</sup> mice transplanted with E17 retinal sheets (Arai et al., 2004). However, this is the first time that successful restoration of cortical response in V1, the primary visual centre of the brain, has been reported following photoreceptor replacement therapy.

In addition to demonstrating that cortical activity is restored following transplantation, we also sought to demonstrate that the visual centers in the brain are capable of integrating the visual information into appropriate behavioural responses. Importantly, we demonstrate restoration of visually-guided swimming behaviours, as assessed using the watermaze test, which shows that visual signals generated by integrated rod precursor donor cells were successfully integrated by the higher visual areas to coordinate visually-guided behaviour. It is well known that retinal signals are amplified along the visual pathway, which may explain why the signal from a limited number of rods is detectable in the cortex but not in the retina when recording ERG responses. Furthermore, clinical studies have shown that functional vision is often maintained despite an absence of detectable ERG responses from the retina (Berson, 2007). Again, this implies that full field flash ERG recordings are not a sensitive method of assessing focal visual function.

Early reports using donor postnatal retinal cells offered suggestions of restoration of visually guided behavior (Klassen et al., 2004; Kwan et al., 1999). However, these studies were limited in a number of ways. For example, improvements in visually
guided behaviour were reported without testing any other functional readouts. In addition, histological assessment of photoreceptor integration and morphology was poor and the number of integrated cells was not quantified, making it very difficult to compare our findings with these earlier reports. Here, we present definitive evidence from the level of the photoreceptor cell through to restoration of visually-guided behaviour that transplanted cell are able to respond to light, and contribute to and restore vision.
CHAPTER 4

APPLICATION OF PHOTORECEPTOR TRANSPLANTATION TO TREAT RETINAL DEGENERATIVE DISEASES

4.1. INTRODUCTION

Degenerative retinopathies including AMD and inherited retinal disorders, both major causes of blindness, are potentially treatable by the transplantation of healthy photoreceptor cells. In Chapter 3, I demonstrated that transplanted cells are capable of responding to light, can transmit the visual information to the brain and can contribute to higher visual function in a model of stationary night-blindness, the Gnat1<sup>−/−</sup> mouse. However, a fundamental question remains as to how broad an application this strategy will have for the very heterogeneous degenerations encompassed within AMD and RP. Moreover, it is anticipated but not known if late-stage disease can be treated or if all degenerations are equally amenable to photoreceptor transplantation. The breadth of application of cell transplantation therapy must be investigated and robust integration must be demonstrated in order to restore vision in the degenerating retina.

Until now, much of the work concerning the feasibility of photoreceptor transplantation as a strategy for retinal repair has used the wildtype retina (Bartsch et al., 2008; Eberle et al., 2011; Gust and Reh, 2011; Kwan et al., 1999; Mizumoto et al., 2003; Pearson et al., 2010; Suzuki et al., 2007; Takahashi et al., 1998; West et al., 2008; West et al., 2009; West et al., 2010) or isolated models of degeneration (Lakowski et al., 2011; Lamba et al., 2009; Maclaren et al., 2006; Mellough et al., 2007; Pearson et al., 2010;
Singhal et al., 2008; Suzuki et al., 2007; Takahashi et al., 1998; Yao et al., 2010) as a recipient. In order for cell therapy to be established as a feasible approach to treat retinal disease, the success of transplantation must also be assessed in different models of retinal degeneration and at different stages of the degenerative process. The degenerating retinal environment is likely to be very different from the wildtype retina and potentially hostile to transplanted cells (Vugler et al., 2007; West et al., 2009), which have to migrate from the site of transplantation, typically the subretinal space, across the inter-photoreceptor matrix and outer limiting membrane (OLM) and into the recipient outer nuclear layer (ONL). In addition, loss of recipient photoreceptors results in a number of concomitant changes in the remaining retinal cytoarchitecture.

Injury and degeneration are often associated with a series of events that culminate in reactive gliosis and the formation of a glial scar, which is represented by lateral GFAP⁺ve processes at the outer edge of the ONL. It has been suggested that reactive gliosis, an inevitable part of the degenerative process, may be inhibitory to migrating cells. This glial scar acts as a reservoir of inhibitory extracellular matrix (ECM) molecules, such as chondroitin sulphate proteoglycans (CSPGs), which are known to prevent axon migration and regeneration in the CNS (Busch and Silver, 2007; Fawcett and Asher, 1999) and prevent the migration of cells transplanted within the damaged region (Bradbury et al., 2002). The glial scar functions to separate intact regions of the CNS from the damaged area and CSPGs are deposited to act as boundary cues for neuronal remodelling. Injury in the retina induces a similar response of the retinal glial cells, namely Müller glia and astrocytes, which undergo reactive gliosis (Cao et al., 2001). In addition to the upregulation of the intermediate filament proteins glial fibrillary acidic protein (GFAP) and vimentin, reactive Müller cells may undergo hypertrophy, presenting a proliferation of fibrous processes and deposition of CSPGs at the outer edge of the retina (Fisher et al., 2005; Inman and Horner, 2007; Lewis and Fisher, 2003). This process of gliosis is characteristic of many retinal disease models (Ekstrom et al., 1988; Fan et al., 1996; Sheedlo et al., 1995), although the temporal relationship
between the onset of gliosis and degeneration may vary between disease models. Importantly, the glial scar may represent a physical barrier to transplanted cell migration; Kinouchi et al (2003) reported an increase in cell integration when transplanting into the vimentin and GFAP double-knockout recipient (Kinouchi et al., 2003).

As degeneration progresses, the structure of the ONL is clearly compromised with widespread loss of photoreceptors and ONL thinning. The OLM, located at the outer edge of the ONL, consists of a series of zonula-adherens junctional complexes formed between photoreceptors and Müller glia and functions to limit diffusion of phototransduction cascade components into the neural retina. Similarly, the outer limiting membrane (OLM) has also been described as a barrier to cell integration and pharmacological disruption results in an increase in the number of cells successful migrating into the host ONL (West et al., 2008). It is possible that the OLM will be compromised as photoreceptors die and there are reports that the OLM is disrupted in the degenerating retina (Campbell et al., 2006; Campbell et al., 2007; Mehalow et al., 2003).

Here we assess photoreceptor transplantation across a number of murine models of photoreceptor degeneration caused by RP. Each model undergoes a progressive loss of rod photoreceptors over a period of time ranging from ~10% loss over 12 months (Gnat1<sup>−/−</sup>) to near complete loss within 3 weeks (PDEβ<sup>att</sup>), and were chosen to represent a range of degeneration speeds. In addition, OLM integrity, glial scarring and chondroitin sulphate deposition will be assessed throughout the degeneration process in relation to transplant outcome. The different models of retinal degeneration used are described below:
Mutations within the human crumbs-like 1 (CRB1) gene can result in retinal degenerative diseases such as retinitis pigmentosa and Leber congenital amaurosis. The CRB1 gene encodes a transmembrane protein that is a key regulator of polarity in epithelial cells. The CRB1 protein localizes to the apical membrane of epithelial cells and in the retina, it is involved in the assembly of the adherens junctions at the OLM. In the Crb1<sup>rd8/rd8</sup> mouse, a single base pair deletion in the CRB1 gene results in a frame shift and premature stop codon producing a truncated protein (Mehalow et al., 2003). There is a progressive fragmentation of the OLM resulting in retinal folding, rosette formation and subsequent photoreceptor cell death. The inner segments lose their ordered arrangement and are 25% shorter than those of wild-type mice at 4 weeks of age and by 10 weeks of age begin to fragment (Mehalow et al., 2003). The outer segment defects are thought to be a secondary consequence of the OLM disruption and illustrates how subtle changes in the microenvironment can impact on endogenous photoreceptor development and morphology. This model permits the study of cell integration in a moderately slow model of degeneration with OLM defects.

Mutations in GNAT1 in humans leads to autosomal dominant congenital stationary night blindness (Peachey et al., 1990). The Gnat<sup>1−/−</sup> mouse model has a targeted deletion in the gene encoding the rod transducin α-subunit resulting in a null protein knock-out (Calvert et al., 2000). In rod photoreceptors, photoexcited rhodopsin activates the G-protein rod transducin, which in turn activates the phototransduction cascade by promoting cGMP hydrolysis by phosphodiesterase. Thus in this model the rods are non-functional. The gross morphology of the retina remains intact, although there is a mild degeneration with age: Calvert et al. (2000) observed a 10% loss of rods by 13 weeks of age and a shortening of the rod outer segments compared to wild-type. No further changes in ONL thickness or segment morphology were observed at 51
weeks (Calvert et al., 2000). Thus, this provides a model of slow degeneration with little cell death and a largely intact retina with relatively normal endogenous outer segment morphology.

*Prph2*<sup>α2/rd2</sup>

Peripherin 2 is a membrane glycoprotein involved in the structural stabilization of the outer segment discs in the photoreceptor. It forms a heterodimer complex with ROM1 to perform this function and is renewed continuously. Mutations in the gene encoding peripherin 2 (*Prph2*) result in retinitis pigmentosa (Travis et al., 1989); the *prph2*<sup>α2/rd2</sup> mouse is homozygote for a null mutation in *Prph2* and fails to develop outer segments completely. Progressive apoptotic cell death has been reported beginning at 2 weeks after birth with the peak of apoptotic activity around postnatal day 18 (Chang et al., 1993). At 2 weeks after birth 11 rows of photoreceptor nuclei are present in the ONL, by 4 weeks this is reduced to 8-9 rows and by 16 weeks only 3-4 rows remain (McNally et al., 2002). This model provides a clinically relevant model of a moderate to fast degenerative environment with no endogenous photoreceptor outer segments.

*Prph2*<sup>+/Δ307</sup>

The *Prph2*<sup>+/Δ307</sup> mouse has a targeted single base deletion at codon 307 of the *rdspерipherin* gene (McNally et al., 2002) similar to mutations found to cause autosomal retinitis pigmentosa in humans. The homozygote *Prph2*<sup>+/Δ307</sup> mouse degenerates very quickly, however in these studies the heterozygote *Prph2*<sup>+/Δ307</sup> mouse is used and the rate of degeneration is moderate. At 2 months, 9-10 rows of photoreceptor nuclei can be observed, whereas by 12 months only 2-3 rows of nuclei still remain. The inner segments are retained, although the membranous discs of the outer segments are severely disrupted in structure and number. The outer segments also appear shorter than wildtype. The inner retina of the heterozygote *Prph2*<sup>+/Δ307</sup> mice was reported normal in appearance, as was the integrity of the OLM (McNally et al., 2002). This
model provides a clinically relevant model to study cell integration in the presence of some endogenous outer segment formation and moderate endogenous photoreceptor cell loss.

*Rho<sup>-/-</sup> mice*

The *Rho<sup>-/-</sup>* mouse carries a targeted disruption of the rhodopsin gene resulting in a null mutant (Humphries et al., 1997). The photoreceptors are lost over a 3 month period. At 24 days post birth, 8-10 rows of photoreceptors nuclei were reported in *Rho<sup>-/-</sup>* mice compared to 10-12 rows in the wildtype. By day 48, ONL thickness had reduced by half and only 1 row of photoreceptor nuclei remained at 90 days post birth. A striking feature of this model is that the outer segments do not form (Humphries et al., 1997). Two previous reports suggest that the OLM is also disrupted in these mice (Campbell et al., 2006; Campbell et al., 2007); although we have found the OLM to be intact at least up to 10 weeks of age (see results).

*PDE6β<sup>rd1/rd</sup>*

The PDE6β<sup>rd1/rd</sup> mouse is a clinically relevant model of recessive RP, carrying a nonsense mutation in the *PDE6β* gene (Danciger et al., 1995). This results in a rapid degeneration of rod photoreceptors beginning 10 days post birth, peaking at P14 with complete loss of rod photoreceptors at P21 leaving a single layer of cone photoreceptors. Outer segment and inner segment growth in the developing retina (P4-P8) is also retarded (Sanyal and Bal, 1973).
4.2. RESULTS

4.2.1. PHOTORECEPTOR TRANSPLANTATION IN DIFFERENT ADULT MODELS OF RETINAL DEGENERATION

Transplanted photoreceptor integration was examined in each of the models at a time when the retina could be considered to be ‘adult’ (6-8 wks; with the exception of PDE6β<sup>rd1/rd1</sup>, which was examined at 3 weeks due to its very rapid degeneration) and compared these to wildtype controls. FACS purified P4-7 Nrl.gfp<sup>+/+</sup> donor cells were transplanted using a single superior subretinal injection of 200,000/μl live cells.

4.2.1.1. Transplanted photoreceptor integration efficiency in adult models of retinal degeneration

Figure 4.1a (grey boxes) demonstrates the number of correctly integrated Nrl.GFP<sup>+</sup>-rod-photoreceptors in each recipient model. Integration into adult Prph2<sup>rd2/rd2</sup> (5067 ± 1310 integrated Nrl.GFP<sup>+</sup>-rod photoreceptors, N = 7), Prph2<sup>Δ307</sup> (3069 ± 659, N = 9), Gnat1<sup>−/−</sup> (3595 ± 649, N = 10) and PDE6β<sup>rd1/rd1</sup> (4451 ± 825, N = 9) recipients was similar to age-matched wildtype controls (4359 ± 711, N = 9) (Figure 4.1a; P>0.05, ANOVA with Dunnett’s correction). Conversely, significantly higher levels of integration were observed in Crb1<sup>rd8/rd8</sup> recipients (10, 763 ± 1535 integrated Nrl.GFP<sup>+</sup>-rod-photoreceptors; 2.4-fold increase; P<0.001; N = 9), compared with wildtype, while integration into the Rho<sup>−/−</sup> mouse was significantly lower (261 ± 24; 17-fold decrease; P<0.05; N = 6; Figure 4.1a).
FIGURE 4. 1. NUMBER OF INTEGRATED PHOTORECEPTORS IS DEPENDENT UPON RECIPIENT RETINAL DISEASE TYPE

(a) Box plots showing the number of integrated Nrl.GFP⁺-rod photoreceptors after transplantation into Gnat1⁻/⁻ (N = 10), Crb1⁺/-B (N = 9), Prph2⁺/-Δ307 (N = 9), Prph2⁺/-B (N = 7), Rho⁻/- (N = 6) and PDE6β⁻/- (N = 9) models of inherited retinal degeneration at 6-8 weeks of age, compared with wildtype controls (N = 9), with the exception of the PDE6β⁻/- model which was transplanted at 3 weeks of age due to its rapid rate of degeneration. Black bars indicate statistical significance (ANOVA with Dunnett’s correction for multiple comparisons). (b) Representative confocal projection images of integrated cells in each model of inherited retinal degeneration (~10µm thick, scale bar 50µm). N.B. in vii, dotted line denotes boundary of ONL/INL and dashed line denotes boundary of ONL.
4.2.1.2. Transplanted cell morphology in models of retinal degeneration: inner/outer segment and synapse formation

The vast majority of previous studies have assessed transplantation outcome on the basis of the total number of cells found within the ONL (Bartsch et al., 2008; Eberle et al., 2011; Gust and Reh, 2011; Lakowski et al., 2010; Lakowski et al., 2011; Lamba et al., 2009; Ma et al., 2011; Maclaren et al., 2006; Tucker et al., 2011; West et al., 2008; West et al., 2010; West et al., 2012b; Yao et al., 2010). However, it is likely that the specific disease aetiology of the recipient will also have a significant bearing upon the health of any transplanted photoreceptors. Photoreceptor survival is critically dependent upon the correct formation and maintenance of synapses and inner/outer segments (Goldberg, 2006; Kennedy and Malicki, 2009). As noted in the introduction, the ability of endogenous photoreceptors to form and maintain inner/outer segments is dramatically different in the models studied. Endogenous photoreceptors in the Gnat1−/− mouse form long segments virtually indistinguishable from wildtype, although by 13 weeks of age there is some outer segment shortening (Calvert et al., 2000). Conversely, inner/outer segments of the endogenous photoreceptor cells in the Rho−/− and PDE6βrd1/rd1 models, if present at all, are extremely short (Calvert et al., 2000; Humphries et al., 1997; Sanyal and Bal, 1973). Therefore, we examined the ability of transplanted Nrl.GFP+ve-rod photoreceptor precursor cells to form synapses and inner/outer segments in the context of the degenerating recipient ONL (Figure 4.2, Table 4.1).
a) Segment formation

First, the number of integrated Nrl.GFP\textsuperscript{\text{\textregistered}}-rod-photoreceptors that had developed segments was assessed (Figure 4.2a). Second, an assessment of the morphological quality of these segments was also made (Figure 4.2c). Over 70 % of Nrl.GFP\textsuperscript{\text{\textregistered}}-rod photoreceptors correctly integrated within the ONL of wildtype, Gnat\textsuperscript{1/\textsuperscript{\text{\textregistered}}} and Crb\textsubscript{1\textsuperscript{rd8/rd8}} recipients developed segments (72 ± 2; 75 ± 3; 77 ± 2 %, respectively; Figure 4.2a, P > 0.05, ANOVA with Dunnett’s correction for multiple comparison, N = 3); these cells adopted typical rod-like morphologies with long segments (Figure 4.2c i, ii, iii). Fewer (60 ± 3\%) Nrl.GFP\textsuperscript{\text{\textregistered}}-rod photoreceptors found within the ONL of Prph\textsubscript{2\textsuperscript{\textDelta307}} developed outer segments, although this reduction was not significant. A significant reduction in segment formation was observed in Rho\textsuperscript{\textsuperscript{\textregistered}} recipients compared to wildtype: only 20 ± 1\% of integrated Nrl.GFP\textsuperscript{\text{\textregistered}}-rod photoreceptors found within the Rho\textsuperscript{\textsuperscript{\textregistered}} recipient ONL developed segments (Figure 4.2a, P<0.001; N = 3) and the segments that did form were very short (Figure 4.2c vi). At 3 weeks of age, the ONL of the PDE6\textsubscript{\textsuperscript{\beta\textsuperscript{rd1/rd1}}} retina is severely reduced to just a single layer of cone photoreceptors in the periphery. Despite this, a significant number of Nrl.GFP\textsuperscript{\text{\textregistered}}-rod photoreceptors were found within the remaining ONL. Gross morphology was markedly different to normal rod photoreceptors, often with enlarged cell bodies and multiple processes (Figure 4.2c, vii), and the number of integrated cells was significantly less than wildtype with 21 ± 5 \% of these cells developing projections orientated towards the RPE, indicative of rudimentary segments (Figure 4.2a, P<0.001, N = 3). Some of these rudimentary segment like structures co-localized with PDE-\textbeta (Figure 4.2c, vii). In Prph\textsubscript{2\textsuperscript{\textDelta2/\textDelta2}} recipients, 48 ± 5\% of Nrl.GFP\textsuperscript{\text{\textregistered}}-rod-photoreceptors found within the ONL formed segments, significantly less than in wildtype (Figure 4.2a, P<0.01, N=3). In addition, the segments formed by Nrl.GFP\textsuperscript{\text{\textregistered}}-rod photoreceptors transplanted into Prph\textsubscript{2\textsuperscript{\textDelta2/\textDelta2}} recipients appeared shorter than those formed in the wildtype recipient.
**b) Synapse formation**

The presence of synaptic boutons, essential for the transmission of light information from the transplanted cell to second-order neurons, was also assessed (Figure 4.2b and d). Spherule synapses typical of rod photoreceptors were formed by over 65 % of integrated Nrl.GFP^{+ve}-rod photoreceptors in *Gnat1^{−/−}* and *Crb1^{rd8/rd8}* recipients (65 ± 5; 66 ± 1 %, respectively), similar to synapse formation observed in wildtype recipients (75 ± 2 %; Figure 4.2b, *P*>0.05, ANOVA with Dunnett’s correction for multiple comparison, *N* = 3 and Figure 4.2d i,ii,iii). Synapse formation in *Prph2^{+/Δ307}* recipients was reduced compared to wildtype with 59 ± 4 % of integrated cells forming synapse spherules, although this reduction was not significant (Figure 4.b, *P*>0.05, *N* = 3 and Figure 2d iv). A moderate but significant reduction in synapse formation was observed in *Prph2^{rd2/rd2}* and *Rho^{−/−}* recipients compared to wildtype, with 45 ± 2 % and 44 ± 5 % of transplanted cells within the recipient ONL developing synaptic boutons, respectively (Figure 4.2, *P*<0.001, *N* = 3, and Figure 4.2d: v,vi). Most severely affected were Nrl.GFP^{+ve}-rod photoreceptors transplanted into *PDE6β^{rd1/rd1}* recipients: just 33 ± 1 % of transplanted cells transplanted photoreceptors sent out processes toward the OPL. (Figure 4.2b, *P*<0.001, *N* = 3 and Figure 2d: vi). In all models, where synapse formation was observed, RIBEYE expression, a marker of rod ribbon synapses (tom et al., 2005) was typically noted (Figure 4.2d).
The morphology and frequency of segment formation by integrated Nrl.GFP⁺ve-rod photoreceptors demonstrated a strong correlation with the ability of the endogenous photoreceptors to form outer segments (Supplementary Table 4.1): those Nrl.GFP⁺ve-rod-photoreceptors integrated within models whose endogenous photoreceptors develop correctly (wildtype, Gnat1⁺c, Crb1⁺dë/dë, Prph2⁺b³⁰⁷) developed new segments and synapses more frequently and of more normal morphology than in those models where the endogenous photoreceptors segment formation is compromised (Prph2⁻d²/d², Rho⁺c, PDE6β⁺d¹/d¹).

Together, these findings show that the cytoarchitecture of the recipient retina plays a significant role in the ability of transplanted rod photoreceptor precursors to assume normal adult rod photoreceptor morphology and elaborate outer segments and form synapses.
FIGURE 4.2. MORPHOLOGY OF INTEGRATED PHOTORECEPTORS IS DEPENDENT UPON RECIPIENT RETINAL DISEASE TYPE

Graphs showing the percentage of integrated Nrl.GFP⁺ve-rod photoreceptors in each model of retinal degeneration that (a) develop outer segments and (b) develop synapses structures (N = 3; ANOVA with Dunnett’s correction for multiple comparisons). Confocal projections (~10 µm thick) showing typical morphology of (c) outer segment length and (d) synapse formation of integrated cells in each model. Integrated cells expressed the rod-specific transcription factor, Nrl (green) and rod α-transducin (c, ii, red), peripherin-2 (c, v, red), rhodopsin (c, vi, red), or PDE-β (c, vii, red) where appropriate. Such markers are absent in the respective endogenous photoreceptors. D, Inserts: high power examples of spherule synapses formed by transplanted cells with host inner retina in each of the models. Most but not all (open arrow heads) co-localized with the ribbon synapse protein RIBEYE (red). N.B. in vii, dotted line denotes boundary of ONL/INL and dashed line denotes boundary of ONL. Scale bar 25µm.
TABLE 4. FORMATION OF SEGMENTS AND SYNAPSES

The relationship between endogenous segment formation and transplanted segment formation. ++++++, formation of many long outer segments; +, few cells developing an outer segment and/or poor outer segment formation in those that do.
4.2.2. THE IMPACT OF DISEASE PROGRESSION ON TRANSPLANTED PHOTORECEPTOR INTEGRATION EFFICIENCY

Photoreceptor cell transplantation is typically proposed as a therapeutic strategy for moderate to severe retinal degeneration (Jacobson and Cideciyan, 2010; Maclaren and Pearson, 2007; Sahni et al., 2011). Disease progression may hinder transplantation efficiency: as disease progresses and photoreceptor cell death ensues, the recipient ONL undergoes thinning and the retinal gliosis is activated. Here we sought to determine what impact disease progression has upon transplanted photoreceptor integration and to assess how long the degenerative recipient retinal environment remains permissive to photoreceptor cell transplantation therapy. Cells were transplanted into the 6 models described above at early, mid and late stage degeneration. These time points were determined by ONL thickness stated within the literature (Table 4.2). The stages chosen can be defined as follows: early = ONL at >70 % thickness of wildtype; mid = 30-70 %; and late = <30 % (Figure 4.3a). The exceptions to this classification were the Gnat1−/− recipients, whose degeneration remains largely stationary over time and the Crb1−/− recipients, which present focal rather than global disorganisation and degeneration (Figure 4.3a). In the first instance, ONL thickness of each model at each chosen time point was measured to confirm the findings presented with the literature; Figure 4.3a and b demonstrate that these time point did fall within the appropriate categories.
<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Early Degeneration (ONL &gt;70% of wildtype)</th>
<th>Mid Degeneration (ONL 30-70% of wildtype)</th>
<th>Late Degeneration (ONL &lt;30% of wildtype)</th>
</tr>
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<td>[6-8 weeks]</td>
<td>[6 months]</td>
<td>[12 months]</td>
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<tr>
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<td>2 months</td>
<td>6 months</td>
<td>12 months</td>
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<td>10 days</td>
<td>-</td>
<td>3 weeks</td>
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**TABLE 4.2. DEFINING DEGENERATION STAGES**

Disease models were chosen as clinically relevant models of RP. Time points were taken to encompass a range of degeneration stages within each model. A standard time point of 6-8 weeks was included to provide information about each model at a time when the retina could be defined as “adult” (*bold, italics*). Stages can be defined as: early = ONL at >70% thickness of wildtype; mid = 30-70%; and late = <30%. Exceptions were *Gnat1⁻/⁻*, which is still 90% of wildtype at 1 yr and *PDE6β<sup>rd1/rd1</sup>*, which is ~90% of wildtype at P10 but reduced to 20% by P21. *Crb1<sup>rd8/rd8</sup>* undergoes focal degeneration, the extent of which broadly follows the criteria above.
FIGURE 4.3. AVERAGE ONL THICKNESS FOR 6 CLINICALLY RELEVANT MODELS OF RP

(a) Average ONL thickness (µm) expressed as percentage of wildtype confirming that each time point picked to represent early, mid, or late degeneration state fell within the categorized thickness: >70% (blue), 30-70% (red) or <30% (green) of wildtype, respectively. (b) Graph representing the absolute values for ONL thickness at their given time point. N = 3 per time point per model.
In wildtype, the number of transplanted Nrl.GFP<sup>**ve</sup>-rod photoreceptors integrating into recipients aged between 6 weeks and 12 months remained constant (Figure 4.4a, 6 week: 4359 ± 711 integrated Nrl.GFP<sup>**ve</sup>-rod photoreceptors, N = 9; 6 month: 4713 ± 945, N = 9; and 12 month: 3386 ± 689, N = 8; ANOVA with Tukey's correction for multiple comparisons). A small but significant decline was observed in the degenerating Gnat<sup>1−</sup> mouse retina. The mean number of integrated cells in early stage (2 months) recipient was 3302 ± 354 (N = 11), reducing to 1548 ± 200 (N = 6) when cells were transplanted into mid stage disease (6 months) recipients and 1269 ± 78 (N = 8) in late stage (12 months) recipients (Figure 4.4b; P<0.05). Unexpectedly, integration in the Prph2<sup>+/Δ307</sup> recipient significantly increased over time (Figure 4.4d; P<0.01): a mean of 3069 ± 659 (N = 9) integrated Nrl.GFP<sup>**ve</sup>-rod photoreceptors was observed in early stage (2 months) recipients, increasing to 5441 ± 1339 (N = 7) and 9534 ± 1992 (N = 7) integrated cells in the mid stage (4 month) and late stage (6 month) recipients, respectively. We also examined this model at 9 months of age and found integration to be similar to that seen in the late stage (7937 ± 1139, N = 9) recipients, demonstrating that in this model transplanted photoreceptors continued to integrate even in the latest stages of degeneration. Integration into the degenerating Crb1<sup>rdb/rdb</sup> mouse presented a bimodal pattern (Figure 4.4c), increasing significantly between early (3 weeks) and mid (6 weeks) stage disease recipients (5534 ± 1007, N = 11 and 10763 ± 1535, N = 9 integrated Nrl.GFP<sup>**ve</sup>-rod photoreceptors, respectively; P<0.01) but a significant decline in integration was observed when transplanted into late stage (12 weeks) recipients (2217 ± 326, N = 11; P<0.001). Despite a loss of >70 % of the endogenous photoreceptors, we observed no significant difference in integration in the Prph2<sup>Δds/Δds</sup> mouse at the stages examined (Figure 4.4e; P>0.05). Mean integration into early (4 weeks), mid (8 weeks) and late stage degeneration (12 weeks) Prph2<sup>Δds/Δds</sup> recipients was 3687 ± 600 (N = 13), 5067 ± 1310 (N = 7) and 4811 ± 772 (N = 12) integrated Nrl.GFP<sup>**ve</sup>-rod photoreceptors, respectively. In contrast, in the Rho<sup>−</sup> mouse, even at the earliest time point examined (4 weeks) when the ONL is still >70 % of wildtype, integration was considerably lower than in any other model (Figure
4.4f). Integrated photoreceptors averaged just $825 \pm 147$ (N = 8) cells in early stage (4 weeks) recipients, $798 \pm 323$ (N = 6) in mid stage (6 weeks) recipients and declined significantly to $195 \pm 11$ (N = 13) integrated Nrl.GFP$^{+ve}$-rod photoreceptors in late disease stage (10 weeks) recipients (P > 0.001). Integration into the $PDE6\beta^{rd1/rd1}$ mouse remained broadly constant between early (P10) and late stage (3 weeks) recipients ($2801 \pm 572$, N = 8 and $4182 \pm 698$, N = 11, respectively; P>0.05, paired t-test; Figure 4.4g), even though the ONL is reduced to a single cell (predominantly cones) layer thick in the periphery by late stage disease.

Thus, very different trends in transplanted photoreceptor integration were observed in the different models of RP as disease progressed. These trends are summarised in Figure 4.4 (black lines). In brief, integration increased as degeneration progressed in the Prph2$^{\Delta307}$ model, and remained constant in the Prph2$^{rds/rds}$ and $PDE6\beta^{rd1/rd1}$, even in the face of severe endogenous photoreceptor cell loss. In contrast, integration efficiency decreased in the Crb1$^{rds/rds}$ and Gnat1$^{-/-}$ models as disease progressed and was already markedly lower in the Rho$^{-/-}$ model than in any other model and continued to decline steeply over time.
**FIGURE 4.4. DISEASE PROGRESSION SIGNIFICANTLY BUT DIFFERENTIALLY AFFECTS PHOTORECEPTOR TRANSPLANTATION EFFICACY ACCORDING TO DISEASE TYPE**

Box plots showing the impact of disease progression upon transplantation outcome in (a) wildtype, (b) Gnat1<sup>-/-</sup>, (c) Crb1<sup>rd8/rd8</sup>, (d) Prph2<sup>Δ307</sup>, (e) Prph2<sup>rd2/rd2</sup>, (f) Rho<sup>-/-</sup>, and (g) PDE6β<sup>rd1/rd1</sup> models of retinal degeneration. White boxes correspond to early degeneration, light grey boxes to mid degeneration and dark grey boxes to late degeneration. a-f: ANOVA with Tukey’s correction for multiple comparisons; g: paired t-test.
4.2.3. ASSESSING THE IMPACT OF THE RECIPIENT
CYTOARCHITECTURE ON TRANSPLANT OUTCOME

The results presented above demonstrate that both disease type and disease progression play a significant role in the success of transplanted photoreceptor integration. Each model degenerates at a different rate and it has previously been hypothesized that the architecture of the remaining host retina is likely to be important for defining the success of transplanted cell integration (Yao et al., 2010).

4.2.3.1. Rate of degeneration

The differences in disease severity encompassed by these six models necessarily mean that although the early, mid, and late degeneration stages were defined by ONL thickness, the rate at which these models degenerate is different. Therefore, the rate of degeneration was measured to assess whether or not the rate of degeneration accounts for the different trends observed in the number of integrated photoreceptor cells that integrated into the recipient retina at different degenerative stages. The rate of degeneration was calculated using the data generated in Figure 4.3b; the overall loss of ONL thickness was divided by the number of days between early and late stage degeneration. This is a crude estimation of the rate of degeneration, which assumes that the process of degeneration occurs in a linear fashion.

The PDE6β<sup>rd1/rd1</sup> mouse had the fast rate of degeneration (white) with an average loss of 3.4 µm per day (Figure 4.5a). The Rho<sup>−/−</sup> and Prph2<sup>2e/rd2</sup> mouse models degenerated at a moderate rate (light grey), with an average loss of 0.5 and 0.8 µm per day, respectively. A slow rate of degeneration (dark grey) was demonstrated in the Prph2<sup>−/Δ307</sup> mouse model, which had an average loss of 0.06 µm per day. There appeared to be no correlation between the rate of degeneration and integration.
efficiency. For example, in the \textit{Gnat1}^{\pm} model degeneration has a stationary rate of degeneration (Figure 4.5a and 4.3b, \textit{black squares}; (Calvert et al., 2000) yet cell integration declines over time (Figure 4.4b). Conversely, a rapid rate of degeneration in the \textit{PDE6\beta^{rd1/rd1}} recipient (Figure 4.5a) was accompanied by little change in cell integration (Figure 4.4g).

4.2.3.2. Density

It has previously been suggested that changes in recipient ONL cell \textit{density} may account for the robust integration efficiency following transplantation of P4 Nrl.GFP^{\pmve} rod precursor cells into aged \textit{Rd9} recipients (Yao et al., 2010). Therefore, we also evaluated ONL cell density over time (Figure 4.5b). No statistical changes in ONL cell density were observed as degeneration progressed in wildtype, \textit{Gnat1}^{\pm}, \textit{Rho}^{\pm}, or \textit{PDE6\beta^{rd1/rd1}} mice. A small but significant reduction in ONL cell density was observed at late stage degeneration \textit{Prph2}^{\pm/\Delta307} mice compared to early stage degeneration (Figure 4.5b, \textit{P}<0.05, ANOVA with Tukey's correction for multiple comparison, \textit{N} = 3). Conversely, a small significant increase in ONL cell density was observed at end stage degeneration in \textit{Prph2}^{rd2/rd2} mice compared to early stage degeneration (Figure 4.5b, \textit{P}<0.05, \textit{N} = 3). Similarly, a small but significant increase in ONL cell density was observed at mid stage degeneration in \textit{Crb1}^{rd8/rd8} mice compared to early stage degeneration (Figure 4.5b, \textit{P}<0.05, \textit{N} = 3). Although some small differences in ONL density were observed in three of the models examined, these changes bear little correlation to the trends observed in integration efficiency. More specifically, the reduction in ONL cell density is mirrored by an increase in cell integration efficiency as disease progresses in the \textit{Prph2}^{\pm/\Delta307} recipient (Figure 4.4d). Conversely an increase in ONL cell density in the \textit{Crb1}^{rd8/rd8} recipient was accompanied by an increased in cell integration efficiency by mid stage disease (Figure 4.4c), yet the increase in ONL cell density in the \textit{Prph2}^{rd2/rd2} recipient was accompanied by no overall changes in cell integration efficiency (Figure 4.4e).
4.2.3.3. ONL threshold

The reduction in photoreceptor number in the recipient ONL may directly affect the efficiency of transplanted donor cell integration. We therefore determined whether there is a threshold, or minimum, ONL thickness that is required for integration success. The mean ONL thickness of each model at each degenerative stage (early, mid and late stages) was plotted against the corresponding mean integration (Figure 4.5c). Regression analysis did not identify a correlation between ONL thickness and the number of integrated rod photoreceptors. This suggests that successful photoreceptor transplantation can be achieved even in a very thinned ONL. For example, integration levels above or similar to wildtype were observed in some (Prph2<sup>+/∆307</sup>, Prph2<sup>rds/ds</sup> and PDE6β<sup>rd1</sup>), but not all models (Rho<sup>−/−</sup>), with an ONL thickness of <30% of wildtype.

These data show that neither the cytoarchitecture of the recipient ONL, as assessed by ONL thickness and density, nor the rate of endogenous photoreceptor loss, are limiting factors for transplanted photoreceptor precursor integration.
FIGURE 4.5. THE IMPACT OF RETINAL CYTOARCHITECTURE ON CELL INTEGRATION

(a) The rate of degeneration, measured as the average loss of ONL thickness per day, in wildtype, Gnat1\textsuperscript{-/-}, Crb1\textsuperscript{rd1/rd1}, Prph2\textsuperscript{rd2/rd2}, Rho\textsuperscript{-/-} and PDE6\textsuperscript{ββ1/ββ1} mice (N = 3 per model). (b) ONL cell density at early, mid and late degeneration stages for all models. ANOVA with Tukey’s correction for multiple comparisons. (c) ONL thickness threshold assessment. Graph showing that there is no relationship between ONL thickness and cell integration. Pearson’s correlation.
4.2.4. THE IMPACT OF THE RECIPIENT MICROENVIRONMENT ON TRANSPLANTED PHOTORECEPTOR INTEGRATION EFFICIENCY

The different microenvironments and degenerative statuses of each of the recipient retinae are likely to account for the differences in integration seen between the different models. Therefore, the microenvironment of each of the models of RP was examined to determine whether changes in these factors could account for the differences observed in integration efficiency as disease progresses. Such examination should also identify factors that could be targets for manipulation to improve integration in end stage disease or models with impaired integration efficiency.

As retinal disease progresses so the microenvironment of the retina undergoes a number of significant changes. As photoreceptor cell death occurs the architecture of the ONL becomes disrupted and it is likely that the OLM, formed between photoreceptors and Müller glia, becomes compromised. In addition, Müller glial cells undergo reactive gliosis, leading to the formation of a glial scar (Bignami and Dahl, 1979; Bringmann et al., 2000; Bringmann and Reichenbach, 2001; Eisenfeld et al., 1984; Fan et al., 1996; Iandiev et al., 2006; Jones and Marc, 2005; Sethi et al., 2005). The glial scar acts a reservoir for the accumulation of CSPGs known to be inhibitory to axonal regeneration (Escher et al., 2008; Inatani et al., 2000; Kennan et al., 2002; Landers et al., 1994). It has been reported that both OLM integrity and glial scaring affect transplantation outcome (Singhal et al., 2008; West et al., 2008). Therefore, we looked to assess glial scarring and OLM integrity in the models of degeneration studied here, and examine whether any changes in these factors over the course of disease progression could account for the differences observed in cell integration trends. To
assess glial scarring, retinal sections were stained for GFAP, vimentin and CSPG (CS-56). A glial scar can be identified by the presence of GFAP+ve processes at the outer edge of the ONL and maybe accompanied by accumulation of CSPG in the IPM. A minimum of 3 independent retinae were analysed for each time point and confocal images were obtained at a standardised central superior location. In addition, global GFAP expression was quantified using western blot analysis. To assess OLM integrity, retina sections were stained for ZO-1. In addition, semi-thin sections were analysed to assess ONL disorganisation, a feature often associated with OLM disruption. The presence and organisation of adherens junctions was assessed using ultra structural analysis.
4.2.4.1. The microenvironment of wildtype recipients in relation to integration

As discussed in section 4.2.3, integration in the wildtype recipient remained constant at all time points examined (Figure 4.4a and 4.6f, black trend line). Glial scarring was assessed by immunohistochemistry (Figure 4.6a and b) and western blot (Figure 4.6d). The intermediate filaments, GFAP (red) and vimentin (green) were used as markers of Müller cell activation and CS-56 (green) was used to assess CSPG expression at the glial scar (Bignami and Dahl, 1979; Bringmann et al., 2000; Eisenfeld et al., 1984; Escher et al., 2008; Inatani et al., 2000; Kennan et al., 2002; Landers et al., 1994). Western blot analysis was performed to quantify GFAP activation was performed to confirm glial activation.

As expected in the absence of degeneration, there was little evidence of glial activation in the wildtype retina at 6 weeks (early) and this did not change with age (Figure 4.6a and d). Expression of GFAP- and vimentin+ve intermediate filaments within the retina was restricted to astrocytes and occasional basal processes of Müller glia. Similarly, CSPGs were sparsely distributed throughout the outer segment region at every time point examined (Figure 4.6b).

OLM integrity was examined by staining for ZO-1, a component of the OLM (Figure 4.6c). At 6 weeks (early) of age, ZO-1 expression appears as a continuous unbroken line, indicating that the OLM is intact and remained so at all time points examined (Figure 4.6c). OLM integrity was confirmed at the oldest time point with electron micrographs and images taken from semi-thin sections (Figure 4.6 ei and ii, respectively). Figure 4.6ei shows adherens junctions (dashed black boxes) at the ultrastructural level neatly aligned and forming between photoreceptor (PR) and Müller glial cells (MC). Similarly, analysis of semi-thin sections showed that the OLM forms a neat continuous line at the outer edge of the ONL (Figure 4.6eii, dashed white line).
Thus, to summarise, the trends in integration efficiency, OLM integrity and glial scaring (Figure 4.6f) remained constant throughout the time frame examined here.
Assessment of the microenvironment at early (6 weeks), mid (6 months) and late (12 months) degenerative stages. (a) Assessment of glial scarring at each time point. Cryosections were stained with glial scarring marker GFAP (red) and vimentin (green). (b) Assessment of glial scarring and CSPG deposition at each time point. Cryosections were stained with GFAP (red) and CSPG (green) (c) Assessment of OLM integrity at each time point. Cryosections were stained with an OLM marker, ZO-1 (red). Scale bar 50µm. (d) Western blot analysis of GFAP expression to assess glial activation. (e) (i) Assessment of OLM integrity at late stage degeneration to the ultrastructural level. Dashed black boxes highlight adherens junctions. (ii) Semi-thin sections demonstrating OLM integrity, indicated by white dashed line, at late stage degeneration. (f) Schematic representation of integration, glial scarring and OLM integrity trend.
4.2.4.2. The microenvironment of the degenerating \textit{Gnat1}^{-/-} recipient in relation to integration

A modest but significant decline in integration efficiency was observed over time when transplanting into early (2 months), mid (6 months) and late (12 month) stage \textit{Gnat1}^{-/-} recipients (Figure 4.4b and 4.7 f, \textit{black} trend line). We observed an increase in glial scarring, as assessed both by immunohistochemistry and western blot (Figure 4.7a and d). GFAP$^{+ve}$ processes were observed to extend throughout the retina and into the ONL and several processes where observed at the outer edge of the ONL by mid and late stage degeneration (Figure 4.7a, \textit{insets} and \textit{white} arrows). In addition, global GFAP expression, as assessed by western blot, was significantly increased at the late stage compared to early stage degeneration (Figure 4.7d, $P>0.05$, ANOVA with Tukey’s correction for multiple comparison; $N = 4$). CSPGs were sparsely distributed throughout the segment region at all time points examined, with a small qualitative increase at late stage disease (Figure 4.7b).

As in wildtype retinas, ZO-1 expression appeared as a continuous unbroken line at every time point examined, indicating that the OLM remains intact throughout (Figure 4.7c). In addition, at the ultrastructural level, adherens junctions were observed forming between photoreceptor cells and Müller glia cells and appeared neatly aligned (Figure 4.7ei, black dashed boxes). In addition, the OLM appeared as a straight line in semi thin section, with no apparent disturbances in ONL structure (Figure 4.7e ii, \textit{white dashed line}).

In conclusion, there is a decline in integration efficiency in \textit{Gnat1}^{-/-} recipients, accompanied by an increase in glial scarring as degeneration progressed (Figure 4.7e) but OLM integrity remained unchanged.
Assessment of the microenvironment at early (2 months), mid (6 months) and late (12 months) degenerative stages. (a) Assessment of glial scarring (white arrows) at each time point. Cryosections were stained with glial scarring marker GFAP (red) and vimentin (green). (b) Assessment of glial scarring and CSPG deposition at each time point. Cryosections were stained with GFAP (red) and CSPG (green). (c) Assessment of OLM integrity at each time point. Cryosections were stained with an OLM marker, ZO-1 (red). Scale bar 50µm. (d) Western blot analysis of GFAP expression to assess glial activation. (e) (i) Assessment of OLM integrity at late stage degeneration at the ultrastructural level. Dashed black boxes highlight adherens junctions. (ii) Semi-thin sections demonstrating OLM integrity, indicated by white dashed line, at late stage degeneration. (f) Schematic representation of integration, glial scarring and OLM integrity trend.
4.2.4.3. The microenvironment of the degenerating \( Crb1^{rd8/rd8} \) recipient in relation to integration

Integration into the degenerating \( Crb1^{rd8/rd8} \) mouse presented a bimodal pattern, increasing significantly between early (3 weeks) and mid (6 weeks) stage disease but declining significantly when transplanted into a late stage disease (12 week) recipient (Figure 4.4c and 4.8f, black trend line).

Although minimal at the early degeneration stage, glial scarring significantly increased between mid and late stage disease as assessed both by immunohistochemistry and western blot (Figure 4.8a and d). We observed few GFAP\(^{+ve}\) processes in the retina at early and mid stage disease, although vimentin expression was high and vimentin\(^{+ve}\) process were observed to extend throughout the retina even at early stages of degeneration (Figure 4.8a). By late stage disease (Figure 4.8a, white arrows and inserts), however, numerous GFAP\(^{+ve}\) fibers extended into the outer retina and along the outer edge of the retina in places, particularly around sites of presumptive rosette formation. These changes were mirrored by a significant increase in global GFAP expression (Figure 4.8d, \( P<0.01 \), ANOVA with Tukey’s correction for multiple comparison, \( N = 4 \)). CSPG expression appeared more dense within the IPM, with deposits being particularly noticeable in the OPL and around rosette formation as degeneration progressed (Figure 4.8b).

As expected in this model (Mehalow et al., 2003), OLM integrity became increasingly compromised with disease progression. OLM disruption was demonstrated by the appearance of increasingly fragmented staining for ZO-1 and the presence of photoreceptor cell bodies in the subretinal space (Figure 4.8c i, white arrows). Upon closer inspection at the ultrastructural level, although some adherens junctions between Müller cells and photoreceptor cells remained (Figure 4.8e i, dashed black boxes), large areas in which adherens junctions were absent were also observed.
(asterisk). Severe disturbances in ONL organization were observed in semi-thin section obtained from late stage disease recipients; the outer edge of the ONL appeared uneven with cell bodies displaced in the subretinal space (Figure 4.8e ii, white dashed line).

In conclusion, a bimodal pattern of integration efficiency is observed in Crb1<sup>rd8/rd8</sup> recipients between early and late stage degeneration, with the highest integration observed when cells were transplanted into mid stage disease recipients. During this same period, OLM integrity is increasingly compromised, a change that is likely to facilitate integration (Figure 4.8f). However, gliosis is also significantly increased, a change that is likely to impede integration. At the mid disease stage, OLM integrity is already markedly compromised but the glial response was not maximal, which may explain the high number of integrated cells demonstrated at this time point.
Assessment of the microenvironment at early (3 weeks), mid (6 weeks) and late (12 weeks) degeneration. (a) Assessment of glial scarring (white arrows) at each time point. Cryosections were stained with glial scarring marker GFAP (red) and vimentin (green). (b) Assessment of glial scarring and CSPG deposition at each time point. Cryosections were stained with GFAP (red) and CSPG (green). (c) Assessment of OLM integrity at each time point. Cryosections were stained with an OLM marker, ZO-1 (red). White arrows indicate breaks in OLM integrity. Scale bar 50µm. (d) Western blot analysis of GFAP expression to assess glial activation. (e) (i) Assessment of OLM integrity at late stage degeneration at the ultrastructural level. Dashed black boxes highlight adherens junctions; asterisks indicate areas where adherens junctions were absent (ii) Semi-thin sections demonstrating OLM integrity, indicated by white dashed line, at late stage degeneration. (f) Schematic representation of integration, glial scarring and OLM integrity trend.
4.2.4.4. The microenvironment of the degenerating Prph2+/Δ307 recipient in relation to integration

Disease progression resulted in a significant increase in integration efficiency when Nrl.GFP+ve -rod precursor cells were transplanted into Prph2+/Δ307 recipients at early (2 months), mid (4 months) and late (6 months) degeneration stages (Figure 4.4d and 4.9f, black trend line).

When assessing the microenvironment of Prph2+/Δ307 mice using immunohistochemistry, we found that GFAP and vimentin were upregulated at all time points examined compared to wildtype (Figure 4.9a). At the earliest stage examined (2 months) GFAP+ve and vimentin+ve processes were observed extending throughout to the outer edges of the retina where they forming a glial scar (Figure 4.9a, white arrows and inset). Similarly, at this early time point CSPG staining appeared condensed and immediately adjacent to the ONL (Figure 4.9b). Strikingly however, although there was no significant decrease in global GFAP expression, as measured by western blot (Figure 4.9d, P>0.05, ANOVA with Tukey’s correction for multiple comparison, N = 4), immunohistochemistry revealed a significant reduction in the number of GFAP+ve processes observed extending into the outer retina, indicating a regression of the glial scars (Figure 4.9a, insets). Similarly CSPG staining appeared to almost completely disperse by late stage disease with very little staining in the interphotoreceptor matrix (Figure 4.9b).

Some disturbances were observed in OLM integrity at all time points examined here. There was some limited disruption in ZO-1 staining at all three time points, indicating some possible breaks in OLM integrity (Figure 4.9c, arrows) although this did not appear to change with time. Ultrastructural analysis of late stage diseased retinae revealed that some adherens junctions remained but these lacked alignment (Figure 4.9e i, black dashed boxes). In the representative electron micrographs shown in
Figure 4.9e i, the asterisk highlights regions where both photoreceptor cell death occurred and where the adherens junctions were absent. This suggests that remodeling of the OLM may occur around regions of cell death. Similarly, semi-thin sections demonstrate that ONL structure and OLM integrity appeared moderately disrupted (Figure 4.9e ii, white dashed line), with the presence of many cell bodies displaced in the interphotoreceptor cell matrix (arrows).

In summary, of all the disease models examined, the Prph2*4.307 was the only model in which transplanted photoreceptor integration increased with disease progression. It is also the only model in which glial scarring, at least within the ONL, decreased. This was accompanied by some remodeling of the OLM, although this did not change with disease progression (Figure 4.9f).
FIGURE 4.9. ASSESSMENT OF THE Prph2^{Δ307} RETINAL MICROENVIRONMENT DURING DEGENERATION

Assessment of the microenvironment at early (2 months), mid (4 months) and late (6 months) degeneration. (a) Assessment of glial scarring (white arrows) at each time point. Cryosections were stained with glial scarring marker GFAP (red) and vimentin (green). (b) Assessment of glial scarring and CSPG deposition at each time point. Cryosections were stained with GFAP (red) and CSPG (green). (c) Assessment of OLM integrity at each time point. Cryosections were stained with an OLM marker, ZO-1 (red). White arrows indicate breaks in OLM integrity. Scale bar 50µm. (d) Western blot analysis of GFAP expression to assess glial activation. (e) (i) Assessment of OLM integrity at late stage degeneration at the ultrastructural level. Dashed black boxes highlight adherens junctions; asterisks indicate areas where adherens junctions were absent (ii) Semi-thin sections demonstrating OLM integrity, indicated by white dashed line, at late stage degeneration. Arrows indicated displace photoreceptor cell bodies. (f) Schematic representation of integration, glial scarring and OLM integrity trend.
4.2.4.5. The microenvironment of the degenerating Prph2<sup>rds/rds</sup> recipient in relation to integration

In the Prph2<sup>rds/rds</sup> model, integration remained constant as degeneration progressed (Figure 4.4e and 4.10f, black trend line). Staining for GFAP and vimentin, showed a marked increase in the number of processes that extended throughout the retina into the ONL, forming a lateral glial scar at the edge of the retina, at mid (8 weeks) and late (12 week) stages of degeneration (Figure 4.10a i, white arrows and inserts). Western blotting demonstrated a striking increase in global GFAP, indicating significant glial activation at the mid and late degeneration stages the in Prph2<sup>rds/rds</sup> retina (Figure 4.4e, P<0.05, ANOVA with Tukey’s correction for multiple comparison, N = 4). Conversely, although CSPG deposition appeared condensed at the outer edge of the ONL at early (4 weeks) and mid (8 weeks) degeneration time points, by late stage degeneration (12 weeks) the CSPGs in the interphotoreceptor matrix appeared more disperse (Figure 4.10b).

ZO-1 expression indicated that some occasional breaks in OLM integrity were present at all degeneration stages (Figure 4.10c i, white arrows), although this occurred with a similar frequency over the degeneration time course. Ultrastructural analysis revealed that although adherens junction were maintained at late stage disease (Figure 4.10e i, black dashed boxes), they appeared elongated in length and Müller glia hypertrophy was apparent. Analysis of semi-thin sections confirmed the disruption of the OLM at late stage disease: the OLM appeared uneven at the outer edge of the retina and several cell bodies were displaced in the interphotoreceptor matrix (Figure 4.910e ii, white dashed line and arrows, respectively).

Thus in summary, integration into Prph2<sup>rds/rds</sup> recipients remained constant between early and late stage degeneration. Although GFAP expression was upregulated, this
was offset by a decrease in CSPG deposition. There was evidence of OLM remodeling at all time points although this did not change with degeneration (Figure 4.10f).
FIGURE 4.10. ASSESSMENT OF THE *Prph2*<sup>−/−</sup> RETINAL MICROENVIRONMENT DURING DEGENERATION

Assessment of the microenvironment at early (4 weeks), mid (8 weeks) and late (12 weeks) degeneration. (a) Assessment of glial scarring (*white arrows*) at each time point. Cryosections were stained with glial scarring marker GFAP (*red*) and vimentin (*green*). (b) Assessment of glial scarring and CSPG deposition at each time point. Cryosections were stained with GFAP (*red*) and CSPG (*green*). (c) Assessment of OLM integrity at each time point. Cryosections were stained with an OLM marker, ZO-1 (*red*). *White arrows* indicate breaks in OLM integrity. Scale bar 50µm. (d) Western blot analysis of GFAP expression to assess glial activation. (e) (i) Assessment of OLM integrity at late stage degeneration at the ultrastructural level. *Dashed black boxes* highlight adherens junctions. (ii) Semi-thin sections demonstrating OLM integrity, indicated by *white dashed line*, at late stage degeneration. *Arrows* indicated displace photoreceptor cell bodies. (f) Schematic representation of integration, glial scarring and OLM integrity trend.
4.2.4.6. The microenvironment of the degenerating \textit{Rho}^{-/-} recipients in relation to integration

Transplanted photoreceptor cell integration efficiency into the \textit{Rho}^{-/-} recipient was markedly worse than observed in other models. Moreover, integration efficiency continued to decline with disease progression (Figure 4.4f and 4.11f, \textit{black} trend line).

Western blot analysis of GFAP expression revealed a striking upregulation of GFAP expression between early (4 weeks) and late (10 weeks) stage disease (Figure 4.11d, \textit{P}<0.001, ANOVA with Tukey's correction for multiple comparison, \(N=4\)). Staining for GFAP and vimentin further shows that there was significant GFAP expression even in the early stages of degeneration and this continued to increase as disease progressed. There was an increase in the number of intermediate filament processes visible in the inner retina with more extending into the ONL at early stage degeneration compared to wildtype (Figure 4.11a, \textit{white arrow}). By late stage degeneration, numerous GFAP$^{+ve}$ and vimentin$^{+ve}$ processes could be seen to extend throughout ONL and these processes appeared to extend laterally along the outer edge of the ONL forming the glial scar (Figure 4.11a i, \textit{white arrows} and \textit{insets}). CSPG expression appeared to condense in the interphotoreceptor matrix as disease progressed; CSPGs formed a thin dense band abutting the ONL at the late stage degeneration (Figure 4.11b).

In contrast to some other models of moderate degeneration, the OLM appeared to remain intact throughout the time course examined here: ZO-1 staining appeared as a strong, continuous unbroken line at the outer edge of the ONL (Figure 4.11c). Similarly, analysis of electron micrographs shows that adherens junctions formed between Müller cell and photoreceptor cells were numerous and neatly aligned, even at late stage disease (Figure 4.11e i \textit{dashed boxes}). Semi-thin sections demonstrated that the OLM remained as a straight continuous line at the outer edge of the retina with no loss of cells into the subretinal space (Figure 4.11e ii, \textit{white dashed line}).
Thus, integration is poor and declines significantly with disease progression in $Rho^{-}$ recipients. This occurs against a background of significantly increasing gliosis and a maintained OLM; both of these factors are likely to impede integration (Figure 4.11f).
FIGURE 4.11. ASSESSMENT OF THE Rho RETINAL MICROENVIRONMENT DURING DEGENERATION

Assessment of the microenvironment at early (4 weeks), mid (6 weeks) and late (10 weeks) degeneration. (a) Assessment of glial scarring (white arrows) at each time point. Cryosections were stained with glial scarring marker GFAP (red) and vimentin (green). (b) Assessment of glial scarring and CSPG deposition at each time point. Cryosections were stained with GFAP (red) and CSPG (green). (c) Assessment of OLM integrity at each time point. Cryosections were stained with an OLM marker, ZO-1 (red). Scale bar 50µm. (d) Western blot analysis of GFAP expression to assess glial activation. (e) (i) Assessment of OLM integrity at late stage degeneration at the ultrastructural level. Dashed black boxes highlight adherens junctions. (ii) Semi-thin sections demonstrating OLM integrity, indicated by white dashed line, at late stage degeneration. (f) Schematic representation of integration, glial scarring and OLM integrity trend.
4.2.4.7. The microenvironment of the degenerating $PDE6\beta^{rd1/rd1}$ recipient in relation to integration

Despite a rapidly progressing degeneration, integration in $PDE6\beta^{rd1/rd1}$ recipients remained constant as disease progressed (Figure 4.4g and 4.12f, black trend line).

Examination of the microenvironment revealed that an extensive glial response was activated by late (3 week) stage disease. Western blot analysis demonstrated a significant upregulation of GFAP expression between early (P10) and late stage degeneration (Figure 4.12d, $P<0.001$, ANOVA with Tukey’s correction for multiple comparison, $N = 4$). Staining for GFAP and vimentin, at early stage degeneration was similar to wildtype, with no intermediate filaments observed within the outer retina (Figure 4.12a). Similarly, CSPG staining appears dispersed throughout the interphotoreceptor matrix (Figure 4.12b). However, by late stage degeneration, a proliferation of GFAP$^{+ve}$ and vimentin$^{+ve}$ process could be seen extending throughout the retina. These processes extended along the outer edge of the ONL forming an extensive glial scar (Figure 4.12a, white arrows and inserts). In addition, CSPGs appeared to condense at the outer edge of the ONL at late stage disease (Figure 4.12b).

Staining for ZO-1 staining appeared markedly fragmented at late stage disease, which suggested extensive OLM disruption (Figure 4.12c). Further analysis at the ultrastructural level (Figure 4.12e i) showed that adherens junctions were present but often formed between two Müller cells (red dashed boxes) alongside the more typical adherens junctions formed between Müller glia and photoreceptor cells (black dashed boxes). This indicated that significant OLM re-modeling had occurred during the time course analysed here. Analysis of semi-thin sections at late stage disease also confirmed OLM disorganization, as the structure of the ONL and outer edge of the retina appeared less ordered than wildtype (Figure 4.12e ii).
To summarise, integration remained constant in $PDE6\beta^{rd1/rd1}$ recipients, even in late stage degeneration. There was a significant and striking increase in glial scarring, while evidence of extensive OLM re-modelling indicated that OLM integrity is compromised during the degenerative process (Figure 4.12f).
FIGURE 4. 12. ASSESSMENT OF THE PDE6B<sup>RD1/RD1</sup> RETINAL MICROENVIRONMENT DURING DEGENERATION

Assessment of the microenvironment at early (P10) and late (3 weeks) degeneration. (a) Assessment of glial scarring (white arrows) at each time point. Cryosections were stained with glial scarring marker GFAP (red) and vimentin (green). (b) Assessment of glial scarring and CSPG deposition at each time point. Cryosections were stained with GFAP (red) and CSPG (green). (c) Assessment of OLM integrity at each time point. Cryosections were stained with an OLM marker, ZO-1 (red). Scale bar 50µm. White arrows indicate breaks in OLM integrity. (d) Western blot analysis of GFAP expression to assess glial activation. (e) (i) Assessment of OLM integrity at late stage degeneration at the ultrastructural level. Dashed black boxes highlight adherens junctions that formed between photoreceptor cells and Müller glia. Dashed red boxes highlight adherens junctions between two Müller glia, indicating a significant degree of OLM re-modelling. (ii) Semi-thin sections demonstrating OLM integrity, indicated by white dashed line, at late stage degeneration. (f) Schematic representation of integration, glial scarring and OLM integrity trend.
4.2.4.8. Summary: the impact of the microenvironment on integration efficiency

The degenerating retina is a complex and changing environment and each of the models examined here presented a unique pattern of changes within their retinal microenvironment as disease progressed. Examination of the changes in OLM integrity, gliosis and transplanted photoreceptor integration in unison allowed some correlations to be drawn that are summarised below.

Integration remains constant with age in the wildtype recipient. Similarly, there was no change in OLM integrity and an absence of glial scarring was demonstrated. Integration remained constant in both the PDE6β<sup>rd1/rd1</sup> and Prph2<sup>rd1/rd1</sup> recipient as disease progressed. The PDE6β<sup>rd1/rd1</sup> model demonstrated a significant and striking increase in glial scarring, as indicated both by CSPG deposition and Müller glia activation, factors that may be inhibitory to transplanted photoreceptor cell migration and integration (Brittis et al., 1992; Kinouchi et al., 2003; Ma et al., 2011; Singhal et al., 2008; Suzuki et al., 2007; Zhang et al., 2004). This model also displayed some evidence of extensive OLM re-modelling suggesting that disturbances in OLM integrity occurred as degeneration progresses. Loss of OLM integrity is known to be favourable to cell integration (West et al., 2008), and this may explain why robust integration was maintained when donor cells were transplanted into late stage degeneration recipients.

The Prph2<sup>rd1/rd1</sup> recipient displayed an increase in GFAP expression, indicative of glial cell activation, which has been suggested to inhibit donor cell integration into the recipient retina (Kinouchi et al., 2003). However, there also appeared to be a decline in CSPG deposition and this may offset this potentially inhibitory effect of GFAP upregulation.

Integration declined over the time course examined here in both the Gnat1<sup>−/−</sup> and Rho<sup>−/−</sup> recipients. Despite very different rates of degeneration, both of these models
maintained OLM integrity but also underwent a significant glial response as disease progressed, both factors likely to impede integration. A bimodal pattern of integration was observed in \(Crb1^{rd8/rd8}\) recipients; a rapid fragmentation of the OLM appeared to be offset with a delayed but significant increase in glial scarring as assessed by both GFAP upregulation and CSPG deposition. Integration efficiency was highest when donor rod precursor cells were transplanted into mid stage degeneration \(Crb1^{rd8/rd8}\) recipients, a time when the OLM was sufficiently disrupted yet the glial response was not fully initiated.

Most encouragingly, an increase in transplanted cell integration was observed in \(Prph2^{-/Δ307}\) recipients as disease progressed and robust integration was shown when donor rod precursor cells were transplanted into late stage degeneration recipients. The trend in integration efficiency coincided with a reduction in glial scarring, which may permit the successful migration of transplanted cells into the ONL. In addition, a moderate yet sustained re-modelling of the OLM was observed in \(Prph2^{rd8/rd8}\) and \(Prph2^{-/Δ307}\) recipients, which may explain the high levels of integration observed in these degenerative models compared to wildtype and \(Rho^{-/−}\) recipients. Similarly, glial scarring was not extensive in \(Gnat1^{-/−}\) and \(PDE6β^{rd1/rd1}\) recipients at the early degenerative stage compared to \(Rho^{-/−}\) recipient retina at the same degenerative stage. This may go some way to explain the higher integration efficiency observed in these former two models. Interestingly, the \(Rho^{-/−}\) model has an intact OLM with extensive glial scarring that occurred early in the degeneration process: these factors may explain the considerably low integration efficiency shown in this model of retinal degeneration.
4.2.5. Manipulating the Microenvironment of the $Rho^{-/-}$ Recipient to Maximise Cell Integration

The data presented in section 4.4 demonstrates that there are significant differences in both OLM integrity and gliosis across the different models of degeneration. Taken together, they offer potential explanations for the different photoreceptor transplantation outcomes. The highest level of transplanted cell integration was seen in the $Crb1^{rd8/rd8}$ recipient model. This model has a disrupted OLM (Mehalow et al., 2003), a factor known to enhance integration efficiency (West et al., 2008) accompanied by an initially low, focal glial response. In contrast, poor integration was observed when transplanting Nrl.GFP$^{+ve}$ rod precursor cells into the $Rho^{-/-}$ mouse, a model with an intact OLM and strong glial response, both factors likely to impede integration (Kinouchi et al., 2003; West et al., 2008). To determine whether these factors are indeed responsible for impeding integration in the degenerating recipient retina, we sought to manipulate them pharmacologically and assess their impact on transplanted Nrl.GFP$^{+ve}$-rod photoreceptor integration. The $Rho^{-/-}$ model (4-6 weeks) was chosen for assessment being the model with the worst transplantation outcome.
4.2.5.1. Chondroitinase ABC

Chondroitinase ABC is an enzyme produced by bacteria that enzymatically digests extracellular CSPGs in vivo (Bradbury et al., 2002; Moon et al., 2001; Morgenstern et al., 2002). We used ChABC to breakdown CSPGs in the retina, a strategy that has been previously used to digest CSPGs in the rat (Bull et al., 2008; Singhal et al., 2008) and in the mouse retina (Ma et al., 2011; Suzuki et al., 2007). Although ChABC has been used extensively in the rodent retina and has been shown to improve integration efficiency when co-transplanted with donor cells into wildtype recipients, none of these groups quantified or verified in vivo digestion of CSPGs following administration of ChABC. There are extensive studies assessing dosing, onset and duration of action of ChABC in the spinal cord of Sprague–Dawley rats (Lin et al., 2008). This study demonstrated ChABC activity 1 day post infusion and showed that ChABC was able to efficiently suppress injury-related up-regulation of proteoglycans digestion with the enzyme remaining activated in vivo up to 10 days post administration (Lin et al., 2008). Based on the findings of Suzuki et al (2007), which demonstrated enhanced synaptogenesis of unsorted Nrl.GFP\(^{+}\) donor cells transplanted into the wildtype MNU-induced recipient model of retinal degeneration, a dose of 0.025 Units / \(\mu\)L was used in our study. To confirm that this dose reduced CSPG content in the Rho\(^{-}\) mouse (age 3-5 weeks), animals were sacrificed at 48 hours post subretinal injection of 1 \(\mu\)L of 0.025 Units / \(\mu\)L ChABC (N = 3). Retinal tissue was processed for immunohistochemistry and western blot. Figure 13a and b demonstrates robust digestion of CSPGs following subretinal injection of ChABC compared to vehicle alone. CSPG content in the interphotoreceptor matrix appears considerably dispersed (Figure 13a) and all three animals showed a reduction in CSPG protein in the ChABC treated eye compared to the contralateral control eye which received vehicle alone (Figure 13b).
FIGURE 4.13. DIGESTION OF CSPGS USING CHABC IN THE Rho<sup>−/−</sup> RECIPIENT

(a) Representative confocal images taken from Rho<sup>−/−</sup> retinal sections stained for CSPG. Treated eyes were injected subretinally with 1 µL of 0.025 Units / µL ChABC (right, +ChABC) or 1 µL of vehicle alone (left, -ChABC). Scale bar 50µm. (b) Western blot analysis of CSPG in three Rho<sup>−/−</sup> mice (aged 3-5 weeks), treated with a subretinal injection of ChABC in one eye (+ChABC). The contralateral control eye received a subretinal injection of vehicle alone (-ChABC). Control retinal extracts that were further treated in vitro with ChABC for 3 hours to achieve maximal digestion to serve as complete digestion controls for the corresponding in vivo treated samples.
Having confirmed that this dose of ChABC reduced CSPG expression in the $Rho^{-/-}$ retina, we next combined ChABC application with photoreceptor transplantation to assess whether enzymatic removal of CSPGs in a model of retinal degeneration could improve transplantation efficiency. Nrl.GFP$^{+/+}$-rod photoreceptor precursor cells were co-injected with either 0.025 U/µL ChABC or vehicle alone into $Rho^{-/-}$ (aged 3-5 weeks) and wildtype recipients. $Rho^{-/-}$ mice receiving ChABC treatment demonstrated a 8-fold increase in transplanted Nrl.GFP$^{+/+}$-rod photoreceptor integration compared with contralateral control eyes ($17,116 \pm 3125 \ (N = 12)$ versus $2051 \pm 248 \ (N = 7)$ integrated cells, respectively; Figure 4.14, $P<0.01$, unpaired t-test). Interestingly, in the wildtype recipient, where there is no gliosis and CSPG expression is diffuse, the effect of ChABC was smaller, leading to a 2.5-fold increase in the number of integrated cells in the ChABC transplanted eye compared to the control eyes ($7558 \pm 1134 \ (N = 9)$ versus $3014 \pm 946 \ (N = 9)$ integrated cells, respectively; Figure 4.14, $P<0.01$).
FIGURE 4.14. INTEGRATION EFFICIENCY FOLLOWING CHABC TREATMENT

Integration efficiency following ChABC treatment (white) in adult wildtype and Rho^{-/-} recipients (aged 3-5 weeks) transplanted with Nrl.GFP^{+ve}-rod precursor cells. Control eyes received Nrl.GFP^{+ve} rod precursor cells alone (black). Unpaired t-test.
4.2.5.2. Targeted OLM disruption using siRNA

We employed the use of siRNAs targeted against ZO-1, to provide a transient yet reversible disruption of the OLM. ZO-1 is an adaptor protein involved in adherens junction formation. Previous reports in the lab demonstrated that the targeting sequence used in the following experiments effectively silences ZO-1 (Sourisseau et al., 2006).

a) Dosing

In the first instance, experiments were performed to establish the optimal dose of siRNA, one that produces a reversible knockdown of ZO-1 at the OLM. Three doses were explored: 10, 15 and 20 µM and administered via subretinal injections to the superior retina in wildtype mice. Animals were sacrificed after 48 hours and the retinae were examined using immunohistochemistry. Figure 4.15a i-iv illustrates that ZO-1 staining remained continuous in animals treated with 10 µM siRNA and appear similar to uninjected wildtype controls (Figure 4.15a i and ii). ZO-1 knockdown was achieved following subretinal injection of both 15 µM and 20 µM ZO-1 siRNA: staining for ZO-1 at the OLM appears fragmented and discontinuous (Figure 4.15a i, ii and vi, white arrows). In addition, this was accompanied by typical characteristics of OLM disruption including disturbances in retinal architecture and displacement photoreceptor cell bodies into the subretinal space (Figure 4.15a iii and iv). The extent of ZO-1 knockdown was greater in the animals treated with 20 µM and ZO-1 staining was absent in a large region around the injection site (Figure 4.15 vi, stars). The use of siRNA longer than 21-nucleotides has been associated with cell death, as a consequence of Toll-like 3 receptors activation (Kleinman et al., 2012). Therefore, we sought to assess whether subretinal administration of ZO-1 siRNA was associated with an increase in cell death. Treatment with the 20 µM dose was associated with a significant increase in apoptotic cell death of the endogenous photoreceptors compared to non-targeting treated contralateral control eyes (1956 ± 459 versus 467 ±
157 apoptotic cells, respectively: Figure 4.15b, <0.05, paired t-test, N = 4), while apoptosis following application of the 15 µM siRNA dose was no different to control injections: 15 µM produces 587 ± 211 apoptotic cells whereas the non-targeting treated contralateral control eye resulted in 589 ± 237 apoptotic cells at 48hrs post injection (Figure 4.15b, , P>0.05, paired t-test, N = 9). Given the moderate ZO-1 knockdown achieved with the 15 µM and the lack of association with apoptotic cell death, all further experiment were performed using the 15 µM dose.
FIGURE 4.15. DOSING OF siRNA TARGETED AGAINST ZO-1

(a) Representative images of retinal sections stained for ZO-1 to assess OLM integrity 48 hours post administration. Different doses of siRNA ZO-1 (ii, 10 µM; iii, 15 µM and iv, 20 µM) were injected into the subretinal injection of the wildtype retina. Animals were culled 48 hours post injection. White arrows indicate areas where the OLM appeared fragmented. Dashed white boxes indicate areas shown in insets. Scale bar 50 µm. (b) Graph showing apoptotic cell death associated with subretinal injection siRNA targeted against ZO-1 (grey boxes) or non-targeting control siRNA (black boxes). Two doses were analysed: 15 µM and 20 µM.
To further characterize the OLM disruption exuded by this dose of siRNA, adult wildtype animals were injected with 15 µM siRNA or 15 µM non-targeting siRNA and sacrificed 48 hours post injection and a number of OLM markers were examined. Figure 4.16 a-c demonstrates that all OLM markers showed a localized OLM disruption. Disrupted staining of pancaderin, Crb1 and β-catenin was demonstrated in all eyes treated with 15 µM ZO-1 siRNA (Figure 4.16: a iii, b iii and c iii, respectively) compared to contralateral non-targeting treated eyes (Figure 4.16: a ii, b ii and c ii, respectively), where staining appeared unbroken and continuous, much like that seen in untreated control eyes (Figure 4.16: a i, b i and c i, respectively). Finally, ultra-structural analysis was used to confirm the disruption of the adherens junction. Eyes treated with ZO-1 siRNA demonstrate a loss of OLM integrity with large distances between the remaining adherens junctions (Figure 4.16d ii, red arrows), whereas the OLM remained intact following treatment with non-targeting siRNA (Figure 4.16d i, red arrows).
Image 1: Uninjected vs. 15μM Non-targeting at 48hr

Image 2: 15μM Non-targeting at 48hr vs. 15μM siRNA ZO-1 at 48hr

Image 3: Electron Microscopy
- i. 15μM Non-targeting at 48hr
- ii. 15μM siRNA ZO-1 at 48hr
FIGURE 4.16. ZO-1 SIRNA KNOCKS DOWN ZO-1 EXPRESSION IN THE OLM

Confocal images demonstrating expression of OLM markers (a) pancadherin, (b) Crb1 and (c) β-Catenin. Images were taken 48 hours post injection of (i) uninjected control retina, (ii) 15 μM non-targeting injected retina or (iii) 15 μM siRNA ZO-1 injected retina. White arrows indicate disruption of the OLM. Scale bar 25 μm. (d) Electron micrographs demonstrating adherens junctions, as indicated by the red arrows, in retina injected with 15 μM (i) non-targeting or (ii) siRNA ZO-1 at 48hrs post injection.
In order for this approach to be a feasible solution to improve transplantation efficiency, any disruption to the OLM must be transient to prevent any long-term damage to the recipient retina (Mehalow et al., 2003). To assess whether disruption of the OLM using siRNA was reversible, gross retinal morphology and ZO-1 staining at the OLM of animals treated with either ZO-1 siRNA or non-targeting siRNA were analysed at 72 hours and 1 week post injection. Figure 4.17a and b demonstrates that staining for ZO-1 was disrupted at 48 hours and 72 hours post injection (Figure 4.17a i and ii) compared to contralateral non-targeting treated control eyes (Figure 4.17b i and ii). Administration of non-targeting control cause a transient detachment of the neural retina observed at 48 hours post injection, which resolved by 72 hours post injection (Figure 4.17b i and ii, respectively). In addition, staining for ZO-1 remained largely intact, however occasional localized disruption was observed at 48 hours post injection (Figure 4.17b i, insert). By 1 week, expression of ZO-1 had largely recovered in those eyes treated with ZO-1 siRNA: staining appeared continuous and unbroken at the OLM similar to non-targeting treated controls (Figure 4.17 aiii and biii, respectively). Figure 4.17c demonstrates H&E staining for eyes treated with ZO-1 siRNA (Figure 4.17c) or contralateral control eyes treated with non-targeting siRNA (Figure 4.17d). Other features characteristic of OLM disruption were observed at 48 and 72 hours post injection of ZO-1 siRNA: ONL folding and displacement of endogenous photoreceptor cells bodies were observed in ZO-1siRNA treated eyes (Figure 4.17c i and ii) but not in contralateral non-targeting treated eyes (Figure 4.17d i and ii). Retinae from ZO-1 siRNA treated eyes appear mostly recovered by one week, although the outer retinal surface remained more uneven than the non-targeting treated control eye (Figure 4.17 c iii and d iii).
FIGURE 4.17. TIME COURSE OF OLM DISRUPTION

Confocal images of wild-type retina following subretinal injections of (a) 15 µM ZO-1 siRNA or (b) 15 µM non-targeting siRNA at (i) 48 hr; (ii) 72 hr and (iii) 1 week post injection. White arrows indicate OLM disruption. Scale bar 25 µm. Retinal sections stained with H&E following injections of (c) 15 µM ZO-1 siRNA or (d) 15 µM non-targeting siRNA at (i) 48 hr; (ii) 72 hr and (iii) 1 week post injection. Scale bar 25 µm.
b) Impact of ZO-1 knockdown on photoreceptor transplantation efficiency

Having confirmed that 15 µM ZO-1 siRNA produced a robust and transient disruption of the OLM, we next combined this approach with photoreceptor transplantation to assess whether targeted disruption of the OLM could improve transplantation efficiency. Nrl.GFP\textsuperscript{+ve}-rod photoreceptor precursor cells were injected into wildtype and Rho\textsuperscript{−} recipients (aged 3-5 weeks) that received pre-treatment with either 15 µM siRNA or 15 µM control non-targeting siRNA. A second control cohort received no pre-treatment. Rho\textsuperscript{−} mice receiving ZO-1 siRNA treatment demonstrated a highly significant 5.5-fold increase in transplanted Nrl.GFP\textsuperscript{+ve}-rod photoreceptor integration compared with eyes that were transplanted with cells alone (9944 ± 1539 (N = 7) versus 1794 ± 357 (N = 7) integrated cells, respectively; Figure 4.18, P<0.001, unpaired t-test). In the wildtype recipient, the magnitude of the effect was similar. Those eyes receiving ZO-1 siRNA treatment demonstrated a significant 4.8-fold increase in the number of integrated cells compared to control eyes that received cells alone (13199 ± 2695 (N = 7) versus 2712 ± 947 (N = 9) integrated cells, respectively; Figure 4.18, P<0.01).

Interestingly, pre-treatment with control scrambled siRNA resulted in a significant increase in integration efficiency compared to eyes that received no pre-treatment receiving only Nrl.GFP\textsuperscript{+ve}-rod cells in both wildtype and Rho\textsuperscript{−} recipients. Similar findings were reported in Chapter 3 where integration efficiency was increased following pre-detachment of the retina using EBSS 48 hrs prior to cell transplantation. Following pre-treatment with non-targeting siRNA, integration efficiency was increased 3.4-fold and 2.4-fold compared to eyes receiving no pre-treatment in Rho\textsuperscript{−} and wildtype recipient, respectively (Figure 4.18, P<0.01 and P<0.05, respectively). However, the there was a significant increase in integration efficiency in ZO-1 siRNA pre-treated eyes compared to control non-targeting treated eyes in both wildtype and Rho\textsuperscript{−} recipients. Eyes pre-treated with ZO-1 siRNA demonstrated a 2-fold and 1.6-fold increase in integration efficiency compared to those eyes pre-treated with control non-
targeting siRNA (Figure 4.18, P<0.05 in both instances). This suggested that the pre-detachment alone causes and increase in integration efficiency.
Integration efficiency following pre-treatment with ZO-1 siRNA 48 hours prior to transplantation in adult wildtype and \( \textit{Rho}^{-/-} \) recipients (aged 3-5 weeks). Control cohorts received either no pre-treatment (cells only, \textit{black}) or pre-treatment with non-targeting siRNA (\textit{grey}). Unpaired t-test.
c) Combined manipulations & functional rescue

Next we sought to examine whether a combination of ZO-1 siRNA and ChABC treatment led to additive effects in integration efficiency and whether the enhancement of transplanted cell integration led to functional improvements in optokinetic head-tracking responses.

In the first instance, Nrl.GFP+ve- rod precursor cells were transplanted into wildtype and $Rho^{-/-}$ recipients (aged 3-5 weeks) following pre-treatment with ZO-1 siRNA 48 hours prior to transplantation and co-injection with ChABC at the time of transplantation. A highly significant 5.8-fold and 8-fold increase in integration efficiency in wildtype and $Rho^{-/-}$ recipients, respectively, was demonstrated in those eyes treated with a combination of ChABC and ZO-1 siRNA compared to those eyes with no treatment (Figure 4.19a and d, P<0.001 in both instances, unpaired t-test). The number of integrated cells was increased from 3014 ± 946 to 17,603 ± 3085 in the wildtype recipient and from 2051 ± 248 to 16,497 ± 3281 integrated cells in the $Rho^{-/-}$ recipient following combined treatment with ZO-1 siRNA and ChABC (Figure 4.19a). Indeed, large areas of transplanted cell integration can be observed in the recipient retina of wildtype and $Rho^{-/-}$ mice (Figure 4.19b). However, combining ZO-1 siRNA and ChABC treatments did not lead to an additive effect on transplantation efficiency compared to the single stand-alone treatment (Figure 4.19 c and d). Although these treatment effects are not statistically significant from one another, the effect of CSPG digestion using ChABC on transplantation efficiency seemed more pronounced in the $Rho^{-/-}$ mouse compared to wildtype (8.3-fold versus 2.5 fold increase in integration efficiency, respectively; Figure 4.19 d). In addition, the effect of OLM disruption using ZO-1 siRNA on transplantation efficiency was comparable between the wildtype and $Rho^{-/-}$ recipients (4.8 fold versus 5.5-fold increase in integration efficiency; Figure 4.19 c and d). These comparisons reflect the differences in the recipient microenvironment; the OLM is intact in both wildtype and $Rho^{-/-}$ recipients, however, there is a striking glial response in the $Rho^{-/-}$ recipient retina that is absent in the wildtype recipient.
Finally, we sought to examine whether the improvements in integration efficiency, achieved using the combined treatment approach, were sufficient to drive optokinetic head-tracking responses. *Rho*<sup>−/−</sup> recipients, which lack rod function, received transplants of Nrl.GFP<sup>−/−</sup>-rod precursors together with combined ZO-1 siRNA and ChABC treatment (N=7). The contralateral control eye received either cells only or no injection. Four weeks post-transplant, animals were tested for optokinetic head-tracking responses in scotopic lighting conditions, as described in section 2.4.4. Neither uninjected mice nor those receiving cells alone demonstrated any consistent head-tracking behavior under scotopic lighting conditions. However, 6/7 of the eyes receiving the combined treatment presented with some head-tracking behavior and detectable contrast thresholds (Figure 4.19e). Furthermore, histological assessment revealed the degree of improvement in the contrast threshold was positively correlated (R<sup>2</sup> = 0.669) with the number of successfully integrated cells recovered after behavioral testing.
FIGURE 4.19. INTEGRATION EFFICIENCY AND FUNCTIONAL RESCUE FOLLOWING COMBINED TREATMENT ADMINISTRATION

(a) Histogram showing integration efficiency following transplantation of Nrl.GFP$^{\text{+ve}}$-rod precursor cells into wildtype and $Rho^\text{−/−}$ recipients (aged 3-5 weeks) treated with a combination of ZO-1 siRNA and ChABC compared to cell only controls. (b) Representative confocal images of cell integration in (i) wildtype and (ii) $Rho^\text{−/−}$ recipients with and without the combined treatment approach. (c) Histogram showing integration efficiency for all treatment regimes in wildtype and $Rho^\text{−/−}$ recipients. (d) Histogram showing fold change improvements for each treatment cohort compared to cell only transplantations. (e) Scotopic rod-mediated optokinetetic head-tracking responses in a subset (N=7) of $Rho^\text{−/−}$ recipients 3-4 weeks post-transplantation with combined treatment of ZO-1 siRNA and ChABC. Plot shows contrast sensitivity against number of integrated rod photoreceptors.
4.3. DISCUSSION

Photoreceptor replacement therapy has been proposed as a treatment for a broad range of retinal degenerations that culminate in death of the endogenous photoreceptor cells and the subsequent loss of vision. Photoreceptor transplantation aims to replace photoreceptors lost as part of the degenerative process that occurs in many retinal diseases, with the ultimate aim of restoring sight. In the first instance, it has been proposed that photoreceptor replacement therapy is a feasible option to treat mid- to end-stage retinal disease whereby the loss of the endogenous photoreceptor has limited the application of alternative approaches such as gene therapy, which relies on the presence and survival of the endogenous photoreceptor cells. A second application of photoreceptor replacement therapy is to treat those diseases in which the underlying cause of the disease is multifactorial and thus currently not amenable to gene or drug therapy. In these latter circumstances the point of intervention could be at early, mid or late degeneration stages. With this therapeutic context in mind, we sought to establish the breadth of application of photoreceptor replacement therapy and we found that transplantation outcome, as assessed by cell integration efficiency and transplanted cell morphology, is very different between disease types. Furthermore, we sought to assess whether photoreceptor replacement therapy could be considered as a feasible treatment strategy for mid- to end-stage disease. We demonstrate that disease progression impacts upon transplantation efficiency in different ways and is dependent on the original disease mutation. Encouragingly, disease stage alone does not govern transplantation outcome and effective transplantation is possible even at late stage in some disease types. Changes in the recipient microenvironment seems to be one of the key determinants of transplant outcome and in those disease types less amenable to transplantation therapy, the microenvironment can be manipulated to improve transplantation success.
4.3.1. Impact of the Recipient Retinal Microenvironment on Integrated Photoreceptor Morphology

Structure plays a key role in the specialized function of the photoreceptor cell. Correct morphological development governs the ability of the photoreceptor cell to respond to light and propagate the visual signal to the second order neurons in the retinal circuitry. Even short reductions in outer segment disk formation can result in a reduction in light sensitivity. Therefore, in addition to assessing transplantation success by determining the total number of transplanted cells found within the recipient ONL, we also assessed the morphological appearance of the transplanted cells based on the presence of synaptic boutons and inner/outer segments structures. We found that the formation of both structures bears a strong correlation to the ability of the endogenous photoreceptor cells to develop typical outer segments structures. More specifically, the development of endogenous rod outer segments is impaired in Prph2\textsuperscript{rd2/rd2}, Rho\textsuperscript{-/-} and PDE\textbeta{6\textbeta}{rd1/rd1} recipients models (Humphries et al., 1997; Sanyal and Bal, 1973; Travis et al., 1989), and the frequency with which integrated cells formed new segments and synapses was also reduced compared to wildtype. Qualitatively, we observed that in those cells that did elaborate segment-like structures, these segments appeared reduced in size and did not always co-localise with the corresponding outer segment proteins absent in each model (Prph2, rhodopsin and PDE-\beta, respectively).

Cross-examination with previous studies reveals a variety of outcomes when assessing inner and outer segment formation and it is difficult to compare between studies as a variety of donor cell sources and recipient models were used. In this study, using one donor cell source and one transplantation technique, we were able to directly compare
inner and outer segment formation frequency following transplantation of Nrl.GFP^ve-rod precursor cells into several different models of retinal disease. Other studies, using different donor cell sources, have demonstrated that not all cells integrated into the degenerating diseased retina express photoreceptor markers such as recoverin and rhodopsin (Klassen et al., 2004; Lamba et al., 2009; Ma et al., 2011). However, as to whether this is a result of the recipient retina or the starting donor cell source is unclear. A study in 2004 using cultured donor RPCs, derived from GFP^ve mice, observed that only a subset of integrated cells transplanted into 1 month old Rd1 recipients co-localised with the photoreceptor marker, rhodopsin (Klassen et al., 2004). The same group also reported that none of the RPC transplanted into 1 month old Rho^-^ recipients expressed rhodopsin (Klassen et al., 2004). Similarly, a study in 2011, also using RPCs, demonstrated that approximately 1% of the cells integrated into 6-8 week old Rho^-^ recipients were positive for recoverin and rhodopsin expression (Ma et al., 2011). Reh TA and colleagues (Lamba et al., 2009) used human embryonic donor cells differentiated towards a retinal fate in vitro and transplanted them into the Crx^-^ mouse. The authors have reported that ~35% of the integrated cells were recoverin positive. However, upon closer inspection of the immunohistochemical data presented in these studies, the morphological appearance of the integrated cells in these studies indicated a complete absence of rod-like morphologies, with transplanted cells appearing in all nuclear layers of the recipient (Klassen et al., 2004; Ma et al., 2011) with no inner or outer segment structures (Klassen et al., 2004; Lamba et al., 2009; Ma et al., 2011). Moreover, quantification of the percentage of integrated cell said to be expressing these photoreceptor markers was based on whether they appeared to co-localise with the GFP^ve cell body of the transplanted integrated cell. This is not the typical site of expression of these proteins. This highlights the importance of examining cell morphology in such studies and that there is a lack of data concerning the ability of the transplanted photoreceptor cells to form inner and outer segment structures. Rather than relying on immunohistochemistry to assess inner/outer segment markers as secondary readout of segment development, in this study we have directly assessed
the primary ability of integrated cells to form segment structures. Following on from the data generated in this study, it would be of significant interest to further assess the expression profile and length of mature outer segments formed by integrated donor cells in each of the models studied here to confirm our qualitative findings. A more informative and reliable method to fully assess this would be to generate a Nrl.GFP X rho.RFP mouse as a donor cell source. This would enable an accurate method to quantify and assess transplanted cells outer segment formation and length using histology and electron microscopy.

The interphotoreceptor matrix provides the necessary developmental cues and structural support for outer segment development and maintenance. Indeed, mature outer segment structures form several connections with components of the interphotoreceptor matrix (Friedlander et al., 1994; Hollyfield et al., 1999; Hollyfield, 1999; Ruoslahti, 1989; Tawara et al., 1988). In the models where endogenous outer segment formation is impaired, the interphotoreceptor matrix also appears reduced in size and thus it is possible that the integrated transplanted rod photoreceptor cells do not have the sufficient structural support to elaborate outer segment structures. This may go some way to explain the reduced frequency and length of outer segments structures observed in these models, namely Prph2<sup>rds/rds</sup>, Rho<sup>−/−</sup> and PDEβ6<sup>rd1/rd1</sup> recipients. When segment develop fails, sensory input is limited. It is interesting to speculate whether this may explain the accompanying reduction in transplanted synapse formation, since endogenous retinal activity is required for synapse formation in the developing retina (Stacy et al., 2005) and there is a marked loss of synapses in the complete absence of sensory input after eye opening (Sernagor, 2005).

The degree of functional rescue will depend on both the total number of integrated cells and the extent to which these cells form complete outer segments and synapses. In those models where the endogenous photoreceptor outer segment development is impaired (Prph2<sup>rds/rds</sup>, Rho<sup>−/−</sup> and PDEβ6<sup>rd1/rd1</sup>), transplanted cells inner/outer segment was also impaired both in length and quantity. As determined in the previous results
chapter, the degree of functional rescue in the Gnat1<sup>−/−</sup> recipient is tightly linked with the number of integrated rod photoreceptor cells. It is therefore likely that greater numbers of integrated rod photoreceptor cells will be required in the Rho<sup>−/−</sup> recipient in order to achieve similar levels of function improvements as those observed in the Gnat1<sup>−/−</sup> recipient, as outer segment formation in the former is impaired. Indeed the optomotor data presented in this chapter supports this: higher numbers of integrated cells where required in the Rho<sup>−/−</sup> recipient to achieve the same contrast sensitivity following transplantation in the Gnat1<sup>−/−</sup> recipient. It is therefore prudent to find a method to improve outer segment formation by the integrated donor cells following transplantation into recipients where the endogenous photoreceptor inner/outer segment is impaired both to promote synaptic connectivity and to maximize photosensitivity and thus the degree of rescue of visual function. The use of polymer structures to provide scaffolds may aid outer segment development of rod precursor donor cells transplanted into the degenerate retina. Several groups have begun to investigate the feasibility and benefit of using polymer scaffolds (Hynes and Lavik, 2010; Tomita et al., 2005; Tucker et al., 2010). Some encouraging improvements in survival and integration of culture mRPC were reported when transplanted on biocompatible polymer scaffolds into wildtype and Rho<sup>−/−</sup> mouse recipients (Tomita et al., 2005). A more recent study indicated that these polymers can also provide a platform from which to release factors to digest the ECM and further improve the integration of mRPCs transplanted into wildtype and Rho<sup>−/−</sup> recipients (Tucker et al., 2010). While the use of scaffolds is of interest, it remains to be seen as to whether mRPC represent the most appropriate source of donor cells. We have previously shown that the correct ontogenic stage of the donor cell population is of paramount importance for successful transplantation outcome (Maclaren et al., 2006). To date, no group has investigated the use of polymer scaffolds to aid transplantation of rod photoreceptor precursor cells, a donor cells source known to retain the intrinsic capacity to migrate, integrate and differentiate into fully mature rod photoreceptors (Maclaren et al., 2006). Furthermore, due to the inherent problems with transplanting mRPC no group has assessed whether polymer scaffolds improve outer
segment formation as the focus has always been to improve survival and differentiation towards a mature rod photoreceptor phenotype. In addition to using polymer scaffolds to aid outer segment development, alterations in the donor cell dissociation regime and the age of the donor cell population may also result in improvements in outer segment development. Kwan et al 1999 used an older donor cell (P7-P9), which was mechanically dissociated retinal prior to transplant. They report good segment development following transplantation into 6-8wk old PDE6βrd1/rd1 recipient mice. Using a slightly older donor where segment formation has already begun may reduce the need for extrinsic development cues from the recipient retina to support outer segment formation. In addition, the enzymatic papain dissociation regime used in our study may cleave some donor cell surface markers that are need for ECM interactions for correct outer segment development, maturation and maintenance.

When assessing transplantation efficiency in severely degenerate recipients, care must be taken when deciding what constitutes an integrated cell. Traditionally, we and others have assessed integration based on the number of cells found within the recipient ONL that adopt a typical rod-like morphology (Bartsch et al., 2008; Eberle et al., 2011; Gust and Reh, 2011; Jiang et al., 2010; Lakowski et al., 2010; Lakowski et al., 2011; Lamba et al., 2009; Ma et al., 2011; Maclaren et al., 2006; Tucker et al., 2011; West et al., 2008; West et al., 2010; West et al., 2012b; Yao et al., 2010). When assessing integration in recipients with end stage degeneration, for example in the PDE6βrd1/rd1 recipient, these assessment criteria were not applicable due to the near complete absence of the recipient ONL and the absence of a typical rod-like morphology adopted by the transplanted cell. In this case we assessed integration based on the number of cells that resided within the remaining layer of the ONL, but that also sent processes towards the OPL. This method is however, reliant on the plane of sectioning and as such our counts are likely to be an underestimate. An alternative strategy to assess integration in these circumstances might be to quantify the number of synapses formed...
with the recipient retina using a donor source that carried a genetic fluorescent marker of synaptic proteins, such as RIBEYE or bassoon.

Here we determined late stage disease as less than 30% of wildtype ONL thickness, however, there may be some interest in assessing transplantation outcome after the death of the entire ONL. When the ONL is completely absent the inner nuclear layer undergoes extensive synaptic remodeling and a period of heightened synaptogenesis (Jones and Marc, 2005), which may promote synapse formation with donor cells transplanted at this stage. To assess synaptic integration under these circumstances a genetic fluorescent synapse marker would be essential.
4.3.2. Impact of the Recipient Retinal Microenvironment on Integrated Photoreceptor Number

Here we demonstrated that both the total number of integrated cells and the morphological appearance of transplanted rod photoreceptor precursor cells change according to disease type and degeneration state. The morphological appearance of integrated cells appears to be governed by the ability of the endogenous photoreceptor to develop outer segments, which in turn is determined by the nature of the original disease mutation. However, the factors controlling cell integration efficiency are more complex. In the first instance we hypothesized that the degeneration stage, and thus the architecture of the remaining host retina would govern transplant outcome. In contrast to what might be expected, integration efficiency was observed to increase, decrease or remain constant with disease progression. Encouragingly this study demonstrates that the recipient retinal tissue cytoarchitecture and disease pathology, as measured by ONL thickness, degeneration speed and ONL density, do not directly impact upon transplant outcome. For example high integration efficiency is apparent in a rapidly degenerating disease model, the \textit{PDE6$^{\text{βrd1/rd1}}$} recipient, and at end stage disease in a moderate degenerating disease, the \textit{Prph2$^{\text{Δ307}}$} recipient.

Following assessment of the recipient retinal microenvironment, it would appear that changes in OLM integrity and the extent of the glial scaring response are two of the major determinants of transplant outcome; integration decreased in those models where OLM integrity was maintained and where gliosis increased with disease progression (\textit{Gnat1$^{-/-}$; Rho$^{-/-}$}) whereas integration increased in a model in which the OLM undergoes re-modeling and the glial scar reduces with disease progression (\textit{Prph2$^{\text{Δ307}}$}). Importantly, we demonstrate that manipulation of these factors resulted in enhanced integration efficiency in the \textit{Rho$^{-/-}$} mouse, suggesting that a tailored manipulation of the retina microenvironment specific to both the disease type and stage
may offer a method to boost integration success in those disease types which are not immediately amenable to photoreceptor replacement therapy. Furthermore, we demonstrate that these manipulations can increase integration efficiency to levels sufficient to permit restoration of visual function.

4.3.2.1. The role of the OLM on transplantation outcome

In this study we demonstrated that OLM integrity can change during retinal degeneration, a phenomenon that has been reported previously for some models (Campbell et al., 2006; Campbell et al., 2007; Mehalow et al., 2003). The outer limiting membrane is composed of a series of adherens junctions formed between photoreceptor cells and Müller glial cells. It is feasible that as the photoreceptor cells die, as part of the disease process, the OLM has to re-model between the remaining cells. In a number of the models studied here, namely the Prph2<sup>Δ307</sup>, Prph2<sup>rd2/rd2</sup> and PDE6β<sup>rd1/rd1</sup> models, there was evidence of OLM re-modeling as indicated by the majority of adherens junctions apparently forming directly between Müller glial cells and photoreceptor cell bodies appeared displaced in the subretinal space. Conversely, in contrast to previous reports (Campbell et al., 2006; Campbell et al., 2007) no signs of OLM re-modeling were observed in the Rho<sup>−/−</sup> model where, despite significant cell death, the OLM adherens junctions were typically observed between photoreceptors and Müller glial. The temporal integrity of the OLM and the degree of re-modeling over time is likely to depend on the nature and kinetics of the endogenous photoreceptor cell death. For example, if there is a defined short rapid period of cell death, as is observed in the PDE6β<sup>rd1/rd1</sup> model, extensive OLM disruption is likely to occur immediately after this cell death peak in a transient manner. For example, in the PDE6β<sup>rd1/rd1</sup> mouse, a model where the peak of cell death occurs at P14 (Sanyal and Bal, 1973), at P10 there were no signs of OLM disruption. However, by P21 areas of extensive re-modeling were observed where the adherens junctions had formed between Müller cells. Similarly, if the endogenous cell death occurs more gradually, as is the case with the
The *Prph2<sup>+/Δ307</sup>* model, the period of OLM disruption and re-modeling may likely be prolonged but less extensive at a given point in time.

It has previously been reported that disruption in OLM integrity in the wildtype recipient results in improvements in integration efficiency (West et al., 2008). Here we confirm the role of the OLM as a key determinant in transplant outcome. Firstly, we demonstrate that integration efficiency is enhanced in the *Crb1<sup>rd8/rd8</sup>* recipient, a model in which the OLM undergoes a progressive fragmentation, compared to donor cell integration observed in wildtype control recipients. Furthermore, we demonstrate that targeted disruption of the OLM using siRNA targeted against ZO-1 increases transplantation efficiency both in wildtype retinae and in the *Rho<sup>−/−</sup>* recipient, a model of degeneration that has an intact OLM (at the time points examine here). To further these studies, it may be prudent to find a more clinically acceptable target for OLM disruption. These studies provide proof-of-principle for the involvement of the OLM in limiting transplanted donor cell integration. However, ZO-1 is also expressed in the RPE and is involved in maintaining RPE cell-cell signaling, adhesion and proliferation (Harhaj and Antonetti, 2004). Indeed, following the use siRNA targeted against ZO-1 in our experiment, some unwanted adverse proliferation of the RPE was observed (data not shown). An alternative target protein that would not produce any secondary adverse affects in the RPE is required. One potential target protein is Crb1; in the eye, this protein is expressed only in the neural retina (van Rossum et al., 2006) and mutations in Crb1 are known to cause OLM disruption (Mehalow et al., 2003). In addition, we know from the data presented in this chapter that the extent and nature of the OLM disruption resulting from a mutation in *Crb1* results in enhanced integration efficiency.
4.3.2.2. The role of the glial scarring on transplantation outcome

Glial scarring is another component that was identified in this study as a key component involved in transplantation outcome. Here we demonstrate that glial scarring is upregulated in many of the models examined here. A significant increase in glial scarring was observed in the Gnat1<sup>−/−</sup> and Rho<sup>−/−</sup> recipients and integration declined as disease progressed. Of note, OLM integrity was maintained throughout the same time period. Interestingly, in the Prph2<sup>rd2/rd2</sup> model, where integration efficiency remains constant as disease progressed, CSPG deposition at the outer edge of the ONL appeared to reduce although GFAP increased. We also observed that in late stage disease in the Prph2<sup>−Δ307</sup> recipient, GFAP<sup>+</sup> processes appear to regress from the ONL region. Kwan et al (1999) also noted a similar phenomenon in the PDE6β<sup>rd1/rd1</sup> severely degenerate retina (6-8 weeks old) using immunohistochemical and ultrastructural analysis. It is feasible that in late stage degeneration, where the ONL is or nearly completely lost, intermediate filament processes regress to protect the inner retinal layer and act as scaffolds for the prolific period of inner retinal re-modeling.

Several other groups have also identified glial scarring, and, more specifically the accumulation of CSPGs, as a potential factor involved in transplantation success both in the retina and the CNS (Bradbury et al., 2002; Bradbury and Carter, 2010; Bull et al., 2008; Huang et al., 2006; Kim et al., 2006; Ma et al., 2011; Moon et al., 2001; Singhal et al., 2008; Suzuki et al., 2007). Our findings corroborate the findings presented by other groups where integration efficiency was enhanced following enzymatic degradation of subretinal CSPGs using ChABC (Bull et al., 2008; Ma et al., 2011; Singhal et al., 2008; Suzuki et al., 2007). The role of CSPGs in axon regeneration inhibition is well documented (Bradbury et al., 2002; Bradbury and Carter, 2010; Dou and Levine, 1994; Emerling and Lander, 1996; Friedlander et al., 1994; Gates et al., 1996; Huang et al., 2006; Jones et al., 2003a; Jones et al., 2003b; Kim et al., 2006; Levine, 1994; Lips et al., 1995; Moon et al., 2001; Morgenstern et al., 2002; Smith-Thomas et al., 1994), however, there are several subtype CSPGs, including neurocan.
and vesicant, and ChABC has a different enzymatic affinity for each subtype. The exact expression profile in each model maybe different and further profiling may facilitate a more targeted enzymatic approach to degrade these specific subtypes and produce a greater improvement in transplanted cell integration. Indeed, a variety of different CSPGs, including neurocan, versican, aggrecan, phosphacan and NG2, are upregulated in the brain at varying rate after injury (Asher et al., 2001; Dobbertin et al., 2003; Dou and Levine, 1994; Lemons et al., 2001) and this is likely to be the case in the retina. Furthermore, degradation of extracellular matrix proteins via induction of matrix metalloproteinase-2 (MMP-2) has been shown to increase neurite outgrowth in retinal explants (Suzuki et al., 2006) and in vivo in the PDE6β<sup>rd1/rd1</sup> mouse (Zhang et al., 2007). Further, when co-injected with RPCs, polymer scaffolds that deliver MMP2 were shown to degrade the ECM proteins, CD44 and neurocan, in the degenerating retina and enhance donor cell integration (Tucker et al., 2010).

Although the role of CSPGs in transplant outcome is well established, the exact role of GFAP and vimentin is unclear. Kinouchi et al (2003) demonstrated enhanced integration efficiency of immature P0 retinal cells transplanted into the GFAP<sup>-/-</sup> Vimentin<sup>-/-</sup> double-knockout recipient (Kinouchi et al., 2003), suggesting that GFAP and/or vimentin normally act to impede migration and integration as we have found here. However, conflicting reports have suggested integration occurs around regions of GFAP accumulation (Nishida et al., 2000; Zhang et al., 2004). In addition, the GFAP<sup>-/-</sup> Vimentin<sup>-/-</sup> mouse has other vascular developmental defects that could account for the differences in integration efficiency observed in this study (Lundkvist et al., 2004; Nakazawa et al., 2007; Verardo et al., 2008). Lundkvist A., et al (2004) demonstrated that the GFAP<sup>+</sup> Vimentin<sup>-/-</sup> mouse responds abnormally to ischemia and that the mechanical integrity of the Müller cell endfeet was compromised, which may suggest some disturbances in membrane integrity. GFAP and vimentin are both intermediate filament proteins and are upregulated in injury and disease to provide structural support to the retina as retinal re-modeling occurs. The exact role of GFAP and/or vimentin in
transplant outcome has yet to be determined: on the one hand, they may provide a structural scaffold for migrating cells or alternatively, they may form a physical barrier at the outer edge of the ONL hindering transplanted cell migration into the recipient ONL. To further elucidate the exact role of GFAP and vimentin in transplantation outcome, these factors would need to be manipulated in the adult retina to avoid any compensatory changes occurring during development.

Through their manipulation, we have shown that both OLM integrity and glial scarring play a role in determining transplanted photoreceptor integration efficiency in the degenerating recipient. We also demonstrate that depending on the changes occurring in the recipient environment, manipulation of one factor may produce a more favorable outcome than the other and that the impact of each intervention is dependent on the recipient microenvironment. For example glial scarring is more prominent in the \textit{Rho}^{-/-} recipient compared to wildtype and accordingly the impact of ChABC on transplant outcome was more pronounced when used to facilitate transplanted photoreceptor integration in the \textit{Rho}^{-/-} recipient than in the wildtype recipient. This data suggests that a tailor approach may be required to optimally manipulate the recipient microenvironment, as some intervention will be more applicable than others in different disease types. Perhaps surprisingly, when these treatment approaches were combined, the effect on transplantation outcome was not additive, at least in the \textit{Rho}^{-/-} recipient. This may be explained by the subretinal injection of ZO-1 siRNA 48 hours prior to transplantation with Nrl.GFP^{+ve}-rod precursor cells and ChABC stimulating an injury response in which CSPGs are upregulated, potentially over and above the existing levels (Kinouchi et al., 2003). Therefore, it may be prudent to investigate alternative strategies to combine these treatments such as injecting the ChABC at the time of ZO-1 siRNA administration or injecting a higher dose at the time of transplantation. That said, the improvement in integration efficiency achieved in this study was sufficient to restore some optokinetic head-tracking responses in the \textit{Rho}^{-/-} mouse. It must be noted that the degree of rescue of optokinetic head-tracking
responses in the *Rho*<sup>−/−</sup> recipient was lower than the degree of rescue achieved in the *Gnat1*<sup>−/−</sup> despite similar levels of integration efficiency. This is most likely to be reflection on the ability of the transplanted cells to develop outer segment and synapse structures once integrated into the recipient retina. We have demonstrated that development of these structures is impaired in the *Rho*<sup>−/−</sup> recipient, and therefore integrated cell sensitivity is likely to be reduced and higher numbers of integrated cells are likely to be required to in the *Rho*<sup>−/−</sup> recipient retina to achieve the same degree of rescue as seen in the *Gnat1*<sup>−/−</sup> recipient.

It is unlikely that these two factors, glial scarring and OLM integrity, alone govern transplant outcome and it is likely that there are a number of other processes that involved. Reactive gliosis itself is a complex process involving many more factors than just GFAP upregulation and CSPG deposition. These include Müller cell hypertrophy and proliferation. Fundamentally, the process of reactive gliosis functions to protect the retina following injury and development of the glial scar may function to stabilize the fragile retinal tissue after injury and cell death (Jones and Redpath, 1998) and to protect the healthy part of the retina from the damaged area (Bush et al., 1999). However, in the initial stages of the glial response to disease or injury, Müller cells produce a variety of neuroprotective growth factors and antioxidants thought to promote neuron survival (Frasson et al., 1999). Indeed, a study from our group in 2012 demonstrated that ectopic expression of insulin-like growth factor (IGF1) using AAV-mediated gene transfer lead to enhanced integration efficiency (West et al., 2012b). If this stage of the glial response coincides with the time of transplantation, donor cell integration into the recipient retina may be enhanced. Other studies have assessed microglial infiltration in the *Prph2*<sup>rd2/rd2</sup> model and have indicated that this may also play a role in transplant outcome (Takahashi, pers comm).

To date, no group has assessed whether vascular changes in the retina affects transplanted donor cell survival. Changes in retinal vasculature as a consequence of disease, may impact upon the long term survival of transplanted photoreceptors as the
failing recipient retinal vasculature may not be capable of supporting the metabolic demand of the large number of donor cells transplanted into the subretinal space (Kwan et al., 1999). Thus far, long term survival of transplanted cells has only been assessed in wildtype recipients (West et al., 2010) where it was demonstrated than the number of integrated cells declines after 4 months post transplantation, an effect that could be partially rescued using immune suppression. The degeneration process, involving a range of biological changes, including microglial activation and extensive pro-inflammatory cell death mechanism, may have a major impact on the immune status of the recipient retina and long term survival of transplanted cells. This issue would need to be addressed by further studies and it is likely that it will need to be assessed in each independent disease model.

To conclude, this study provides the first comprehensive assessment of photoreceptor transplantation in several clinically relevant models of retinal degeneration. We have demonstrated that transplant outcome is influenced by local changes in the host retinal microenvironment and that OLM integrity and glial scarring in particular play a major role in determining the ability of transplanted cells to integrated into the recipient retina and form mature rod-like morphologies. Importantly, we demonstrate that by understanding the recipient microenvironment and administering tailored manipulations of this environment, significant improvements in transplantation success can be achieved.
CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

The studies presented in this thesis aimed to address two key questions in the field of photoreceptor replacement therapy; whether integrated Nrl.GFP<sup>+</sup>-rod precursor donor cells can contribute to meaningful vision and to assess whether photoreceptor replacement therapy is suitable for the treatment of a wide breadth of retinal degenerative diseases.

5.1. OVERALL CONCLUSIONS

To address the first question, we performed a rigorous assessment of the functional capabilities of integrated Nrl.GFP<sup>+</sup>-rod precursor donor transplanted into the Gnat1<sup>−/−</sup> mouse model of stationary night-blindness, which lacks rod photoreceptor function. We first had to optimize the transplantation procedure in order to achieve levels of integration sufficient to test function. We assessed the functional capabilities of integrated photoreceptors from a bottom upwards approach, from the ability of the integrated cells to form outer segments and synapses, through to the level of the visual cortex to examine whether visual signals generated by transplanted rod photoreceptor precursors were detected and integrated by the higher visual centers into meaningful vision. We present definitive evidence that integrated Nrl.GFP<sup>+</sup>-rod precursor donor cells are capable of (1) robustly integrating into the Gnat1<sup>−/−</sup> recipient retina and can express key phototransduction cascade proteins in a light-dependent manner; (2) can respond to light with photokinetics similar to wildtype rods; (3) can form typical rod photoreceptor synapse structures; (4) can propagate these signals to higher visual areas in the brain and (5) can restore rod-mediated visual-guided behaviour.
In chapter 4, I described the first comprehensive assessment of photoreceptor replacement potential by performing transplants of Nrl.GFP<sup>+</sup>-rod precursor donor cells into murine models of RP. This is the first study of its kind, directly comparing integration efficiency following a standardized transplantation protocol into 6 clinically relevant models of retinal degeneration. We have demonstrated that transplanted cell integration efficiency and morphology is governed by disease type. Following transplantation of Nrl.GFP<sup>+</sup>-rod precursor donor cells into the adult recipient retina we showed that integration occurs with a higher efficiency some models (Crb<sup>1<sup>rdB</sup>/rdB<sup>rdB</sup>) and low efficiency in other models (Rho<sup>-/-</sup>), compared to those donor cells transplanted into adult wildtype recipients. Furthermore, the ability of integrated cell to form inner/outer segment and synapse parallels the ability of the endogenous photoreceptors to form segment structures.; By transplanting Nrl.GFP<sup>+</sup>-rod precursor donor cells at different stages of the degenerative process (early, mid and late stage degeneration), we demonstrated that disease progression impacts upon transplanted cell integration efficiency differentially in the recipient models studied here. Integration was observed to increase (Prph2<sup>+/Δ307</sup>), decrease (Gnat1<sup>-/-</sup>, Rho<sup>-/-;</sup> Crb<sup>1<sup>rdB</sup>/rdB</sup>) or remain constant (Crb<sup>1<sup>rdB</sup>/rdB</sup>; PDE6β<sup>β/rd1</sup>/rd1<sup>rd1</sup>) as disease progressed and this trend was governed by changes in the recipient microenvironment, namely glial scarring and OLM integrity. Encouragingly, we found that the extent of degeneration, as measured by ONL thickness and density, does not impact upon transplantation outcome and we observed robust integration even when donor cells where transplanted into late stage disease recipients in some models (Prph2<sup>+/Δ307</sup>; Crb<sup>1<sup>rdB</sup>/rdB</sup>; PDE6β<sup>β/rd1</sup>/rd1<sup>rd1</sup>). Importantly, we show that in a model with poor integration efficiency, the Rho<sup>-/-</sup> recipient, integration can be increased by transiently manipulating the recipient microenvironment to a level sufficient to restore some visual function.
5.2. PROSPECTS FOR CLINICAL APPLICATION

5.2.1. Donor cell source

The studies presented in this thesis demonstrate that it is possible to restore vision by rod photoreceptor transplantation. The donor cells used in these investigations were freshly isolated rod photoreceptor precursor cells as we considered them to be optimal for assessing whether the strategy of photoreceptor replacement therapy can improve visual function. However, the future application of photoreceptor replacement therapy to treat human disease must also be considered and the generation of a suitable donor cell source must be established. Several groups have shown in vitro differentiation of retinal cells from both mouse and human ES and iPS cells (Hirami et al., 2009; Ikeda et al., 2005; Lamba et al., 2006; Lamba et al., 2009; Meyer et al., 2006; Osakada et al., 2008; West et al., 2012a), including cells expressing markers including Nrl, indicating maturation toward rod precursors (Lamba et al., 2009; Tucker et al., 2011; West et al., 2012a). It is hoped that these cell sources will provide a readily available, easily scalable and ethically sound donor cell source.

The first issue to address is one of safety, as accidental transplantation of any undifferentiated cells may lead to tumour formation in the eye (Arnhold et al., 2004; Chaudhry et al., 2009; Hara et al., 2006). West et al. (2012) recently demonstrated that there is a high tumorogenic risk when transplanting a mixed population of early stage cultures of differentiated mouse-derived ES retinal cells. However, by prolonging the differentiation protocol and refining the donor cell selection process, tumorogenic risk was virtually eliminated (West et al., 2012a).

Another consideration is whether these donor cell types are capable of integrating into the recipient retina. Reports by Lamba et al. 2009 indicated robust integration and function rescue following transplantation of human ES-derived retinal cells into Crx−/− mouse recipients. However, a recent report showed that no integration was observed following transplantation of mouse ES-derived retinal cells into the wildtype or
degenerating recipient, even when the transplanted population was >60% Crx\textsuperscript{+ve} (West et al., 2012a). The differences in integration efficiency reported in these studies may arise from the differences of differentiation efficiency of human and mouse ES cells in vitro. It is also possible that at least some of the cells apparently integrated in the Lamba et al. (2009) report were endogenous cells labeled as a consequence of using viral vectors to label transplanted cells, a phenomenon that was observed in the report by West EL et al. (2012). Further studies assessing whether more mature ES-derived retinal progenitor cells differentiated in vitro will retain integration-competency are essential. In addition, the use of 3-dimensional differentiation methods, which better models in vivo development, may provide a more reliable culture system to generate integration-competent donor cells (Eiraku et al., 2011) and early findings in the lab indicate this to be so (West, Gonzalez et al., pers comm).
5.2.2. Treatment of end stage disease

Although we have gone some way to address the breadth of application of photoreceptor transplantation therapy as a means to treat retinal degenerative disorder, further work will be required to establish the degree of functional rescue achievable in different disease types. We demonstrated restoration of optokinetic head-tracking responses in the Rho\textsuperscript{−/−} recipient when high integration efficiencies were achieved. In several models, particularly those with poor endogenous photoreceptor segment formation, the ability of the transplanted cells to form new inner/out segments is impaired (Humphries et al., 1997; Sanyal and Bal, 1973; Travis et al., 1989) and it is therefore likely that far higher numbers of successfully integrated cells will be required to restore the same degree of functional rescue as that observed in the Gnat1\textsuperscript{−/−} mouse. Indeed, the degree of rescue of optokinetic head-tracking responses achieved following photoreceptor transplantation in the Rho\textsuperscript{−/−} mouse was reduced compared to the degree of rescue achieved in the Gnat1\textsuperscript{−/−} recipient, when comparable numbers of transplanted cells were integrated.

Several groups are exploring methods, such as polymer scaffolds, which may enable donor cells to elaborate inner/outer segments in environments in which endogenous inner/outer segment formation is severely compromised or absent (Tucker et al., 2010). There is significant interest as to how useful this strategy may be for the treatment of end stage disease where the entire ONL is completely degenerate and when extensive inner retinal re-modeling occurs. In such cases, so-called bioscaffolds may be necessary to provide the architectural support required for the photoreceptors to form the appropriate laminar structures.

Furthermore, assessing donor cell integration in the severely degenerate retina is problematic and a more reliable method to assess donor cell synaptic connectivity is required. In addition, it is becoming increasingly understood that severe degeneration is typically accompanied by a significant degree of synaptic remodeling. The use of a genetically linked fluorescent synapse marker may be useful to quantify donor-host cell
synaptic integration but also to assess whether normal synaptic connectivity is retained. Anterior-grade viral tracers, which are capable of crossing the synaptic cleft, may offer an indication as to whether functional circuitry is conserved and/or restored in end stage disease following transplantation of photoreceptor donor cells. This technique has already been used successfully in the hamster retina. Norgren RB et al (2003) injected Herpes simplex virus type 1 and type 2 (HSV) into the vitreous to examine the optic pathway from the retina to the brain. Transfection of the donor cell population with a fluorescently labeled anterior-grade viral tracer could be used to trace the donor cell connectivity by fluorescently labeling multiple second order neurons in the recipient retina visual network.
5.2.3. Longevity and immune rejection

Prolonged treatment effects, and therefore prolonged integrated cell survival, must be demonstrated if photoreceptor replacement therapy is to be established as a therapeutic option for the treatment of human retinal diseases. The work described in thesis followed transplanted cells for a period of up to 6 weeks post-injection. The eye has traditionally been described as an ‘immune privileged’ site that will allow survival of grafted tissue for indefinite periods of time. However, work by our group has demonstrated that despite survival of integrated cells up on 12 months post transplant, the number of integrated cells began to decline significantly from 4 months post transplant and that this effect could be attributed to the activation of the adaptive immune response (West et al., 2010). Although this problem could be significantly improved following administration of cyclosporine A, continuous immune suppression is not ideal in a clinical setting. It is hoped that autologous donor cell sources, such as iPS-derived donor cells or adult stem cell-derived donor cells, could circumvent this need. Alternatively, closely immunologically matched donor cells differentiated from cell bank human ES cell lines characterized by human leukocyte antigens (HLA) may also provide a suitable donor cell source (Taylor et al., 2005).
5.2.4. Transplantation of cone photoreceptor cells

Currently, much of the work in the field of photoreceptor transplantation therapy has focused on replacement of rod photoreceptor cells, and it is unclear whether replacement of cone photoreceptor by transplantation is possible. Colour vision and high central visual acuity is mediated by cone photoreceptors and diseases that result in loss of cone photoreceptors, such as LCA, can lead to severe visual impairment that dramatically impact on patients’ quality of life. As such there is much interest as to whether the principles of rod photoreceptor transplantation can be applied to achieve successful cone photoreceptor replacement.

The ontogenic stage of rod photoreceptor donor cells was critical for defining the integration competent donor population and indeed, it is the post-mitotic rod precursor cells that retain the ability to integrate into the adult recipient retina (Maclaren et al., 2006). In 2010, we demonstrated that embryonic donor cells from Crx.GFP transgenic donor mice, which genetically labels both rod and cone photoreceptors, were able to integrate and differentiate into mature cone photoreceptors in the adult recipient retina. Although the total cell integration remained low, these proof-of-concept experiments indicate that the ontogenic stage of cone photoreceptor cells is also critically important for integration competency (Lakowski et al., 2010). The low numbers of integrated cone photoreceptors may partly be explained by a lack of environmental cues for donor cone cell migration. In development, cones are born far earlier than rod photoreceptor cells in a much more immature retinal microenvironment. As such, the adult recipient retina may lack the environmental cues to support extensive migration of transplanted cone photoreceptors. Indeed, many transplanted Crx.GFP\textsuperscript{\textasciitilde} donor cells differentiated into cone photoreceptor cells in the subretinal space (Lakowski et al., 2010), supporting the hypothesis that donor cone precursor cells have a more limited migratory capacity that transplanted rod precursor cells. Further studies will be required to assess mechanism of photoreceptor donor cell migration and integration with the aim to improve transplanted cone precursor cell integration efficiency.
In addition, transplantation of cone photoreceptors as a means to treat cone-rod dystrophies presents a number of issues. Reconstruction of the human macular by transplanting cone photoreceptors into the subretinal space behind the macular region is a high risk procedure as it may cause damage to any surviving cone photoreceptors existing in the macular and may consequently severely affect any remaining central vision. Therefore, this approach may only be a suitable strategy in end stage cone degeneration where the potential risk-to-benefit ratio supports the risks associated with surgical intervention. In addition to the risks associated with surgical intervention, there are a number of other factors that are likely to impede this line of research. In the first instance, the mouse retina does not contain a macular region and therefore this may not be an appropriate environment to demonstrate the feasibility of cone transplantation as a method to treat human cone dystrophies. Other species that have a macular region may be more appropriate, such as the opossum or cat.


cell lines derived from adult human retina exhibit neural stem cell characteristics. Stem Cells 25, 2033-2043.


