Retinal transplantation in a rodent model of Retinitis Pigmentosa

Anthony S L Kwan

Institute of Ophthalmology

University College London

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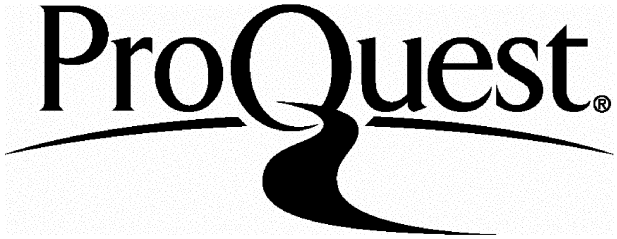

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Preface

The studies described in this dissertation are my own work and were carried out in the Department of Pathology, Institute of Ophthalmology, University College London (UCL). None of these experiments are substantially the same as any that I have submitted for a degree, diploma, or other qualification at any other university. I further state that no part of my dissertation has already been, or is currently submitted for any such degree, diploma, or certificate.

Acknowledgements

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To my wife, Julia, and my son, Alex

Abbreviations

ACAID	anterior chamber associated immune deviation
ANOVA	analysis of variance
AMD	age related macular degeneration
βPDE	β-subunit of phosphodiesterase
bFGF	basic fibroblast growth factor
cd/m^2	candela/metre square
CNS	central nervous system
CNTF	ciliary neurotrophic factor
cGMP	cyclic guanosine monophosphate
DAB	diaminobenzidine
DCT	dark compartment time (in seconds)
DNA	deoxyribonucleic acid
E	embryonic
ERG	electroretinogram
GCL	ganglion cell layer
GFAP	glial fibrillary acid protein
H_2O_2	hydrogen peroxide
HBSS	Hank's balanced salt solution
HRP	streptavidin-horseradish peroxidase
INL	inner nuclear layer
IPL	inner plexiform layer
LDPT	light-dark preferential test
mfERG	multifocal electroretinogram
mRNA	messenger ribonucleic acid
MHC	major histocompatibility complex
NGF	nerve growth factor
ONL	outer nuclear layer/photoreceptor layer
OPL	outer plexiform layer
PBS	phosphate buffered saline

PKC	protein kinase C
PLP	periodate/lysine/paraformaldehyde fixative
PLR	pupillary light reflex
PN	postnatal
PSTH	post-stimulus time histogram
RCS	Royal College of Surgeons
rd	retinal degeneration
RetNeT™	Retinal Information Network
RF	receptive field
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
SC	superior colliculus
SIRP	signal-regulatory protein
TPL	transplanted layer

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Abstract

Over the past two decades, studies in rodents with photoreceptor degeneration have explored the use of retinal transplants to reconstruct the outer retina. More recently studies on transplantation have moved into human trials before any evidence of the functional inprovement of retinal transplants in animal experiments has been established.

In the present work, I have pursued the possibility of using mechanically dissociated cells as donor material and have focused on three issues - the appearance of the graft itself, its interface with the host retina and the potential for functional restitution after transplantation. These studies have been conducted in an animal model of retinitis pigmentosa (RP), the retinal degeneration (rd) mouse, which is a direct homologue of one of the autosomal recessive forms of RP in humans.

A technique was developed to transplant neonatal retinal microaggregates into the subretinal space of the mouse eye. The histology of the grafted eyes was examined and compared with normal and dystrophic mouse retinae without transplants. Different retinal cellular components were labelled with appropriate antibodies and the results showed that microaggregate transplants could produce a rod photoreceptor-rich cell layer. Evidence of host-graft connections was seen both in terms of synaptic protein labelling and in an increase in the number of synapses observed ultrastructurally. Such evidence was further supported by cross-species (rat to mouse) transplant experiments. Retinal graft function was assessed by light and dark preferential behaviour and measurement of retinal thresholds. Results were encouraging and showed some restoration of visual function following transplantation

in a few animals but statistically *not* significant. It was a worthwhile investigation but results have to be interpreted with caution. Further investigation with a larger number of animals is recommended. This work has clinical relevance to the development of transplantation as a treatment for human retinal degeneration.

Chapter 1 – Introduction

A major cause of visual impairment in humans in the Western world is the progressive loss of photoreceptors that occurs in two main groups of diseases - age related macular degeneration (AMD) and inherited photoreceptor dystrophies which include RP.

Inherited photoreceptor dystrophies comprise a variety of disparate genetically determined conditions and differ from one to another in their mode of inheritance, their pattern of visual loss and retinal appearance but they have the same outcome namely photoreceptor loss (Bird, 1995). A superficial classification divides the dystrophies into peripheral degeneration, in which the rods are the primary target of the disease, and central degeneration, in which the cones are the primary target of the disease. So far more than 100 genes have been implicated as reported in Retinal Information Network or RetNet™ (Daiger *et al.*, 2001), a catalogue of genes causing inherited retinal diseases. Many of the genetic defects are thought to be intrinsic to the photoreceptors, for example rhodopsin gene and peripherin/RDS gene, but a small number are due to genetic defects in the retinal pigment epithelial (RPE) cells (Gu *et al.*, 1997; Morimura *et al.*, 1998), whose primary function is to phagocytose shed outer segments and provide metabolic and trophic support for photoreceptors.

The aetiology of AMD is less clear than RP although it appears to be due to RPE dysfunction and changes in Bruch's membrane such as lipid deposition, protein cross-linking and reduced permeability to nutrients and these lead to secondary photoreceptor loss (Green and Enger, 1993; Young, 1987; Zarbin, 1998). Diet, environment and hereditary factors all play a role in the course of the disease. It

accounts for approximately 50% of all cases of registered blindness in England and Wales (Evans, 1995; Evans *et al.*, 1996).

In neither disease has a definitive cure been identified, but in RP four main approaches are being explored - metabolic treatment (e.g. Vitamin A) (Berson *et al.*, 1993), growth factor application (Faktorovich *et al.*, 1990; LaVail *et al.*, 1998), gene therapy (Ali *et al.*, 2000; Bennett *et al.*, 1996) and surgery which includes transplantation (del Cerro *et al.*, 1997; Lund *et al.*, 2001a). All four present potential treatments and are designed to prevent the degenerative process from advancing and preserve residual vision. The biggest problem, replacing photoreceptors once they are lost, can only be solved by transplantation, the other option is visual prosthesis but this technology is in a very early stage of development. One tremendous advantage in exploring possible treatments has been the availability of animal mutants for experimental work. These mutants are closely parallel to or directly homologous with human diseases. More recently a number of transgenic animals have been produced with defective genes for human forms of retinal disease.

A series of transplantation studies has explored the potential for replacement of lost photoreceptors in experimental animals, using a variety of donor tissue preparations. These have included microaggregates (Gouras *et al.*, 1994; Jiang and del Cerro, 1992a), cell suspensions (del Cerro *et al.*, 1989; Juliusson *et al.*, 1993), retinal sheets obtained from vibratome sectioning or excimer laser preparation of immature or mature retina (Aramant *et al.*, 1999; Huang *et al.*, 1998), and whole or large strips of retinae taken from embryos (Ghosh *et al.*, 1999). In previous attempts with microaggregates or dissociated cells, the main problems were the formation of rosettes and loss of laminar order (Juliusson *et al.*, 1993). They also tended to form

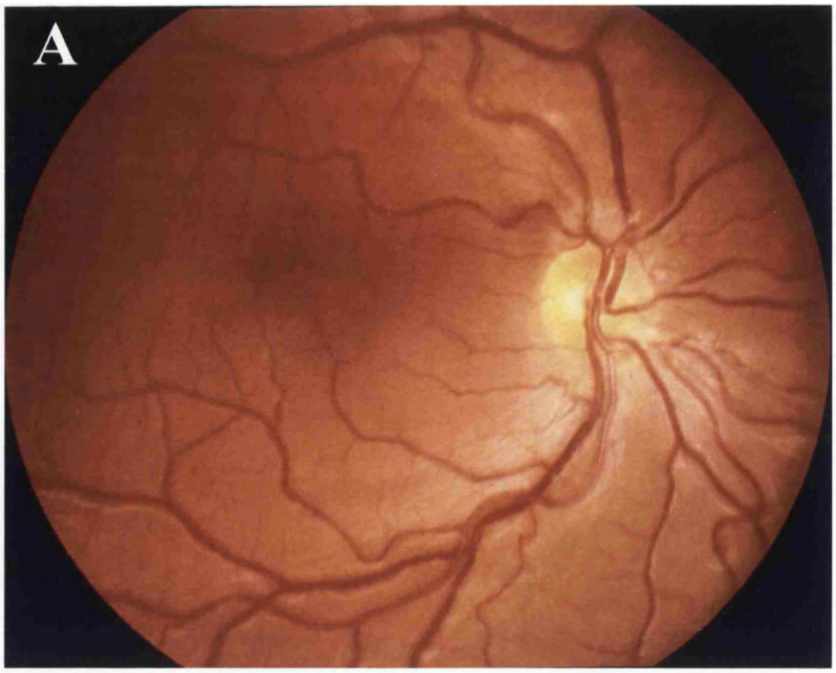
clumps rather than continuous sheets in the subretinal space (Gouras *et al.*, 1992; Gouras *et al.*, 1994). The interface with the host also presented problems; in one study there was evidence of a glial scar that might limit synaptic interactions (Gouras *et al.*, 1994). The use of sheets of retina solves the problem of laminar order and continuity but is difficult to employ in small rodents like mice. Whole or large pieces of embryonic retinae provide a better interface with the remaining host retina although how complete or how normal synaptic integration may be is not yet clear. In none of the studies is there clear evidence that such grafts improve visual responsiveness over preoperative levels. While this work has been extended to patients, there is as yet no objective evidence of improved function. These investigations, while providing useful evidence on graft viability, have nevertheless attracted critical comments and the argument is compelling for more comprehensive studies on animal models before contemplating further clinical experiments (Berson and Jakobiec, 1999; Bok, 1993; Sharma and Ehinger, 1997a).

1.1. Retinitis Pigmentosa

The commonest type of inherited photoreceptor dystrophies is RP. RP, a type of peripheral degeneration, is characterised by primary loss of rod function, defective vision in the mid-zone of the visual field and morphological changes in the post-equatorial fundus. About 1 in 3500 to 1 in 4000 people are affected (Berson, 1993; Bunker *et al.*, 1984). An estimated 1.5 million people are affected around the world. The disease can be subdivided on the basis of inheritance, with about 60% having autosomal recessive disease, 10-25% autosomal dominant, and 5-18% X-linked (Bird, 1995). There is no known definitive treatment for the disease, except in some rare forms of RP, Refsum disease and Bassen-Kornzweig syndrome (abetalipoproteinemia). In Refsum disease, with an elevated serum phytanic acid due to a deficiency of phytanic acid oxidase, treatment with a low-phytol, low-phytanic acid diet has resulted in the lowering of serum phytanic acid and stabilisation of retinal function. In a-beta-lipoproteinemia, large doses of Vitamin A are used to treat patients with the ineffective transportation of fat-soluble vitamins, this results in reversal of the electroretinogram (ERG) and prevention of retinal degeneration (Berson, 1993). The symptoms and signs of RP are well known. Affected patients typically report impaired dark adaptation, night blindness, and difficulty with mid-peripheral visual field. As the condition progresses, they develop a tendency to blue blindness, lose far peripheral field of vision, and eventually central vision. In more advanced stages, signs include attenuated retinal vessels, intraretinal "bone-spicule" pigment, and waxy pallor appearance of the optic disc (Figure 1A,B). Some patients become blind as early as 30-year-old, the majority being legally blind by the age of 60 (Berson, 1993).

Figure 1

(A) Colour fundus photography of a normal human retina. (B) Colour fundus photography of a patient with RP showing the bone-spicule pigmentation (ARROW) in the mid-periphery, RPE atrophy and early vascular attenuation. (C) A simplified diagram illustrating the complex process of apoptosis and the main factors involved.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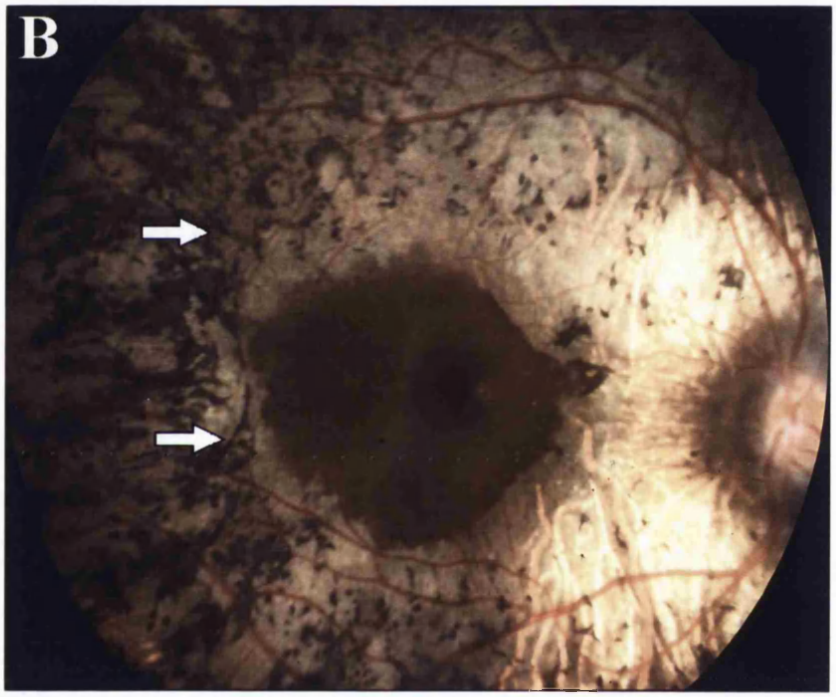
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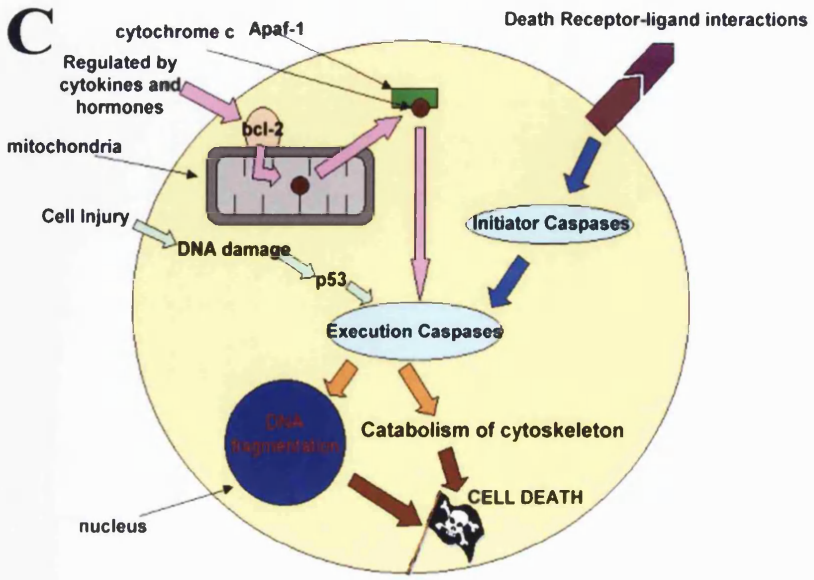
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cytochrome c
Apaf-1
Death Receptor-ligand interactions
Regulated by cytokines and hormones
bcl-2
mitochondria
Cell Injury
DNA damage
p53
Initiator Caspases
Execution Caspases
DNA fragmentation
Catabolism of cytoskeleton
CELL DEATH
nucleus

1.1.1. Morphological changes in RP retina

Photoreceptors – rods

In all genetic forms of RP, the earliest histopathological change in the rods is shortening of their outer segments, which can be demonstrated with immunochemistry using rhodopsin antibodies. Death of the rods usually begins in the mid-peripheral (equatorial) retina and progresses with time to involve the macula and more peripheral retina with the far periphery spared until the end stage of the disease. This sparing is thought to be due to the low levels of incident light in the far periphery (Li *et al.*, 1994). The majority of rods in this area are immunoreactive for basic fibroblast growth factor (bFGF) (Li *et al.*, 1997), a protein that promotes rod cell survival in the dystrophic rodent retina (Faktorovich *et al.*, 1990; Steinberg, 1994). Moreover the pronounced degeneration of the inferior retinal regions may reflect increased sensitivity of photoreceptors with this mutation to the damaging effects of light (Heckenlively *et al.*, 1991). These effects may be greatest in the inferior retina because light is usually derived from overhead sources including the sun and indoor illumination.

Photoreceptors – cones

Rod cell death in RP retinae is usually accompanied by changes in the neighbouring cones, including outer segment shortening, increased cytoplasmic density, axonal elongation, and ultimately, cone cell death (Milam *et al.*, 1996). It is thought that cone cell death may be triggered by toxic by-products or metabolic changes from rod cell death (Bird, 1992) or result from the loss of trophic factors normally derived from the rods (Mohand-Said *et al.*, 1998; Mohand-Said *et al.*, 2001). bFGF has been

shown to be present in rods but absent from cones in normal human retina (Li *et al.*, 1997), and may represent a rod-derived trophic factor for cones provided that this protein is secreted by rods and cones have bFGF receptors. Indeed one theory of retinal transplantation is for transplanted retinal cells to secrete and restore these rod-derived factors which may be essential for the survival of remaining cones (Mohand-Said *et al.*, 2000). When all rods and most of the cones have died, the macula, the area involved in detail vision, usually retains a monolayer of cone somata with very short or absent outer segments. They are not enough to sustain normal vision and the quality of life of the patients is significantly reduced.

Neurites

There is evidence that peripheral rods in RP retinae sprout long, axon-like neurite processes (Milam *et al.*, 1996). These rod neurites, which contain rhodopsin on their surface membrane, project for considerable distances and bypass the dendrites of horizontal and rod bipolar cells (the normal targets of rod axons in the retina). The neurites are closely associated with the hypertrophied processes of Müller cells that have undergone reactive gliosis in response to photoreceptor cell death. These changes suggest that mature photoreceptors have remarkable plasticity. Such changes are seen in some animals with inherited retinal degeneration, e.g. cats, pigs, but so far have not been recognised in the rodent models (Li *et al.*, 1998; Milam *et al.*, 1998).

Such plasticity is important when retinal transplantation is to be considered, particularly since one objective of retinal cell grafting is cell replacement and new synapses must be established between the transplanted normal photoreceptors and the host's inner retinal cells. The changes in microenvironment in human RP that

encourage neurite sprouting may lead to similar changes in the transplanted photoreceptors. This may affect the integrity of the visual circuitry. Certainly more research is required in this area before any meaningful clinical trial can begin in retinal transplantation.

Subretinal space

In early RP, the volume of the subretinal space shrinks as the photoreceptor outer segments shorten. Following death of all photoreceptors, the retinal space is lost, due to migration of RPE cells into the inner retina and extension of hypertrophied Müller processes outward to the region of Bruch's membrane. These changes may hinder subretinal injections of donor retinae and corrective genes in viral vectors (Milam *et al.*, 1998).

The subretinal space also contains the interphotoreceptor matrix, a complex mixture of glycoproteins and glycosaminoglycans secreted by the RPE and photoreceptor cells that border it. It is thought that with the rod cell death, the composition of interphotoreceptor matrix is altered which might make it unfavourable for the remaining cones and also for the survival of transplanted normal photoreceptors. It has been shown that the level of interphotoreceptor retinoid binding protein, a protein secreted mainly by rods and which participates in retinoid transportation between RPE and photoreceptors, decreased with the death of rods. This may contribute to the shortening of cone outer segments and ultimately to the death of cones (Milam *et al.*, 1998).

Other retinal cells

Müller cells undergo reactive gliosis in different retinal degenerations. The histological changes include cellular hypertrophy, migration from the inner nuclear layer (INL) to the outer nuclear layer (ONL or photoreceptor layer), and increased levels of glial fibrillary acid protein (GFAP) - an intermediate filament protein found mainly in astrocytic glia in normal human retina. In advanced RP, a thickened layer of Müller cell processes replaces degenerated photoreceptors, and this may become a barrier to synapse formation between host neurones and transplanted retina. However, there is some evidence that photoreceptor axons can penetrate a glial barrier in the dystrophic mouse retina (Gouras *et al.*, 1994). This deserves further investigation in larger animals and even primates.

GFAP-positive astrocytes are found near blood vessels in the retinal nerve fibre layer and in the anterior portion of the optic nerve head in humans. In advanced RP, many cells in the INL belong to astrocytes that have undergone reactive hyperplasia. Proliferation of these cells contributes to the formation of epiretinal membranes (Milam *et al.*, 1998).

Microglia, stellate cells associated with blood vessels in the inner layers of the retina, play a role in phagocytosis of apoptotic (programmed cell death) retinal cells in normal or diseased eyes. They are thought to participate in removing diseased photoreceptors as blocking their migration prolongs photoreceptor survival in the Royal College of Surgeons (RCS) rats (Thanos and Richter, 1993). Their role in RP is unclear.

Many inner retinal neurones are preserved in RP. Two separate studies have shown that in patients with advanced RP, the maculae retain between 78% to 88% of the cells in the INL (Santos *et al.*, 1997; Stone *et al.*, 1992). This suggests that loss of photoreceptors causes little transneuronal degeneration in the INL. GABA-positive amacrine cells appear to sprout new processes out of the inner plexiform layer (IPL).

Ganglion cell loss parallels the loss of photoreceptors in RP. In advanced RP, about 30% to 75% of the ganglion cells near the maculae are histologically intact (Santos *et al.*, 1997; Stone *et al.*, 1992). Ganglion cell loss may be due to transneuronal loss (Newman *et al.*, 1987), decrease in blood supply (Li *et al.*, 1995) or compression of axons by retinal vessels (Wang *et al.*, 2000). Any of these mechanisms may compromise ganglion cells and the blood supply of the retina and therefore are an important consideration for therapies based on retinal transplantation or rescue. However, if cochlear implants in patients who have hearing loss are any guide, they have been clinically successful in patients who have lost 90% of spiral ganglion cells. (Fayad *et al.*, 1991; Linthicum, Jr. *et al.*, 1991). In extramacular regions of RP patients, the remaining inner retinal neurones, as a percentage of normal cell counts, far outnumber the percentage of spiral ganglion cells required for a cochlear implant to restore auditory function (Humayun *et al.*, 1999).

Retinal blood vessels

Attenuation of the retinal blood vessels is a hallmark of RP. This was originally thought to be due to diminished ganglion cell metabolism after photoreceptor degeneration. It is now known that migration of RPE cells into the inner retina produce significant alterations to the retinal vessels (Li *et al.*, 1995). These RPE cells secrete a form of extracellular matrix that resembles Bruch's membrane near blood vessels and occludes the blood vessels with time. Blood flow is reduced by 78% even though the vessels retain their normal regulatory response to increased oxygen (Grunwald *et al.*, 1996). The vascular endothelial cells next to the migrated RPE cells develop fenestration and leak, this clearly disrupts the normal blood-ocular barrier and may affect the immune privilege of the eye. This should be considered when cellular transplantation is considered.

RPE, Bruch's membrane and choriocapillaris

At an advanced stage of RP, when most of the photoreceptors are dead, RPE cells are found to be detached from Bruch's membrane and to have migrated to perivascular sites in the inner retina. This produces the clinical appearance of so-called bone spicule pigmentation of the retina (Figure 1B). Underlying choriocapillaris often disappeared, but it is still unclear whether this is due to the loss of photoreceptors or RPE or both. Some evidence suggests that choriocapillaris loss is due to RPE loss (Del Priore *et al.*, 1996), since choriocapillaris undergo atrophy within a week of RPE debridement in pigs.

The cause of RPE migration is not known. It has been suggested that it is due to loss of by-products from photoreceptors which may affect the ability of RPE cells to

adhere to Bruch's membrane. The relocation of RPE cells to perivascular regions may reflect their affinity for vascular basal laminae. This migration has implications for transplantation therapy, as it may be necessary to replace both RPE and photoreceptors if transplantation is performed at late stages of the disease (Milam *et al.*, 1998).

Bruch's membrane is affected by the normal ageing process with thickening of the membrane and deposition of drusen (Young, 1987). As in normal retinae, these ageing changes in Bruch's membrane are also present in RP retinae (Meyer *et al.*, 1982). Moreover some patients with RP have widespread deposits of abnormal material between RPE and the inner collagenous layer of the Bruch's membrane (Kuntz *et al.*, 1996). This again may jeopardise the survival of the transplant if transplantation is to be performed at a late stage of the disease.

1.1.2. Potential Treatments for RP

Dietary/Drug

Modification of diet may be useful in some specific forms of retinal dystrophy. Examples include dietary control in Refsum disease (a phytanic acid deficiency); vitamin A supplementation for a-beta-lipoproteinaemia (Ehlers *et al.*, 1981) and vitamin B6 supplementation for gyrate atrophy (Kennaway *et al.*, 1989), but not all patients benefit from the treatment, suggesting disease heterogeneity. Such heterogeneity may also have been a factor in a randomised, controlled, double-blind clinical trial on the use of vitamin A and vitamin E in the treatment of RP (Berson *et al.*, 1993), where only a subset of patients appeared to benefit from vitamin A

supplementation. Moreover the beneficial effect was only demonstrated in ERGs; there was no improvement in visual acuity. Recently, D-cis-diltiazem, a calcium-channel blocker that also acts on light-sensitive cyclic guanine monophosphate (cGMP) gated channels, has been shown to rescue photoreceptors and preserve visual function in a rodent model of RP (Frasson *et al.*, 1999). There was initial enthusiasm but subsequent studies found that diltiazem did not work with other models of retinal degeneration (Bush *et al.*, 2000; Pearce-Kelling *et al.*, 2001) and it seems that diltiazem only works on some subgroups of retinal degeneration. Further studies will be required to assess whether this drug has any clinical application.

Growth factors

Growth factors are known to play a key role in the survival and differentiation of neurones in the central nervous system (CNS) and peripheral nervous system (Levi-Montalcini, 1987). A number of growth factors (e.g. bFGF) and, to a lesser extent, their receptors have been identified in the retina or in retinal culture (Bugra and Hicks, 1997; Carwile *et al.*, 1998; Gao and Hollyfield, 1996). There is some variability among the studies, but this is undoubtedly due in part to use of different developmental stages, use of different animals and use of techniques with different sensitivities when studying factor and receptor localisation. Axokine, a mutein of ciliary neurotrophic factor (CNTF), was one of the most promising growth factors as it was shown to delay retinal degeneration in animal models (Chong *et al.*, 1999) and a Phase I clinical trial was initially planned for patients. However, a parallel trial of its use in the CNS for weight loss was stopped after occasional herpes reactivation as reported and a trial in transgenic P347L opsin pig was halted too due to lack of photoreceptor rescue, occasional cataract formation and CNTF-related ERG

abnormalities (Hauswirth and Beaufrere, 2000). In view of these findings, there are still many practical issues to be resolved including the mode of delivery, ways of maintaining the growth factor level at a therapeutic dosage, and the type of growth factors to be used. Studies into human RP patients have not gone to phase I clinical trials, although a number of avenues are presently being explored.

Gene Therapy

A number of studies have been undertaken in experimental animals (see Chapter 1.2.4.) but no gene therapy treatments have been attempted on human RP patients yet. Ideally there are 4 prerequisites that should be met for any genetic therapy targeted to the human eye, namely (1) a gene delivery technique that is efficient and non-toxic, (2) the genetic and biochemical basis of the ocular disease is known so that an appropriately matched therapeutic approach can be selected, (3) expression of the therapeutic gene can be controlled, and (4) animal model experiments have shown in principle that the therapy can work with no/minimal side-effects. In terms of safety, most of the animal studies have reported no detection of virus in the blood or in any other organs, suggesting that infectivity does not spread. However, Dudus *et al.* (1999) found evidence of a persistent transgene product in the CNS after introduction into the eye, raising the possibility that virus might spread beyond the site of injection. Recent fatalities in human gene therapy experiments (Lehrman, 1999; Marshall, 1999) argue for caution in the use of the current vector approaches and for the development of new vectors.

As with the growth factor studies, most of the replication defective adenovirus transfer studies in animals reported a loss of label and loss of photoreceptors after

relatively short periods of time. The reasons for this are not known: possibly the transgene is unstable, exposure to the virus is eventually toxic, or alternatively, the transgenes may persist but become ineffective because other regulatory elements are lost. A further explanation is that the cells themselves are eventually removed by some form of immune rejection (Reichel *et al.*, 1998).

Until safe vectors, producing stable, long-term expression of factors can be identified, this is unlikely to be a suitable treatment, especially since to be maximally effective it should be instituted early in life. In addition, the complex genetics of RP makes it difficult to establish commercially viable treatments when homologous genes are introduced, especially since each patient group will often be quite small, e.g. the most common subtype in the USA, P23H, represents about 23% of the whole (Gal *et al.*, 1997).

Transplantation/Surgery

Transplantation in animal models started long ago (see Chapter 1.2.4.), and the principle is the same in attempting to prevent/cure the disease: - to rescue/preserve photoreceptors or to reconstruct/replace lost photoreceptors. To date all the human trials have been concentrated on the replacement principle at the end-stage disease as the risk to the patient is smaller than in rescue therapy where surgery has to take place earlier while numerous host photoreceptors are still present. The first article reporting on the results of retinal transplantation in humans was published in 1997 (Kaplan *et al.*, 1997). In this report, two patients with advanced RP received a sheet of adult human photoreceptor cells harvested from autopsy eyes. There was no perception of light by either patients twelve months after transplantation. In January 1995, a multi-

centre, phase I clinical study (safety study) began and some RP patients received unilateral subretinal transplants of a fetal neural retinal cell suspension (Das *et al.*, 1999). There was a 3-year follow-up of a cohort of 14 patients with transplants. It demonstrated that the procedure was safe over the study period. None of the patients experienced any clinically observable signs of inflammation, infection, or graft rejection. One patient developed retinal detachment. The global ERG remained undetectable in all the patients, but authors claimed that the visual acuity of three patients changed from light perception to hand motion at the 1 year follow-up and in one patient it had changed from light perception to 6/60. These improvements were reportedly sustained for months or years and no comparable change occurred in the unoperated eye of any patients. However these changes in visual acuity could be due to spontaneous fluctuations (Berson, 1995; Grover *et al.*, 1997), a placebo effect, or functions of remaining host photoreceptors (Berson and Jakobiec, 1999). The first histological result of a human neural retinal transplant in a patient with AMD, showed survival of the transplanted cells in the subretinal space with no evidence of inflammation or rejection under light microscopy but well-developed photoreceptor outer segments were not present (del Cerro *et al.*, 2000). It was claimed that long-term transplant survival, 3 years in this case, could be achieved, but some would argue that it was impossible to identify the donor cells. Radtke *et al.* (1999) transplanted intact sheets of fetal retina (15 and 17 weeks of gestation) into the subretinal space of two patients with autosomal recessive RP. Both patients reported a subjective improvement in vision after the transplant, and one patient showed a transient multifocal ERG (mfERG) response in the transplanted area at 4 months. However, the mfERG response could not be recorded at 6 and 9.5 months after transplantation. The overall follow-up period for both patients was less than 14 months, and thus long-term effects of the transplant are unknown. Moreover such

mfERG activity is not sufficient to establish that functional connections have developed since the transplanted cells could differentiate into both bipolar cells and photoreceptors, thus the transplant alone may produce both components of the ERG, namely an a-wave originating from the photoreceptors and a b-wave originating from bipolar cells. Humayun and coworkers (Humayun *et al.*, 2000) performed subretinal transplants of human fetal retinal microaggregate suspensions without postoperative systemic immunosuppression in eight RP patients with bare light perception vision and one AMD patient also with bare light perception vision. Three of the eight RP patients demonstrated possible improved light sensitivity during the initial months of follow-up. However, visual improvement disappeared between 3 and 13 months of follow-up. After transplantation, no subject showed any changes in the ERG recordings or scanning laser ophthalmoscope macular perimetry relative to their preoperative baseline. Although a definite positive effect on visual function could not be demonstrated, the apparent high tolerance for graft tissue is promising for future efforts in the field of neural retinal transplantation.

An alternative approach to replacing photoreceptors with cells is to use implanted microphotodiodes to carry a visual signal to the cells of the inner retina (Eckmiller, 1997; Humayun *et al.*, 1996; Peachey and Chow, 1999; Rizzo *et al.*, 2001). Using various animal models, photodiode arrays have been placed either subretinally (Peyman *et al.*, 1998) where they would be expected to stimulate bipolar cells or epiretinally where they laid adjacent to the vitreal surface to stimulate cells of the ganglion cell layer (GCL) (Eckmiller, 1997). These artificial implants appear to remain stable and are biocompatible. In humans, direct pattern stimulation of the surface of the retina can elicit simple light perception (Humayun *et al.*, 1996), but long-term implantation of functioning photodiode/electrode arrays has yet to be

reported. Much still has to be done to ensure that a significant signal can be presented to cells of the inner retina and that the progressive deterioration of the inner retina (which normally follows outer retinal cell degeneration) does not compromise efficacy. It has yet to be seen how far the CNS can interpret signals delivered through such prostheses to elaborate visual images. Presently the best that might be expected from such endeavours would likely be a fairly crude image, but this may be sufficient to permit navigational vision.

1.2. Mouse Model of Retinal Degeneration

In the search for better treatments of retinal disease studies on animal models of inherited retinal degeneration have increased dramatically over the last two decades (Hafezi *et al.*, 2000; Litchfield *et al.*, 1997). Advances in molecular genetics, particularly the development of "knockout" gene technology, have provided researchers with the tools to develop hypothetical animal models of human disease seemingly at will. Despite these advances it is a spontaneous genetic mutation, the rd mouse, known for over 70 years, that is one of the most studied animal models of human RP.

1.2.1. Origin of the retinal degeneration (rd) mouse

In 1923 Keeler discovered a strain of mice (CBA/Ki) lacking all photoreceptors which he termed "rodless" (gene symbol r). Keeler went on to publish a number of papers describing the morphological, behavioural, physiological and genetic characteristics of the rodless mouse (Farber *et al.*, 1994; Keeler, 1966). Unfortunately this strain became extinct in 1939 when he discontinued his colony due to a lack of interest in the animal model. Later Keeler found that mice with the same phenotype as rodless were surprisingly common throughout Europe and the United States. In 1951 one of these strains (C57BL/6) was described by Brückner who concluded that the retinal changes seen in this strain were similar if not identical to the rodless lesions (Farber *et al.*, 1994). This strain was named as "retinal degeneration" (gene symbol rd).

A few years later Dunn (1954) found that C3H mice carried traits allelic to the disease in Brückner's C57BL/6 mice. These traits were also present in inbred strains developed by Sidman and Green (1965). Like the rodless the rd mutation is inherited in an autosomal recessive manner, and Sidman and Green (1965) had located the defective gene in Chromosome 5. Following extensive studies of the rd mutant throughout the world, Keeler wrote that he considered the mutation to be identical with that of his original stocks of CBA/Ki rodless mice (Keeler, 1966). Recently, recombinant deoxyribonucleic acid (DNA) technology analysis (polymerase chain reaction) of DNA from 70-year-old sections of rodless retina have confirmed Keeler's suspicions (Pittler and Baehr, 1991).

1.2.2. Morphological changes of rd phenotype

Keeler's original interpretation of the rodless disease defined it as dysplasia (a failure in the development of photoreceptor cells from birth), however subsequent investigations in the 1950's and 60's revealed that the initial development of visual cells in the rd mouse is normal and the lesion occurs only after the neural retina has differentiated and started to grow outer segments (Farber *et al.*, 1994).

Development of the normal mouse retina

On the first postnatal day the normal (+/+) mouse retina has three layers; a GCL, an IPL and a thick layer of mitotic neuroblasts. By postnatal day 4 the IPL and outer plexiform layers (OPL) become clearly distinct and the neuroblasts become recognisable as photoreceptors, showing well-delineated inner segments. At about day 8 photoreceptors have developing photoreceptor outer segments. From this point

on there is a steady growth of visual cells until around day 25 when adult dimensions are reached. At this stage, the ONL of the mouse retina (containing photoreceptor nuclei) is typically around ten rows deep (LaVail and Sidman, 1974). The majority of cells produced postnatally differentiate as rods and this cell division and differentiation cease by day 11 postnatally (Young, 1985). By the time adulthood is reached the normal mouse retina will consist of around 97% rods and 3% cones (Carter-Dawson and LaVail, 1979). In the inner retina, bipolar cells, amacrine cells, Müller cells and horizontal cells constitute 41%, 40%, 16% and 3% of the inner retina respectively (Jeon *et al.*, 1998).

The rd mutation

Retinal degeneration in the rd mouse is now known to be caused by a mutation in the gene encoding the β-subunit of rod cGMP phosphodiesterase (βPDE), an enzyme involved in phototransduction (Bowes *et al.*, 1990). The nature of the primary lesion in rd gene remains unclear. After the initial identification of the rd gene as βPDE, two specific defects were found in the rd gene. Sequence analysis uncovered a large piece of viral DNA from a xenotropic murine leukemia virus (Xmv-28) in the promoter region (Bowes *et al.,* 1993). Bowes and coworkers suggested that blindness in this mouse strain is a result of infection of a "founder" mouse many decades ago with a virus, which resulted in a random insertion of viral DNA into a gene (βPDE) required for photoreceptor survival. This viral insertion has rendered the βPDE gene incapable of being transcribed into messenger ribonucleic acid (mRNA). Genetic studies by Pittler et al.(1991) revealed that rd degeneration may have an alternative explanation, a nonsense mutation in the protein-coding region of βPDE. At the time of this writing, it remains unresolved as to which defect in the rd gene causes the death of

the photoreceptors. Interestingly, both the viral DNA insertion and the point mutation are present in every strain of rd mouse tested and are widely distributed through many common inbred strains and recently captured wild mouse strains from different parts of the world. Several investigators have reported inadvertently generating blind laboratory animals by backcrossing normally sighted mice that were heterozygous for rd (rd/+). The finding of two distinct mutations in many strains suggests that the blinding rd defects in mice are ancient. The persistence of this blinding mutation in many mouse strains for decades suggests that vision is not essential for survival of the species. Regardless of the exact nature of the lesion, the rd mutation causes disruption in the transcription of the β-subunit resulting in a failure of cGMP phosphodiesterase to assemble into a functional complex (Farber *et al.*, 1994; Farber, 1995).

Mechanism of photoreceptor cell death

Defects in the βPDE complex and subsequent accumulation of cGMP in rd retinae appears to play a major role in the degeneration of rod photoreceptors. If the PDE inhibitor isobutylmethyxanthine is applied to cultures of Xenopus eye rudiments, there is an accumulation of cGMP and a loss of photoreceptors (Lolley *et al.*, 1977). Similarly, observations made from cultures of human retina with PDE inhibitor by Ulshafer revealed a selective degeneration of rod photoreceptors but a preservation of cones (Ulshafer *et al.*, 1980). Whilst it is clear that an accumulation of cGMP precedes photoreceptor death, the exact processes which link these two events are unknown. A possible explanation is that the chemical changes, triggered by the rd mutation, cause cell death through a necrotic mechanism such as toxic alterations in the photoreceptor matrix. An alternative hypothesis is that cell death occurs through

the activation of apoptotic mechanisms. It is generally accepted that developing neurones have an intrinsic capacity to die through a process of programmed cell death or "apoptosis". Apoptosis is particularly associated with cell pruning and failed synaptogenesis during neuronal development and a number of lines of evidence suggest this to be the mechanism of photoreceptor death in the rd retina. Apoptosis is a complicated process and involves a number of factors (Figure 1C) but they are all outside the scope of this thesis. Apoptosis tends to affect isolated cells rather than patches of tissue, and is rarely accompanied by inflammation. Furthermore, apoptotic cells are usually phagocytosed by adjacent cells without the involvement of circulating macrophages (Portera-Cailliau *et al.*, 1994). These findings are all consistent with observations made during degeneration of the rd retina. A particular hallmark of apoptotic cell death which distinguishes it from other types of cell death is the cleavage of DNA at internucleosomal linker regions by an endonuclease enzyme which produces DNA fragments in multiples of around 200 base pairs. Using agarose gel electrophoresis investigators have found good evidence of these hallmark DNA fragments in the rd retina (Chang *et al.*, 1993; Lolley, 1994). It appears that DNA fragmentation occurs during the period of photoreceptor degeneration. If apoptosis is the primary mechanism of photoreceptor death in the rd retina then the question of how it is triggered by a defect in the βPDE needs to be raised. There is some evidence relating to the role of cGMP in regulating the apoptotic process in cells of the silk moth but as yet no evidence of such a relationship has been found in neurones (Lolley, 1994; Lolley *et al.*, 1994).

The effect of the rd mutation on rod photoreceptors

Differences between the normal and rd retina have been reported as early as postnatal day 4 (Carter-Dawson *et al.*, 1978; Farber *et al.*, 1994). At this time a delay in the separation of the ONLs and INLs, as well retarded growth of the photoreceptor inner segments, has been observed. The first clear sign of morphological disruption becomes apparent around postnatal day 8 with swelling of the mitochondria and the appearance of vacuolar inclusions in the inner segments. This is rapidly followed by disorganisation of the outer segment disc stacks. At around day 10 photoreceptor nuclei become pyknotic and this is followed by rod cell death. Rod outer segments in the rd retina do not enlarge beyond a rudimentary length, reached by day 12. By postnatal day 21 a single sparse row of photoreceptor nuclei remains (Carter-Dawson *et al.*, 1978; Farber *et al.*, 1994); this is compared with ten rows observed in the normal mouse retina at the same period.

The effect of the rd mutation on cone photoreceptors

The single row of photoreceptor nuclei that remains in the rd retina from around day 21 is composed largely of cone photoreceptors. Cone photoreceptors therefore degenerate at a much slower rate than rods (Carter-Dawson *et al.*, 1978; Jimenez *et al.*, 1996; LaVail and Sidman, 1974; LaVail *et al.*, 1997). Carter-Dawson *et al.* (1978) have found that although 98% of the rod photoreceptor nuclei have degenerated by around day 17 the majority of cone nuclei survive. The photoreceptor population in the rd retina therefore quickly approaches 100% cones. There follows a period of steady cone degeneration between days 17 and 36 when approximately half of the cone population is lost. The remaining cones degenerate slowly, by 4 months

of age the central retina contains only about 1.5% of the original cone nuclei population, the majority of which are still observable after 18 months but no ultrastructurally visible traces of outer segments can be detected (Carter-Dawson *et al.*, 1978). Immunocytochemical methods using polyclonal antibodies to cone-opsin have detected immunoreactive cells beyond 600 days (Jimenez *et al.*, 1996). LaVail's group has recently provided histological evidence of cone receptors well into the second year of life (LaVail *et al.*, 1997). It is therefore becoming increasingly likely that some cone nuclei will survive in the rd retina for the entire life span of the animal. The mechanism of cone cell death is also thought to be due to apoptosis, although early researchers were unsure of the mechanism as the rd mutation only affected the rod photoreceptors. Recent work has shown that rod photoreceptors in normal retina release a diffusible factor stimulating cone survival, so when there is a significant loss of rod photoreceptors, the level of diffusible factor is reduced to a level that cone survival is compromised and cones begin to degenerate (Mohand-Said *et al.*, 1998; Mohand-Said *et al.*, 2001). Regardless of whether the cones survive in rd mice, with the loss of their outer segments and change in morphology there is always a question of whether they are capable of functioning normally in the dystrophic retina.

The spatio-temporal pattern of photoreceptor degeneration

Photoreceptor degeneration does not occur uniformly over the retina. Rod photoreceptors in the posterior retina degenerate more rapidly than those in the periphery. Using light microscopy and electron microscopy (EM), Carter-Dawson *et al.* (1978) found that by day 36, no rod nuclei were visible in the posterior retina, whereas some rod nuclei persisted through to day 65 in the far periphery and

moreover there was no differences in the rate of rod degeneration in the superior and inferior retina. However, studies using immunolabelling of rod-opsin showed that degeneration of rods is markedly less in the dorsal retina than the ventral retina, so that rod-opsin immunoreactive cells disappear from ventral retina between 100-200 days of age and by 365 days there are no rod-opsin immunoreactive cells (Garcia-Fernandez *et al.*, 1995; Jimenez *et al.*, 1996). LaVail *et al.* (1997) suggested that this hemispheric difference may have been biased due to examination of relatively few retinae, combined with the fortuitous selection of mice with relatively uniform counts in the superior and inferior hemispheres.

The spatio-temporal distribution of cone degeneration follows a similar pattern to that seen in the rod system with regards to the posterior-peripheral gradient. At eighteen months of age the posterior retina contains around 1.5% of the original cone population but it rises up to 5% in the far periphery (Carter-Dawson *et al.*, 1978). This posterior-peripheral gradient has since been confirmed by a number of studies (Jiménez *et al.*, 1996; LaVail *et al.*, 1997). The pattern of hemispheric difference of cone degeneration is less clear. Using immunostaining techniques both Garcia-Fernandez, *et al.* (1995) and Jiménez, *et al.* (1996) have reported by 100-day-old a higher number of cone-opsin immunolabelled photoreceptors were found in the dorsal hemisphere than in the inferior retina. This hemispheric difference persisted to 365 days with the number of cone-opsin immunoreactive cells remaining almost constant in the dorsal retina between 100 to 600 days of age but no cone-opsin immunoreactive cells were found in the inferior retina from day 100. This finding was confirmed independently by another group which studied the distribution of peanut agglutinin lectin (PNA)-labelled cones (Ogilvie *et al.*, 1997). However using morphological criteria LaVail *et al.* (1997) found

there to be an almost continuous monolayer of cone nuclei in the inferior hemisphere with significantly more cones surviving than in the superior hemisphere which typically showed only scattered nuclei. The authors argued that immunocytochemical studies failed to label significant numbers of cones leading to the difference in findings.

The effect of the rd mutation on synaptogenesis

As synaptic organisation of the OPL takes place after the maturation of horizontal cells and before the completion of bipolar cell differentiation in the rd mouse retina, dyad configurations (between horizontal and photoreceptor cell terminals) are present in the rd retina by postnatal day 4 but triad configurations (two lateral processes, a synaptic ribbon and medial bipolar dendrites in rod photoreceptors) do not form (Blanks *et al.*, 1974). This failure is probably due to the defect in the photoreceptors rather than in bipolar cells since bipolar cells are normal and viable at the early degenerative stage (Blanks and Bok, 1977).

Persistent plasticity and compensatory synaptic growth in the rod terminals following photoreceptor degeneration can have important functional implications, especially in terms of connectivity of transplanted cells (Sanyal *et al.*, 1992). Rod neurites sprouting *in vivo* suggests that mature photoreceptors retain remarkable plasticity. Rods form neurites *in vitro* when cultured on Müller glia (Gaudin *et al.*, 1996). However in patients with RP, electron microscopy revealed that the rod cells sprout long axon-like neurite processes which projected for considerable distances into the inner retina, bypassing the dendrites of horizontal and rod bipolar cells, and failing to make functional synapses correctly with the INL. This

is seen in dystrophic retinae of longer lived animals (cat and pigs) but has yet to be identified in rodent models of retinal degeneration (Milam *et al.*, 1998). The concern for transplantation studies is that transplanted rod photoreceptors may respond in the same way as the host photoreceptors and sprout neurites that would not connect to the INL in the normal fashion. This warrants further investigation.

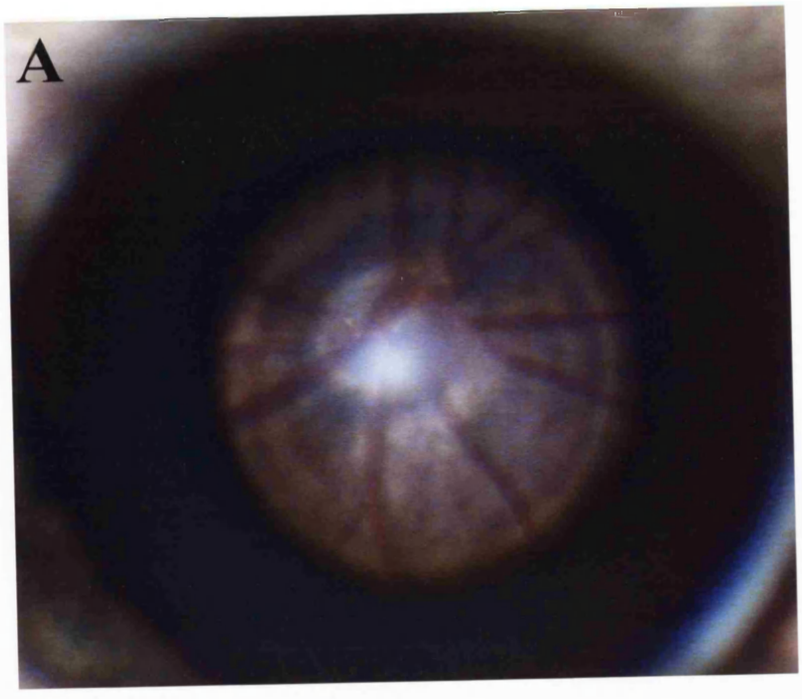
The effect of the rd mutation on RPE, Choroid and other retinal cell layers

With the loss of photoreceptors, a series of changes occurs in the surrounding cells and structure of the rd mouse retina in the subsequent months. By eight-week-old, there is a loss of RPE where photoreceptors are absent. From 6 months onwards, small foci of RPE cells migrate into the retina and are seen in association with local vascular complexes in the outer retina layer (Figure 2A,B). Similar, but more pronounced, changes are seen in RP. At eight-week-old choriocapillaris adjacent to degenerating RPE lose their fenestration and this change increases with time. By 20 months 5-10% of the entire choriocapillaris is missing. Loss of related arterioles does not occur until about 2 years. (Neuhardt *et al.*, 1999).

Retinal blood vessels begin to reduce in number from 14 days, which is thought to be an adaptation to the metabolic changes associated with the disease (Blanks and Johnson, 1986; Matthes and Bok, 1984). By three months of age there is a consistent and substantial loss of fine capillaries from the deep retinal plexus of vessels and there is associated dilatation of the drainage venules. Eventually tortuous vessels and neovascular formations are seen in the outer retina, associated with RPE cells (Ogilvie *et al.*, 1997; Wang *et al.*, 2000).

Figure 2

(A) Fundus photography of a 3-month-old normal mouse showing normal red reflex from the retina and blood vessels. (B) 3-month-old dystrophic rd mouse retina. Note the attenuated blood vessels (ARROW), pale optic disc and pigment clumping in the retina; in the periphery (C) Classical visual cliff apparatus for testing animals and babies. Note the edge of the cliff (ARROW).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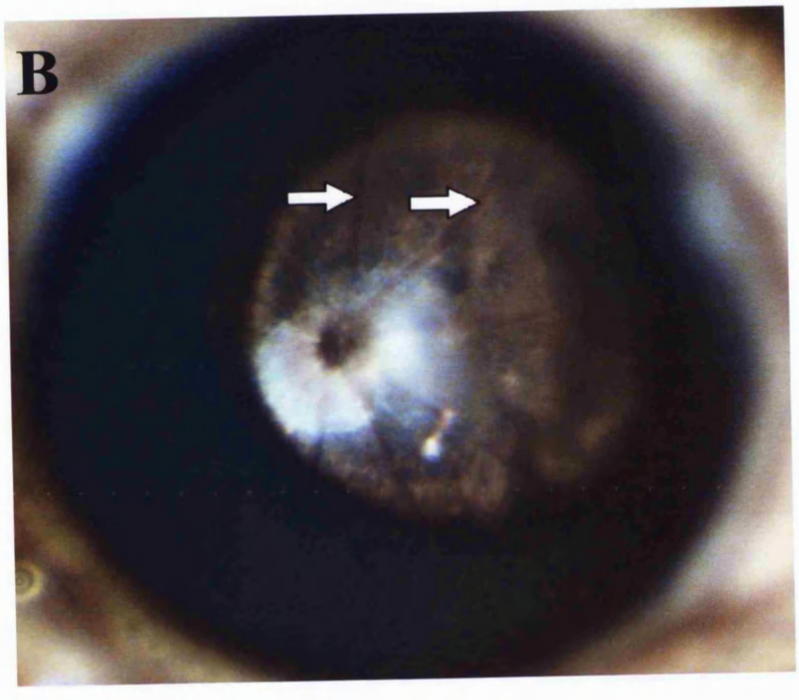
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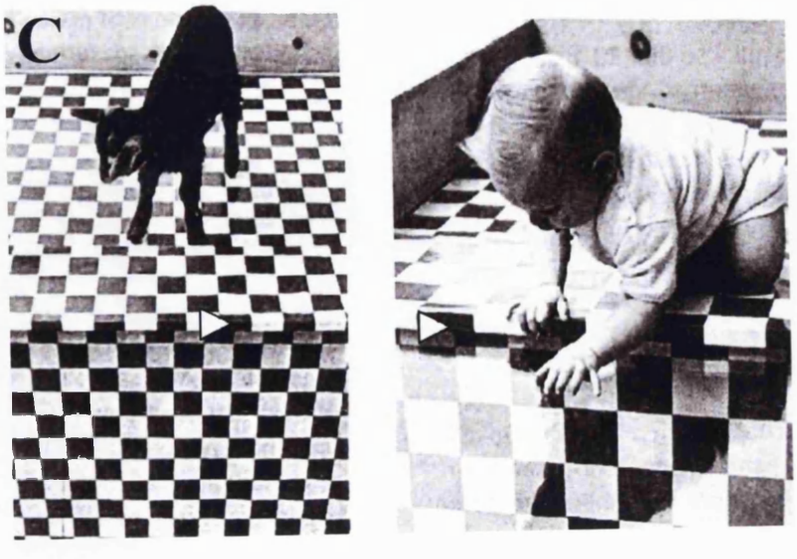

C

Mild secondary loss of bipolar cells in the INL is evident by 41 days (Blanks *et al.*, 1974), but overall survival is not hindered by photoreceptor cell death (Ogilvie *et al.*, 1997). However, a recent study has shown that there is significant morphological alteration to the bipolar cell architecture, with loss of dendrites and loss of the specific receptor for rod-bipolar transmission, the metabotropic glutamate receptors (Strettoi and Pignatelli, 2000). Horizontal cells are still present in the adult rd retina (3.5 months of age), but are slightly reduced in numbers. The cell bodies and first order dendrites become hypertrophic and small and terminal dendrites completely disappear. Grafstein *et al.* (1972) reported that between 3 and 6 months of age, the rd retina shows fewer ganglion cells and these are 10-20% smaller and show an abnormal rate of axoplasmic transport. These findings are confirmed in part by a recent study from the Lund group (Wang *et al.*, 2000). In response to retinal injury Müller radial glia express GFAP and have the corresponding mRNA. In the rd mouse, disruption of neurone-glia interactions causes activation of the GFAP gene in Müller cells and the accumulation of GFAP progresses from cells in the peripheral retina to the centre.

Circadian rhythms and photoreception in the rd mouse

Despite widespread destruction of the photoreceptor cells rd mice as old as 767 days retain the capacity to phase shift their circadian locomotion rhythms in response to brief pulses of light (Foster *et al.*, 1991). Furthermore, this sensitivity is indistinguishable from those seen in age-matched +/+ and rd/+ controls. This response is abolished by bilateral enucleation suggesting that the cells mediating circadian regulation must reside within the eye (Provencio *et al.*, 1994). These studies imply that the photic input pathway to the circadian system of the rd mouse is

unaffected by the mutation. If surviving cones were mediating these circadian events a decline in the sensitivity of the circadian system would be expected with age, paralleling the loss of cones. Provencio *et al.* (1994) have investigated this hypothesis but found no relationship between the age of the mouse and circadian sensitivity suggesting that cone cells are not involved in circadian entrainment. Recently, a new group of photoreceptors has been identified which are responsible for the regulation of the circadian system (Lucas *et al.*, 1999).

Ocular immune response: the effect of the rd mutation and transplantation

Kaplan and Streilein (1977) demonstrated that rats treated with allogeneic lymphoid cells in the anterior chamber (AC) accepted for a prolonged interval of time orthotopic skin allografts syngeneic with the cells injected into the AC. Moreover rats of one inbred strain produced specific serum antibodies against the injected cells. This demonstrated that a systemic immune response could be evoked by antigen inoculation into the AC and the response was characterised by weakened cell-mediated immunity. This was the beginning of the notion that immune privilege is actively, rather than passively, maintained. Different antigens have since been injected into the AC and the immune responses investigated. In general, a stereotypic pattern of immune response has been observed, now termed Anterior Chamber Associated Immune Deviation (ACAID) and its characteristics are well-described (Streilein, 1990; Streilein, 1999). A number of factors can affect the stability of this immune privilege. Vascular integrity is vital since artificially disrupting the outer blood-retinal barrier leads to loss of the immune privilege (Wenkel *et al.*, 1999). The length of the disease process can also affect immune privilege since it is lost in older rd mice (Welge-Lussen *et al.*, 1999). Moreover a systemic response identical to

ACAID can be evoked when antigens are placed in the vitreous cavity or in the subretinal space. Experiments suggest that prolonged survival of neuroretinal or RPE grafts in the subretinal space is possible but this immune privilege is not for an indefinite period (Jiang *et al.*, 1993; Jiang *et al.*, 1995; Wenkel *et al.*, 1999). Geneicity of donor and recipient and the match of major histocompatibility complex (MHC) haplotypes (Zhang and Bok, 1998), especially class I, can affect the length of immune privilege, as does the upregulation of MHC class II expression in graft cells (Kohen *et al.*, 1997). The question of immune privilege and transplant rejection is a particularly important one for clinical use. Different mechanisms of cell rejection, such as acute lymphocyte-mediated rejection or delayed hypersensitivity mechanisms make this a highly complex problem that requires much more investigation in the retina.

1.2.3. Visual function of rd mice

Behavioural studies

Lindzey and Winston (1962) tested the maze learning capability and effects of pre-training in inbred strains of mice. The subjects were expected to run through an alley T-maze and the number of errors made was recorded. Trials were repeated and the error scores were compared. It was shown that a particular strain of inbred mice, C3H, showed higher error scores and poorer learning curves than other inbreds. This strain was recognised to be visually impaired by 25 days of age (Noell, 1958) and it is now believed to be the same as rd mice. The main concern regarding this test is that there was no clear procedure to prevent smell being used as a cue and it might have played a part in the level of performance. Wimer and Weller (1965) studied black-white discrimination learning in a water maze, and it was found that three strains of mice lacking in photoreceptors made more incorrect responses than mice with normal retinae. Fox (1965) tested and found that rd mice had no depth perception on the visual cliff. Most of these studies tested mice and ages well beyond the early retinal degeneration period, and the rd strains' poor performance was generally attributed to their apparent blindness. Nagy and Misanin (1970) examined visual perception in rd mice using pattern perception on the visual cliff task and activity measurement in an open field at different levels of illumination. In the visual cliff task (Figure 2C), the arena was made of a 30-inch square glass plate with a black and white chequered pattern board directly underneath one half and the same pattern board 16 inches beneath the other half. A raised runway of the same chequered pattern was placed on the arena and the subjects were placed on this runway. The latency and side of descent were recorded. The results suggested a gradual decrease in the number of mice descending to the shallow side as a function of age and by 40 days (5.7 weeks)

the frequency matched that of a random event. However, it was assumed that mice had good depth perception and no control was tested in this experiment. In the open field experiment, the arena was a field of 36 x 36 inches square that was divided into 36 six-inch squares. Under constant illumination (high: 100 or low: 1 foot-candle) and a steady background noise level of 63 dB, a subject was placed in the arena and the number of squares crossed was measured for a 2 minute period. This was repeated each day for 4 consecutive days. The results demonstrated that high illumination relatively depressed activity through 100 days of age. They argued rd mice had the ability to discriminate light and dark until 100 days of age. However, heat from the lamp at high illumination might also have affected the result and the absence of normal animals in the study make this results less convincing.

Nagy and McKay (1972) studied the open-field behaviour of young rd mice with the open square field described above. Two levels of illumination (10.76 lux and 1076.4 lux) were used. Animals between the age of 10 and 30 days were used. It was found that there was an increase in activity at low luminance and the effect did not disappear by 30 days, indicating some level of light discrimination at this young age. From these experiments, it is now believed that during the second postnatal week while the retinal degeneration renders these mice relatively insensitive to certain kinds of visual cues, such as utilised in visual cliff discrimination, it does not produce total blindness.

Provencio *et al.* (1994) assessed the circadian rhythms and visual capacity of rd mice. The association of a bright white light stimulus (6.8uW/cm^2) with a mild closely temporally coupled electric shock (0.05mA) was used as a behavioural assay for visual photoreception. It was found that rd mice (>180 days of age) could not be

conditioned to avoid an electrical shock in response to a preceding pulse of bright light indicating that the surviving cones were unable to function for this behavioural task.

Mrosovsky and Hampton (1997) investigated the amount of time normal, rd, and enucleated mice spent in each area when they were given a choice of dark or illuminated compartments of about 37.5 cm x 38 cm with an opening (8 cm x 5 cm) connecting them. The illuminated side has a light level of about 300-lux. The age of rd mice ranged between 10 to 19 months. The subjects were placed in the arena for a 22-hour period and the time spent in the light and dark areas was measured. Results showed that both normal and rd mice preferred the dark area. However since the animals were left in a confined space for such a long time, the sense of smell may have influenced the time spent in the compartments and moreover at this level of bright illumination, the cone population was likely to be the determinant factor. A final criticism is that this was a cross-sectional study without any repetition of the experiment which may lessen the significance of the test.

Electrophysiological studies

As in the normal mice, ERG is first recordable in rd mice during the second week of life. The ERG of a 11-day-old rd mouse was comparable to that of an age-matched normal mouse, but between day 12 to 18 the a-wave stopped developing (Noell, 1965), and the threshold was elevated by 2 log units as early as day 12 (Noell, 1958). The maximum amplitude of the ERG of the rd mouse was reached between 14 and 15 days of age. By 20-28 days of age, there was no recordable ERG or visual evoked potential (VEP) in the rd mouse (Bonaventure and Karli, 1969; Noell, 1958;

Yamazaki and Suga, 1969). This study used the C57BL mouse background unlike the other electrophysiology studies which used the C3H background, but this result proved that the rd phenotype did not alter with different backgrounds (Provencio *et al.*, 1994). In terms of the pupillary light reflex (PLR), it did exist but it appeared to have abnormal latency (Parapuram *et al.*, 1995).

Retinal (Visual) threshold in rd mice has been investigated. Bonaventure and Karli (cited in Bonaventure *et al.*, 1985) found the visual threshold in rd mice to be 5 log units higher than in normal mice. In an extensive series of studies performed by Drager and Hubel (1975; 1976; 1978), receptive field properties, topography and retinal threshold responses were measured in normal and rd mice. The topographical study measured the visual responses of the retina in the superior colliculus (SC) to a light square 5 - 10° wide and 2.2 log units brighter than background at 70 - 100 points over the retina. This revealed a progressive concentric loss of vision with age in the rd mice. A central scotoma of about 100° was evident from about 24 days of age and the peripheral ring of visual field that had a reduced response disappeared with time. This result is confirmed by our study (see Chapter 3.2.1.). Response pattern in the retina of rd mice, measured from the SC, appeared to be the same as those in normal mice as long as the signal was strong enough, i.e. responses were off-centre, on-centre, on/off with or without a suppressive surrounding zone. The sizes of the receptive fields were about normal except in the younger rd mice where the field was slightly bigger. Mice have no macula and have a poorly developed area for fine detail vision as compared to larger animals like cats. Moreover even with morphologically normal cones in the retina, Purkinje shifts appeared to be absent as measured by behavioural methods (Bonaventure *et al.*, 1985) and ERG criteria (Drager and Hubel,

1978) in normal mice. It is unlikely that such a shift is present in rd mice, especially since rd and normal mice have a similar spectral sensitivity. Drager and Hubel (1978) were unable to elicit such a shift either, but they found that both normal and rd mice had a spectral sensitivity peaking at about 505 μm with no obvious change between light and dark adapted state. In the last part of their experiment, they characterised the relative sensitivity of rods and cones to white light by determining increment thresholds. It was found at low background levels that the threshold stimulus was significantly higher than in normal animals, but at higher background levels this figure approached that of normal mice. In dark adaptation, no Kohlrausch break (rod-cone break) was observed in either normal or rd mice, however in rd mice, after about 60 minutes, the threshold had reached a plateau which remained stable at a level of 2-3 log units above the threshold reached by the normal mouse. This indicated the rod contribution to dark adaptation was largely missing. These changes in the rd mice, namely 'night blindness' in clinical terms, are consistent with the histological findings of the rd retina.

Summary

A number of studies have investigated the visual functional capability of the rd mouse; however, they give inconsistent results. There are discrepancies between different electrophysiological and behavioural tests. As there is no standardised way of testing the function of these animals, especially in the behavioural tests, a range of light intensities, temperatures, animal ages, and testing conditions have been used; all these may contribute to the differences between their results.

1.2.4. Experimental treatments in rd mouse

The ultimate goal of research into retinal degeneration in the rd mouse is to provide therapeutic and curative strategies that can be used to alleviate the symptoms of human (and animal) eye disease. Morphological, biochemical and genetic observations from the rd retina have led to two principal approaches in attempting to prevent/cure the disease: - to rescue/preserve photoreceptors or to reconstruct/replace lost photoreceptors. These methods, like those attempted in human RP, include drug treatment, growth factors, gene therapy, and retinal transplantation.

Drug treatment

As there is an abnormally high level of cGMP in the rd mouse retina, D-cis-diltiazem, a calcium-channel blocker that also acts on light-sensitive cGMP-gated channels, has been shown to rescue cone photoreceptors and preserve their visual function in terms of ERG amplitude at 25 and 36 days in the rd mouse (Frasson *et al.*, 1999). This work has yet to be confirmed by other investigators and meanwhile other studies using this drug on other animal models of retinal degeneration were negative (Bush *et al.*, 2000; Pearce-Kelling *et al.*, 2001). Given the genetic heterogenieity of RP in humans, this form of treatment may only be beneficial in patients with mutations in phosphodiesterase genes or probably other proteins of the phototransduction cascade. Further studies will be required to assess whether this drug has a wider application.

Growth factors

In the retina, bFGF has been shown to slow the progression of photoreceptor degeneration in RCS rats (Faktorovich *et al.*,1990). This was the first time an

inherited retinal degeneration had been slowed significantly by a pharmacological agent. As a result there has been a surge in studies examining the possible therapeutic role of growth factors in animal models of retinal degeneration. Lambiase and Aloe (1996) have shown that intraocular and retro-ocular injections of nerve growth factor (NGF), one of the most highly characterised neurotrophins, given to young rd mice resulted in the preservation of the ONL, when compared with untreated subjects. LaVail *et al.* (1998) later showed that intra-vitreal injections of CNTF, but not brain-derived neurotrophic factor, leukaemia inhibitory factor, bFGF or insulin-like growth factor II, could slow the retinal degeneration in the rd mouse. The results with Axokine were equivocal. New trophic factors are continuing to be identified that have an effect on photoreceptor rescue in rd mice, the most recent ones include glial cell line-derived neurotrophic factor (Frasson *et al.*, 1999) and pigment epithelium-derived factor (Cayouette *et al.*, 1999). Recently, it was shown that growth factors in combination, rather than individually, rescued rd mouse photoreceptor in organ culture. These results demonstrate that trophic factors promote photoreceptor survival through a synergistic interaction (Ogilvie *et al.*, 2000). The protective action of growth factors seen in the above studies may be due to their inhibition of apoptotic cell death which has been reported in the rd mouse retina (Porter-Calliau *et al.*, 1994). NGF has been shown to block apoptosis in rat embryo sympathetic neurones and chicken immune cells *in vitro* and it is possible that the effect of growth factors in the rd mouse occurs through a similar mechanism (Lambiase and Aloe, 1996). Questions remain over the long term ability of growth factors to provide photoreceptor rescue, these include possible toxic side effects, finding a safe mode of delivering the growth factors, and establishing the correct choice of growth factor for each particular degeneration. Until these are addressed the therapeutic use of growth factors in human eye disease is likely to be limited.

Gene therapy

Firstly gene therapy can be applied by gene augmentation. As retinal degeneration in the rd mouse is caused by a mutation in the gene encoding the βPDE, Lem *et al.* (1992) expressed the normal bovine homologue of βPDE in transgenic mice homozygous for the endogenous rd allele and showed that photoreceptors could be rescued. This indicated that the expression of normal βPDE was sufficient to restore cGMP PDE activity and prevent photoreceptor degeneration. Furthermore, the photoreceptors showed normal morphological features up to the age of three months, even ultrastructurally. Unfortunately, examination of mice aged between four and seven months from one transgenic line (RP-33), revealed that a progressive degeneration was taking place in these retinae (Farber *et al.*, 1994). In terms of somatic retinal gene therapy, Bennett *et al.* (1996) showed that subretinal injections of a recombinant replication-defective adenovirus that contained the murine cDNA for wild-type βPDE resulted in βPDE transcripts and increased PDE activity, delaying photoreceptor cell death by 6 weeks. This finding was the first to demonstrate cell rescue by *in vivo* gene transfer, thus supporting the feasibility of treating an inherited retinal degeneration by somatic gene therapy even though the effect was not lasting.

The second approach to gene therapy is by anti-apoptosis therapy. As apoptosis appears to be the common death pathway for RP animal models, its manipulation by genetic means appears logical. The bcl-2 gene (an anti-apoptotic gene) significantly enhanced photoreceptor survival in the rd mice if a wild-type copy of the βPDE gene was also delivered (Bennett *et al.*, 1998).

The third approach is through growth factor gene delivery. As described earlier, particular growth factors have the capability to delay photoreceptor degeneration. Cayouette and Gravel (1997) had shown that following intravitreal injection of an adenoviral vector encoding a NGF/CNTF fusion gene into one eye of rd mice, many strong CNTF-immunoreactive profiles were detected in various cell types of the injected eyes. Semiquantitative analysis of the corresponding retinae reveals that the ONL retains significantly more rows of photoreceptor nuclei than that of eyes treated with a control (LacZ) vector, or untreated, for at least 18 days after vector administration.

The last approach is probably the most exciting and involves ribozyme therapy. The problem with dominantly inherited mutations is the production of inappropriate gene products disturbing metabolism and causing cell death. Ribozymes, small RNA molecules, act by cleaving mutant transcripts in an allele-specific manner while leaving the wild-type transcripts intact. LaVail *et al.* (2000) recently showed that in a rat model of autosomal dominant RP, the P23H transgenic rat, ribozyme rescue appears to be a potentially effective long-term therapy with effects lasting up to eight months post-injection. It is also effective in late-stage therapy.

Intraocular transplantation

In the literature the earliest attempt to transplant into the eye was in 1873, when Van Dooremaal placed a variety of cells, including human labial mucosa into the anterior chambers of rabbit eyes (cited in del Cerro *et al.*, 1997), the purpose was to assess the survival of various tissues. Over the years, the anterior chamber served as a site for transplantation studies because the clear cornea allowed observation of grafted tissue

and the unusually low immune response in the anterior chamber was beneficial. However it was not until 1959, that the first neural retinal transplants into eyes were performed (Royo and Quay, 1959), where foetal rat retinae were implanted into the anterior chambers of the eyes of the mothers. The aim was to study the survival and development of retinal tissue. Photoreceptors differentiated and many of them formed rosettes, where cells group in a circular fashion with their inner segments and rudimentary outer segments directed towards the luminal centre. The next advance was the discovery that immunological closeness of donor and host tissue was not apparently necessary for successful transplantation in the eye (del Cerro *et al.*, 1987). This experiment reconfirmed the concept of the immune privilege of the anterior chamber, however it is now known that it is not entirely correct and further studies are required.

Turner and Blair (1986) performed the first transplantation of retinal neurones to the posterior segment when they transplanted neonatal rat retina into a specially lesioned site in the adult rat retina. Physical continuity between the graft and host was seen and the graft developed a laminar structure. Since then a number of laboratories around the world have attempted neural retinal transplantation and reported positive findings with respect to photoreceptor transplant survival in the rd mouse. Initially Gouras *et al.* (1991) transplanted reporter gene labelled normal mouse photoreceptors into rd mouse retina with up to 4 weeks survival and showed that rudimentary outer segments were formed. Silverman *et al.* (1992) transplanted sheets of mature mouse photoreceptors harvested by vibratome into rd mouse retina and showed that they survived for 3 months. Using photoreceptors labelled by a transgenic transporter gene and two different cell preparations of neonatal cells, Du *et al.*(1992) found surviving grafts at 2 months post-operation and EM revealed that some surviving

photoreceptors showed relatively mature outer segments. Silverman *et al.* (1994) has reported maintenance and regeneration of cone outer segments in 4-month-old rd mice following transplantation of photoreceptors at 4 weeks of age. This was later supported by further works from Sahel's group (Mohand-Said *et al.*, 1997; Mohand-Said *et al.*, 2000) who showed that rod photoreceptor transplants increased the survival of host cones. These findings suggest that photoreceptor transplants have a certain rescue, as well as reconstruction, potential. Long-term survival of grafted photoreceptor varies tremendously (del Cerro *et al.* 1997). Gouras *et al.* (1994) have shown that allogeneic photoreceptor transplants survived for at least 7 months in the rd retina without immunosuppression. However, Jiang and del Cerro (1992a), using the reciprocal transplantation paradigm, found that grafts of normal foetal retinae into rd eyes faired less well than normal grafts to normal retina and they had a lower percentage of survival with time. It was also suggested that donor age influenced the success of retinal grafts (Aramant *et al.*, 1988) and also the immune privilege of allogeneic retinal grafts is not indefinite (Jiang *et al.*, 1995).

1.3. Background of experimental retinal transplantation in rodent models

1.3.1. Donor cells

In retinal degeneration, photoreceptors are mainly involved. It is not always necessary to aim for replacing all retinal layers. In the past, many types of retina to retina transplantation have been performed and tissue fragments, cell suspensions, enzymatically isolated photoreceptors, and whole sheets of photoreceptors have been shown to survive transplantation. Fragments of donor retina can be prepared relatively easily, donor tissue is often divided into smaller pieces using microscissors. Long-term results in terms of overall morphology have been good with this preparation. However, this is more suitable for larger animal models, since the transplantation instrument has to be proportionately larger than that used for transplanting dissociated cells. Dissociated cell grafts may integrate better with the host retina. This preparation involves treating the donor retina with enzymes and triturating to a cell suspension. However, the use of enzymes may leave the resultant photoreceptors with disturbed morphology, namely disruption of outer segments (Gouras *et al.*, 1992). In the brain, cell suspensions for transplantation have been enriched by cell sorting with a flow cytometer (Notter *et al.*, 1988). Sheets of photoreceptors or whole retina have been successfully isolated and transplanted into the subretinal space, however the technique is not readily repeatable by other research groups and is again limited to larger animal models. The organisation of the cells in different types of retinal transplant has been found to vary significantly. Juliusson *et al.* (1993) transplanted E17 to E19 Sprague Dawley rat retina in suspended and fragmented forms and studied cellular organisation 28 days later by immunohistochemistry. Rhodopsin labelling revealed transplanted photoreceptors

arranged in rosettes in tissue fragment transplants, whereas in cell suspension transplants, photoreceptors were heterogeneously distributed. The donor age is known to affect the survival of the graft. Aramant *et al.* (1988) found that rat retina could be successfully transplanted within a large time span extending up to 2 weeks into postnatal life. However, in terms of organisation and survival, the success rate started to fall significantly after one week postnatally, and grafts of postnatal day 21 tissue degenerated within one to two days after transplantation. Recently Ghosh and Ehinger (2000) showed that, with sufficiently atraumatic techniques in tissue preparation and transplantation, there was almost no upregulation of MHC antigens on retinal neurones. They suggested that adult donor tissue should survive transplantation if these criteria were met.

1.3.2. Techniques of transplantation

In rodent models, early attempts concentrated on injecting cell suspensions and cell aggregates into the subretinal space. These preparations have the advantage of requiring only a small injection tool thus limiting the damage to the recipient eye. A transvitreal approach has the advantage of being more precise in delivering cells to the subretinal space because it permits direct visualisation (Wongpichedchai *et al.*, 1992). However, such an approach is particularly difficult, if not impossible, in mouse eyes because of the size of the lens and its curvature and vitrectomy is not technically feasible (Sharma *et al.*, 1997b). Moreover the transvitreal approach requires a retinotomy in order to deliver the cells, and the retinotomy cannot be closed intra-operatively due to technical difficulties associated with the small eye. The retinotomy often leads to delay in reabsorption of subretinal fluid and may increase the time needed for the iatrogenic retinal bleb (detachment) to settle. As a

result, graft survival can be jeopardised by apoptosis in the host retina triggered by prolonged detachment (Berglin *et al.*, 1997). The transcleral technique has the advantage of a direct approach to the subretinal space, but there is nearly always some reflux of donor cells from the scleral entrance site that reduces the size of the graft and the success rate of transplantation. There is also a likelihood of donor cells entering the choroid and a risk of breaching the RPE layer, jeopardising the blood-retinal barrier (Al Amro *et al.*, 1999). Full- and partial-thickness retina sheet transplantation has also been attempted. These have the advantage of correctly orientating the graft prior to transplantation. With the more widespread use of the vibratome in the preparation of the retinal sheet, it became clear that a better way of delivering the donor cell sheet was required. Early attempts with this technique had limited success in rodents (Silverman and Hughes, 1989) and were rarely reproducible by other investigators but recently with a specially designed, patent-pending instrument, the results from retinal sheet transplantation in a rat model have been more encouraging (Seiler *et al.*, 1998). In larger animals like monkeys and rabbits, the surgical procedures and techniques used can be translated into clinical use. This has largely evolved around pars plana vitrectomy and retinotomy for introduction of the graft (Ghosh *et al.*, 1999). Whichever the technique, the common problems encountered include reflux of donor cells, damage to the recipient ocular structure, ocular haemorrhage, persistent retinal detachment, vitreous loss and surgery performed with poor visibility.

1.3.3. Animal behavioural tests

The only behavioural tests performed on rodents with retinal transplants were in rats with light-damaged retina (del Cerro *et al.*, 1991 and 1995). The test involved the principle of light conditioned suppression of startle responses, however the two studies showed opposite results and it is still unclear whether transplantation improved vision in these rats with light damaged retinae.

To our best knowledge, there has been no behavioural tests performed on rd mice with retinal transplantation. A few studies (see Section 1.2.3.) have suggested that the rd mouse is able to show a discriminatory response to high and low luminance levels which is indistinguishable from that in the normal mouse, in spite of widespread photoreceptor destruction (Mrosovsky and Hampton, 1997; Nagy and McKay, 1972). In all these experiments, however, very high levels of (*bright*) illumination have been used, and it appears that this is sufficient to elicit activity in the remaining cone photoreceptors and mediate the behavioural response. This interpretation is supported by the observation that bilateral enucleation abolishes the behavioural response (Dyer and Hammond, 1975).

Therefore it would be useful to device a simple behavioural test that assess the visual function of rd mice and then to use it to assess the effect of retinal transplantation. The visual function of any mice can be assessed by their preference for light or dark environments. As nocturnal animals mice have a natural preference for dimly illuminated areas. The light/dark paradigm is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of the animals. This paradigm is commonly used in assessing anxiolytic compounds in

mouse models (Young and Johnson, 1991). Transition frequency and time between light or dark compartments have been reported to be an index of activity-exploration because of habituation over time, and the time spent in each compartment to be a reflection of aversion/preference (Hascoet *et al.*, 2001). Therefore given a choice between a light or dark compartment it is the time that a mouse spends in each compartment that will be used to assess its light or dark preference. This principle forms the basis of the behavioural experiment in this thesis. In transplantation experiments, the light as a conditioned stimulus has been used for the assessment of intracerebral retinal graft function (Coffey *et al.*, 1989).

1.3.4. Electrophysiology test

Original studies from the Lund group showed that functional connectivity including PLR could be established with retinae transplanted into the brain (Coffey *et al.*, 1989; Klassen and Lund, 1987 and 1990). However there are only a few studies that have used electrophysiological methods to examine the effects of neuroretinal transplantation into the retinae of animal models of retinal degeneration. Silverman *et al.* (1992) found some recovery of the PLR and VEP after such transplants into light-blinded Fisher rats. Recent work has suggested that the PLR may not be a good predictor of retinal recovery as there was no correlation between the size of the remaining photoreceptor population and the extent of pupillary constriction (Kovalevsky *et al.*, 1995). Although VEP is a objective measure of functional recovery, detection of a full-field flash VEP does not establish that the measured response is coming from the precise area of the transplantation. Adolph *et al.* (1994) transplanted embryonic mammalian donor retina into the subretinal space of a mature host. After isolating the graft to avoid contamination of graft responses by host retinal

activity, transient ON or ON-OFF spike-like responses and local ERG were recorded simultaneously via a single electrode on the graft surface. Woch *et al.* (2001) reported that following co-transplantation of foetal retina and RPE into the subretinal space of RCS rats, 19 out of 29 RCS rats with transplants showed visually evoked responses recorded from, and restricted to, a small area of the SC that corresponded topographically to the portion of the retina with the transplant. No recordings were found in age-matched RCS rats. Recently Radner *et al.* (2001) measured the light-driven responses of retinal ganglion cells in rd mice with neural retinal transplants and showed the preservation of light-driven retinal ganglion cell responses in 3 of 10 eyes five weeks after receiving a transplant at 13 days of age. They argued that this demonstrated connectivity between the transplant and host retina. However, these responses were recorded at the retinal level and it is difficult to say whether the responses had reached the higher visual centres, namely the SC or the visual cortex. SC has a topographic representation/map of the retina, which shows little indication of magnification or distortion in rodents (Siminoff *et al.*, 1966). The presence of a clear map of the retina and relatively simple response properties make it an effective site in which to examine both the effects of photoreceptor loss as well as how transplantation affects this process. Recently Sauvé *et al.* (2001) has demonstrated a technique to measure the visual threshold of RCS rats in the SC, and the technique has been successfully used to assess the effect of Schwann cell transplantation in RCS rats (Lawrence *et al.*, 2000). Briefly, a light point source will be presented in front of the eye from a specially designed visual field testing apparatus, similar to human perimetry testing. Single and multiunit recordings will be covering the full extent of the SC of the subject. The threshold sensitivity will be recorded at each point. Compilation of visual thresholds at each point will give a map encompassing the whole SC, presented as the corresponding retinal representation. The advantage of

measuring visual function via the SC is that it will show higher order visual function restoration after cell transplant. This technique forms the basis of the retinal threshold measurement in this thesis.

While the animal experiments are still ongoing, transplantation studies using human retinal tissue has begun. Human embryonic retinae were grafted into the subretinal space of adult rats with encouraging histological results (Aramant and Seiler, 1994; Ehinger *et al.*, 1991; Seiler and Aramant, 1994). With these results, admittedly still inconclusive, retinal transplantation proceeded, probably unwisely, into human subjects.

1.4. Experimental Aims

An important and logical question which follows from rd mouse research, is whether the retinal graft can integrate well with the dystrophic retina at an advanced stage of the disease where most of the photoreceptors are lost (replacement theory) and whether grafted retinal tissue can re-established functional circuitry with the host retina sufficient to allow restoration of some form of visual function. In order to address these questions, **firstly** I aim to develop a technique of transplantation into the subretinal space of rd mice. **Secondly** I plan to analyse the histological features of the retinal graft and the host-graft interface at short survivals (up to six week). The integrity of the graft will be studied using mainly light microscopy. **Thirdly** I aim to study the visual function of dystrophic rd mice, normal mice and rd mice with retinal grafts to see whether the transplants will re-establish some visual function at short survivals. I will assess the visual function of the mouse in two ways. (1) I will study the mouse's innate behavioural response to light with a light-dark preferential test (LDPT). (2) The other method is an electrophysiological test, retinal threshold measurement. It is hoped that the results from these studies will contribute towards the information needed to assess the suitability of retinal transplantation as a clinical tool for treating various forms of retinal disease.

Chapter 2 - Methodology and technique development

2.1. Animal Source

All animal care and handling during this study conformed to the requirements of the Home Office (U.K.) regulations for the care and use of laboratory animals and the U.K. Animals (Scientific Procedures) Act (1986). Mice were bred and maintained in a colony at the Institute of Ophthalmology, London. Recipient mice were homozygous for the rd locus (C57BL/6J, rd/rd) and donor mice were non-dystrophic syngeneic normal mice (C57BL/6J, -/-). For rat to mouse transplant experiment, normal donor rats are also bred and maintained in a colony as above. All animals were reared from birth in cyclic light (12 hour on / 12 hour off) with a room temperature of 21°C.

2.2. Donor tissue preparation

Firstly as syngeneic grafts are well known to be least immunogenic and have better survival even without immunosuppression (Jiang and Streilein, 1992b; Larsson *et al.*, 1999), syngeneic mouse retinae harvested from C57BL/6J normal mice were used and no immunosuppressants were used in my experiments. Secondly, as mentioned above, donor age influences the survival of retinal grafts (Aramant *et al.*, 1988) and donor age extending up to 2 weeks into postnatal life had been successfully transplanted. We had chosen to use donors of age around 1-week-old as their relatively larger eye compared to those from younger animals would provide abundant donor material.

As the mouse eye is considerably smaller than a rat eye, two common methods of donor tissue preparation were tested. The enzyme dissociation method was tested first, as it would yield a cell suspension that could be transplanted with a smaller needle/cannula. The papain dissociation method was employed, this was originally described by Townes-Anderson *et al.* (1988) and later used in several transplantation studies (Gouras *et al.*, 1992, Du *et al.*, 1992). Donor mice aged 7-9 days (PN 7 – 9) received tribromoethanol (230mg/kg) intraperitoneally as an anaesthetic prior to decapitation and enucleation. Eyes were collected in Ca2+-free and Mg2+-free Hank's balanced salt solution (HBSS; Gibco BRL Life Technologies, UK) at 4°C. The cornea, iris, lens and vitreous were dissected away from the globe and neural retina was removed from the resultant scleral cup and placed in HBSS at 4°C. At this early age, the RPE is only loosely adherent to the photoreceptors and on inspection few pigment cells adhered to the isolated retina. The retina was transferred into a papain solution from a proprietary papain dissociation system (Worthington

Biochemical Corporation, USA) for a pre-determined time at 37°C in an incubator with 95% O_2 : 5% CO_2 as this would both oxygenate and buffer the retina at physiological pH. The retina was then rinsed and washed gently with HBSS three times before it was triturated ten times with a fine glass pipette. The resultant solution was a cloudy cell suspension. Different enzyme incubation times (10, 15 and 20 minutes) were tested but all yielded preparations with poor cell viability as tested by exclusion of trypan blue vital dye (viability of 69%, 69% and 62% respectively). With such poor viability, a microaggregate preparation was tested.

The microaggregates that most studies have used involved cutting up the isolated retina with microscissors resulting in a solution with large pieces of retinal tissues fragments (Gouras *et al.*, 1992; Juliusson *et al.*, 1993). However, these aggregates were considered too big for transplantation into the mouse eye. Therefore we decided to triturate the aggregate solution. Gentle trituration was performed using a fire-polished fine glass pipette (internal diameter of 1mm) for about 10 times to further separate the fragments and develop a mixture of microaggregates and cell suspension. The preparation was allowed to settle at 4°C for 30 minutes, then excess supernatant was removed. The viability of the tissue was assessed by exclusion of trypan blue vital dye (>95% viability). Cells were kept at 4°C in HBSS until transplantation. A number of different media has been used by different groups (Ehinger *et al.*, 1991; Ivert *et al.*, 1998; Jiang and del Cerro, 1992a; Mohand-Said *et al.*, 1997). We have selected HBSS because it facilitates the formation of a subretinal bleb (detachment) by reducing the retinal adhesiveness transiently (Faude *et al.*, 1999). It is also absorbed from the subretinal space more quickly than other media (Negi *et al.*, 1986) thus facilitating the resolution of the retinal bleb formed after transplantation and

subsequently reducing the risk of rosette formation in the graft. For the following experiments, this preparation of microaggregates is used.

2.3. Transplantation Technique

My aim has been to develop a technique that permits direct visualisation of the fundus during transplantation with minimal reflux of grafted cells from the injection site, and a short recovery period of retinal detachment following the procedure. Two techniques initially described by Lazar and del Cerro (1992) and Whiteley *et al.* (1996) have been modified. Mice were anaesthetised with tribromoethanol (230mg/kg) intraperitoneally and the eyes were dilated with 1% tropicamide (Alcon Laboratories, UK). The head was secured by a nose bar, and to allow easy access, the eye was partially protruded from the orbit by tying gently with a releasable suture (6.0 Vicryl) behind the equator of the globe. This allowed good exposure of the intended injection site and stabilised the globe. A short cylindrical-shaped ring (3 mm in length) was cut from a small piece of a clear rubber tubing/ring (internal diameter of 10 mm). This was placed on the eyelids with the protruded eye in the centre, thus the ring formed a "cylinder" with the eye in the base. When the cylinder was filled with 2% sterile hypromellose solution, the solution formed a relatively smooth and flat surface covering the whole globe (Figure 3A). The whole unit behaved like a contact lens and under the operating microscope the fundus could be visualised. Fundus photographs of control mice and dystrophic mice during the transplantation procedure were acquired through a Nikon Coolpix 950 digital camera. The method was derived during the development of the transplantation technique and it involved removing the original operating microscope eyepiece (WILD-Heerbrug microscope) and replacing it with a Leica periplan 10x eyepiece (from a conventional

light microscope). The eyepiece was fitted to the digital camera by screwing the eyepiece into the lens thread. For stable fitting of the Leica eyepiece, an aluminium spacer ring, custom made at the Institute of Ophthalmology, was fitted in between the eyepiece and the operating microscope tube. The details of the method have been published (Kwan, 2000).

A 25-gauge needle was used to dissect away the conjunctiva and sclera, exposing the choroid in the dorso-temporal position. Donor material for injection was drawn into a sterile, self-drawn fire-polished glass pipette (internal diameter 200 to 250 μm) which was connected to a 10 μl Hamilton syringe via a polyethylene tube. Two microlitres of the material was slowly injected through the sclerotomy into the subretinal space of the eye. A successful subretinal injection was indicated by the formation of a dorso-temporal retinal bleb that involved about 1/3 of the retina (Figure 3B,C). During the injection, the cornea was punctured (corneal paracentesis) using a 30-gauge needle to release aqueous fluid. This step helped to reduce the raised intraocular pressure due to the subretinal bleb formation, thereby reducing the amount of reflux of injected material from the entry site. The releasable suture behind the globe was removed. The animal was subsequently placed in an incubator to recover. As no retinotomy was made, theoretically the retinal bleb/detachment should settle quickly.

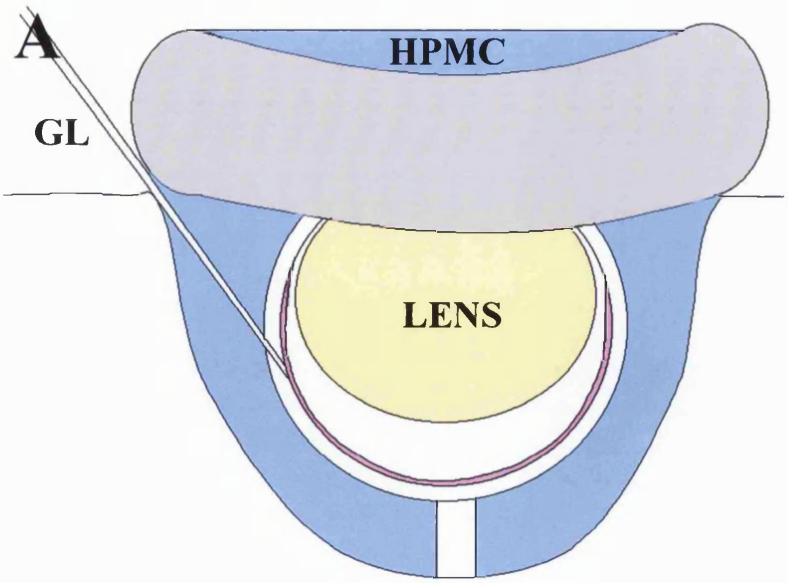
In preliminary experiments, firstly I employed the above modified technique and injected methyl blue ink into the subretinal space of three normal mice. Histological sections of the retinae immediately after the injection showed that the blue pigment was correctly located in the subretinal space. Figure 4A shows a Cresyl violet

staining of a wax section from a 6-week-old normal mouse retina immediately after subretinal injection of methyl blue ink. It shows a detached retina at the site of transplant, but the blue ink (ARROW) can be clearly seen in the subretinal space lying above the RPE. Secondly I injected retinal microaggregates prepared as above into the subretinal space of normal mice. A total of 18 normal mice were used as recipients. Subretinal injections were performed in both eyes, therefore a total of 36 subretinal injections were performed. Eyes were collected for histology one week after transplantation. Evidence of correct placements of subretinal grafts was seen in 23 eyes (63.8%). Thirteen eyes had failed injections either due to intravitreal placement of the grafts (6 eyes, 16.7%) or no evidence of graft survival (7 eyes, 19.4%). Figure 4B shows Cresyl violet staining of a wax section from a 8-week-old normal mouse retina one week after subretinal transplantation of retinal microaggregates obtained from 7-day-old normal mice. There was no sign of retinal detachment and the transplanted cells were seen clearly lying between the host ONL and RPE.

Overall this technique provides a fairly consistent results clinically after the initial practice run of about 20 eyes, there was, however, a steep learning curve. It took a while to get used to operating in small rodent eyes especially for one who is accustomed to operating in human eyes. No immediate complication, including traumatic cataract, significant haemorrhage and tissue damage, was seen during the practice run. No complication, including infection and cataract, was observed at one week following the practice run.With this encouraging result, I proceeded to transplant into rd mice.

Figure 3

(A) Schematic diagram of the transplantation procedure. 2% hypromellose (HPMC) provides a fairly flat surface in front of the cornea and thus acts as a contact lens that allows visualisation of the fundus through an operating microscope. A fine glass pipette (GL) is inserted via the pars plana into the subretinal space and is ready for transplantation. Note the clear rubber ring (RING) with HPMC acts like a contact lens and the large natural lens (LENS) of the mouse eye. (B) Colour fundus photography of a 6-week-old rd mouse during a successful subretinal transplantation. A retinal bleb (localised retinal detachment) can be seen and it is pale and white in colour because it contains retinal microaggregates. A retinal vessel (ARROW) can be seen on the surface of the bleb which indicates that the graft is lying in the subretinal space. (C) Colour fundus photography of another 6-week-old rd mouse during another successful subretinal transplantation.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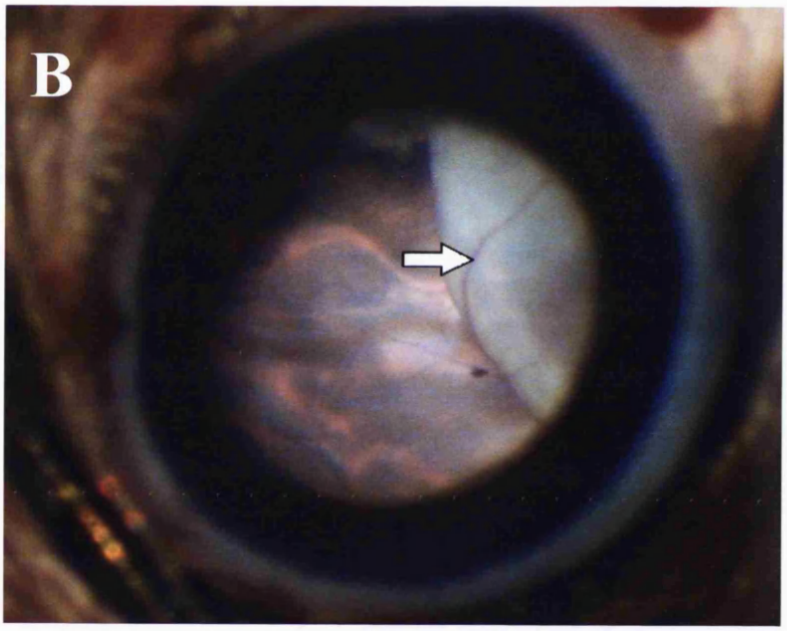
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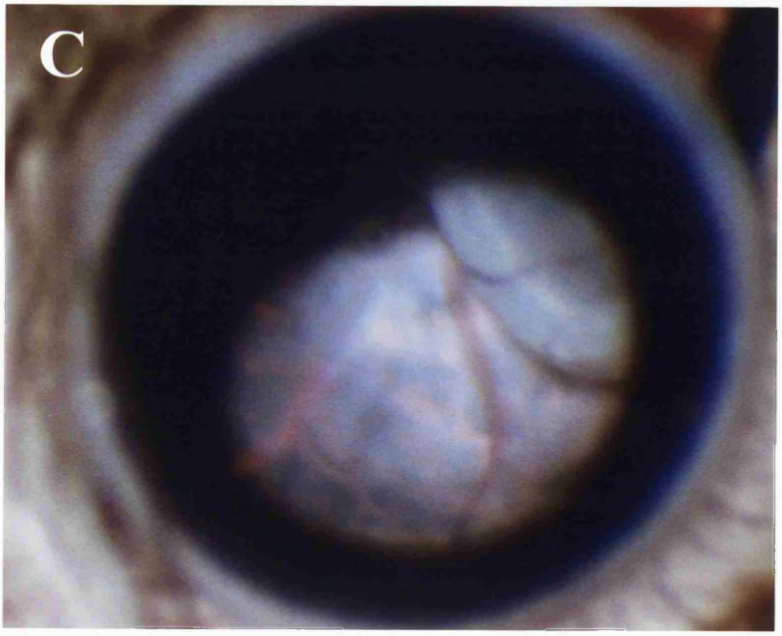
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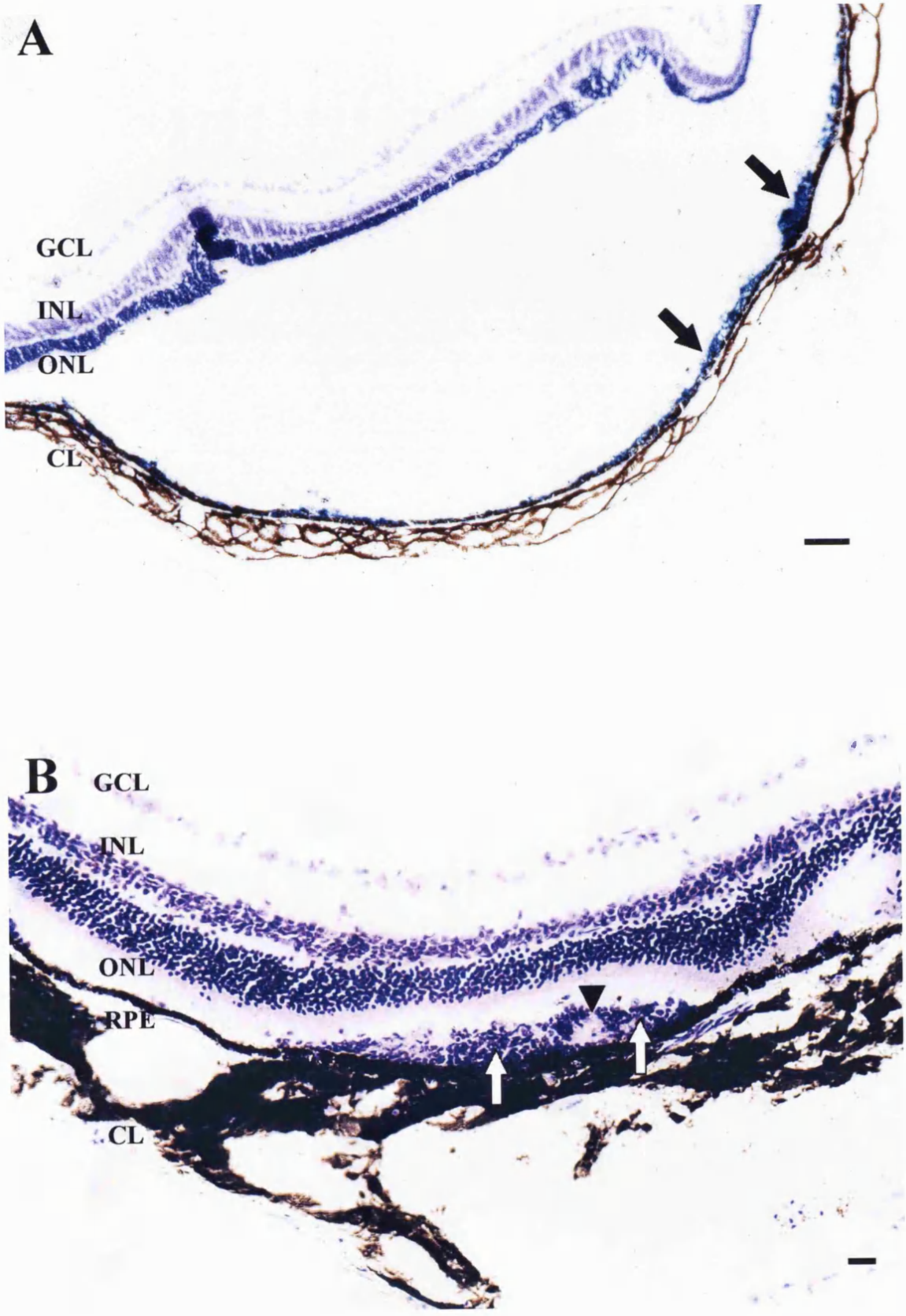
Figure 4

(A) Cresyl violet staining of a 6-week-old normal mouse retina immediately after injection of methyl blue ink into the subretinal space. There is a retinal detachment at the site of injection, but the blue ink (ARROW) can be clearly seen in the subretinal space lying above the RPE. Scale bar = 0.1 mm. (B) Cresyl violet staining of a 8-week-old normal mouse retina one week after subretinal transplantation of syngeneic 7-day-old normal mouse retinal microaggregates. The subretinal fluid has been reabsorbed and there is no retinal detachment. The transplanted cells (ARROW) can be seen lying between the ONL and RPE. Rosettes are indicated by ARROWHEAD. Scale bar = 0.01 mm.

GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear (photoreceptor) layer; RPE, retinal pigment epithelium; CL, choroidal layer.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2.4. Histology

2.4.1. Baseline study and mouse-to-mouse transplantation

Baseline Study

A baseline study was performed on normal and dystrophic mouse. Eyes from 9 normal mice and 9 dystrophic rd mice, 7-day-old (n=6), 6-week-old (n=6) and 12-month-old (n=6) were studied for light microscopy (Table 1). Retinal sections were studied and labelled with antibodies. The sections will be labelled for rhodopsin (for rods), protein kinase C (PKC, for rod photoreceptor related bipolar cells), GFAP (for astrocytes and activated Müller cells), RT97 (neurofilaments in ganglion and horizontal cells and their axons), calbindin (for horizontal cells and some amacrine cells), parvalbumin (for subtypes of amacrine cells and ganglion cells), F4/80 (for microglia and macrophages), P84 and 6G3 (for species-specific synaptic protein). They were compared with previous published reports and subsequent transplantation results.

Table 1. No. of animals used in baseline histology

	Normal mice	Dystrophic mice
7 day old	3	3
6 month old	3	3
12 month old	3	3

Transplantation studies (Mouse to mouse)

Transplantation studies (Mouse to mouse) used normal mouse retinae as donor material and dystrophic rd mice as recipients (See Flow Diagram below). A total of 66 six to eight-week-old dystrophic rd mice received bilateral subretinal transplants of neonatal (PN 7 - 9) syngeneic normal mouse retina as described above (a total of 132 eyes), this included all those that were tested in LDPT (n = 36) and electrophysiology tests (n =18). The animals were sacrificed at 2 (n = 30), 4 (n = 20), and 6 weeks (n = 16) after transplantation. After harvesting the eyes, 110 eyes (n = 55) were processed for light microscopy and 22 eyes (n = 11) were processed for electron microscopy.

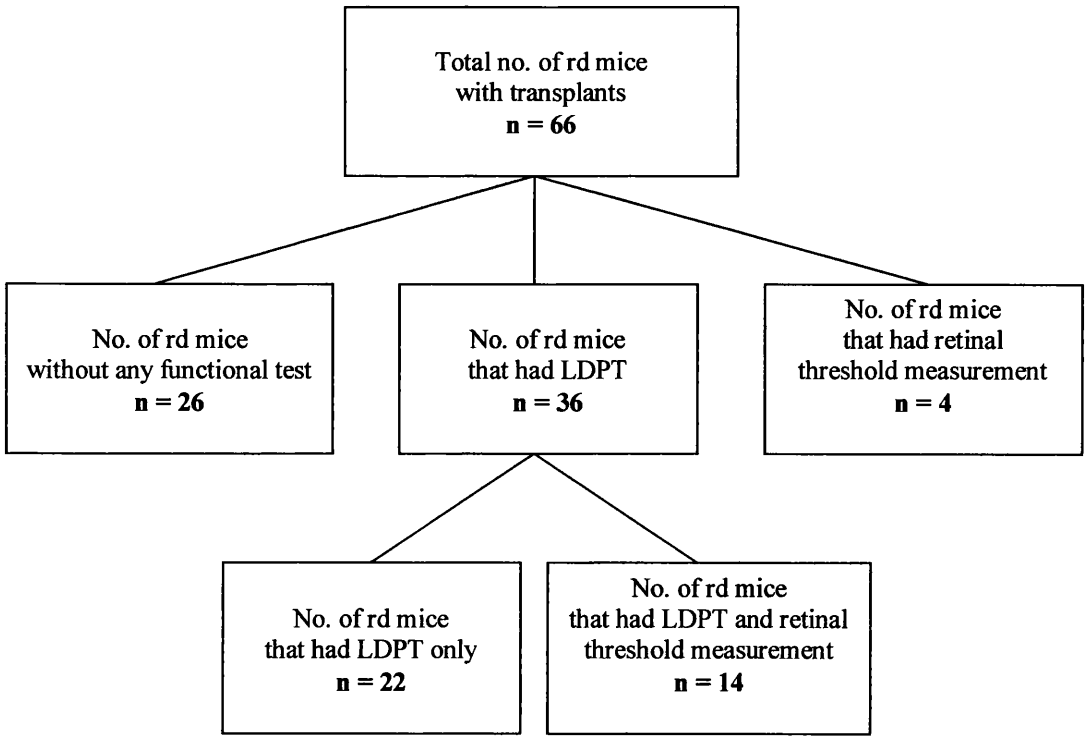


Table 2. No. of animals sacrificed (mouse to mouse transplant)

Weeks	Total no. of animals sacrificed at each time point (No. of animals that had LDPT before sacrificed)	
2	30	(14)
4	20	(15)
6	16	(7)
Total	66	(36)

Control Experiment

For sham control, the process of surgery and injection of carrying medium as used in other studies is not used here because in rd mice there is no significant host photoreceptor cells left for preservation. Therefore the sham control has to include cells for transplantation. Some studies used fibroblasts as sham with partly the reason that they might secret growth factors to preserve the remaining photoreceptors and again it may not be appropriate for the study. Final cerebellar homogenates was chosen as the material of cell sham control because it could test the possibility that any functional recovery was not specific for the retinal microaggregate graft but instead could be mimicked by other non-visual neural cells. (de Cerro et al., 1995) In my study, a total of 6 dystrophic rd mice (n = 6) received bilateral subretinal grafts (12 eyes) of cerebellar homogenates derived from 7-day-old syngeneic normal mice (PN = 7). The homogenates were prepared in the same way as retinal microaggregates. LDPT tests were carried out on these animals and eyes were harvested two weeks post-transplantation and processed the same way as above.

Light microscopy, immunohistochemistry and electron microscopy

For light microscopy, animals were perfused under tribromoethanol terminal anaesthesia with phosphate-buffered saline (PBS) and then 4% periodate/lysine/paraformaldehyde fixative (PLP) (see Section 2.4.4.) (McLean *et al.*, 1974). A suture was placed at the superior conjunctiva for orientation and the eyes were enucleated. The cornea was perforated and the eye was post-fixed in PLP for 1 hour and rinsed in PBS solution. The eyes were embedded in low-melting point polyester wax. Eight-micron sections were cut serially, mounted on charged glass slides and stored at 4°C until used. One series of sections from each eye was stained with Cresyl violet in order to locate the area of transplant, and selected slides from the other series of the same eyes were labelled using specific antibodies.

Sections were de-waxed briefly in absolute and 95% alcohol and washed with PBS (See Appendix I). The slides were incubated in 5% de-fatted milk (a blocking agent) to reduce non-specific antibody binding for 30 minutes. The following primary antibodies were used as appropriate:- rhodopsin (for rod photoreceptor, rho4D2, 1:3000, generous gift from Dr. R. Molday, University of British Columbia, Canada), PKC (for rod bipolar cells, MC5, 1:100, Santa Cruz Biotechnology, Inc., USA), RT97 (for horizontal cell and retinal ganglion cell axons; monoclonal anti-200-kd neurofilament protein, 1:500, generous gift of Dr. R. Morris, KCL Guy's Hospital, London, UK), GFAP (for astrocytes and reactive Müller cell processes, 1:1000, Sigma-Aldrich Co. Ltd. UK), Calbindin (mainly for horizontal cells and some subtypes of amacrine cells, 1:200, Sigma-Aldrich Co. Ltd. UK), Parvalbumin (for subtypes of amacrine cells, Serotec Ltd., Oxford, UK), F4/80 (for murine microglia and macrophages, Serotec Ltd., Oxford, UK), P84 (for mouse specific synaptic

protein, 1:1, generous gift from Dr C. Lagenaur, University of Pittsburgh, USA.) and 6G3 (for rat specific synaptic protein, 1:1, generous gift from Dr C. Lagenaur, University of Pittsburgh, USA) were applied at the appropriate concentrations diluted in antibody diluent (a mixture of PBS with 1% Bovine serum albumin). Slides were incubated in a humidified chamber with the primary antibodies overnight at 4°C. After washing in PBS for 3 x 10-minute, they were incubated with the secondary antibodies diluted in antibody diluent and 1% rat or mouse serum for 30 minutes. They were washed in PBS 3 x 10-minute and reacted with an immunohistochemical avidin-biotin-peroxidase complex system (standard Elite ABC kit, Vector Laboratories). After washing in PBS 3 x 10-minute, they were developed in a mixtured 3-3' diaminobenzidine solution (DAB; Sigma-Aldrich Co. Ltd. UK) activated with hydrogen peroxide and aqueous nickel ammonium sulphate for 3 minutes, washed in PBS and distilled water, dehydrated through a graded series of alcohol, cleared in xylene and mounted in DePeX (Merck/BDH, Littleworth, UK). The nickel ammonium sulphate changes the brown DAB product to black, making it easier to detect and to photograph.

For electron microscopy, after animals were deeply anaesthetised with tribromoethanol terminal anaesthesia, 0.5ml of fixative was injected into the posterior chamber of the eye. The fixative consisted of 2.5% paraformaldehyde, 2.5% glutaraldehyde and 0.01% picric acid in 0.1M cacodylate buffer (pH 7.4). The dorsal pole of each eye was marked with a suture; the eyes were removed and left in the same fixative for 24 hours at 4°C. After washing in cacodylate buffer, the lenses were removed, the retinae were post-fixed in 1% osmium tetroxide for 1 hour. After dehydration through a graded series of alcohols to epoxypropane, they were

embedded in TAAB embedding resin (TAAB laboratories Aldermaston, UK). Semi-thin sections were stained with 1% Toluidine blue in 1% borate buffer, and ultra-thin sections were contrasted with alcoholic uranyl acetate and lead citrate. Grids were viewed on a Jeol 1010 electron microscope.

2.4.2. Rat-to-mouse transplantation

In the final experiment, in order to study the host-graft interface, normal rat retinae of 7 to 9-day-old were used as donor material and transplanted into dystrophic rd mice. Once again the donor tissue was prepared the same way as above. Cryostat sections were prepared from these eyes and the host-graft interface can then be studied by the labelling the sections with rat-specific antibody, designated 6G3, that reacts with a component of the photoreceptor synaptic array, synaptic protein, but does not cross-react with mouse tissue. Thus, rat tissue transplanted to rd mice (maintained on an immunosuppressant) can be identified using such an antibody in addition normal EM would be employed to assess the synapses at the host-graft interface. A total of 14 two to three-month-old dystrophic rd mice received bilateral normal retinal grafts from 7-day-old normal rats (PN = 7). No functional tests were performed on these subjects. Ad libitum oral cyclosporin was used for immunosuppression (210 mg/litre of drinking water) from 2 days before transplantation until they were sacrificed. After one week 6 were sacrificed (3 for cryostat sections and 3 for electron microscopy study) and the other 8 subjects were sacrificed after the second week (4 for cryostat sections and 4 for electron microscopy study).

In these rat to mouse transplants, cryostat sections were prepared for light microscopy. Animals received an overdose of sodium pentabarbitone (Euthatal,

Rhońe Mérieux, Harlow, UK; 200 mg/ml), perfused intracardially with PBS and the eyes removed and frozen in optimal cutting temperature compound (OCT; Tissue-Tek, Miles, Raymond Lamb, London, UK) in liquid nitrogen vapour before sectioning on a cryostat. Slides were kept in -70°C freezer until used. Antibodies where appropriate were applied as before.

2.4.3. Reagents and reactions

2,2,2 **Tribromoethanol for anaesthesia**

Solution A:	2,2,2 Tribromoethanol	4.67 g
	100% ethanol	3.3 ml

Sonicate solution A until it is dissolved. Heat 250 ml of saline to 70°C and add solution A to saline. Shake the mixture well and store in foil wrapped bottle to prevent light degradation.

Dosage: 0.5 ml / 100 g body weight

Phosphate Buffered Saline (PBS) – (Stock – 10x working solution)

Distilled water (dH_20)	1800ml
Potassium Chloride (KCL)	4g
Potassium Phosphate Monobasic (KH_2PO_4)	4g
Sodium Chloride (NaCl)	160g
Dibasic Sodium Phosphate (Na_2HPO4)	22.8g

Mix the chemicals in the above order, make up to 2000ml with dH_2O. Dilute 1:9 parts for use as 0.1M solution and pH to 7.3

Periodate/lysine/paraformaldehyde fixative (PLP)

Solution A: Dissolve 13.7 g of lysine monohydrochloride in 375 ml of dH_20

Solution B: Dissolve 1.4 g of anhydrous Na_2HPO_4 in 100 ml of dH_20

Add A and B together and store at 4°C

Solution C: Dissolve 20 g of paraformaldehyde in 200 ml of dH_2O plus 3 drops of NaOH (0.1M)

Warm solution to 60°C to dissolve the content. Store at 4°C

Prior to use: Mix the above solutions, top up to 1 litre with PBS, adjust pH to 6.8 and then add 2.14g of sodium periodate

0.5% Cresyl Violet

Cresyl Violet stain	5g
Sodium Acetate	2g
dH_20	1 litre

Filter solution. Adjust pH to 3.4 with glacial acetic acid

Cresyl Violet staining for wax section

De-wax	100%	Ethanol	15 seconds
	95%	Ethanol	15 minutes
	(Dry in current of cool air for 30 minutes)		
Rehydrate	70%	Ethanol	2 minutes
	dH_20		2 minutes
Stain	0.5% Cresyl Violet		3 minutes
Differentiate	dH_2O		2 minutes
	70%	Ethanol	2 minutes
	95%	Ethanol/Acetic acid	15 seconds
	95%	Ethanol	2 minutes
	100%	Ethanol	2 minutes
	100%	Ethanol	2 minutes
De-fat	Histoclear Solution		4 minutes
	Histoclear Solution		4 minutes
Cover	coverslip with DePeX		

Immunohistochemistry

Retinal histological wax sections were mounted on gelatin double-subbed slides or charged slides and stored sealed in polythene bags at 4°C until labelling. They were de-waxed as for Cresyl violet staining before being processed as follows:-

Blocking stage (to reduce non-specific antibody binding with the tissue)

Incubate slides in 5% de-fatted milk (in PBS) at room temperature for 30 minutes

Primary antibody application

Pour off blocking solution, add primary antibody diluted to the appropriate concentration with antibody diluent (see Table 7). Incubates in primary antibody at 4°C overnight in a humidified chamber.

Washes

Wash slides in PBS 3 times in 10-minute intervals to remove primary antibodies.

Secondary antibody application

Add biotinylated secondary antibody solution. The secondary antibody should recognise the primary antibody. It was made up from an ABC Elite kit (Vector Labs., Peterborough, U.K.), antibody diluent and appropriate serum 30 minutes prior to application (see Table 7). Incubate for 30 minutes at room temperature.

Washes

Wash slides in PBS 3 times in 10-minute intervals to remove secondary antibodies.

Streptavidin-Horseradish Peroxidase (HRP) Complex

Streptavidin, which binds strongly to biotin, is complexed with HRP at this stage

Add 100 µl of Solution A and 100 µl of Solution B (ABC Elite kit) to 2.5 ml of PBS (for coverage of 10 slides), mix well and stand for 30 minutes prior to application. Cover the slides and incubate in room temperature for 30 minutes.

Washes

Wash slides in PBS 3 times in 10-minute interval to remove the ABC complex.

Diaminobenzidine – Nickel Intensification (DAB –Ni+) reaction (To visualise HRP)

Solution A	Nickel Ammonium Sulphate	1.39 g
	PBS buffer	50 ml
Solution B	DAB	100 mg
	PBS buffer	150 ml

Inside the fumehood, solution A is stirred until all the crystals dissolved and solution B is sonicated until all the DAB has dissolved. Add solution A to solution B and filter. Add 1.0 ml of 3% H_2O_2 and mix well. Place sections into the solution for 2 minutes or until desirable staining intensity is achieved. Dispose and clean all glassware and working surfaces with bleach after staining.

Counter-staining

Cresyl violet counter-staining was performed in some cases as above and all the slides were dehydrated and mounted.

P84, 6G3 and PKC Immunohistochemistry

These antibodies penetrate the sectioned material less readily and in order for them to attach to the appropriate antigenic sites, permeablisation step was required in the slide preparation. After de-waxing and drying in a cool current of air for 30 minutes the slides were covered with a solution of 0.05% Triton X-100 in PBS and 10% Horse Serum (Blocking serum for non-specific antibody staining). The slides were incubated for 30 minutes at room temperature. They were washed in PBS 3 times in 10-minute intervals. Primary antibody application (P84, 6G3 or PKC) and subsequent steps were followed as above

Table 3. Antibodies used and concentrations

Primary antibodies	Raised in	Primary antibody concentration	Secondary antibodies	Secondary antibody concentration	Serum
GFAP	Rabbit	1 : 1000	Anti-rabbit	1 : 150	Rat
αPKC	Mouse	1 : 100	Anti-mouse	1 : 150	Rat
Calbindin	Mouse	1 : 200	Anti-mouse	1 : 150	Rat
F4/80	Rat	1 : 10	Anti-rat	1 : 150	Mouse
Rhodopsin	Mouse	1 : 3000	Anti-mouse	1 : 150	Rat
P84	Rat	1 : 1	Anti-rat	1 : 150	Mouse
RT97	Rat	1 : 500	Anti-rat	1 : 150	Mouse
6G3	Mouse	1 : 1	Anti-mouse	1 : 150	Rat
Parvalbumin	Mouse	1 : 1000	Anti-mouse	1 : 150	Rat

2.5. Light-dark preferential test (LDPT)

2.5.1. Pilot study

As nocturnal animals mice have a strong preference for low illumination areas (Hascoet and Bourin, 1998; Hascoet *et al.*, 2001). However, in order to display light avoidance behaviour the animal first needs to discriminate between light and dark illumination. In the present study a *low* level of illumination (*dim* illumination) is used. This will allow us to investigate the discriminatory response of the rd mouse in more detail at near threshold levels and also provides us with a behavioural paradigm within which it should be possible to identify functional improvements in rd mice with successful photoreceptor transplants.

In collaboration with Dr. Peter Coffey from, Department of Psychology, University of Sheffield, behavioural tests have been devised to assess the visual function of rodents. A pilot study was performed by Mr. P Oliver (BSc thesis, Department of Psychology, University of Sheffield) in Sheffield in which animals were placed in a circular open field arena. The arena was constructed from a 250 x 50 cm aluminium sheet, screwed together to form an open-ended cylinder of 80 cm in diameter. This was placed over a Formica base that had previously been marked out into 3 equal segments. A three-segment cover was placed on top of the cylinder. This was composed of equally sized segments of black opaque and clear Plexiglas plus an open section. The cover was interchangeable allowing the segment that came under shade to be changed for each trial. This was a precautionary measure to rule out extraneous odours as external cues. Illumination was provided by a 500 watt halogen lamp, which was suspended approximately 1 metre above the centre of the arena and

controlled by a rheostat providing 3 different levels of illumination. Animals were placed in the arena (through the open segment) for four 5-minute sessions: one under red light conditions and three levels of white light illumination (5, 560, 1020 and 1250 lux). The experiment aimed to establish whether animals were capable of exhibiting light-dark preference behaviour by seeking out the dark segment, which would be expected for normally sighted rodents. The total time spent under the dark segment was recorded. Results showed that there was a luminance dependent reduction in the amount of time spent in the dark segment in 6-week-old dystrophic rd mice as compared to 6-week-old normal mice. These behavioural tests provided a simple statement of baseline visual performance for rodents and a quick screening method to assess the efficacy of transplants (Coffey *et al.*, 1998; Kwan *et al.*, 1998). The results of this experiment have shown that 53-day-old rd mice were capable of displaying light avoidance behaviour if the stimulus was of a high enough intensity (1250 lux). Furthermore it demonstrated that at high illumination levels, this ability was not significantly different from age-matched normal mice. At low intensity illumination levels however, rd mice had problems in using changes in illumination to guide their behaviour. This difference was particularly evident with red illumination (5 lux).

2.5.2. A computerised tracking system – Tru Scan™

In order to assess accurately the time that a mouse spent in a light or dark environment at a low luminance level, a computerised tracking system, Tru Scan™ developed by Coulbourn instruments (USA), was used. It had a square cage arena measuring 10.2 inches x 10.2 inches, and surrounding the cage was a photobeam (infra-red) square sensor which could detect in two dimensions the position of an

animal placed inside the cage (Figure 5A). The photobeam sensor had beams that were 0.6 inches apart. The co-ordinates of the animal were continually updated every second and were fed via a relay box into a computer. The arena was divided into a light and a dark segment with the light/dark or retreat box enclosing half the arena and which had a doorway allowing the animal to enter or leave. This box was a complete 5-sided box that reached fully to the floor with no distracting light gap or holes at the bottom for photobeams. Instead the box was constructed from the same plastic filter material as for the beam filters on the sensor rings. This material was opaque to visible light (appearing black) but transparent to the infra-red beams. Since the entire wall of the box passed the photobeams, it was possible to locate the animal inside the box at any time. The doorway, measuring 4 cm x 4cm was centred on the arena when the box was placed on one side of the arena (Figure 5B). Therefore when an animal was placed in the arena with the retreat box in place, it could freely roam between the light and dark area. The floor of the arena comprised a removable tray. The retreat box and the floor tray were cleaned thoroughly between tests to remove odour as a behavioural cue. The sensor ring was connected to a relay station via a 9-pin cable. The relay station gathered all the information and fed it into a Windows™ PC computer (Intel Pentium 266 MMX, 64 MB RAM) via an interface ISA card (L18-16S/C). The Tru Scan™ software analysed the data and provided a read-out of the time an animal spent in the light or dark segment over the study period and a "track"-type plot of the co-ordinates during the study period.

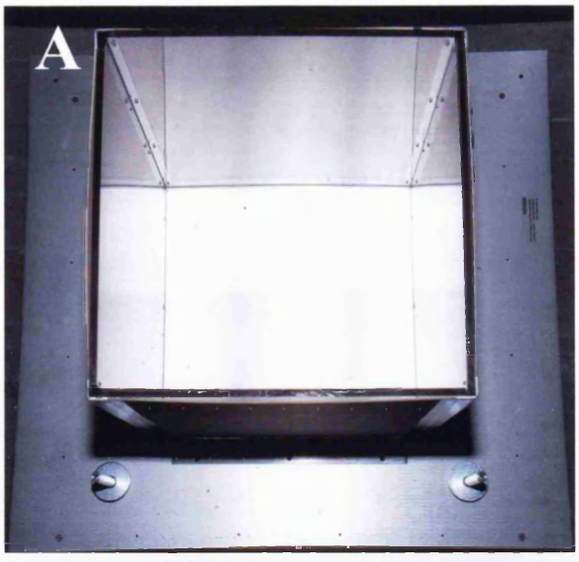
One luminance level was used and the light source was provided by Schott KL1500 fibre-optic lamp (15V, 150W) via its two fibre-optic extensions (Figure 5C). A white plastic diffuser sheet was installed 10 cm below the two fibre-optic extensions, and

this provided even illumination of the floor of the arena on the light side which was measured as 2 units (cd/m^2) of luminance with a Minolta chroma meter CS100, whilst the dark segment had a zero reading. A pair of speakers driven by an untuned AM radio signal was placed beneath of the arena. This generated white noise which helped to eliminate other background noises as behavioural cues. This equipment was located in an air-conditional darkroom, and the room was kept at a constant temperature of 21°C, the same as the normal animal storage facility. The temperature in the two compartments were also measured and both were recorded at 21°C. The light compartment could maintain room temperature because the fibre-optic lamps provided the light source and the light bulb that generated the light was placed well away from the testing arena.

A ***pilot study*** was set up initially to measure the minimum length of time required to assess the mice in the arena before consistent results were achieved. Six normal and 6 dystrophic 6-week-old mice were used. They were placed separately in the arena for 15 minutes, 30 minutes and 60 minutes on separate occasions randomly for three consecutive days (a total of 9 tests per mouse). The time they spent in the dark segments was analysed. Results indicated that for each mouse the 15-minute testing period led to variable times being spent in the dark segments. More consistent results were obtained with the 30-minute and 60-minute testing periods. The variability probably reflects the time it took for the animal to initially settle in the arena. The first 15 minutes or so may be required to familiarise themselves with their surrounding before exhibiting their light-dark preference behaviour. Therefore a 30-minute testing period was chosen for subsequent studies.

Figure 5

Tru Scan™ apparatus. (A) View of the arena from above showing the 4 walls and the white removable tray at the bottom. (B) View from above with the dark compartment in place. The doorway between the light and dark compartments can be seen (ARROW). (C) The apparatus is situated in an isolated darkroom, the fibre-optic light source (ARROW) and diffuser (ARROWHEAD) are in place and the computer is linked to the arena via a relay box.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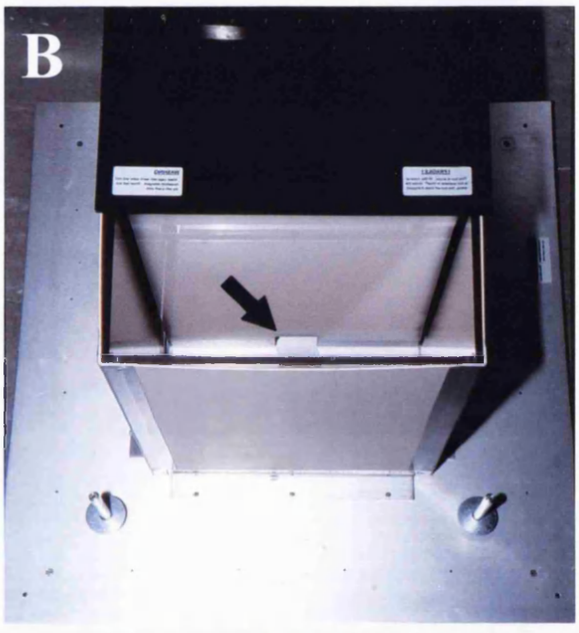

B


C

2.5.3. Experimental Design

Baseline Study

To assess whether the normal and dystrophic mice have a preference for the dark compartment or a difference in their preferences, the dark compartment time (DCT) was measured. It was defined as the time spent in the dark compartment over a pre-determined period. Two baseline studies were set up to measure the DCT of normal and dystrophic rd mice with respect to age prior to a transplantation study.

The first was a *cross-sectional study*, 4 dystrophic rd mice and 4 normal mice were randomly included for each age group, namely 6 weeks, 3 months, 6 months and 12 months, producing a total of 16 dystrophic rd mice and 16 normal mice. Two separate DCT measurements were made for each mouse (RUN 1 and RUN2) two weeks apart. For the test period of 30 minutes (1800 seconds), an animal without any preference for either the light or dark compartment would spend an equal amount of time in both compartments, namely 900 seconds.

Table 4. No. of animals used in baseline cross-sectional study of LDPT

	6 weeks	3 months	6 months	12 months
Normal mice	4	4	4	4
Dystrophic mice	4	4	4	4

The second was a *longitudinal study*, in which a total of 6 normal mice and 6 dystrophic rd mice were randomly selected at birth. Tru Scan™ tests were performed at 6 weeks, 3 months, 6 months and 12 months of age. In the normal group, DCT results were obtained in all the animals at 6 weeks (n = 6) and 3 months (n = 6) but at 6 months and 12 months three of the mice had died and only 3 recordings were obtained at these time points. In the dystrophic groups, DCT results were obtained from all the animals at all the time points except one animal had died by twelve months and so only 5 recordings were made at 12 month.

Table 5. No. of animals used in baseline longitudinal study of LDPT

	6 weeks	3 months	6 months	12 months
Normal mice	6	6	3	3
Dystrophic mice	6	6	6	5

Transplantation study

Finally, a transplantation study examined DCT in dystrophic mice that received retinal implants or sham injections. All animals were tested before transplantation and post-transplantation, and DCT was measured using the Tru Scan™ test. A total of 36 dystrophic rd mice received bilateral retinal transplant by the method described above. DCT was measured again at 2 (n = 36), 4 (n = 22) and 6 (n = 7) weeks post-operatively and some of the subjects were sacrificed after DCT measurements for histological analysis (see Table 2). Prior to performing any statistical analysis on the transplant study LDPT results, the retinae of all animals that received retinal implants

were first examined for photoreceptor survival. On histological analysis, mice with evidence of surviving retinal transplants and their DCT results were analysed as a separate group.

A total of 6 dystrophic rd mice received cerebellar homogenates in their subretinal space and acted as sham controls, again Tru Scan™ measurements were performed prior to and 2 weeks post-transplantation.

Table 6. No. of rd mice in transplant and sham control groups in LDPT

	Pre-op	2 week	4 weeks	6 weeks
Transplant group	36	36	22	7
Sham control	6	6	-	-

2.5.4. Statistical analysis and strategy

On the advice of statisticians from the Department of Psychology, University of Sheffield, ANOVA (analysis of variance) was the chosen statistical method for data analysis. ANOVA is a powerful parametric statistical method that applies to categorical independent variables associated with continuous dependent variables. It is a natural and logical extension of the t-test because it compares means between groups. It has greater application than the t-test because comparison between two or more groups can be made. ANOVA uses the F probability distribution and, like the t-test or chi-square test, degrees of freedom must be taken into account. Here both one-

way and two-way ANOVA analyses are required and multiple factors influences are also taken into account. Here it is used to test the null hypothesis of no difference between different groups.

An analysis of variance (ANOVA) was performed on the data using a commercial statistical package (StatView, SAS Institute Inc, USA). In the cross-sectional study, a 3-factor ANOVA with one repeated measure (Group [normal versus dystrophic] x Age [6 weeks, 3 months, 6 months, 12 months] x Test [RUN 1, RUN 2]) was used to analyse the data. Test was the repeated measure. For the longitudinal study, a 2-factor ANOVA with one repeated measure (Group [normal versus dystrophic] x Age [6 weeks, 3 months, 6 months, 12 months]) was used to analyse the data. Age was the repeated measure. The aim was to examine whether normal sighted mice would spend significantly more time in the dark compartment compared to visually impaired dystrophic rd mice. The hypothesis was there was no difference in the time they spent in the dark compartment.

The transplant animals were separated into two groups, a histology-positive group and a histology-negative group, based on the presence or absence of graft post-transplantation. Using these criteria, a 2-factor ANOVA with one repeated measure (Group [histology-positive, histology-negative, Sham] x transplantation [pre-transplantation, post-transplantation]) was used to analyse the data. Pre- and post-transplantation measurements were the repeated measure. The hypothesis was that there was no difference in DCT between the histology-positive group and the others.

2.6. Retinal threshold measurement

Dr Y. Sauvé performed the retinal threshold measurements in our electrophysiology laboratory at the Institute of Ophthalmology. My involvement was in preparing the animals and in helping to collect the data and assisting Dr Sauvé with the experiments. Animals were anaesthetised with urethane (1.25 g/kg, intraperitoneally), the head held by a nose bar and the test eye stabilised with three equidistant subconjunctival sutures attached to the fixation frame. This provided maximal eye stability without substantially obstructing the animal's field of vision. In order to eliminate the potential effects of variations in resting pupil size, full dilation was obtained with topical Tropicamide (1.0%; Alcan Laboratories Ltd, UK). A non-correcting contact lens protected the cornea. Body temperature was monitored and maintained at 37°C. A translucent hemisphere (Ganzfield bowl, 110 cm in diameter) was centred on the optic disc projection of the tested eye as visualised by an ophthalmoscope (Figure 6A). A one-hour period of adaptation at 0.02 cd/m^2 was implemented prior to recording to ensure rod activation. Single- and multi-unit recordings were made from the superficial layers of the SC to a depth of 100 - 300 μm using glass-coated tungsten electrodes (custom made in Dr Sauvé's laboratory; resistance: 0.5 MΩ); care was taken to record sufficiently superficially to avoid the larger field cells of the deep stratum griseum superficiale and stratum opticum. Preliminary studies in which the SC was exposed by aspiration of the overlying cortex indicated that recording sites (localised by stereotaxic positioning) covered the full extent of the SC with the exception of its medial bank, which was located just under the superior sagittal sinus. No cortical ablations were made in this study in order to avoid possible alterations of SC light-evoked responses. The microelectrode penetrations were made along a rectilinear grid of 100 μm steps. At sites where a

response to whole-eye illumination was found, an attempt was made to define a discrete receptive field (RF) for that unit. The visual field was systematically searched by moving spots of light (38mm diameter with luminance of up to 4.5 log cd/m^2 higher than background) across the surface of the translucent hemisphere (background luminance kept at 0.02 cd/m^2). Once a response could be elicited in this manner, the RF was located and its centre defined. The RF was then illuminated with a flashing light centred on the RF (spot of 38 mm in diameter with 1 second duration at 3 seconds intervals). Neutral density filters were added progressively (minimal steps of 0.1 log filters; unfiltered stimulus was 4.5 log illumination above background) to establish the minimal illumination required to elicit a response.

Threshold values for a total of about 76 points (usually separated by 200-μm recording steps in the SC) were presented as maps with topological reference to the retina (Figure 6B). In area where there was no response even with the highest setting, symbol "n" is designated or an auditory response when there was no response with light, symbol "a" is designated in the results.

To further characterise the electrical signal recorded, thus responsiveness of the RF illumination, a post-stimulus time histograms (PSTH) can be assembled over 30 consecutive stimuli (1-second duration, 3-second intervals). To maximise the response a stimulus of 4.5 log unit above background is used. PSTH reveals the latency of the ON-response (seconds), the activity of the response in number of spikes of electrical signal and the duration of the ON-response.

Baseline study

A total of 3 normal mice (one 6-week-old, two 3-month-old) and 9 dystrophic mice (five 8-week-old, one 9-week-old, three 3 -month-old) was used for the baseline study. A 76-point retinotopic map was plotted across the SC as described above. For operational reasons, all the baseline recordings were performed on the right eye. As the electrophysiological test was a terminal experiment (no recovery from anaesthesia was allowed according to Home Office regulations), animals were sacrificed after each experiment and the eyes were harvested for histology as described above.

Transplanted group

A total of 18 dystrophic rd mice that received "good" retinal transplants, as defined by good intra-operative subretinal graft appearance observed through the operating microscope, were included in the study. All the threshold testing was performed 2 to 3 weeks after transplantation when the mice were between 8 to 11 weeks of age (Table 7). All the animals received bilateral transplants and only the side with the "good" transplant was tested, therefore a total of 18 retinotopic threshold maps were obtained (Figure 11).

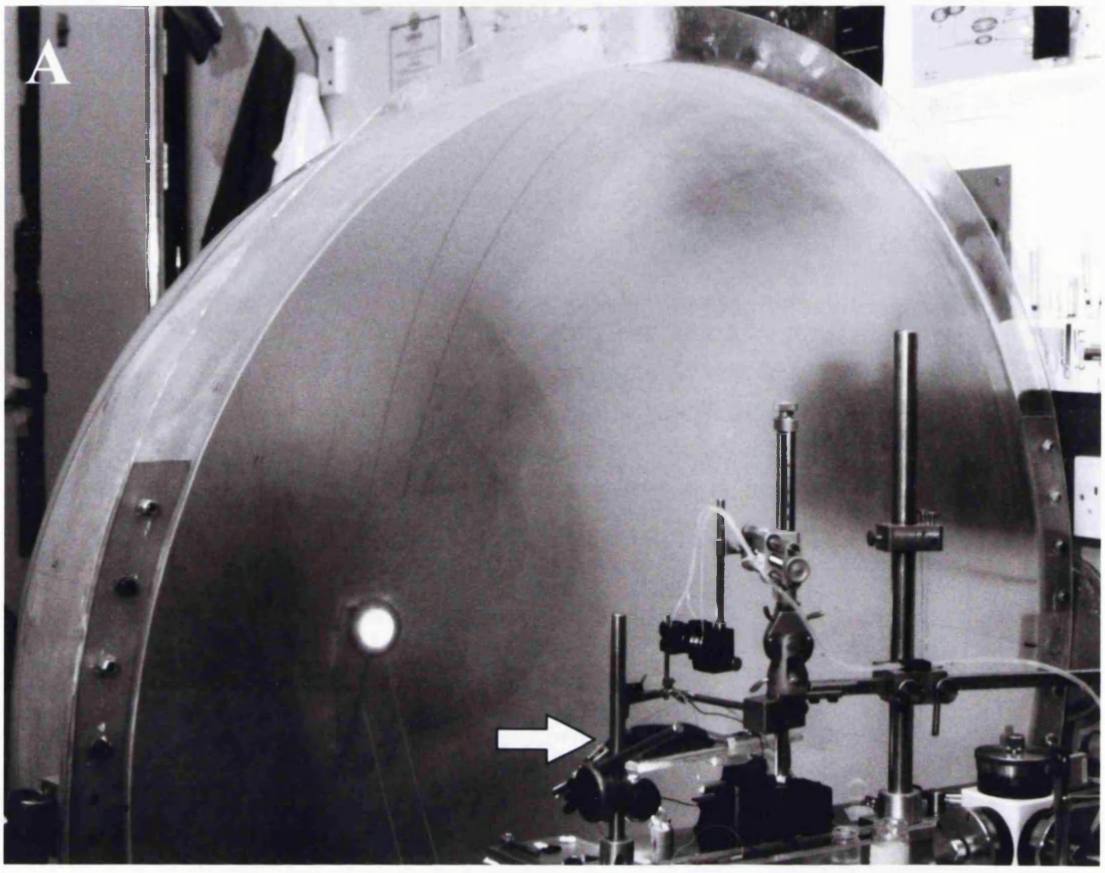
Table 7. Retinal threshold measurement time points for transplanted animals.

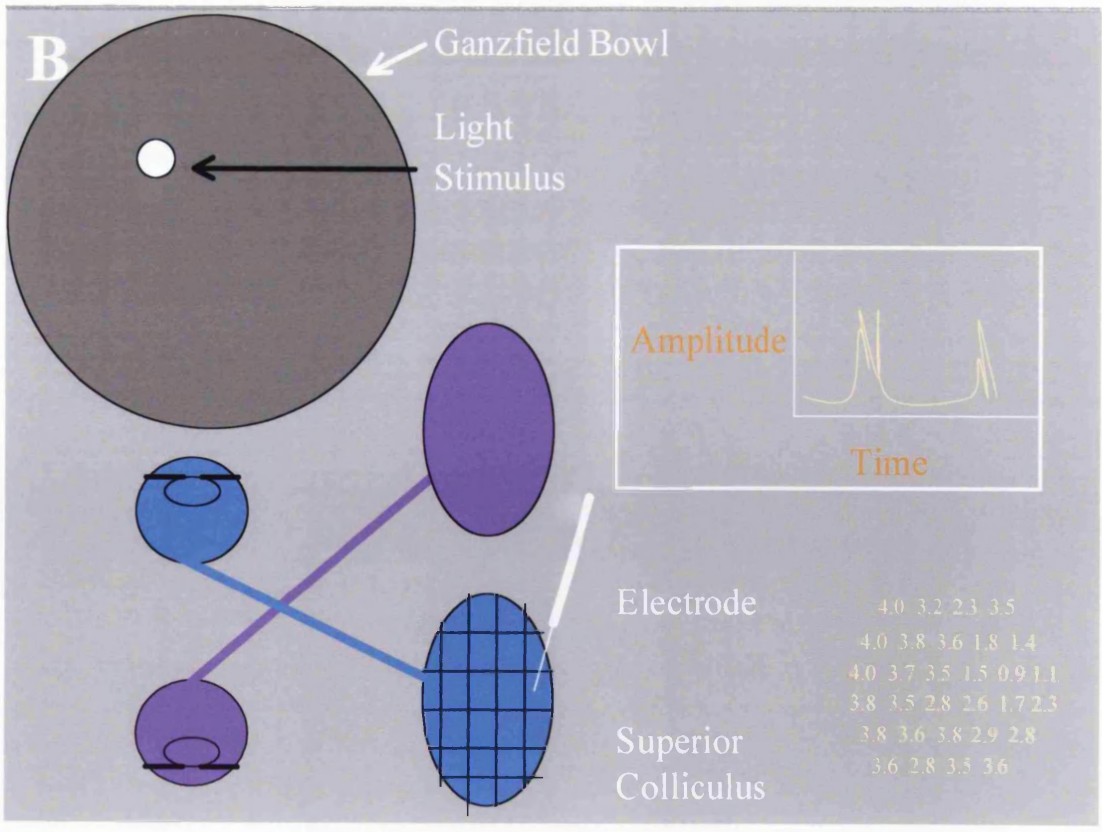
(Subject codes relate to animal from Figure 11)

Subjects	Side	Age at time of tests (weeks)	Weeks after transplantation
XIII	R	10	3
XIV	L	10	3
XV	L	10	3
XVI	R	11	3
XVII	R	11	3
XVIII	R	11	3
XIX	R	8	2
XX	R	8	2
XXI	R	8	2
XXII	L	9	3
XXIII	L	8	2
XXIV	L	8	2
XXV	L	11	3
XXVI	R	10	2
XXVII	L	9	3
XXVIII	R	9	3
XXIX	L	9	3
XXX	R	9	3

Figure 6

Retinal threshold measurement apparatus. (A) The retinal threshold measurement apparatus is seen. The light source is mounted on guide wires behind the semi-transparent hemisphere (the ganzfield bowl). The subject (ARROW) is mounted with a nose bar and positioned at the centre of the hemisphere. (B) Schematic diagram showing the electrode penetrating into the superficial layer of the superior colliculus for threshold measurement. When a sufficient light stimulus is presented and the retinal threshold is reached at a particular point of the retina, an electrical signal of on- and off- responses can be recorded. Threshold is measured by inserting different neutral density filters in front of the tested eye until no recording can be made. A 76-points retinotopic map is eventually obtained


A


B
Ganzfield Bowl
Light
Stimulus
Amplitude
Time
Electrode
Superior
Colliculus
4.0 3.2 2.3 3.5
4.0 3.8 3.6 1.8 1.4
4.0 3.7 3.5 1.5 0.9 1.1
3.8 3.5 2.8 2.6 1.7 2.3
3.8 3.6 3.8 2.9 2.8
3.6 2.8 3.5 3.6

Chapter 3 - Results

3.1. Light-dark preferential test results

3.1.1. Baseline study of normal and dystrophic mice

In the ***cross-sectional study***, Figures 7A and 7B show the results of RUN 1 and RUN 2 respectively (see also Table 8 and 9). Mean DCT of normal and dystrophic mice were compared at all the age groups. On average, normal mice (range 564 to 1397 seconds, 31% to 77.6%) spent more than 900 seconds in the dark compartment in both RUN 1 and RUN 2 except for the 6-week-old group in RUN 1 which spent 811.0 ± 77.9 seconds (41.5 ± 4.3%) in the dark compartment. The dystrophic rd mice on average (range 444 to 951 seconds, 24.7% to 52.8%) spent consistently less than 900 seconds in the dark segment in both RUN 1 and RUN 2 in all age groups (Figure 7). These results show a large variation in the dark compartment times. The difference between the normal and dystrophic group was apparent from the earliest time point tested (Figures 7).

Table 8. Cross-sectional study for LDPT - RUN1 (mean DCT time in seconds ± standard error of the mean)

	6 weeks	3 months	6 months	12 months
Normal mice	811.0 ± 77.9	1102.5 ± 64.6	1087.8 ± 51.7	1009.8 ± 47.1
Dystrophic mice	633.5 ± 83.5	646.0 ± 23.5	706.5 ± 48.9	688.3 ± 15.9

Table 9. Cross-sectional study for LDPT - RUN 2 (mean DCT time in seconds ± standard error of the mean)

	6 weeks	3 months	6 months	12 months
Normal mice	930.8 ± 46.7	1256.3 ± 106.4	1149.0 ± 87.3	1088.8 ± 60.0
Dystrophic mice	625.3 ± 37.1	756.5 ± 66.9	544.8 ± 34.5	758.8 ± 22.7

Statistical analysis showed a significant difference in the amount of time the normal and dystrophic animals spent in the dark compartment (Cross-sectional study: $F=131.80$, $df=1,3$ $p<0.0001$). The normal group showed a significant increase in the time spent in the dark compartment from 6 weeks to 3 months as compared to the dystrophic group ($F=3.3$, $df=3,24$ $p=0.038$).

In ***longitudinal study***, normal mice (range 564 to 1257 seconds, 31.3% to 69.8%) on average spent more than 900 seconds in the dark segments at all the time points and in contrast on average the dystrophic mice (range 541 to 907 seconds, 30.1% to 50.1%) consistently spent less than 900 seconds in the dark segment at all the time points (Table 10, Figure 8).

Again statistical analysis showed a significant difference in the amount of time the normal and dystrophic animals spent in the dark compartment (Longitudinal study: $F=80.64$, $df=1,6$ $p<0.0001$). The difference between the normal and dystrophic group was apparent from the earliest time point tested (Figures 8). Graphically, in the longitudinal study (Figure 8), there was an increase in the amount of time both groups

spent in the dark compartment as the animals aged, however this was not significant ($F=3.01$, $df=3,18$, $p>0.05$).

Table 10. Longitudinal study for LDPT (mean DCT time in seconds ± standard error of the mean)

	6 weeks	3 months	6 months	12 months
Normal mice	967.2 ± 68.3	961.0 ± 99.3	1133.0 ± 269.7	1379.3 ± 204.1
Dystrophic mice	666.5 ± 49.7	726.0 ± 26.5	713.2 ± 42.4	839.4 ± 31.6

These results rejected the null hypothesis of no difference between normal and dystrophic animals in the light-dark preferential test. Therefore a simple light-dark preferential test to assess the time spent in the dark compartment can distinguish between normally sighted mice and visually impaired dystrophic rd mice through the observation that normal mice spend more time in the dark compartment than dystrophic mice..

3.1.2. Effects of retinal transplantation

Pre-operatively in the total transplant group, the mean DCT was 769.8 ± 34.0 seconds (42.8 ± 1.9%). Post-operatively this average was slightly increased to 889.8 ± 35.3 seconds (49.4 ± 2.0%) at 2 weeks, 877.8 ± 78.5 seconds (48.8 ± 4.4%) at 4 weeks, and 866.8 ± 104.3 seconds (48.2 ± 5.8%) at 6 weeks (Table 11, Figure 9). This increase in DCT was more obvious in mice with evidence of subretinal grafts

(histology-positive group, n=10) where the DCT increased from 795.1 ± 36.3 seconds (44.2 ± 2.0%) preoperatively to 991.5 ± 53.2 seconds (55.1 ± 3.0%) at 2 weeks (n=10), 1085.8 ± 276.7 seconds (60.3 ± 15.4%) at 4 weeks (n=4) and 913 seconds (50.7%) at 6 weeks (n=1) postoperatively. Histological analysis showed no graft survival in any of the sham controls. The sham controls had a mean DCT of 742.5 ± 42.9 seconds (41.3 ± 2.4%) preoperatively and 843.3 ± 96.1 seconds (46.9 ± 5.3%) at two weeks after transplantation. Table 12 shows the mean DCT results of the histology-positive transplant group, histology-negative group and sham control pre-operatively and at 2 weeks postoperatively (Figure 10).

Table 11. All transplant group and sham control (mean DCT time in seconds ± standard error of the mean)

	Pre-op	Post-op 2/52	Post-op 4/52	Post-op 6/52
All transplant group	769.8 ± 34.0	889.8 ± 35.3	877.8 ± 78.5	866.8 ± 104.3
Sham control	742.5 ± 42.9	843.3 ± 96.1	-	-

The results in Figure 10 show that all the groups have a larger DCT at two weeks post-transplantation. There was a significant increase in the amount of time spent in the dark compartment post-surgery (Pre/Post: $F=6.26$, $df=1,39$, $p<0.05$; Figure 24) with the increase being more evident in the histology-positive group. However, this increase was not significantly different between the groups (Group x Pre/Post: $F=0.50$, $df=2,39$, $p=0.63$). Probably due to the small "n" numbers in each group and large individual variations, there were no significant differences between the

histology-positive group, histology-negative group and sham animals (Group: F=1.4, df=2,39, p=0.25). In conclusion, these findings show that while there is a significant increase in the amount of time spent in the dark compartment post-transplantation, which is more evident in the histology-positive group, but the difference between groups does not achieve significance.

Table 12. Histological positive, negative transplant and sham control groups (mean DCT time in seconds ± standard error of the mean)

	Pre-op	Post-op 2/52
Histology-positive group (n=10)	795.1 ± 34.0	991.5 ± 35.3
Histology-negative group (n=26)	760.0 ± 44.7	860.7 ± 42.2
Sham control (n=6)	742.5 ± 42.9	843.3 ± 96.1

Figure 7(A)

Baseline cross-sectional study (RUN 1) comparing dark compartment times (DCT, seconds) between normal and dystrophic rd mice. (Error bars = standard error of the mean)

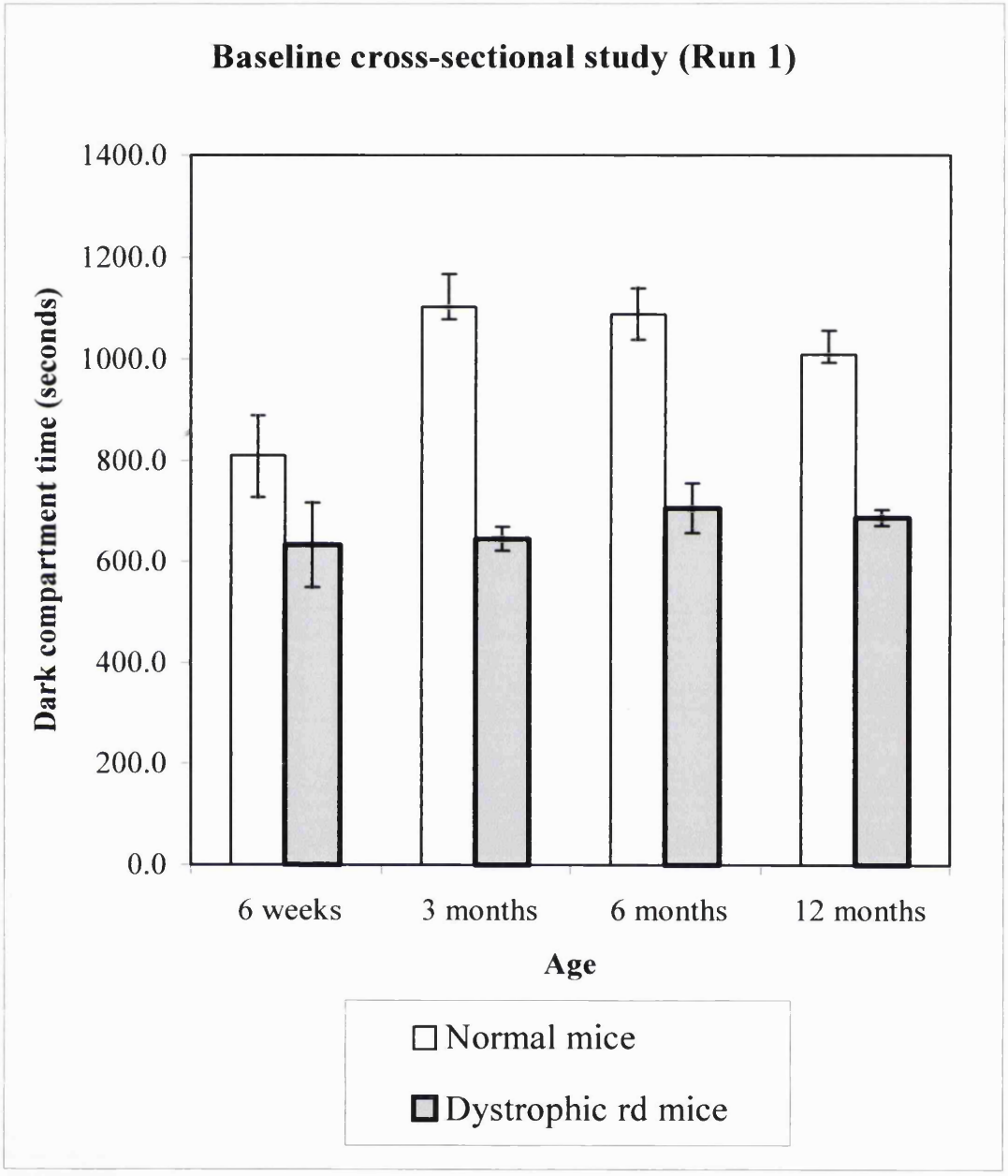

Baseline cross-sectional study (Run 1)
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
Dark compartment time (seconds)
6 weeks
3 months
6 months
12 months
Age
Normal mice
Dystrophic rd mice

Figure 7(B)

Baseline cross-sectional study (RUN 2) comparing dark compartment times (DCT, seconds) between normal and dystrophic rd mice. (Error bars = standard error of the mean)

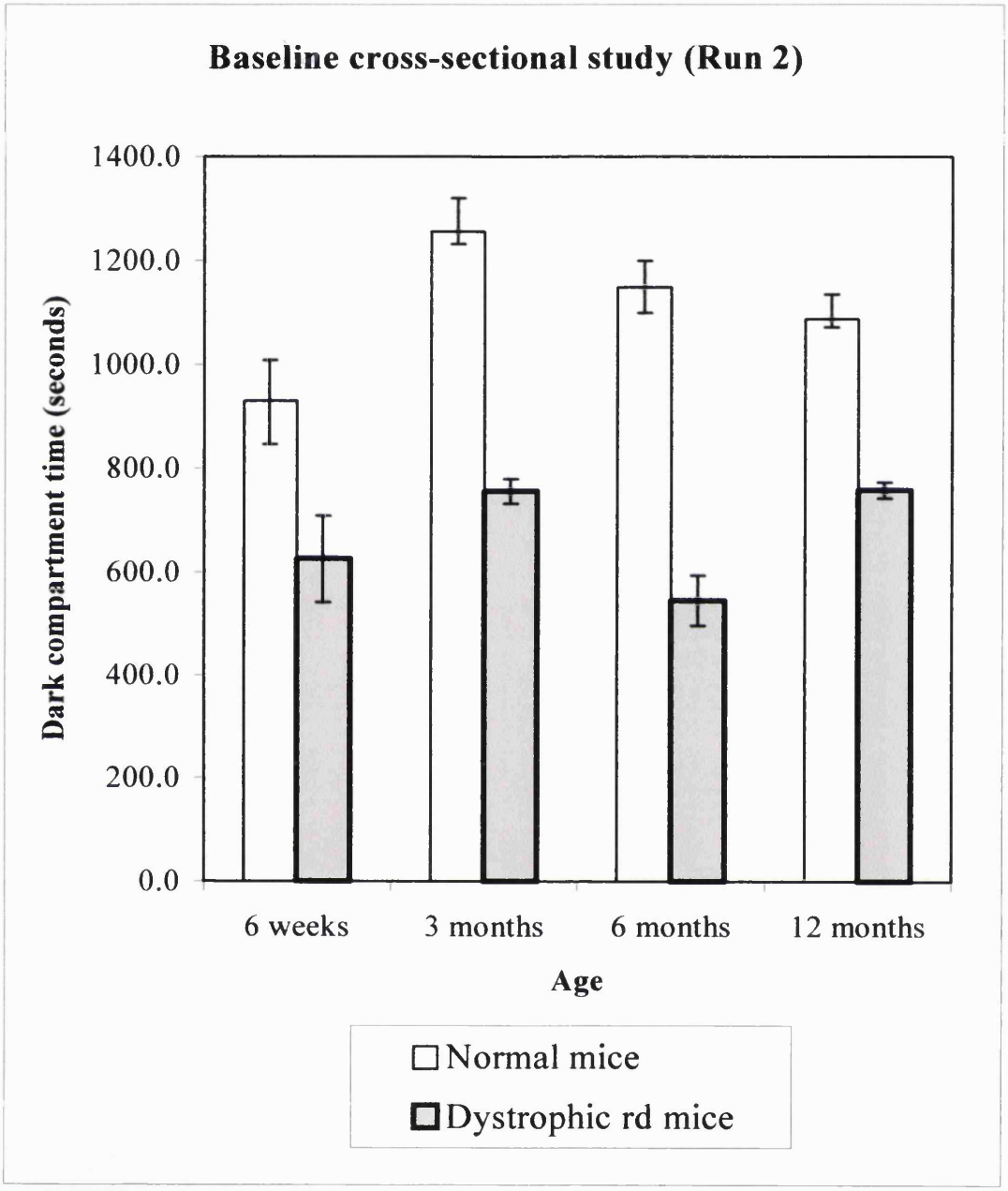

Baseline cross-sectional study (Run 2)
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
Dark compartment time (seconds)
6 weeks
3 months
6 months
12 months
Age
Normal mice
Dystrophic rd mice

Figure 8

Baseline longitudinal study comparing dark compartment times (DCT, seconds) between normal and dystrophic rd mice. (Error bars = standard error of the mean)

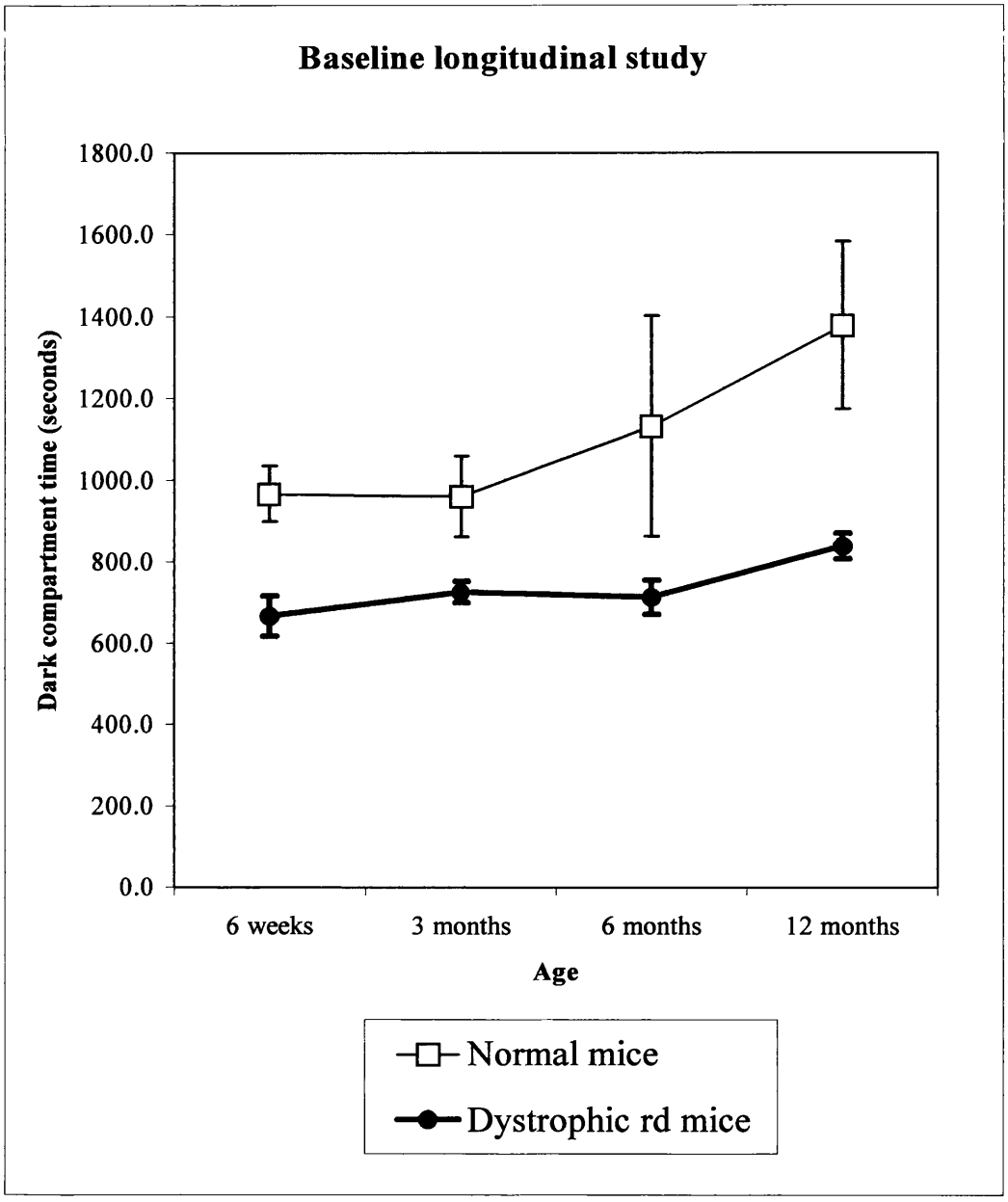

Baseline longitudinal study
1800.0
1600.0
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
Dark compartment time (seconds)
6 weeks
3 months
6 months
12 months
Age
Normal mice
Dystrophic rd mice

Figure 9

Pre- and post-transplantation LDPT results showing dark compartment times (DCT) between sham control group and all transplant group. (Error bars = standard error of the mean)

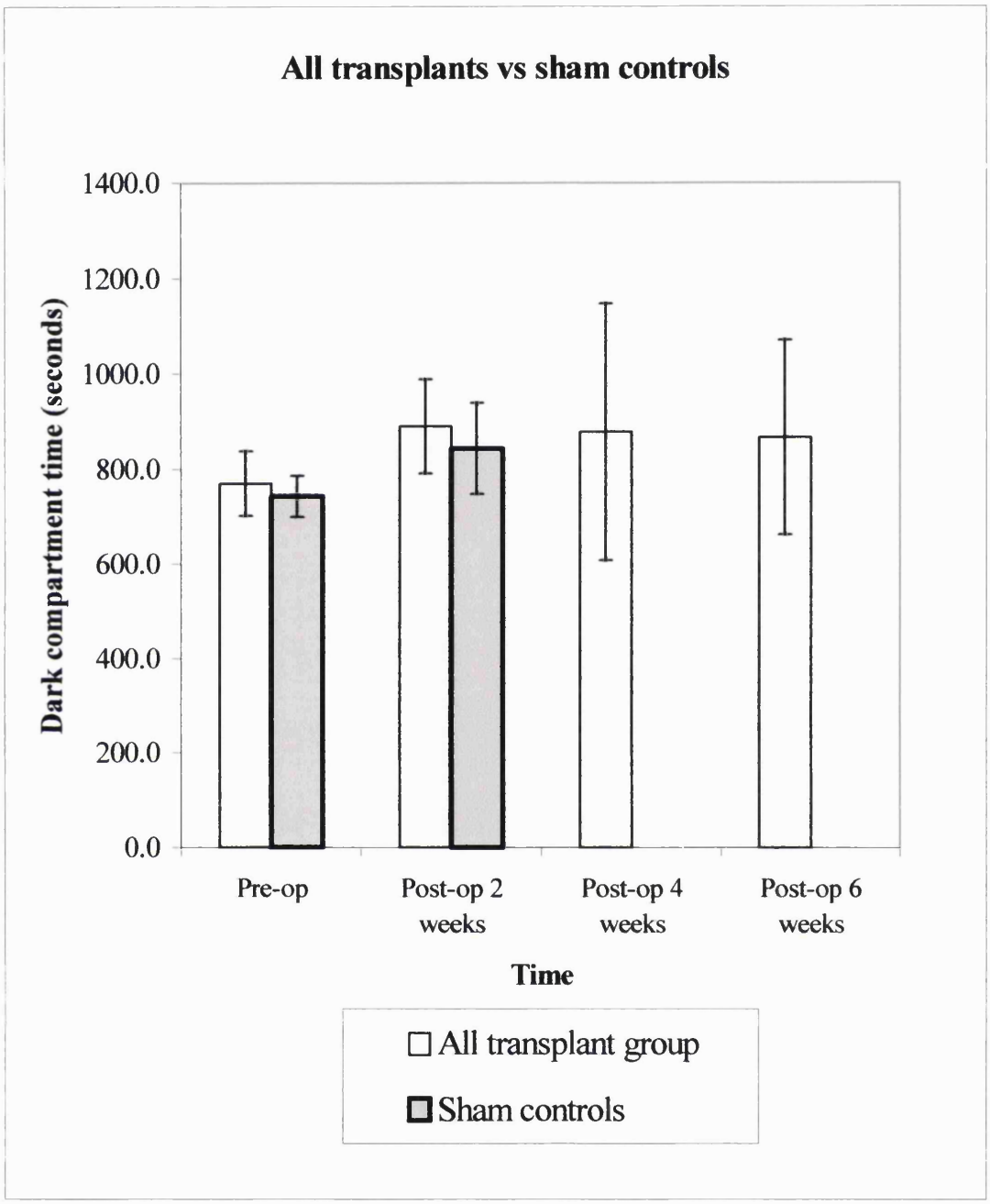
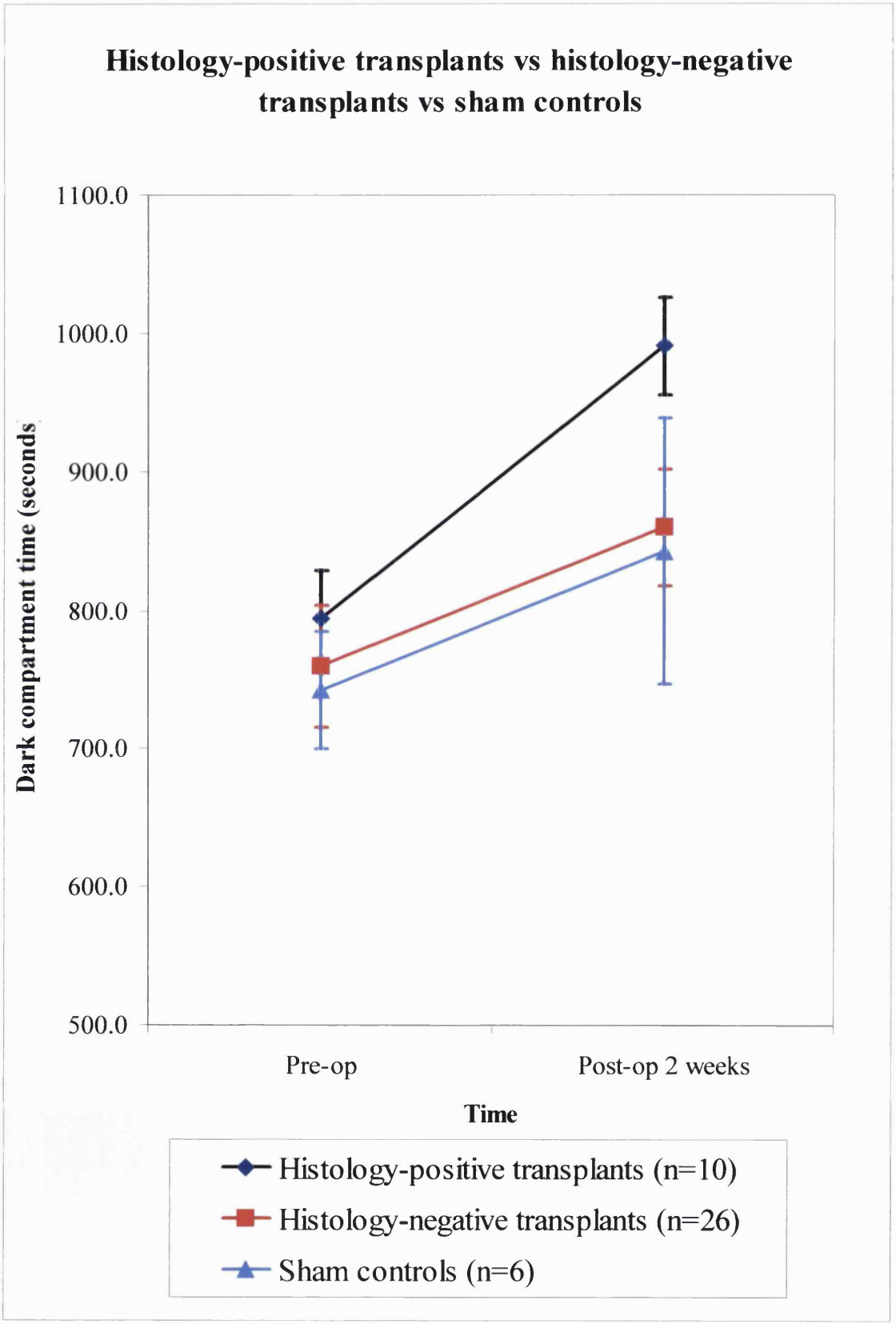

All transplants vs sham controls
Dark compartment time (seconds)
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
Pre-op
Post-op 2 weeks
Post-op 4 weeks
Post-op 6 weeks
Time
All transplant group
Sham controls

Figure 10

Pre- and 2-week post-transplantation LDPT results showing dark compartment times (DCT) between sham control, histology-positive and histology-negative groups. (Error bars = standard error of the mean)


Histology-positive transplants vs histology-negative transplants vs sham controls
1100.0
1000.0
900.0
800.0
700.0
600.0
500.0
Dark compartment time (seconds
Pre-op
Post-op 2 weeks
Time
Histology-positive transplants (n=10)
Histology-negative transplants (n=26)
Sham controls (n=6)

3.2. Electrophysiology

3.2.1. Baseline study of normal and dystrophic mice

The individual results are shown in Figure 11 (Subjects I to XII). In agreement with Drager and Hubel (1978), retinal thresholds for the 3 normal mice (Subject I to III) can be measured easily at all 76 points and the sensitivity ranged from 0.6 to 1.6 log units. In the dorso-temporal quadrants, this value ranged from 0.6 to 1.1 log units. This indicated that a response could be achieved by quite low luminance stimulation. In contrast, the retinal threshold for the dystrophic mice was diminished significantly by 8-week-old and the responses ranged from no response (symbol "n") in all 76 points in one dystrophic mouse (Subject V) to some mild responses limited to the dorso-nasal retina (e.g. Subject VIII). Moreover, the light level required to elicit a response in the dystrophic mice is much greater than for the normal mice at the corresponding points of the retinae, most of the responses could only be elicited when the light level was more than 4.5 log units. In one 8-week-old dystrophic mouse (Subject VIII), a lower threshold of 3.3 log units was recorded but this was confined to the far periphery of the dorso-nasal retina. This relative preservation of threshold sensitivity in the dorso-nasal retina was consistent with previous findings (Drager and Hubel, 1978). In the dorso-temporal retina, where the transplant would be placed, the threshold value ranged from no response to a maximum of 3.8 log unit in Subject IV.

Figure 11. Individual retinal threshold measurement maps

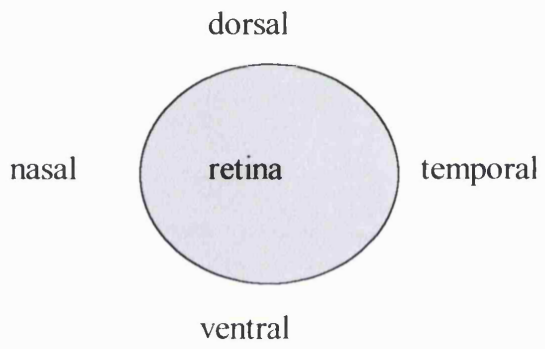
Values in log unit above background level

n = no response

a = auditory response

The maps from left or right eyes have been transposed to give the same orientation.

76-points retinotopic maps



Subject I, normal mouse (3-month-old)

			1.4	1.2	1.0	0.8			
		1.4	1.2	1.1	0.9	0.8	0.8		
	1.5	1.2	1.1	0.9	0.9	0.8	0.8	0.8	
1.5	1.3	1.2	1.0	0.9	0.9	0.9	0.9	0.9	1.0
1.2	1.2	1.2	1.0	0.8	0.9	1.0	1.0	1.0	1.1
1.1	1.3	1.5	1.2	1.0	0.9	0.9	1.0	1.0	1.1
1.0	1.4	1.7	1.4	1.1	1.0	0.8	0.9	1.0	1.1
	1.4	1.2	1.1	1.0	0.9	1.0	1.0	1.0	
		1.0	1.1	1.1	1.1	1.0	1.0		
			1.3	1.5	1.3	1.1			

Subject II, normal mouse (3-month-old)

			1.2	1.1	0.9	1.0			
		1.1	1.0	0.9	0.9	0.9	0.9		
	0.9	0.9	0.9	0.9	0.8	0.7	0.7	0.7	
1.1	1.1	1.1	1.0	1.0	0.9	0.8	0.7	0.7	0.7
1.3	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.7
1.6	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.8	0.8
1.8	1.6	1.3	1.2	1.1	1.0	0.9	0.9	0.9	0.9
	1.3	1.2	1.1	1.0	0.9	0.9	0.8	0.8	
		1.3	1.2	1.1	0.9	0.8	0.7		
			0.9	0.9	0.8	0.7			

Subject III, normal mouse (6-week-old)

			0.9	0.8	0.8	0.7			
		1.0	0.9	0.9	0.8	0.7	0.8		
	1.3	1.0	1.0	0.9	0.8	0.7	0.8	0.8	
1.7	1.4	1.1	1.0	0.9	0.8	0.7	0.7	0.8	0.8
1.8	1.5	1.1	1.0	0.9	0.8	0.7	0.7	0.7	0.8
1.7	1.4	1.2	1.1	1.0	0.9	0.8	0.8	0.8	0.8
1.6	1.4	1.2	1.1	1.0	0.9	0.8	0.8	0.8	0.8
	1.5	1.4	1.2	1.1	1.0	0.9	0.8	0.8	
		1.5	1.3	1.1	1.0	0.9	0.9		
			1.4	1.3	1.2	1.1			

Subject IV, control rd mouse (8-week-old)

			3.9	3.8	3.8	>4.5			
		>4.5	4.0	4.1	>4.5	n	n		
	>4.5	>4.5	4.3	>4.5	>4.5	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject V, control rd mouse (8-week-old)

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	n	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject VI, control rd mouse (9-week-old)

			a	a	a	a			
		a	a	a	a	a	a		
	n	n	a	a	a	a	a	a	
n	n	n	n	a	a	a	a	a	n
n	n	n	n	n	a	a	a	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject VII, control rd mouse (8-week-old)

			>4.5	n	n	n			
		>4.5	>4.5	n	n	n	n		
	>4.5	>4.5	>4.5	n	n	n	n	n	
4.0	>4.5	>4.5	>4.5	>4.5	>4.5	n	n	n	n
3.6	>4.5	>4.5	>4.5	>4.5	>4.5	n	n	n	n
>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	n	n	n	n
>4.5	>4.5	n	n	n	n	n	n	n	n
	>4.5	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject VIII, control rd mouse (8-week-old)

			>4.5	>4.5	>4.5	n			
		>4.5	>4.5	>4.5	>4.5	n	n		
	3.3	3.5	3.6	>4.5	n	n	n	n	
>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	n	n	n
n	n	>4.5	>4.5	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n		
		n	n	n	n	n	n		
			n	n	n	n			

Subject IX, control rd mouse (3-month-old)

			n	>4.5	n	n			
		>4.5	>4.5	>4.5	n	n	n		
	>4.5	>4.5	>4.5	>4.5	n	n	n	n	
>4.5	>4.5	>4.5	>4.5	n	n	n	n	n	n
>4.5	>4.5	>4.5	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n		
		n	n	n	n	n	n		
			n	n	n	n			

Subject X, control rd mouse (8-week-old)

			>4.5	>4.5	>4.5	n			
		>4.5	>4.5	>4.5	n	n	n		
	4.1	4.3	4.4	>4.5	n	n	n	n	
>4.5	>4.5	>4.5	>4.5	n	n	n	n	n	n
>4.5	>4.5	>4.5	n	n	n	n	n	n	n
>4.5	>4.5	n	n	n	n	n	n	n	n
>4.5	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XI, control rd mouse (3-month-old)

			a	a	a	a			
		a	a	a	a	a	a		
	a	a	a	a	a	a	a	a	
a	a	a	a	a	a	a	a	a	a
a	a	a	a	a	a	a	a	a	a
a	a	a	a	a	a	a	a	a	a
a	a	a	a	a	a	a	a	a	a
	a	a	a	a	a	a	a	a	
		a	a	a	a	a	a		
			a	a	a	a			

Subject XII, control rd mouse (3-month-old)

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	>4.5	>4.5	n	n	n	n	
n	n	>4.5	>4.5	>4.5	>4.5	n	n	n	n
n	n	n	>4.5	>4.5	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XIII, transplanted rd mouse

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	n	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XIV, transplanted rd mouse

			n	n	n	n			
		>4.5	>4.5	n	n	n	n		
	3.9	4.2	4.5	>4.5	n	n	n	n	
>4.5	>4.5	4.5	>4.5	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XV, transplanted rd mouse

			n	n	n	n			
		n	n	>4.5	>4.5	>4.5	n		
	n	n	n	>4.5	4.1	3.5	4.1	n	
n	n	4.5	>4.5	>4.5	3.5	2.4	3.1	3.8	n
n	n	4.5	>4.5	>4.5	4.0	3.3	3.7	4.2	n
4.1	4.3	4.5	>4.5	>4.5	4.4	4.2	4.4	4.5	n
4.1	4.3	>4.5	>4.5	>4.5	4.5	4.4	>4.5	n	n
	4.4	>4.5	>4.5	>4.5	>4.5	>4.5	n	n	
		>4.5	>4.5	>4.5	>4.5	>4.5	n		
			>4.5	>4.5	>4.5	>4.5			

Subject XVI, transplanted rd mouse

			a	n	n	n			
		a	a	n	n	n	n		
	a	a	a	a	n	n	n	n	
a	a	a	a	a	n	n	n	n	n
a	a	a	a	n	n	n	n	n	n
a	a	n	n	n	n	n	n	n	n
a	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XVII, transplanted rd mouse

			a	a	a	n			
		a	a	a	n	n	n		
	a	a	a	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XVIII, transplanted rd mouse

			n	n	n	n			
		>4.5	>4.5	>4.5	>4.5	>4.5	n		
	3.4	3.3	3.1	3.6	4.0	4.3	4.5	>4.5	
3.6	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5
3.8	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5	>4.5
>4.5	>4.5	>4.5	>4.5	>4.5	n	n	n	n	n
>4.5	>4.5	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XIX, transplanted rd mouse

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	n	n	n	n	n	n	
>4.5	>4.5	n	n	n	n	n	n	n	n
4.0	>4.5	n	n	n	n	n	n	n	n
>4.5	>4.5	>4.5	>4.5	n	n	n	n	n	n
>4.5	>4.5	5.5	>4.5	n	n	n	n	n	n
	>4.5	>4.5	>4.5	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XX, transplanted rd mouse

			>4.5	>4.5	4.3	4.2			
		>4.5	>4.5	4.5	4.4	>4.5	>4.5		
	>4.5	>4.5	3.8	4.2	4.5	>4.5	>4.5	>4.5	
>4.5	>4.5	>4.5	4.2	>4.5	>4.5	>4.5	>4.5	>4.5	n
>4.5	>4.5	>4.5	4.5	>4.5	n	>4.5	>4.5	>4.5	n
>4.5	>4.5	>4.5	>4.5	>4.5	n	>4.5	>4.5	>4.5	n
>4.5	3.7	>4.5	>4.5	>4.5	n	n	n	n	n
	>4.5	>4.5	>4.5	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXI, transplanted rd mouse

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	n	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXII, transplanted rd mouse

			>4.5	n	n	n			
		>4.5	>4.5	n	n	n	n		
	>4.5	n	n	n	n	n	n	n	
>4.5	>4.5	n	n	n	n	n	n	n	n
>4.5	>4.5	>4.5	n	n	n	n	n	n	n
>4.5	>4.5	>4.5	n	n	n	n	n	n	n
>4.5	>4.5	n	n	n	n	n	n	n	n
	>4.5	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXIII, transplanted rd mouse

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	n	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXIV, transplanted rd mouse

			>4.5	n	n	n			
		>4.5	>4.5	n	n	n	n		
	>4.5	>4.5	n	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXV, transplanted rd mouse

			>4.5	>4.5	>4.5	n			
		>4.5	>4.5	>4.5	n	n	n		
	>4.5	>4.5	>4.5	>4.5	n	n	n	n	
>4.5	>4.5	>4.5	>4.5	n	n	n	n	n	n
>4.5	>4.5	>4.5	n	n	n	n	n	n	n
>4.5	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXVI, transplanted rd mouse

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	n	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXVII, transplanted rd mouse

			n	n	n	n			
		>4.5	n	n	n	n	n		
	>4.5	>4.5	n	n	n	n	n	n	
>4.5	>4.5	>4.5	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXVIII, transplanted rd mouse

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	n	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	>4.5	>4.5
n	n	n	n	n	n	>4.5	>4.5	>4.5	>4.5
	n	n	n	n	>4.5	>4.5	>4.5	>4.5	
		>4.5	>4.5	>4.5	>4.5	>4.5	>4.5		
			>4.5	>4.5	>4.5	>4.5			

Subject XXIX, transplanted rd mouse

			n	n	n	n			
		n	n	n	n	n	n		
	>4.5	n	n	n	n	n	n	n	
>4.5	>4.5	n	n	n	n	n	n	n	n
>4.5	>4.5	n	n	n	n	n	n	n	n
>4.5	>4.5	n	n	n	n	n	n	n	n
>4.5	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	4.5	
		n	n	n	n	n	n		
			n	n	n	n			

Subject XXX, transplanted rd mouse

			n	n	n	n			
		n	n	n	n	n	n		
	n	n	n	n	n	n	n	n	
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
n	n	n	n	n	n	n	n	n	n
	n	n	n	n	n	n	n	n	
		n	n	n	n	>4.5	>4.5		
			n	n	n	>4.5			

3.2.2. Effects of retinal transplantation

Fifteen (83.3%) retinotopic maps were similar to those of the baseline dystrophic rd mice with either no recordable response or a small area of response at the dorso-nasal peripheral retina with a large threshold of >4.5 log units. A few mice (Subjects XXVIII, XXIX, XXX) showed some response at the infero-temporal peripheral retina with high thresholds (>4.5 log unit). However in 3 subjects (Subject XV, XVIII, XX) showed lower threshold responses (i.e. increased sensitivity) in the areas of map corresponding to the transplant locations, i.e. dorsal and dorso-temporal retinae. In one mouse (Subject XV), a region with a threshold of 2.4 log unit was seen in the dorso-temporal retina; this is more than 2 log units above the corresponding area in a dystrophic rd mouse without a graft.

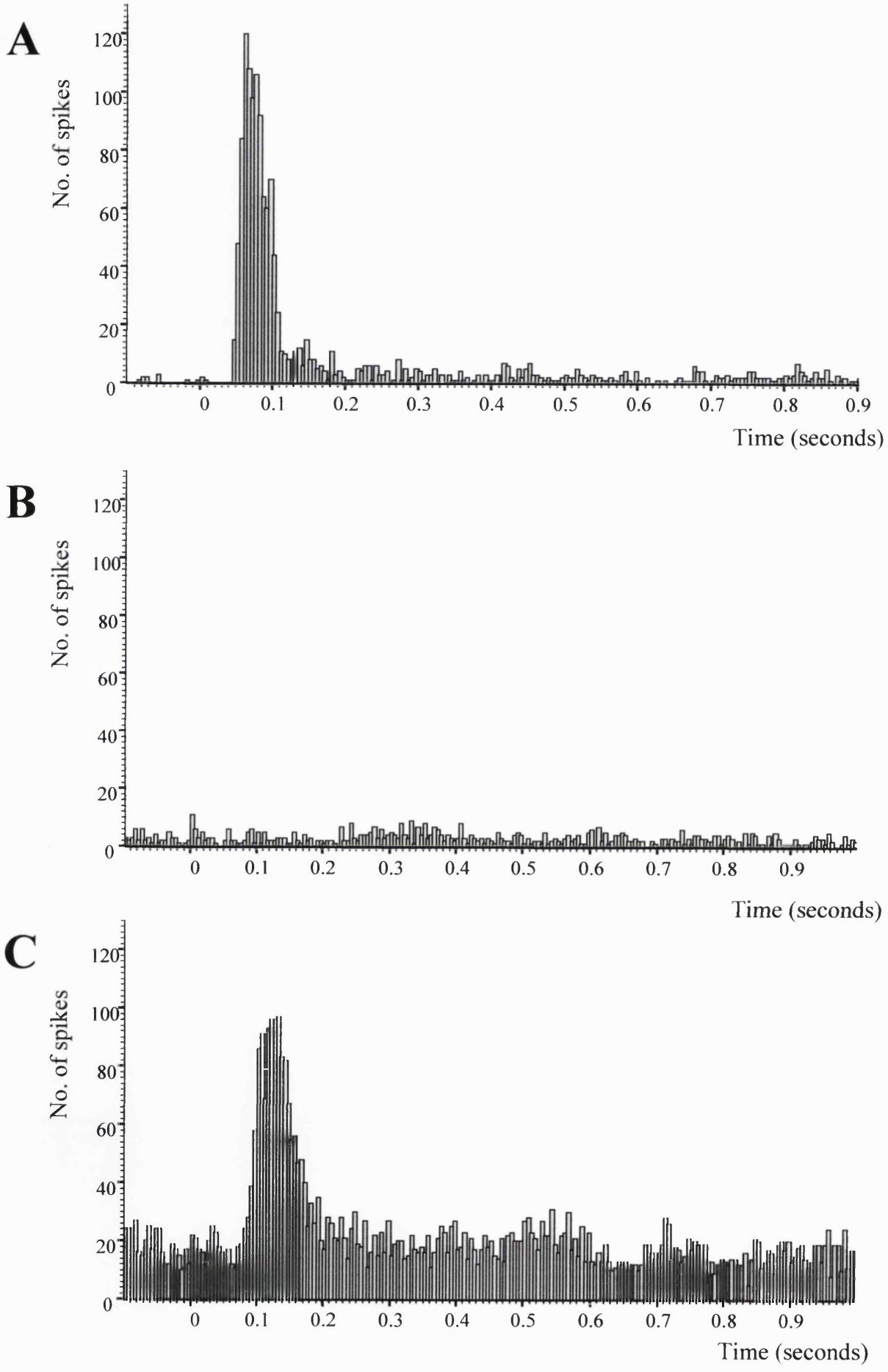
A typical PSTH from a normal mouse (example from Subject II) showed a latency of 0.05 seconds, a spike count of about 120 at its peak and duration of 0.1 second (Figure 12A). The PSTH of visual responses recorded at the site in the SC of Subject XV, where the 2.4 log unit threshold was measured, were compared with PSTH from a comparable area in the dorso-temporal retina in a control 8-week-old dystrophic mouse with no transplant. Stimulation of the centre of the classical RF alone (4.5 log cd/m^2 above background illumination of 0.02 cd/m^2) produced no response in the control dystrophic mouse with no transplant (Figure 12B) but stimulation of the centre of the classical RF in Subject XV gave notable a ON-response (Figure 12C). This response in Subject XV was slightly different from one obtained from a normal mouse, as it had a longer latency of 0.1 second, a small spike count of 100 at its peak and a longer duration of 0.15 second. Moreover there was a higher spontaneous electrical activity at baseline with spike counts ranging from 10 to 30. All these

demonstrated that a clear signal was generated from the transplanted area in Subject XV and the differences in the characteristics of the signal imply a functioning, but imperfect, synaptic connections had been generated through the graft.

The significance of these findings was confirmed when histological analysis showed evidence of subretinal grafts in the 3 animals with improved visual threshold sensitivity and no evidence of grafts in the other 15 mice which showed no recovery in threshold measurements. Failed grafts could be regarded as sham injections and confirmed that if there was no graft present there was no reduction in retinal thresholds.

Figure 12

Post-stimulus time histograms of a normal mouse, a dystrophic mouse and the rd mouse with transplant (Subject XV in the retinal threshold measurement) three weeks after transplantation. Note the longer latency, longer duration of the ON-response and the smaller maximum spike counts in the transplanted mouse compared to those of the normal mouse. (Each bin in the x-axis is in 5 ms interval)


A
No. of spikes
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Time (seconds)
B
No. of spikes
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Time (seconds)
C
No. of spikes
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Time (seconds)

3.3. Histology results

3.3.1. Baseline study of normal and dystrophic retinae

Cresyl violet staining

At 7 days of age Cresyl violet staining revealed cell bodies from the GCL and the two retinal nuclear layers and the ONL was now recognisable as photoreceptors, showing early signs of inner segments. Figure 13A is a photomicrograph of Cresyl violet stained section of a 7-day-old normal mouse retina, at a time when donor retina is usually harvested, which shows no observable outer segments attached to the ONL. Dystrophic retinae of the same age had exactly the same appearance but in the subsequent weeks the difference between the two becomes more evident. By 6-week-old, the difference between the two groups was very clear. As illustrated in Figure 13B the normal retina has the three distinct retinal cellular layers and the long outer segments associated with the 7- to 8-cell thick photoreceptor layer. However, in the dystrophic retinae (Figure 13C), it was evident that there was a significant reduction in the thickness of the photoreceptor layer with only a thin single layer of sparsely distributed photoreceptors present. The 12-month-old retinae material resembled 6-week-old material except that in the dystrophic mice, the photoreceptors were even more sparsely distributed. All these observations are in keeping with the findings published in the original detailed study of rd mouse retina (Carter-Dawson *et al.*, 1978). From these results we can be sure that at the intended time of transplantation, at 6- to 8-week-old, dystrophic rd mice would have only a single sparsely populated layer of photoreceptors and any increase in the appearance or number of photoreceptor layers could be attributed to the transplant.

Immunostaining of 6-week-old normal and dystrophic retinae

Rhodopsin consists of a single polypeptide chain of 348 amino acids and is the photosensitive protein found in rod outer segments of vertebrates responsible for dim light vision. Rhodopsin labelling in normal mouse retinae revealed positively labelled rod photoreceptor cell bodies and intensely labelled rod photoreceptor outer segments (Figure 14A). The labelling was evenly distributed throughout the whole of the ONL. There were no rhodopsin-positive cells in the GCL or in the INL. In the dystrophic retina, no rhodopsin positive cells were seen anywhere in the retinae (Figure 14B), suggesting that the remaining cells in the ONL were cone photoreceptors. These results were consistent with previous finding (Carter-Dawson *et al.*, 1986).

GFAP is present in intermediate filaments of astrocytes and reactive Müller glia cells. In the normal retina (Figure 15A) a few processes from astrocytes were positively labelled for GFAP and organised in relatively regular palisade-like fashion running from the inner limiting membrane a short distance into the IPL. In the dystrophic retina, radial GFAP-positive processes crossed the IPL and the INL. In the region that used to be the ONL, GFAP processes, arranged more randomly, filled the area (Figure 15B). These processes are probably mostly from reactive Müller glia whose cell bodies were located in the INL and whose processes run radially through the retina. Müller cells show increased GFAP expression in response to focal or generalised retinal injury, such as laser photocoagulation (Humphrey *et al.*, 1993) and light damage (Eisenfeld *et al.*, 1984) as well as in genetically determined retinal degeneration, such as the RCS rats (Eisenfeld *et al.*, 1984), the recessive feline model

of retinal degeneration (Ekstrom *et al.*, 1988) and human RP (Milam and Jacobson, 1990).

RT97 labels 200 kilo-Dalton neurofilament protein (found in mature neurones and axons). In the retina it labels axons and sometimes dendritic processes and cell bodies of horizontal cells and retinal ganglion cells. In the normal retina, ganglion cells and their axons showed positive labelling for RT97 in the GCL, the positively labelled axons running parallel and near to the surface of the GCL; horizontal cells and their axons were also positively labelled and evenly distributed in the INL (Figure 16A). There was no positive labelling in the ONL. However this particular RT97 antibody is known to cross-react with photoreceptor outer segments, and in the normal mouse retina the outer segments were positively labelled for RT97. In the dystrophic retina, the labelling pattern was the same as the normal retina in the GCL and INL, except that the number of positively labelled cells appeared to be fewer. Again there was no labelling in the ONL, but the cross-reactive labelling of outer segments was absent in the dystrophic retina (Figure 16B) because of the lack of outer segments and photoreceptors in 6-week-old rd mouse.

PKC in one isoform or another is believed to be a second messenger used in neurotransmission by both rod and cone bipolar cell systems and ON- and OFF-centre systems in the retina. One particular isoform, αPKC, is known to be specifically associated with rod bipolar cells in the mouse (Haverkamp and Wassle, 2000). In the normal retina, αPKC labelling showed numerous rod bipolar cells in the INL. They were characteristically located at the photoreceptor layer side of the INL. At high magnification, it showed numerous dendritic processes extending from the

rod bipolar cells into the photoreceptor layer (Figure 17A). In the dystrophic rd mouse retina, there were fewer PKC positive bipolar cells and there were no observable dendritic branches extending into the remains of the ONL (Figure 17B).

Calbindin is a calcium-binding protein and its distribution in the retina has been well characterised (Peichl and Gonzalez-Soriano, 1994). There are differences in its distribution in different species, but in the mouse it is found in horizontal cells, subset of amacrine cells, and some ganglion cells (Haverkamp and Wassle, 2000). In the normal mouse retina, calbindin-positive horizontal cells were seen distributed evenly through the INL, and the dendritic processes from the horizontal cells extended towards the ONL (Figure 18A). A few calbindin-positive ganglion cells were seen in the GCL, and a few amacrine cells were also noted in the IPL. In the dystrophic retina, the labelling pattern was similar to normal retina but the number of calbindin-positive cells was reduced, especially in the INL, where there is a reduction in calbindin-positive horizontal cells and their dendritic processes were also reduced in size and numbers (Figure 18B). Calbindin positive amacrine cells and ganglion cells appeared as in the normal retina.

F4/80, a murine macrophage-specific antigen, can be found on microglia cells and macrophages (Hume *et al.*, 1983). In the normal retina, a few F4/80 positive cells were found near blood vessels and sporadically in ONL and the plexiform layers (Figure 19A). In the dystrophic retina, a similar pattern was found (Figure 19B) with some of the cells having a pronounced stellate configuration which, may be a sign of activation. These F4/80 positive cells are thought to be microglial cells, since macrophages are normally larger and rounded and are not normally found in healthy retina.

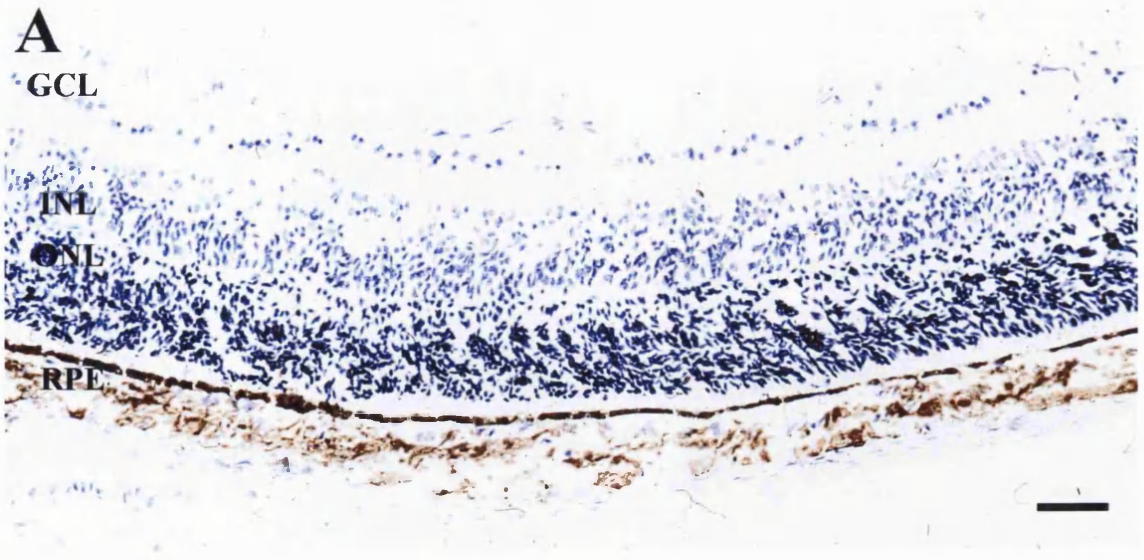
Parvalbumin, a selected calcium-binding protein thought to buffer intracellular calcium, is localised in selected neurones in the nervous system including the retina. In the normal mouse retina, parvalbumin-positive cells were seen in the INL and GCL, indicating selected amacrine and ganglion cells (Figure 20A). This is consistent with other published findings (Haverkamp and Wassle, 2000; Wassle *et al.*, 1998). In the dystrophic mouse retina, the labelling was similar to the normal retina except that there appeared to be fewer parvalbumin positive cells (Figure 20B).

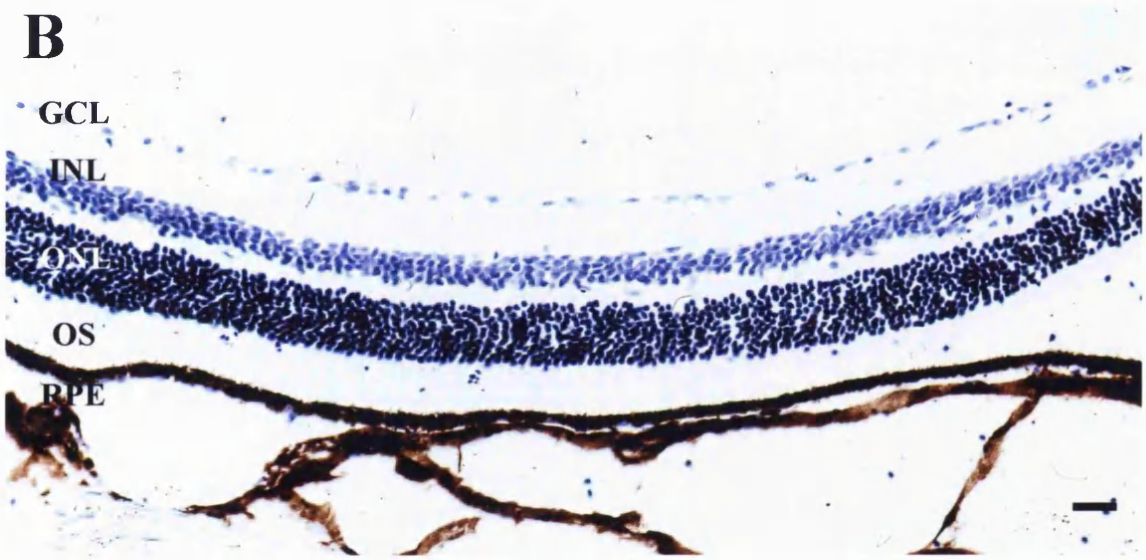
The **P84** antibody used in this study was important for examining the distribution of synapses. P84 is a member of a signal-regulatory protein family (SIRP) and has been identified as a neuronal membrane glycoprotein (Comu *et al.*, 1997; Jiang *et al.*, 1999). In mouse retina, signalling activities of P84 are localised to sites of synaptic contact in the retina (Mi *et al.*, 2000). P84 labelling is found in the plexiform layers and it is specific to mouse, no labelling is seen in rat retina. It is suggested that it may play a key role in intracellular signalling both during synaptogenesis and in mature synapses. In the normal mouse retina, it specifically labelled the synaptic regions, namely the IPL and OPL (Figure 21A). In unoperated dystrophic rd mice, a discontinuous line of labelling was seen at the outer border of the retina (Figure 21B). It tended to lie deep to residual cones and probably reflected the distribution of synapses associated with them.

Figure 13

Light micrograph of (A) Cresyl violet staining of a typical 7-day-old normal mouse retina. The distinction between the inner and outer retinal cell layers can just be visualised but the photoreceptor inner and outer segment layers are absent. (B) Cresyl violet staining of a typical 6-week-old normal mouse retina. The three retinal layers can clearly be seen. The nuclei in the outer nuclear layer (photoreceptor layer) are typically darker stained than the other two retinal cell layers and there is now a clear gap between the outer nuclear layer and the RPE layer where the inner and outer segments of photoreceptors can be found. (C) Cresyl violet staining of a typical 6-week-old dystrophic rd mouse retina. This is the time when transplantation is performed in this study and the most striking feature is the absence of the darkly stained outer nuclear (photoreceptor) layer except for a few sparsely distributed cone photoreceptors (ARROW).

Scale bar = 0.01 mm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear (photoreceptor) layer; OS, outer segments; RPE, retinal pigment epithelium.


A
GCL
INL
ONL
RPE


B
GCL
INL
ONL
OS
RPE

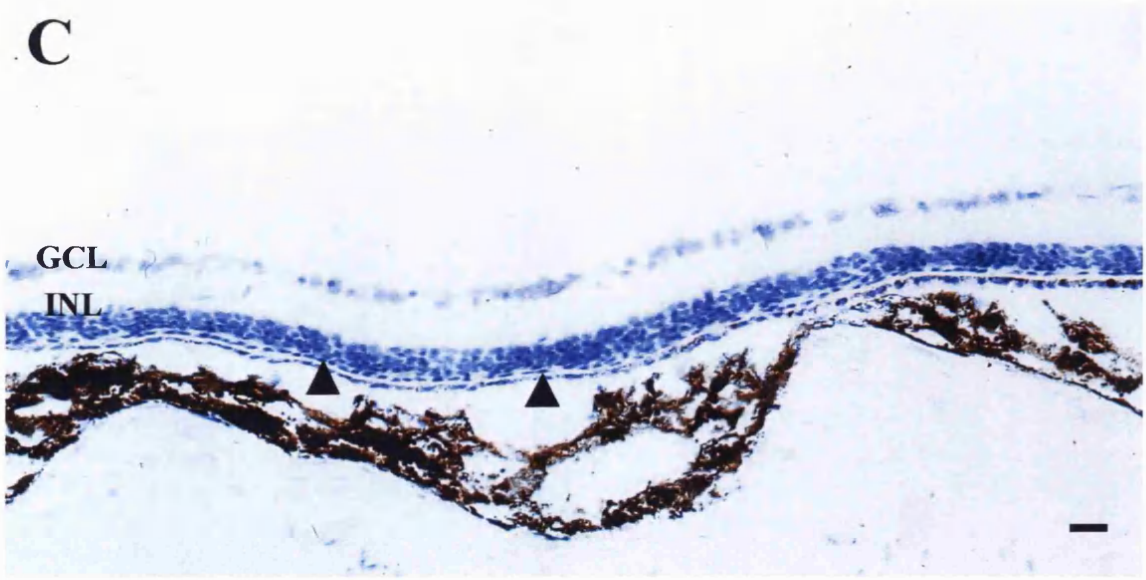

C
GCL
INL

Figure 14

Black and white light micrograph of Rhodopsin labelled retina. (A) Rhodopsin labelling of an 8-week-old normal mouse retina. The outer nuclear layer and outer segments are positively labelled. (B) Rhodopsin labelling at the edge of a subretinal retinal microaggregate transplant two weeks after transplantation into a 7-week-old dystrophic rd mouse. Note that the cells in the graft are predominantly rhodopsin-positive (ARROW) which indicates that they were rod photoreceptor cells. No rhodopsin positive cells are seen in the area with no transplant (*). (C) High magnification of a rhodopsin-labelled transplanted photoreceptor layer with short outer segments (ARROWHEAD) strongly labelled and aligned in a regular fashion adjacent to the host RPE.

Scale bar = 0.01 mm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments; TPL, transplanted cell layer.

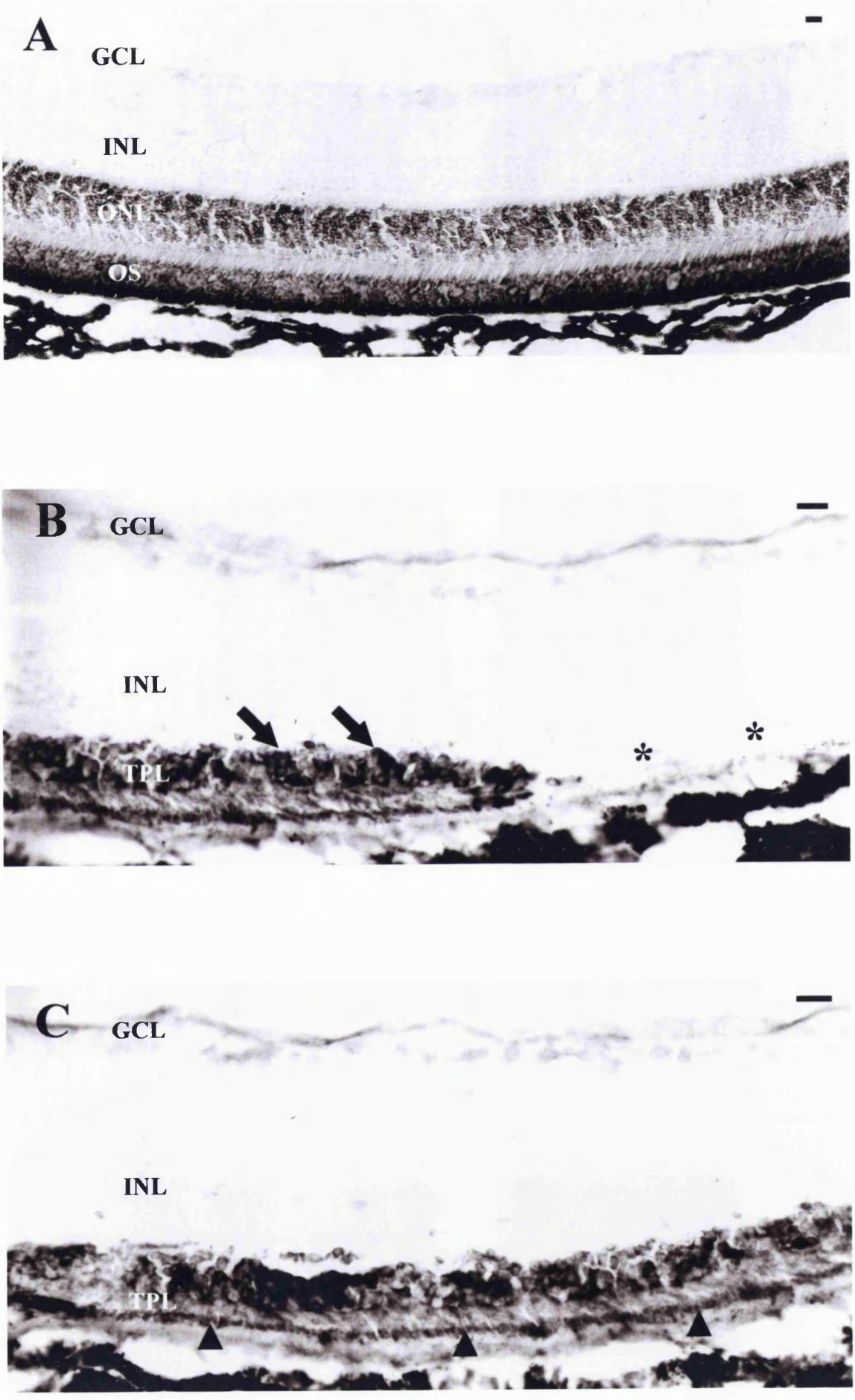
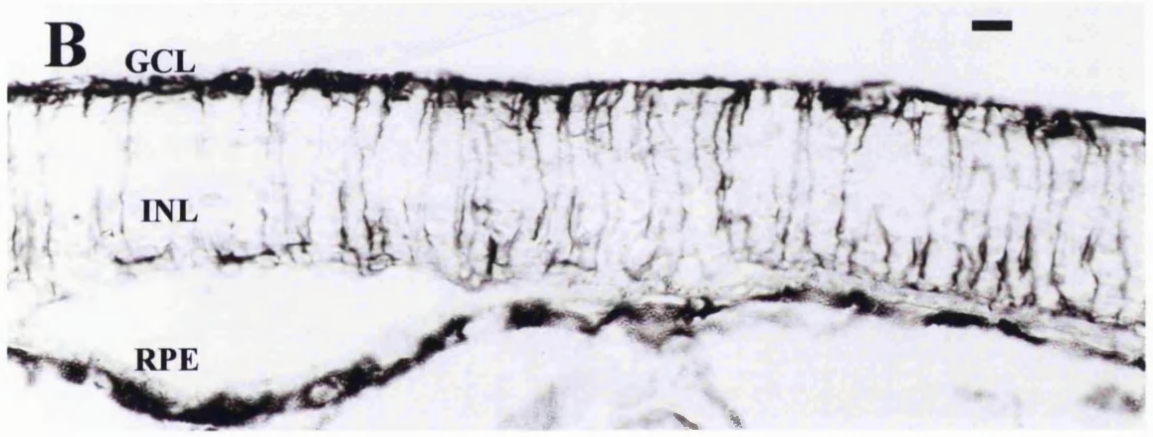

A
GCL
INL
ONL
OS
B
GCL
INL
TPL
*
*
C
GCL
INL
TPL

Figure 15

GFAP labelling of retina. (A) In a normal 6-week-old mouse retina, only the astrocyte processes associated with the ganglion cell layer are reactive. (B) GFAP labelling in a 6 week-old dystrophic rd mouse showing activated Müller cell processes in a regular and linear array and traversing through the entire retinal thickness. (C) Two weeks after transplantation in a 6-week-old rd mouse. In the host retina away from the graft, the labelling resembles that of the control dystrophic animal but in the transplanted layer (TPL) activated Müller cell processes show a widespread and irregular distribution (ARROW) associated with the region of the microaggregate transplant. However, in the area where grafted outer segments are in close apposition to the host RPE, the labelling pattern is aligned regularly (*). There is an incomplete glial barrier at the host-graft interface.

Scale bar = 0.01mm. GCL, host ganglion cell layer; INL, host inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; TPL, transplanted cell layer.


A
GCL
INL
ONL
RPE


B
GCL
INL
RPE

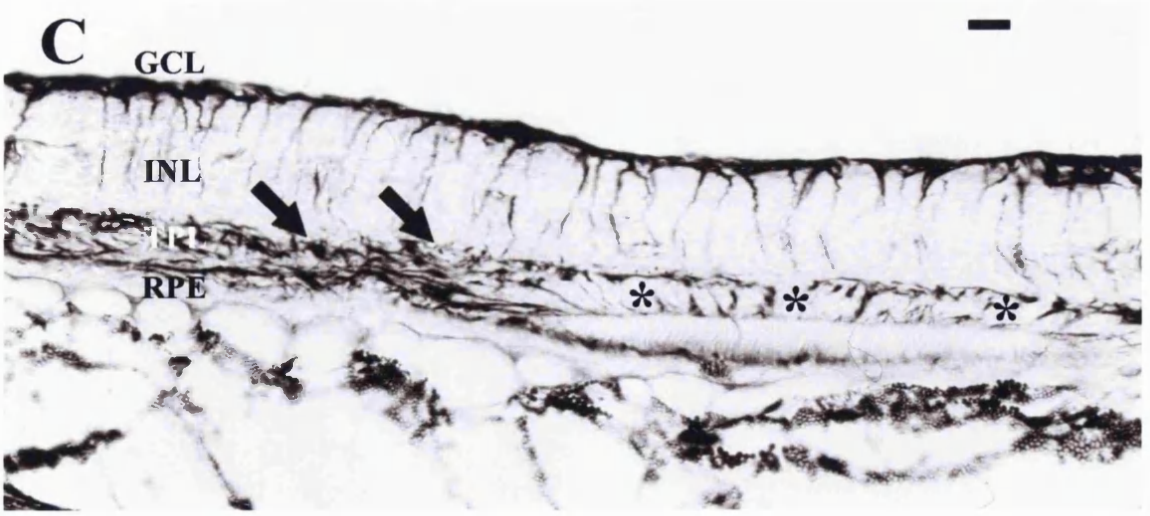

C
GCL
INL
RPE

Figure 16

(A) RT97-labelled section showing positively labelled ganglion cells (CLEAR ARROWHEAD), their axons (BLACK ARROW) and horizontal cell processes (CLEAR ARROW) in a 6-week-old normal mouse retina. The cross-reactive labelling of the outer segments (OS) can be visualised. (B) In the dystrophic retina of a 6-week-old rd mouse, ganglion cell and horizontal cell axons positively labelled but there is no labelling of the outer segments. (C) Retina of a 8-week-old mouse with transplant. Ganglion cells and horizontal cells are labelled similar to A and B above and only a few positively labelled cells can be seen in the transplanted layer.

Scale bar = 0.01 mm. GCL, host ganglion cell layer; INL, host inner nuclear layer; ONL, outer nuclear layer; OS, outer segments; TPL, transplanted cell layer.

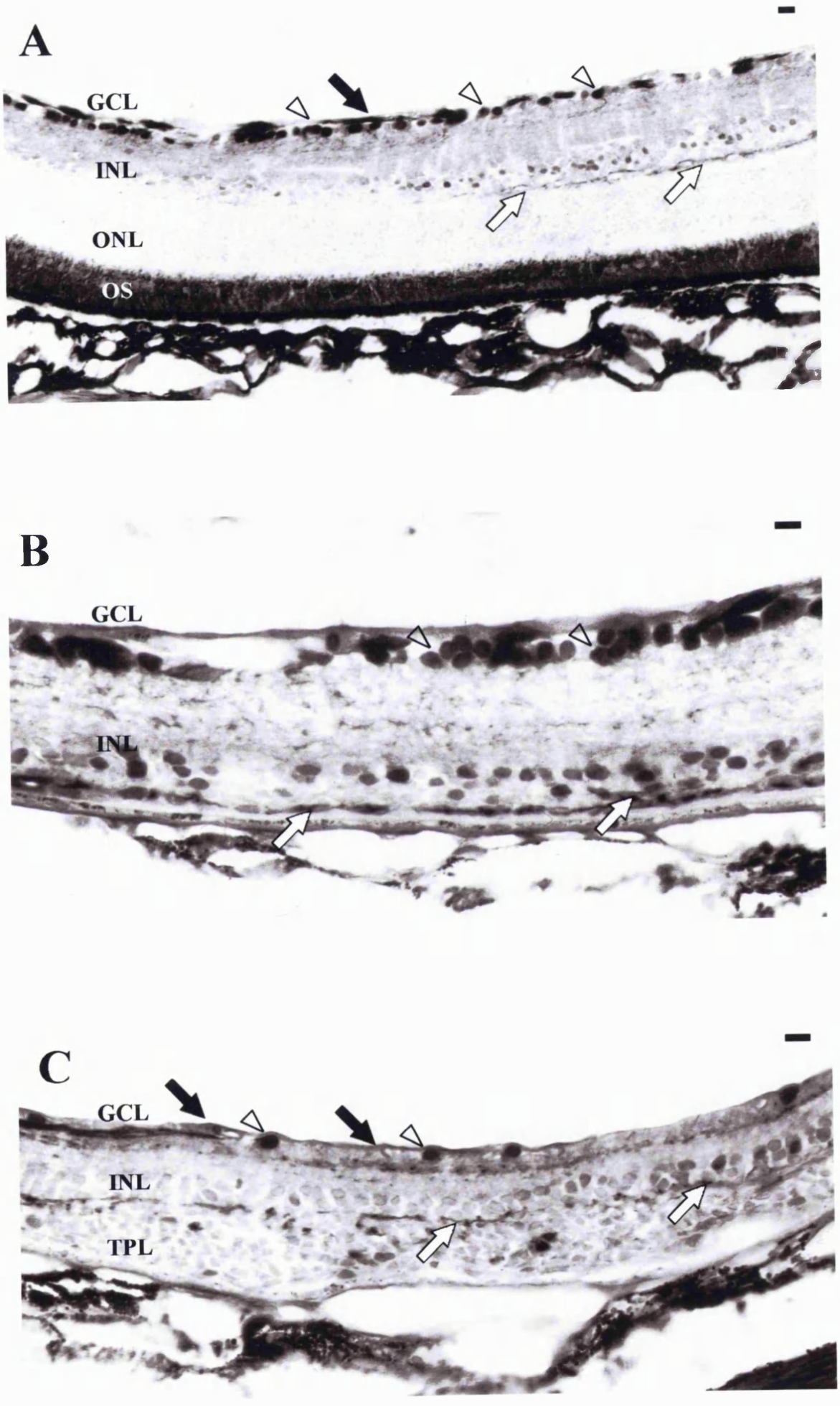
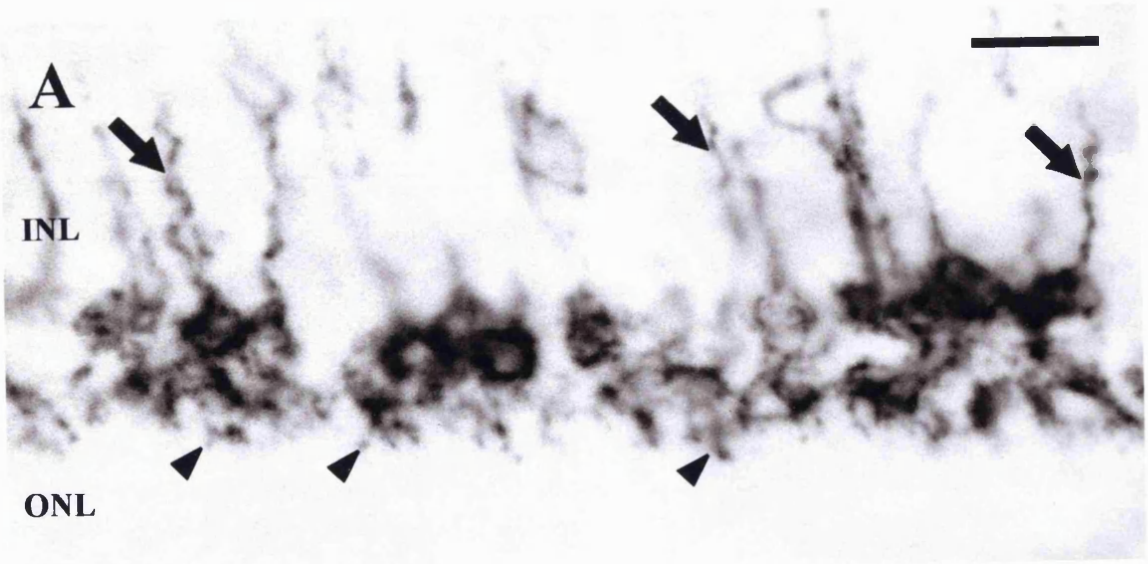
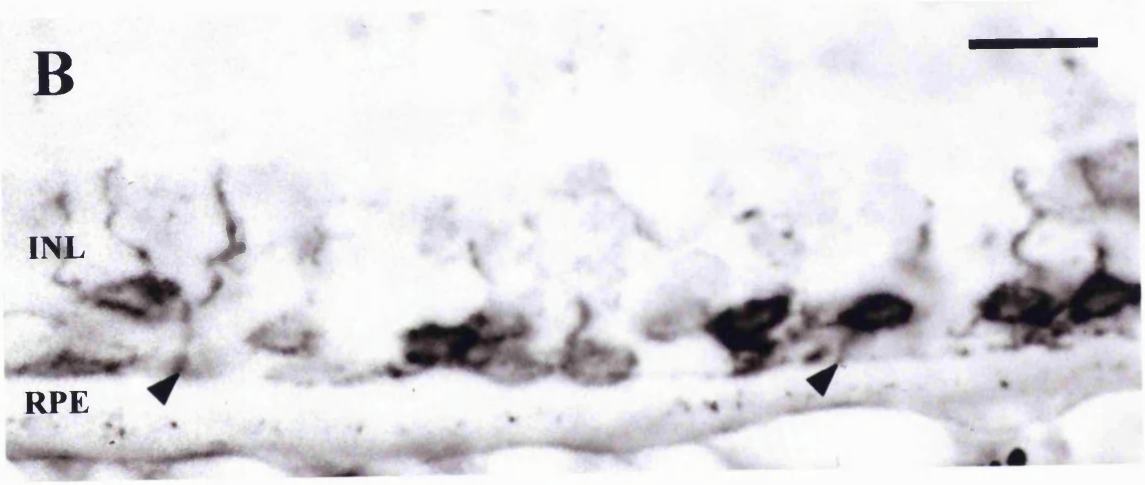

A
GCL
INL
ONL
OS
B
GCL
INL
C
GCL
INL
TPL

Figure 17

PKC-labelled retinal sections of (A) normal 8-week-old mouse retina showing axonal processes (ARROW) of rod bipolar cells extending into the inner plexiform layer and a large number of dendrites (ARROWHEAD) extending into the outer plexiform layer; (B) dystrophic 6-week-old rd mouse retina showing a reduced number of rod bipolar cells and only a few dendritic processes (ARROWHEAD) next to the RPE; (C) retina with transplant in a 7-week-old rd mouse 2 weeks after transplantation showing an increased number of PKC-positive cells in the inner nuclear layer and more dendrites (ARROWHEAD) extending into the graft.

Scale bar = 0.01 mm. INL, host inner nuclear layer; ONL, host outer nuclear layer; RPE, host retinal pigment epithelium; TPL, transplanted cell layer.


A
INL
ONL


B
INL
RPE

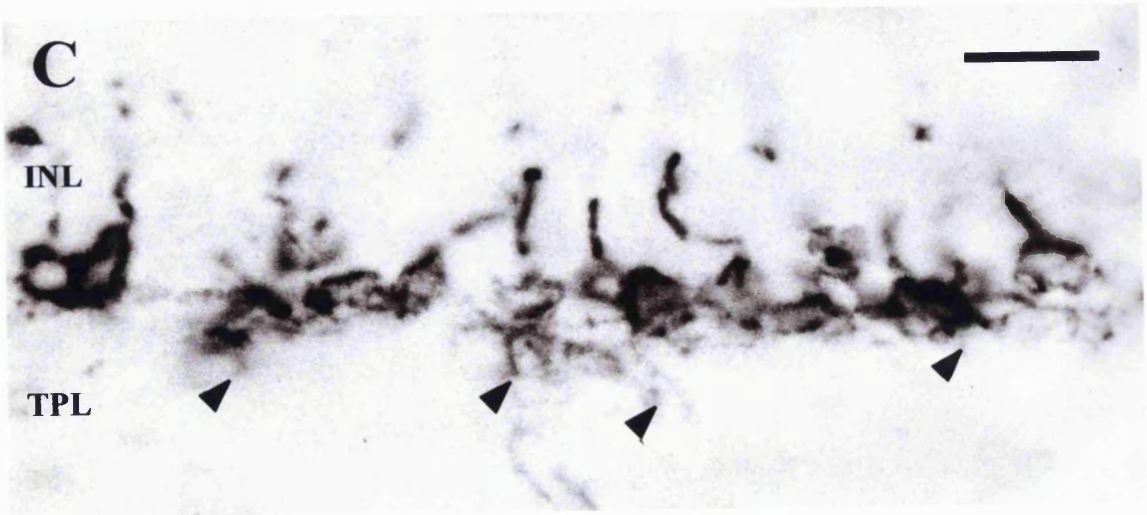
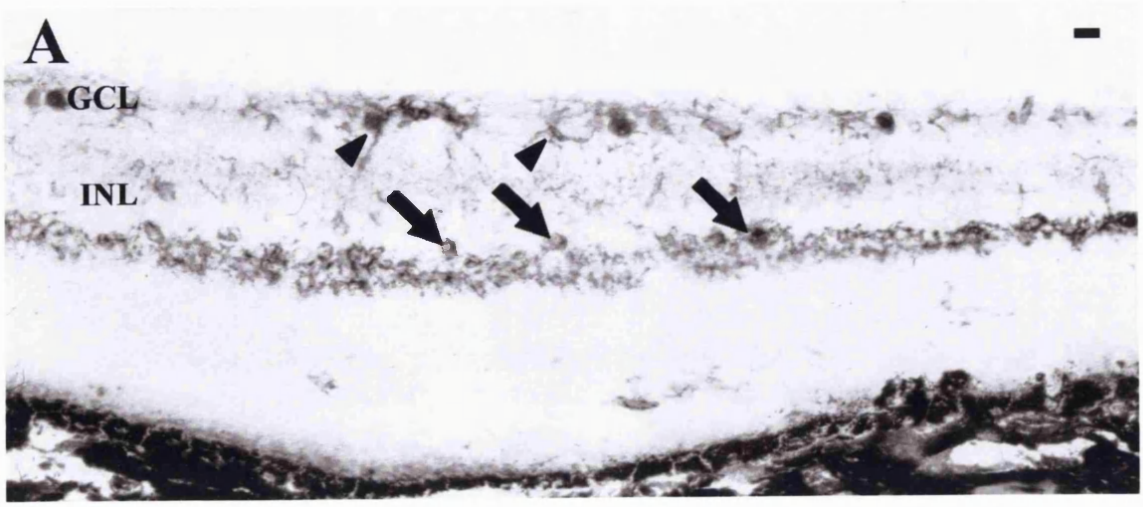
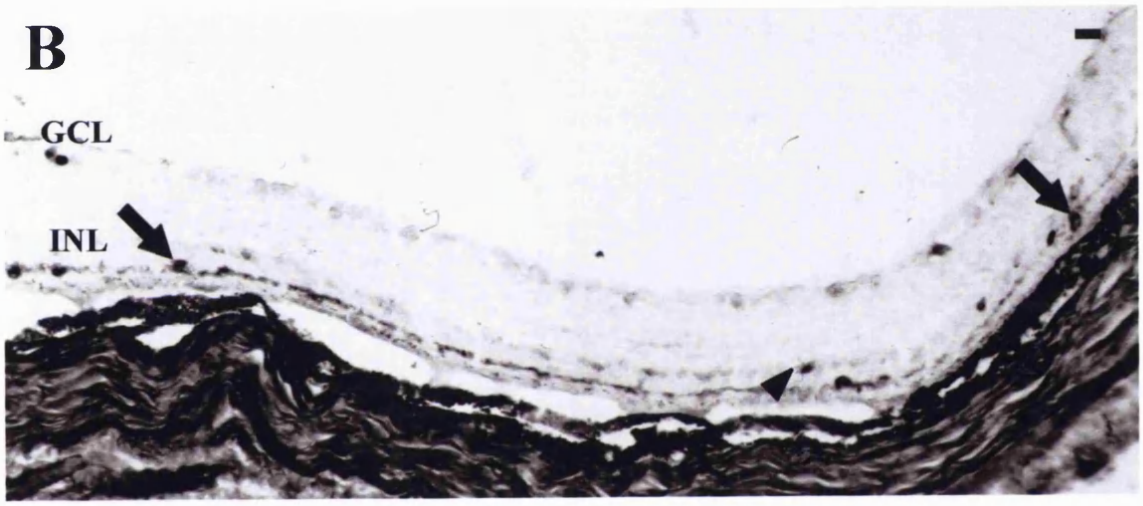

C
INL
TPL

Figure 18

Calbindin labelling of 6-week-old normal retina (A) shows positively labelled horizontal cells (ARROW) and a few amacrine cells (ARROWHEAD) in the inner nuclear layer, occasional ganglion cells are also labelled. (B) In the dystrophic rd retina from a 6-week-old mouse, there is a reduction in positively labelled cells compared to normal retina. (C) At the edge of transplant in a 8-week-old rd mouse, calbindin labelled horizontal cells (ARROW) and amacrine cells (ARROWHEAD) can be seen in the host retina. There are no positively labelled cells in this section of the graft (TPL,*).

Scale bar = 0.01 mm. GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; TPL, transplanted layer.


A
GCL
INL


B
GCL
INL

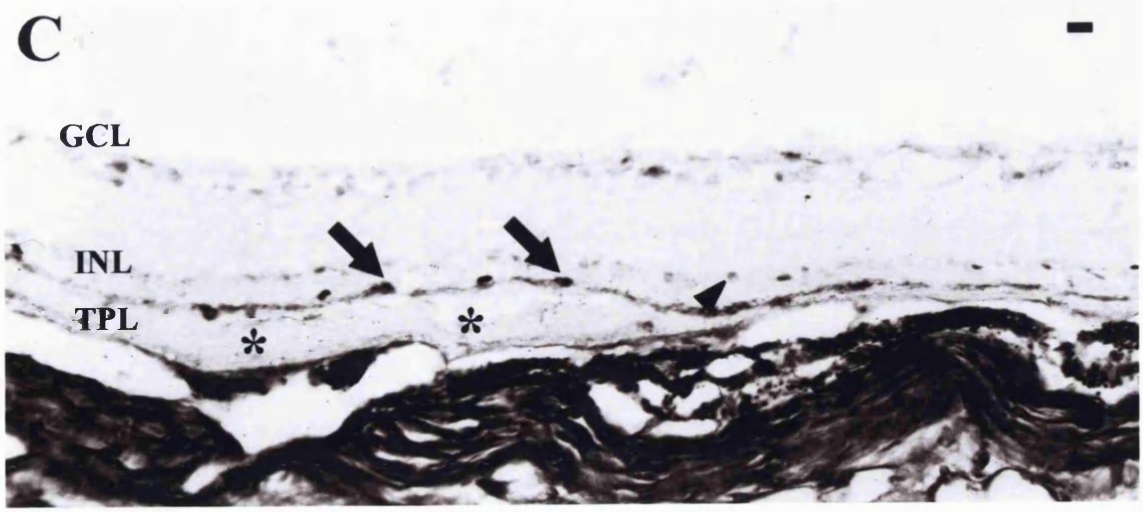

C
GCL
INL
TPL

Figure 19

(A) F4/80 labelling of a 6-week-old normal mouse retina with Cresyl violet counter-staining showing a few positively labelled microglia cells (ARROW) around retinal vessels. (B) F4/80 labelling of a 6-week-old dystrophic rd mouse retina without Cresyl violet labelling showing positively labelled microglial cells (ARROW) in the inner nuclear layer. (C) Cresyl violet staining of an area of transplant in a 6-week-old rd mouse two weeks post-transplantation. There is no obvious photoreceptor layer left but an area of fibrosis (*) can be seen and this is associated with a number of large pigmented cells (ARROWHEAD), believed to be macrophages (see Figure 19).

Scale bar = 0.01 mm. RPE, host retinal pigment epithelium; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

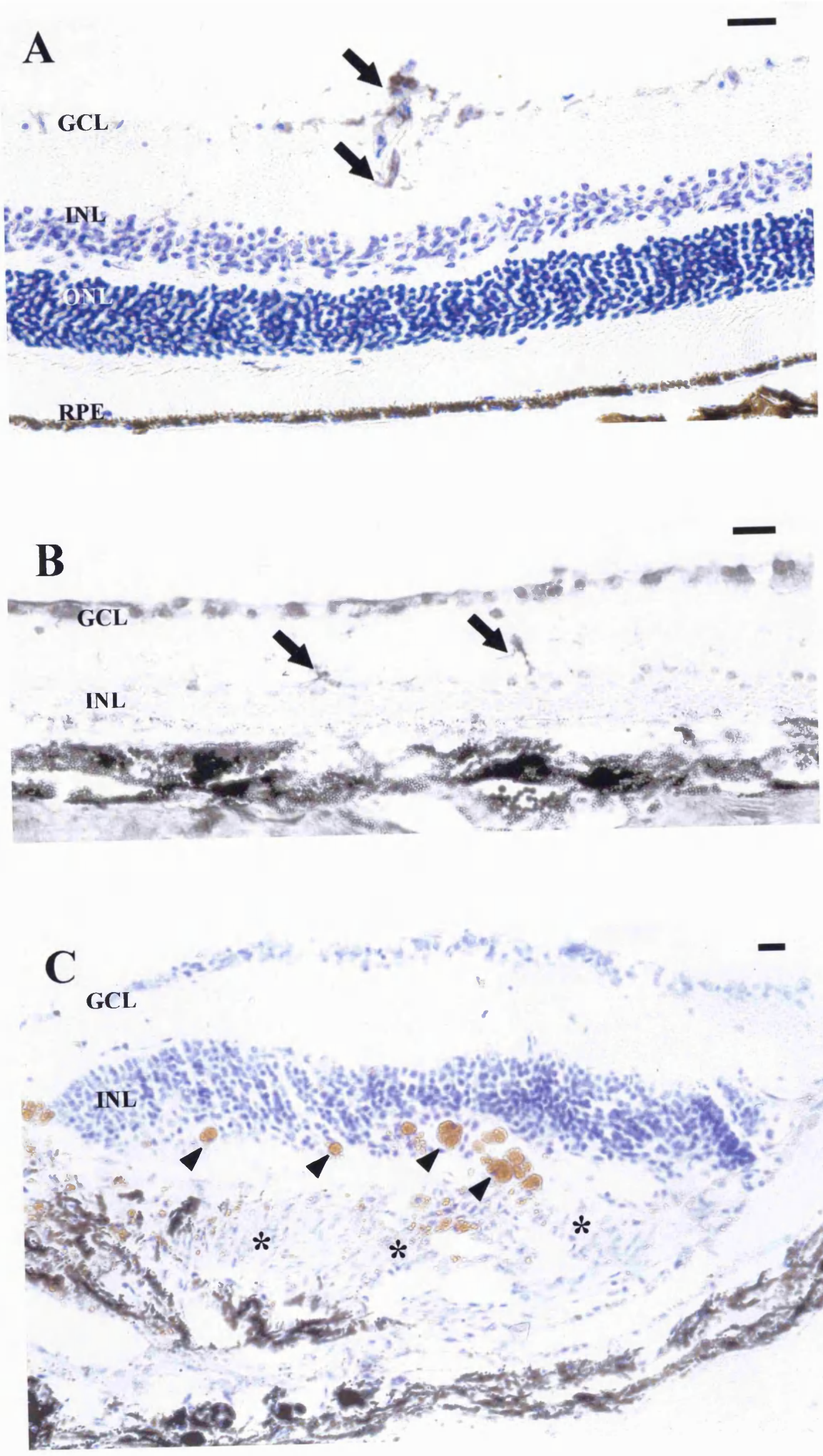
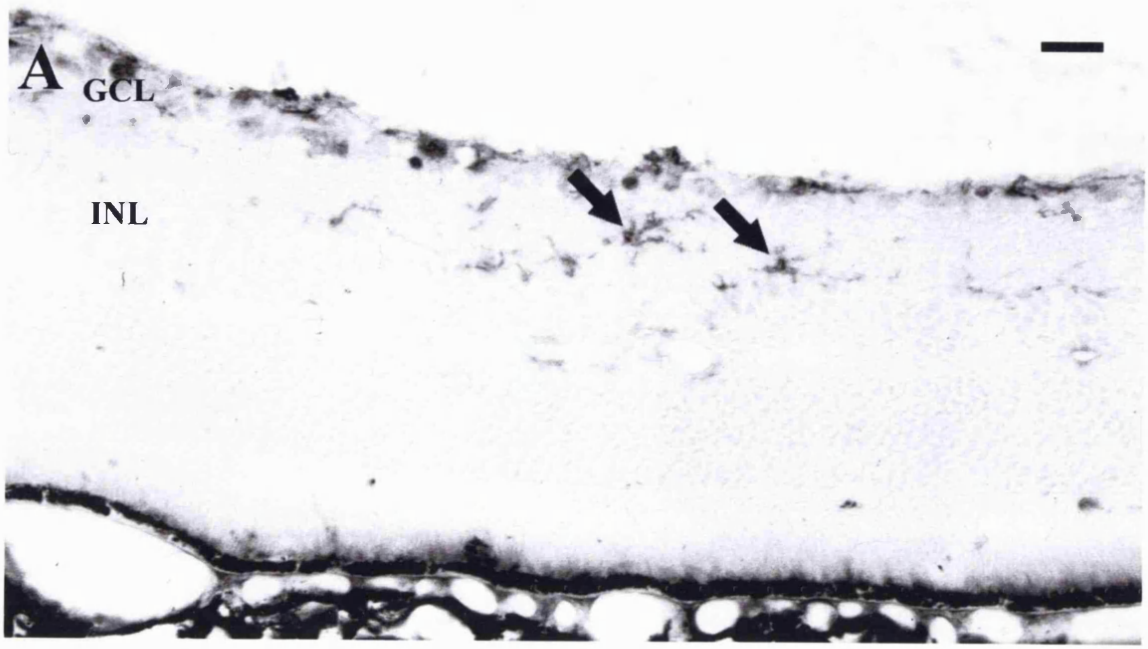
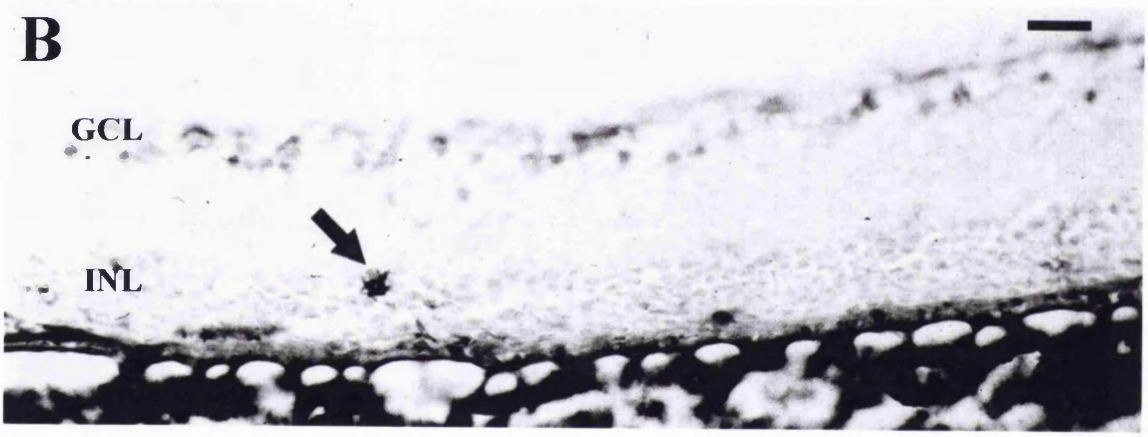

A
GCL
INL
ONL
RPE
B
GCL
INL
C
GCL
INL

Figure 20

Parvalbumin labelling of (A) 6-week-old normal mouse retina shows some amacrine cells (ARROW) in the inner nuclear layer. (B) In a 6-week-old dystrophic retina, there are few parvalbumin-positive amacrine cells (ARROW) in the inner nuclear layer. In (C), only a few parvalbumin-positive cells (ARROW) are observed in the grafted area (*) of a 8-week-old rd mouse.

Scale bar = 0.01 mm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.


A
GCL
INL


B
GCL
INL

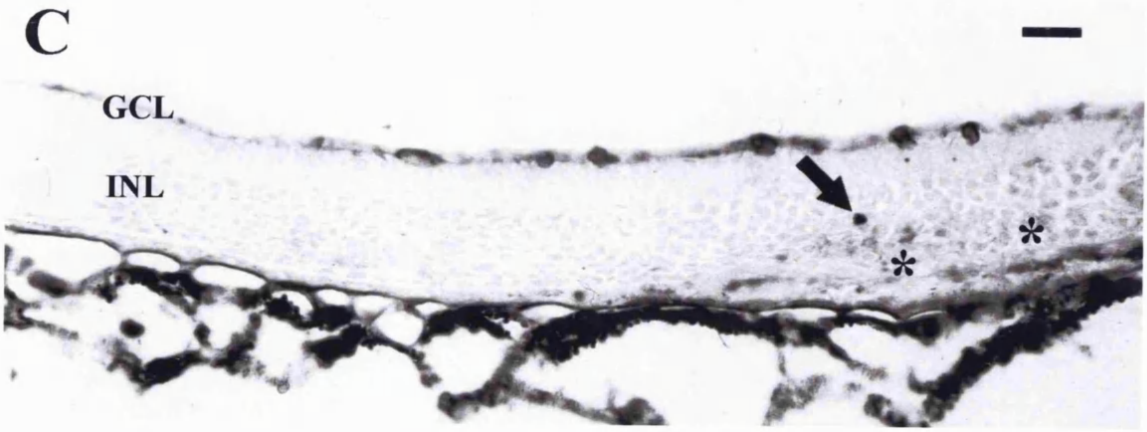
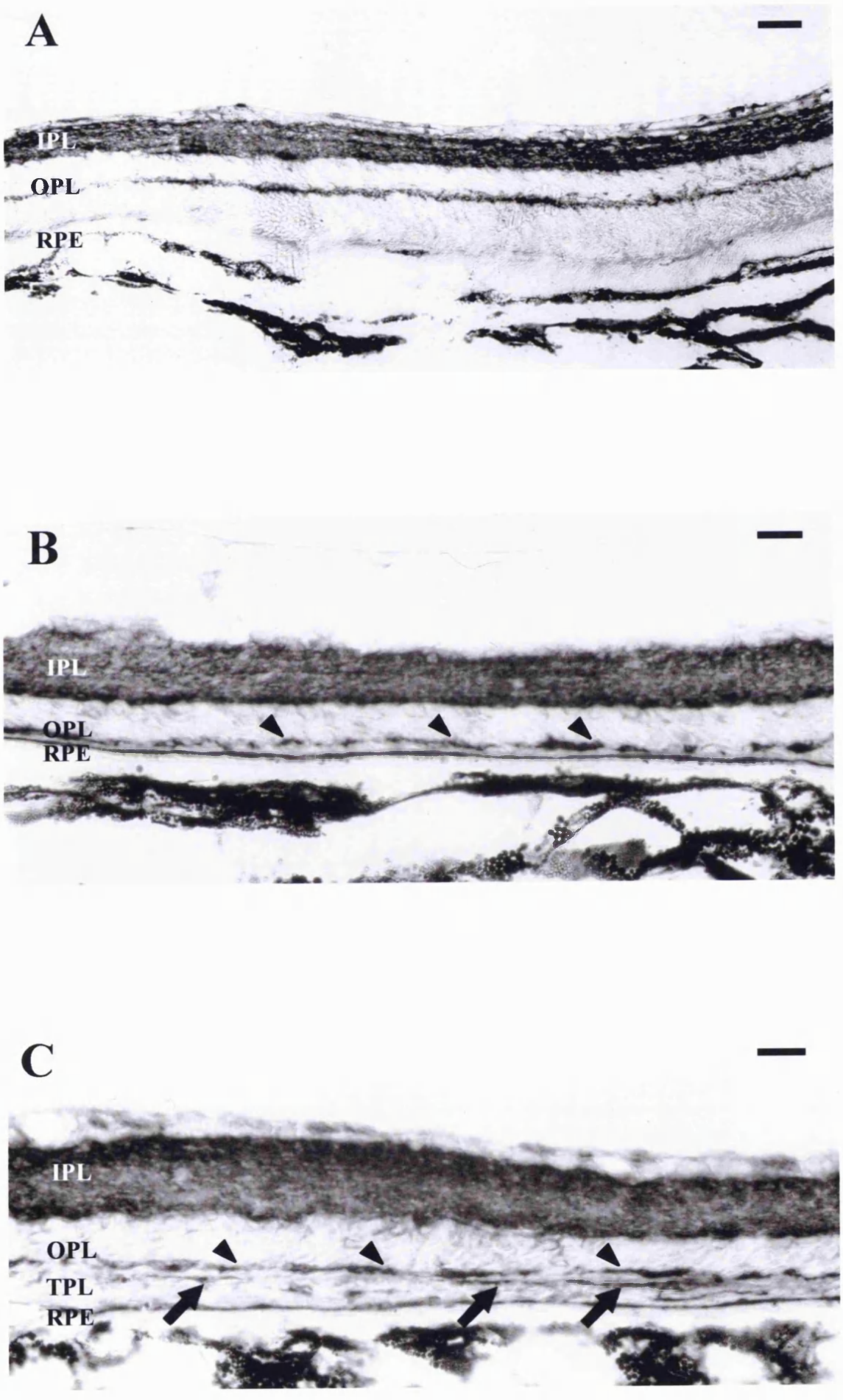

C
GCL
INL

Figure 21

P84 labelling of (A) a normal retina of a 6-week old mouse, the inner and outer plexiform layers are positively labelled. (B) A dystrophic retina from a 6-week-old rd mouse showing the inner plexiform layer and a discontinuous outer plexiform layer (ARROWHEAD) next to the darkly stained and continuous RPE layer. (C) Transplanted retina from a 8-week-old rd mouse showing the same discontinuous labelling of the host outer plexiform layer (ARROWHEAD) and a new synaptic lamina at the host-graft interface (ARROW).

Scale bar = 0.01mm. RPE, host retinal pigment epithelium; IPL, inner plexiform layer; OPL, outer plexiform layer; TPL, transplanted cell layer.


A
IPL
OPL
RPE
B
IPL
OPL
RPE
C
IPL
OPL
TPL
RPE

3.3.2. Transplanted retina

Mouse-to-mouse transplantation

Evidence of graft survival was seen in 26 eyes (19.6% of the total number of eyes, n = 20), of which 15 (n = 12) survived for 2 weeks, 6 (n = 5) for 4 weeks and 5 (n = 3) for 6 weeks. Of these, grafts were confined to the subretinal space without retinal detachment in 10 eyes (7.6% of the total) with 8 (n = 8) survived for 2 weeks and 2 (n = 2) for 4 weeks (Table 13). In the other 16 eyes (12.1% of the total), there was leakage of grafted cells into the vitreal cavity and associated retinal detachment. There was evidence of fibrosis alone in the subretinal space in 13 eyes (9.8% of the total) and this was usually associated with the presence of large cells that had the morphology of macrophages in the Cresyl violet stained sections (Figure 19C). There was no evidence of grafts in the other 93 eyes (70.5%); presumably the grafts had either effluxed after the transplantation or degenerated (apoptosed) without inducing an overt phagocytic or fibrotic response. In sham control, no evidence of graft survival was seen in any of the eyes but subretinal fibrosis was seen in 2 of the grafted retina similar to that seen in retinal transplants.

Table 13. Mouse to mouse transplant results at different survival times (no. of eyes)

	2 weeks	4 weeks	6 weeks	Subtotal
Subretinal grafts with no detachment	8	2	0	10
Grafts with retinal detachments or leakage of grafts into vitreous	7	4	5	16
Subretinal fibrosis	5	5	3	13
No evidence of grafts	40	29	24	93
Total	60	40	32	132

Cresyl violet staining of subretinal grafts

In the 10 mouse-to-mouse subretinal grafts where there was no fibrosis or retinal detachment, the grafts appeared as distinct and continuous sheets occupying usually about one third of the total retinal area and in some cases as much as one half. In most cases grafts were in close contact with host RPE and host retinae (Figure 22 and 23). They were about 4-6 cells thick except towards the borders of the grafts where they were usually reduced to 2 cells thick. Much of each graft consisted of cells with darkly stained nuclei similar in appearance to the darkly stained ONL in normal mouse retina (Figure 13A). No signs of acute inflammation were seen. Occasional rosette formations were seen in the grafts with outer segment material in the centre, but gradually the outer segment material was irregularly configured (Figure 23). More commonly was the presence of lengths of retina with outer segments in close apposition to the host RPE (Figure 22B). Previous studies using whole retinae and microaggregate grafts suggested that outer segments were more likely to be regularly aligned if they are in direct contact with the host RPE (Gouras *et al.*, 1994). At the

graft-host RPE interface, host RPE appeared intact, and RPE cells appeared more full and rounded (Figure 22C), especially where outer segments were in contact with them, in contrast to areas where there was no transplant. This might reflect increased metabolic activity associated with the presence of photoreceptors.

Antibody labelling of the subretinal grafts

Rhodopsin antibody labelling showed that most of the cells within the grafts were rhodopsin-positive (Figure 14B) and evenly distributed in the grafted layer. At the edge of the transplant, the rhodopsin-positive labelling stopped abruptly demonstrating clearly the edge of the graft and in the host retina, beyond the border of the graft there were no rhodopsin-positive cells. Rhodopsin labelling of age-matched dystrophic rd retinae failed to show any positive cells. Therefore, rhodopsin-positive rods were introduced either as post-mitotic or progenitor cells during the transplantation process. Ultrastructurally the cells in the graft mostly had the typical chromatin pattern of rods. At the host RPE/graft junction, where outer segments could be seen in Cresyl violet staining, rhodopsin labelling revealed short outer segments which were rhodopsin-positive and were arranged in a regular fashion adjacent to the host RPE (Figure 14C).

GFAP antibody labelling showed that reactive glial filaments, presumably in Müller cell processes, traversed the host retinae towards the grafts similar to that seen in control dystrophic retina (Figure 15C). In some parts of the transplant there is evidence of randomly orientated GFAP processes. However, where grafts with outer segments adjacent to the host RPE, the GFAP positive processes are fewer in number

and becomes aligned with blocks of photoreceptors. There is some evidence of a thin reactive glial scar between the host and the transplant but it is not complete.

RT97 antibody labelling showed positively labelled ganglion cells and horizontal cells with their axons in the host retina, and in the graft there were a few positively labelled cells (Figure 16C). This may indicate that a few horizontal cells and possibly ganglion cells survived transplantation.

PKC antibody labelling showed PKC positive bipolar cells in the host retinae, there were a few sparsely distributed positive cells in the graft. This indicated that rod bipolar cells (one of the predominant cell types in the INL) were not a major component in the grafts (Figure 17C).

Calbindin (Figure 18C) and **parvalbumin** (Figure 20C) labelling showed positively labelled horizontal, amacrine and some ganglion cells in the host retinae, similar to that of control retinae. In the grafts a few positive cells were seen which were generally locally and sparsely distributed single cells, none of the grafts showed any evidence of a systematic laminar distribution of any of these cell types.

In those grafts where fibrosis was seen (Figure 19C), it was often associated with the presence of large rounded pigmented cells in the subretinal space (see also Figure 25A). These cells were not seen in the 10 good mouse to mouse subretinal grafts nor in indeed in the 3 rat to mouse subretinal grafts. The pigmentation suggests that these cells are macrophages laden with haemosiderin, possibly deriving from regional haemorrhage at the time of surgery. Using the **F4/80** for macrophages and microglia antibody, these cells were indeed positively labelled confirming these as

macrophages. In the vicinity of these cells there were positively labelled cells which had a stellate configuration (Figure 25B), which are likely to be activated microglia with their dendritic processes.

Immunohistochemistry of the graft-host retina interface

In Cresyl violet stained retinae of control dystrophic rd mice, there was a single discontinuous layer of loosely distributed cells along the outer border of the retina. These were the remaining cones as previously described. In grafted retinae, these cells could still be identified between graft and host with cell-free spaces either side (Figure 22C). One concern was that a glial barrier, situated between the graft and host, might fill this space and limit synaptic integration. However, although there was some evidence of glial scarring (Figure 15C) the scar was discontinuous and this was confirmed with electron microscopy. In the rhodopsin-labelled sections, the positively labelled cells were seen up to the interface region but although fine processes were sometimes seen at the border, no examples were seen of rhodopsin positive processes extending beyond the interface zone. RT97 labelling of control dystrophic retinae without transplants showed tangentially running processes of horizontal cells coursing along the outer surface of the retina; a similar appearance was seen in transplanted retinae, with no evidence of breaching of the interface (Figure 16C). By contrast, PKC-labelled rod bipolar cells did show evidence of dendritic growth at the interface (Figure 17C). In normal animals, PKC antibody labelled a clearly defined and uniform group of bipolar cells. Most of these had cell bodies close to the OPL, with occasional ones lying deeper in the INL. Dendritic processes distributed in the OPL. They had an axon that continued unbranched through the retina with dilatations along its course and a large club-like ending in the IPL close to the layer of retinal

ganglion cells. This was typical of rod bipolar cells, although it is possible that some of the more deeply located somata could be a subtype of cone bipolar cell. In unoperated dystrophic rd mice there were several differences compared with the transplanted group. First there were very few dendritic branches and these tended to run tangentially at the outer border of the retina. Second, the number of bipolar cell bodies appeared reduced and they were discontinuously distributed along the outer border of the retina. Third, the club-like endings in the IPL were less evident and they seemed to have smaller arbours than in normal retinae. This appearance was also seen in the retinae of grafted animals away from the area of the transplant. In the region adjacent to the graft there was clear evidence of bushy processes on the apical surface of some of bipolar cell somata. In addition there appeared to be more PKC-positive cells in the outer border of the INL. Whether these were new cells migrated from the graft or host cells that had changed their labelling characteristics was not clear.

In **P84** synaptic antibody labelling, as mentioned earlier, in the unoperated dystrophic rd mice, a discontinuous line of positive labelling was seen at the outer border of the retina, between the INL and the remaining cones in the OPL (Figure 21B). It tended to lie deep to the residual cones and probably reflected the distribution of synapses associated with them. A similar pattern of labelling was also seen in grafted retinae; it did not seem to be more extensive than in controls or in retina adjacent to the graft. Most importantly, at the interface between graft and host retinae, an additional continuous line was seen lying outside the cone layer (Figure 21C). This suggested that there was a separate synaptic interface between graft and host. This interface could also be seen in semi-thin sections and in EM of adjacent sections.

EM of this region showed that it contained abundant synapses, some of which were associated with terminals appearing similar to typical rod spherules, suggesting that they might indeed belong to transplanted cells. The terminals contained synaptic ribbons and formed frequent complex synaptic arrays, involving both dyad and triad synapses (Figure 26). Similar synapses were not seen in the comparable location in adjacent retina away from the transplant, or in control non-dystrophic rd mouse retinae.

Rat-to-mouse transplantation

Cryostat sections of the eyes were obtained. Subretinal grafts were seen in 3 eyes (10.7%, n = 3) from three animals, all at 1-week survival. These grafts also formed a continuous layer in the subretinal space of the dystrophic mice just like those found in the mouse-to-mouse transplant. However there were some striking differences between them and the mouse to mouse transplants, namely numerous rosettes were observed (Figure 24A), there was a lack of outer segments aligning with the host RPE and the grafts were generally thicker. Again there was no sign of acute inflammation.

6G3, also a member of SIRP family, is rat specific synaptic protein. 6G3 antibody labelling was negative in mouse retina but it labelled rat IPL and OPL (Figure 24B). In rat-to-mouse subretinal transplants, 6G3, rat specific synaptic membrane antibody, showed no labelling of the IPL and OPL of the host mouse retinae as expected, but it was positive at the host-graft junction (Figure 24C). Similar to the P84 labelling at the host-graft interface in the mouse to mouse transplant, the 6G3 labelling was most intense between the host retina and the graft, indicating an increase in synaptic membrane protein expression around the grafted cells at the host-graft interface. This

might be interpreted as possible synaptic activity between the graft and host and neuronal processes were involved.

Figure 22

(A) Cresyl violet staining of a subretinal transplant in a 8-week-old dystrophic rd mouse showing the edge of the transplant (ARROW) at 2 weeks post-transplantation. Note that in the region of retina where there is no transplant (right of the ARROW), the outer nuclear layer contains only dispersed cells. The transplanted layer (left of the ARROW) with widespread photoreceptors with slightly pink stained outer segments (ARROWHEAD) is clearly seen. Scale bar = 0.1 mm. (B) High magnification of the edge of the above transplant. Scale bar = 0.05 mm. (C) High magnification of Cresyl violet stained area of transplanted cells with outer segments, from the same mouse as in A and B, in close apposition to the host RPE (ARROWHEAD) which have a fuller appearance than RPE cells away from the graft. Note the host-graft interface with a single layer of host residual cone photoreceptors (ARROW). Scale bar = 0.01 mm.

GCL, host ganglion cell layer; INL, host inner nuclear layer; TPL, transplanted cell layer; OS, inner and outer segments of transplanted cells; RPE, host RPE.

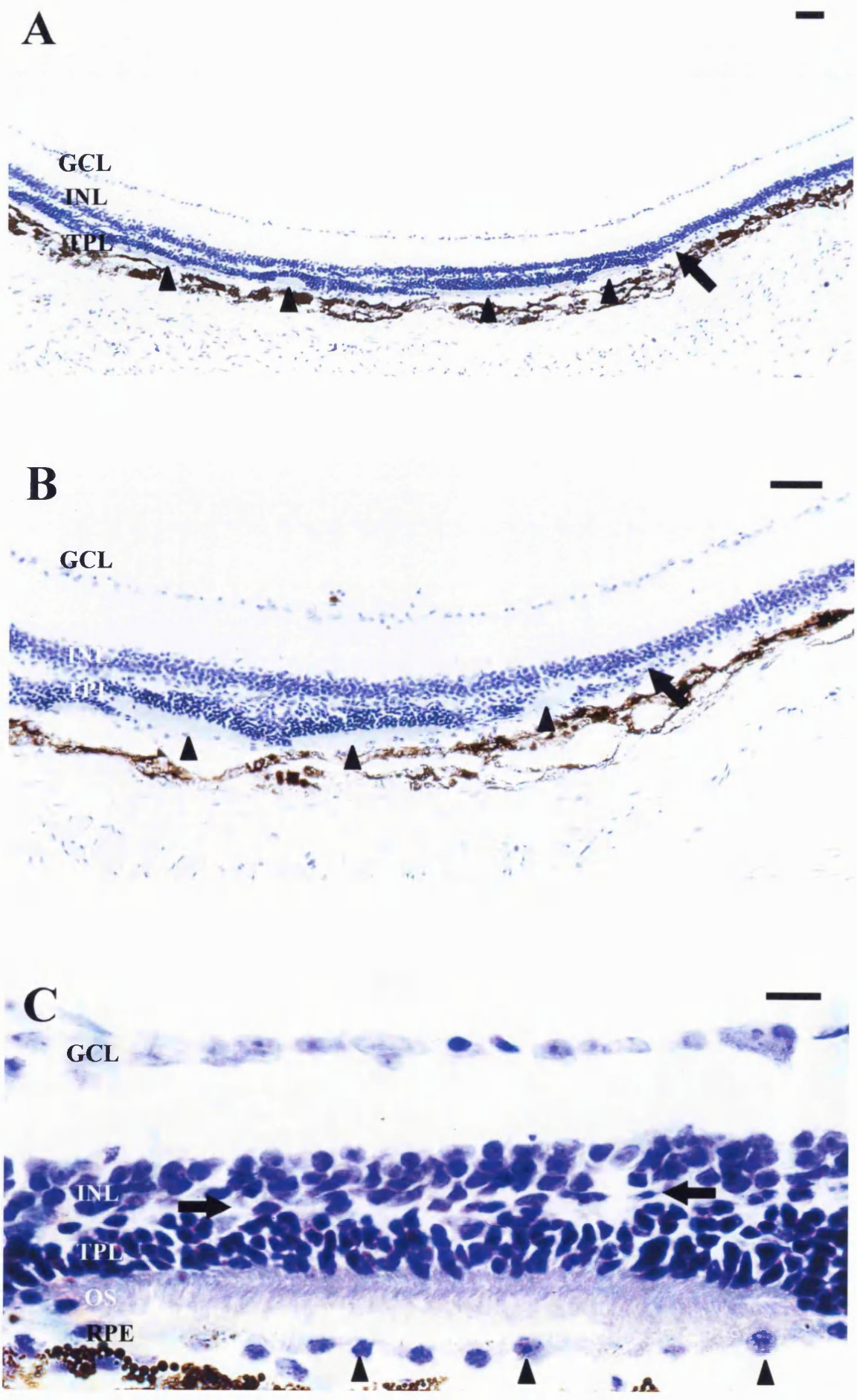

A
GCL
INL
IPL
B
GCL
C
GCL
INL
IPL
OS
RPE

Figure 23

Cresyl violet staining of further examples of successful retinal grafts into dystrophic rd mice at different survival time. The areas with successful transplants are shown between the two ARROWHEADS at (A) 2 weeks, (B) 2 weeks and (C) 4 weeks after transplantation. They have three retinal cell layers as described in Figure 6. The areas vary in size but all are found in the dorso-temporal subretinal space and no discernible retinal detachment can be seen. A few rosettes are present (ARROW).

Scale bar = 0.1 mm.

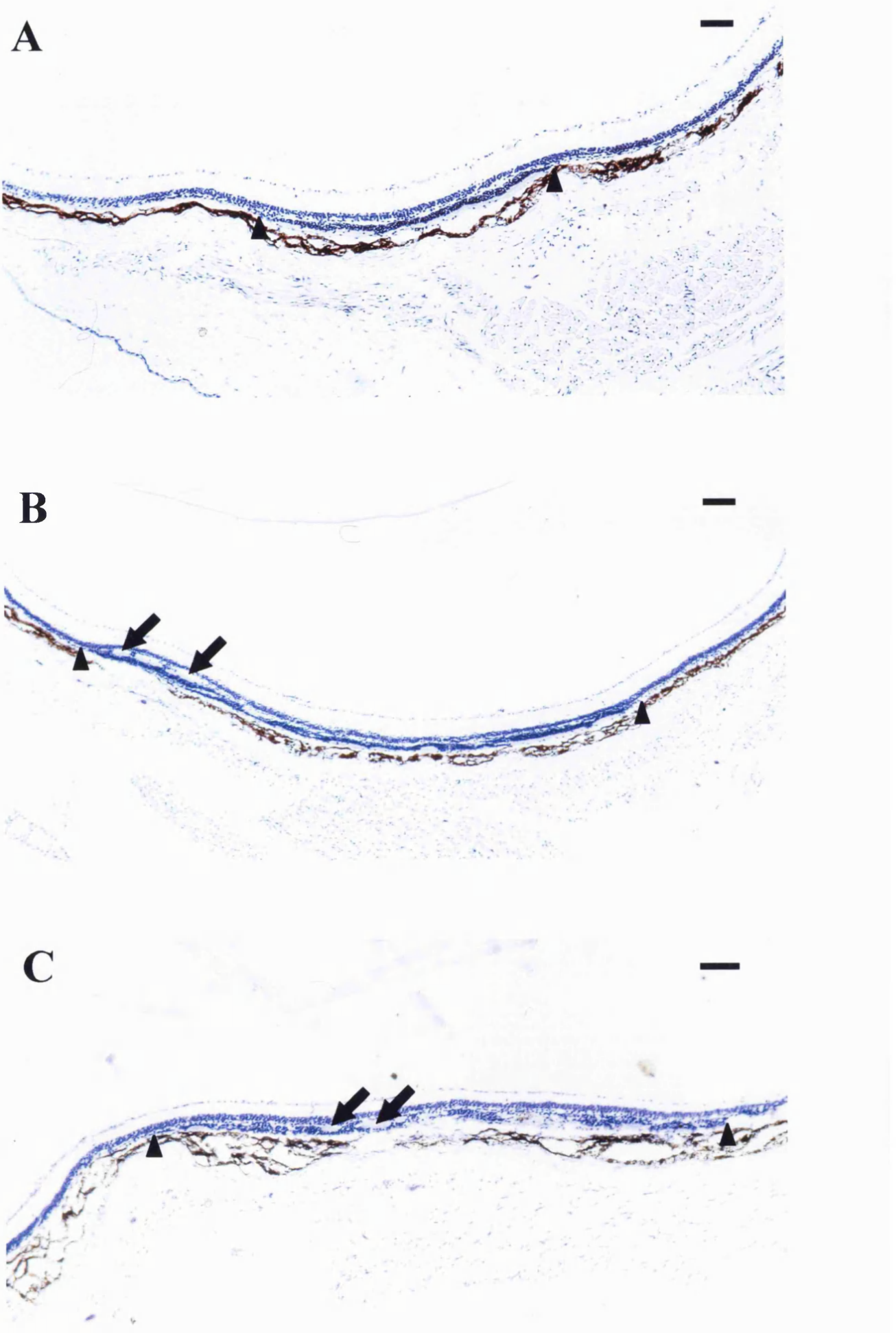

A
B
C

Figure 24

Cresyl violet staining of rat to mouse subretinal transplant. (A) Subretinal graft of neonatal rat retinal microaggregate into a 3-month-old dystrophic rd mouse two weeks after transplantation. A large graft is seen in the subretinal space between the two ARROW and it is thicker than those seen in mouse to mouse transplants. Moreover a large number of rosettes (ARROWHEAD) can be seen. Scale bar = 0.1 mm. (B) Cryostat section of a 6-week-old normal rat retina showing 6G3 labelling of IPL and OPL, similar to the P84 labelling pattern of normal mouse retina as in Figure 15. Scale bar = 0.01 mm. (C) High magnification of 6G3 labelling at the host-graft interface shows increased labelling (ARROW). Note that inner and outer plexiform layers are not labelled in the host retina (mouse) as 6G3 antibody is species-specific and it only labels rat synapses. Scale bar = 0.01 mm.

GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments; TPL, transplanted layer.

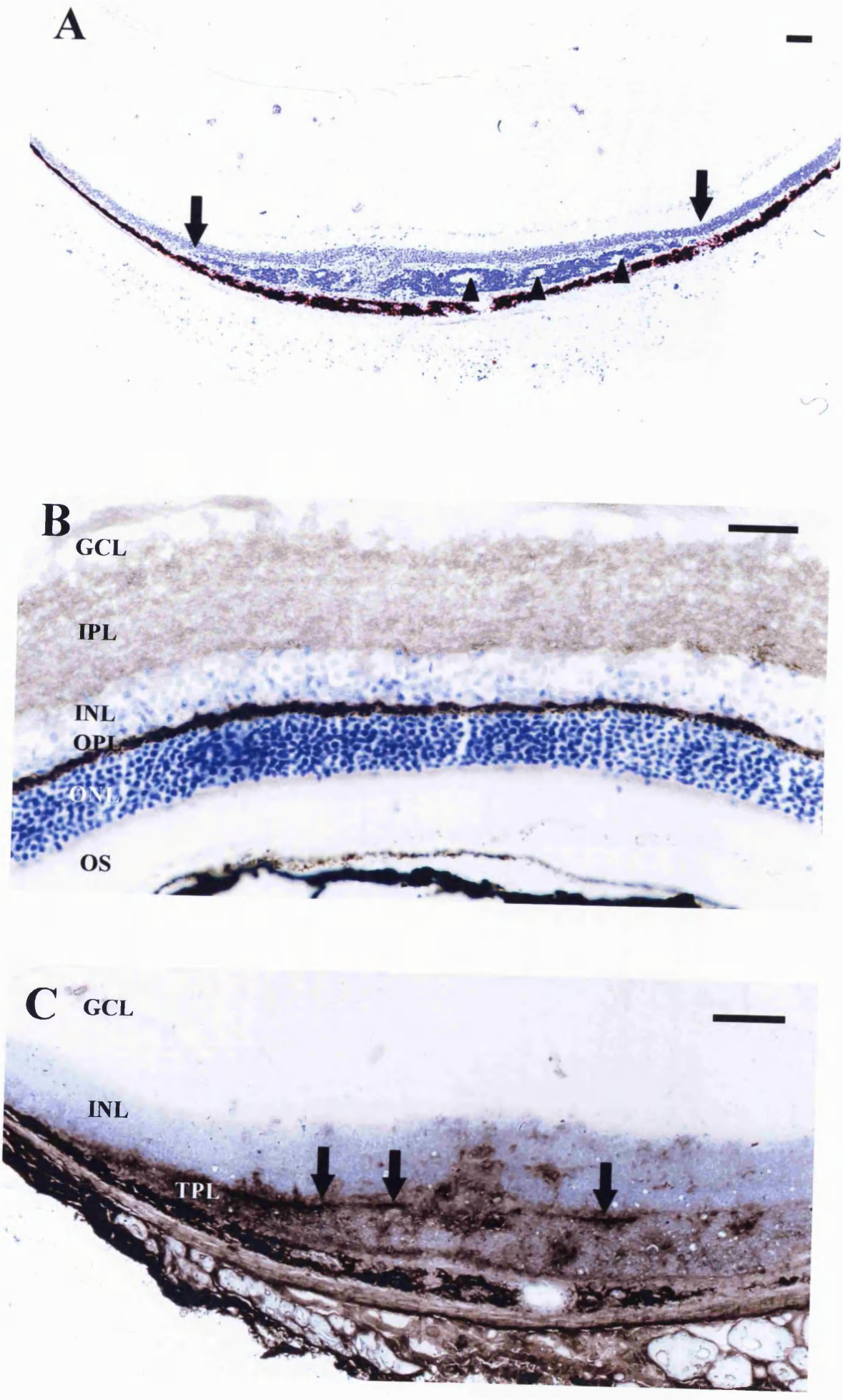
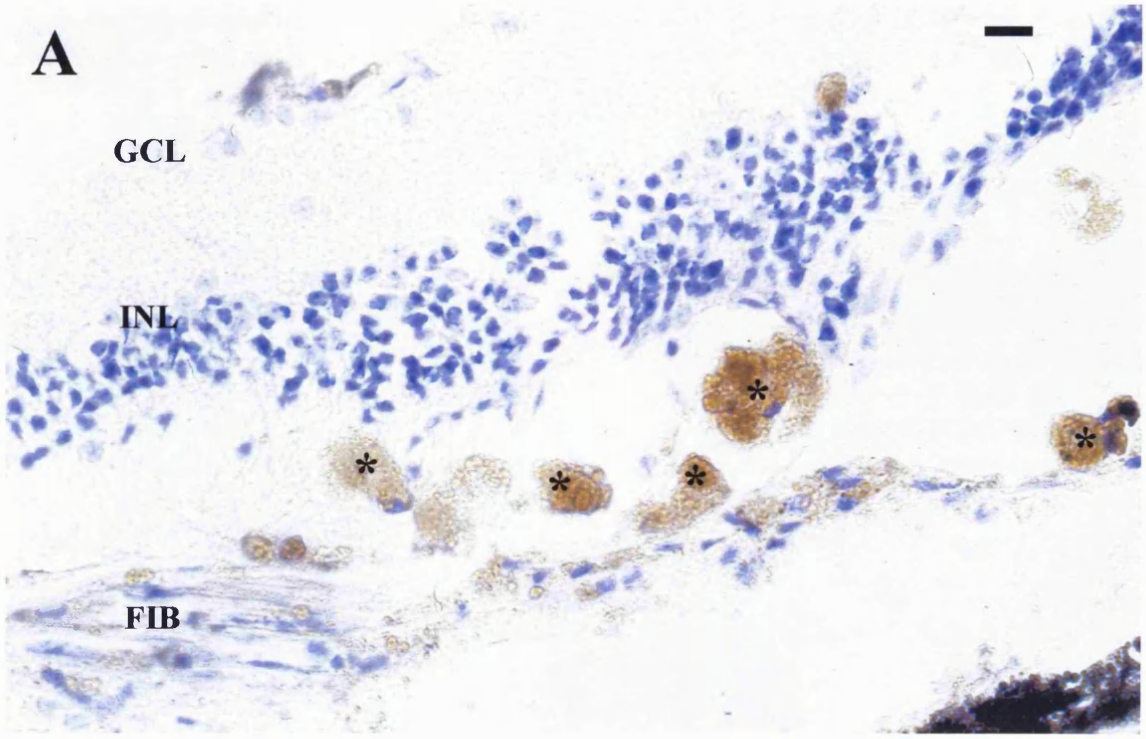

A
B
GCL
IPL
INL
OPL
ONL
OS
C
GCL
INL
TPL

Figure 25

(A) High magnification of Cresyl violet stained area of mouse to mouse transplant associated with fibrosis with several large pigmented cells (*). (B) F4/80 labelling shows positively labelled macrophages (*) which correspond to the large pigmented cells in the Cresyl violet sections and together with activated microglia (ARROW).

Scale bar = 0.01 mm. GCL, ganglion cell layer; INL, inner nuclear layer; FIB, subretinal fibrosis.


A
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INL
FIB

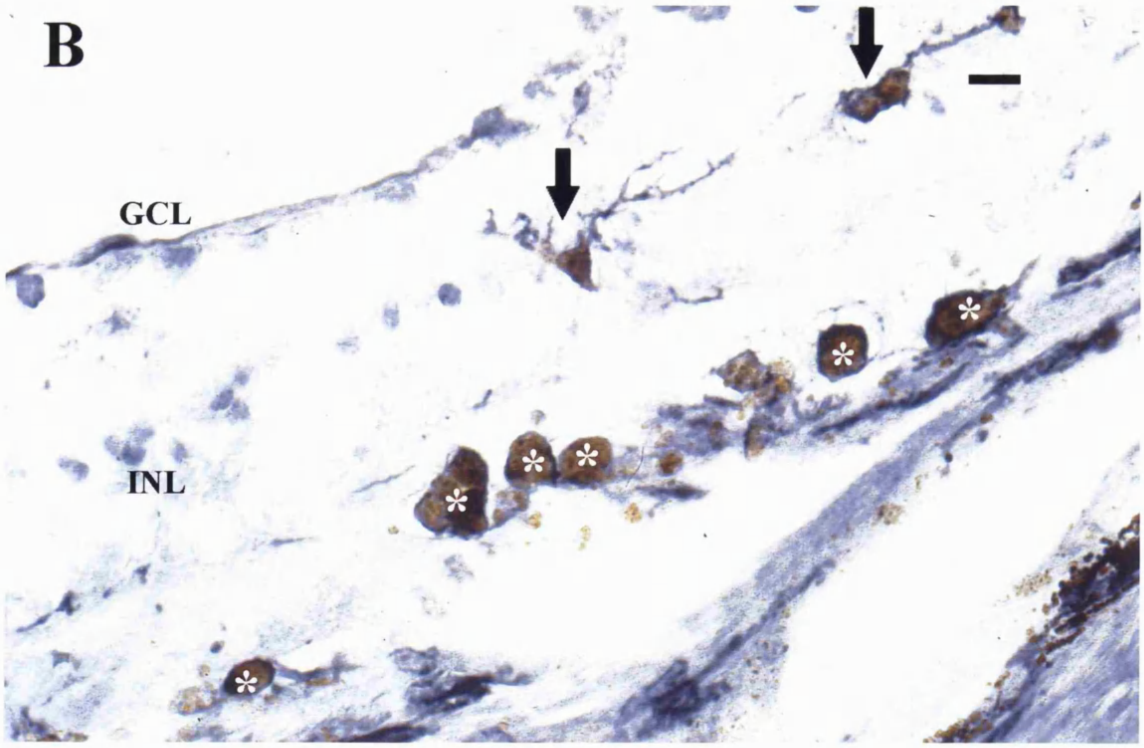
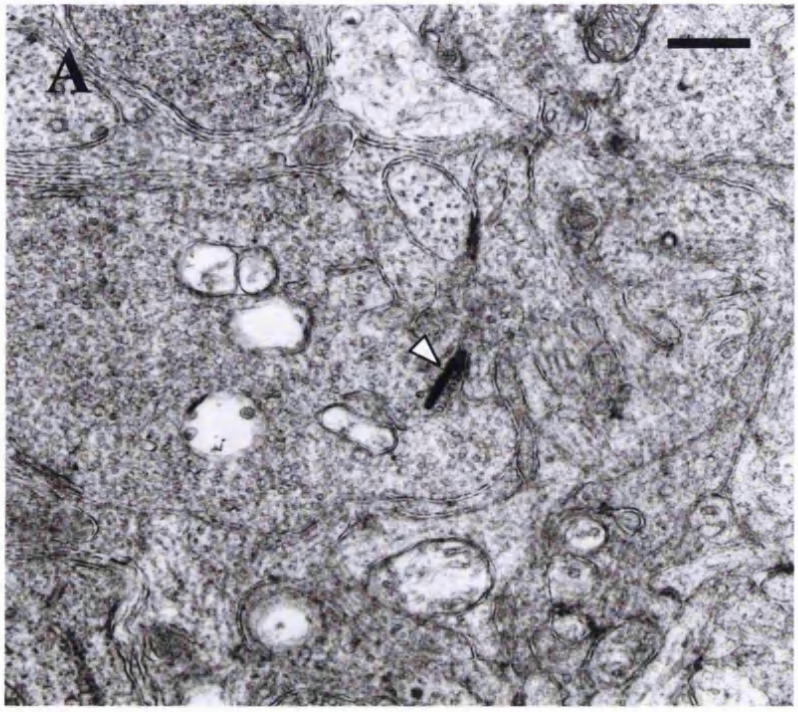
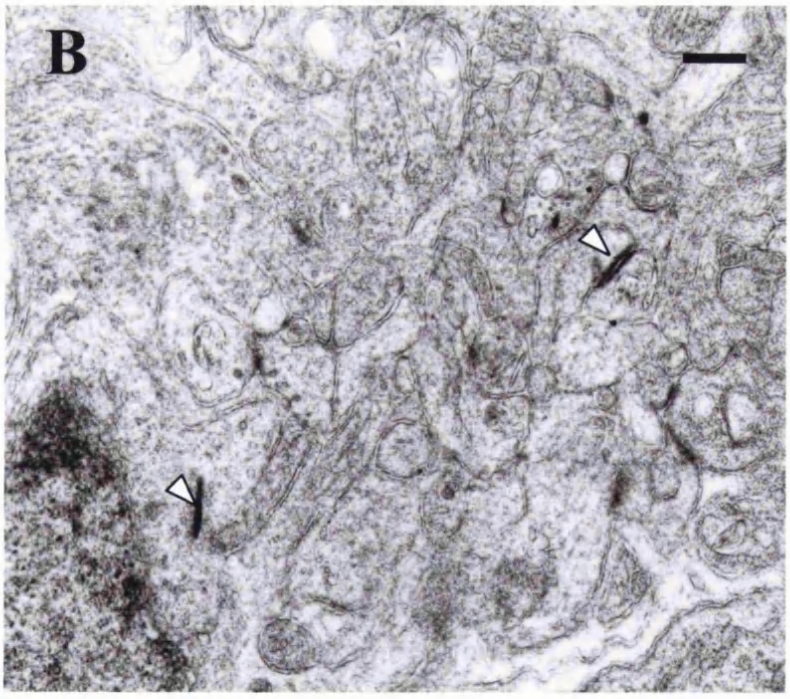

B
GCL
INL

Figure 26

Transmission electron microscopy. (A) and (B) are transmission electron micrographs showing two typical photoreceptor terminals at the graft/host interface of a 8-week-old rd mouse, corresponding to the position of the new synaptic lamina. (A) shows a typical rod spherule. Scale bar = 500nm. (B) shows a perinuclear synapse. Synaptic ribbons can be seen in each (ARROWHEAD). Scale bar = 200 nm.


A


B

4.0. Discussion

Since the transplantation process and retina could be visualised through the operating microscope using this technique, it was possible to obtain a good intra-operative indication of whether the grafts had been successfully placed in the subretinal space. This, for example, helped in the selection of suitable animals for post-operative, labour-intensive, electrophysiological tests. The aim was to measure the effect of subretinal grafts in dystrophic mice, therefore if microaggregates were effluxed from the sclerotomy site or injected into the vitreous, those experimental animals would not be chosen for retinal threshold measurements. The 'blind' injection method that other studies have used would not be helpful in this selection process (del Cerro *et al.*, 1989; Gouras *et al.*, 1991; Jiang and del Cerro, 1992a). One might have to perform measurements on experimental animals that had no transplant in the first place. Here evidence of grafts in the eyes was seen in 26 of the 132 dystrophic eyes that received transplants. This is relatively low number considering in the pilot experiment of ink injection and microaggregates injection into normal mouse eyes achieved good success rate (63.8%, See Section 2.3.). When it came to performing transplantation in dystrophic mice, there was a noticeable difference to the transplantation process and results. The initial part of the technique was similar to injection in normal mice, however when the insertion of the glass pipettes and the subsequent injections of the microaggregate were performed, it was noted that there was a higher incidence of inadvertent perforations of the retinal tissue and leading to injection of microaggregates into the vitreous cavity. This observation was more frequent in the early sessions of transplantation and after the first 15 to 20 injections it was not as frequent. This cause of these perforations of retina was attributed to the thin retinal tissues in the dystrophic mice which were more prone to surgical trauma than those in

normal mice. This showed a steep learning curve in the transplantation technique in dystrophic animals. It was also for the above reasons that in the early part of the study the dystrophic mice with microaggregate transplant were not used in the LDPT and retinal threshold tests.

As previously mentioned, there are other factors that could jeopardise graft survival, including donor age, apoptosis, transplantation trauma leading to up regulation of MHC complexes, breakdown of the blood-retinal barrier and vascular remodelling. We believe our transplantation technique has achieved reasonable results in the small mouse eye.

Histological results showed that early postnatal retinal cells (PN 7 – 9) that had been gently and mechanically dissociated survived for at least 6 weeks after transplantation into the subretinal space. In 10 eyes, the grafts integrated with the remaining host retina and the replacement photoreceptors formed outer segments over part of their area, particularly where they lay adjacent to the host RPE. Previous studies using dissociated cells or microaggregates have failed to achieve the large areas of coverage seen here and generally showed frequent rosette formation with only small and discontinuous areas in which outer segments were lined up against the host RPE (Gouras *et al.*, 1992; Gouras *et al.*, 1994; Juliusson *et al.*, 1993; Sharma *et al.*, 1997b). The differences may be attributable to a number of causes. First, by taking retinal cells at a late stage in histogenesis, there is a likely emphasis on photoreceptors being the dominant graft cell. Second, the mild mechanical dissociation without use of enzymes gives a better yield of viable cells with possibly healthier morphology and the use of Ca^{2+} and Mg^{2+} free medium ensures a greater level of dissociation. Thirdly by lowering the intraocular pressure through

decompressing the eye with a corneal puncture, the amount of cellular efflux from the injection site is probably significantly reduced and a better coverage of a larger area of retina by the grafted cells can be achieved. Furthermore, since the approach used here did not involve a retinotomy to gain access to the subretinal space, the post-operative retinal blebs settled quickly, thus improving the chance of cell survival and integration.

Perhaps the most promising observation is the development of relatively large areas of transplant with photoreceptors whose outer segments lie against the host RPE. In the ten successfully grafted eyes, on average the graft was found in 9 slides in each of the 4 series of slides that were cut. Each slide contains 10 wax sections of 8 μm thick each. If one assumed that the area of transplant is circular in shape, the average diameter of each grafted site would be 9 x 4 x 10 x 8 μm or 2880 μm. The estimated area of a graft is π x $(2880/2)^2$ μm^2 or 2073600 μm^2. As the estimated total retinal surface area from mouse retinal flat mount is about 15000000 μm^2 (Wang *et al.*, 2000 and personal communication), the average graft occupied about 13.8% of the subretinal space. In previous studies of photoreceptor transplantation into rd mice, this has only been achieved in small, localised areas in a somewhat discontinuous fashion (Gouras *et al.*, 1994; Silverman *et al.*, 1992). It has been suggested that the integrity of the Müller cells may be necessary for maintaining orientation and graft survival (Gouras *et al.*, 1994), the present findings of ordered outer segments and ordered Müller cell processes seen with GFAP would suggest that such a relationship is present. Some of the previous studies have had difficulty showing unequivocally that there is synaptic integration between graft and host in rd mice (Jiang and del Cerro, 1992a; Silverman *et al.*, 1992). Here there is quite strong evidence that this did

occur. First, the space between graft and host was not completely occupied by a glial scar. Second and more crucially, was the appearance of a new synaptic lamina, seen in light microscopic sections labelled with the P84 antibody for synaptic protein. This was distinct from the synaptic layer attributable to the remaining cones. Moreover 6G3 labelling for synaptic protein in the rat to mouse transplant also showed stronger labelling at the host-graft junction than within the graft. This again suggests the formation of synapses between host and graft rather than labelling of synaptic protein within graft. Ultrastructurally, it was evident in the mouse to mouse transplants that there were many synapses in the region where the new synaptic lamina was seen by light microscopy, and many of them appeared more like typical rod spherules than cone pedicles. Both the location and appearance of synapses therefore argue for a degree of reconstructed circuitry. This finding is supported by previous studies on other animal models where (1) ultrastructural analysis of areas of host retina, which were in apposition to the photoreceptor transplant, revealed the presence of numerous ribbon-type synapses, whereas such synapses were rare in unreconstructed retina (Gouras *et al.*, 1991; Silverman *et al.*, 1992; Zucker *et al.*, 1994); and (2) synapses could be found along transplanted embryonic retinal cell processes that had grown into the host IPL. This suggested that neuronal fibres originating from embryonic retinal transplants could form synapses in the host retina (Aramant and Seiler, 1995). There is one concern in the present study, however and that relates to the fact that there appear to be more bipolar cells at the graft-host interface in grafted mice. If some of these had migrated from the graft into the host retina, then it is possible that some of, the observed synapses could be between donor photoreceptors and donor bipolar cells. This in itself may not be a major obstacle, because it is still possible that the bipolar cells themselves could recreate circuitry downstream. However, evidence

for the formation of connections is not on its own sufficient: they must transmit a sensible signal to the host CNS.

In this study, the transplants were studied over a relatively short time period extending up to 6 weeks. While one study showed that some retinal grafts could survive for a long time in rd mice (25% at 7 months) (Gouras *et al.*, 1994), other work has shown deterioration of grafts with time. Jiang and del Cerro (1992a) found that 60% of syngeneic retinae grafted into the eyes of rd mice were present 3 days postoperative, but this number gradually dropped to around 10% at 30 days. Our survival rate was comparable to their results. Studies of the vascular changes following photoreceptor loss in the rd mouse have shown that the deep plexus is differentially lost with time (Blanks and Johnson, 1986; Matthes and Bok, 1984; Wang *et al.*, 2000). This raises the possibility that if a large graft is added, as in our study, the circulation may not respond sufficiently quickly to accommodate the increased metabolic demands and some level of degeneration may ensue. This is currently under investigation. Moreover recent studies have shown that there are associated changes in the RPE, choriocapillaris, ganglion cells and their axons, and aberrant vascular formations in rd mice (Neuhardt *et al.*, 1999; Nishikawa and LaVail, 1998; Wang *et al.*, 2000): all these may affect the success of retinal transplantation. Apart from these factors, graft rejection would undoubtedly play a part in the long-term survival of retinal graft. The loss of the immune privilege in older rd mice (Welge-Lussen *et al.*, 1999) and the questionable integrity of the blood-retinal barrier following retinal injection/transplant due to the small size of the mouse eye are likely to jeopardise the long-term survival of retinal grafts in rd mice (Al Amro *et al.*, 1999). Therefore, to investigate the long-term effects of retinal graft in

RP model, one may have to study a larger animal model with possibly a lower degeneration time scale.

The purpose of setting up the LDPT was to device a behavioural test that can distinguish between normal and dystrophic mice and then to use it in an attempt to distinguish successfully transplanted mice from unsuccessfully transplanted mice and control animals. The LDPT results showed that at low luminance, normal mice preferred a dark environment as they stayed fairly consistently on average more than 50% of the time in the dark compartment. These findings were true for both the longitudinal and cross-sectional studies and were statistically significant. The results for dystrophic mice were more complicated than anticipated. If rd mice were "blind" when they reached 6-week-old, they were expected to have no preference for the light or dark compartments and therefore should spend an equal amount of time in the light and dark compartments (i.e. 900 seconds each). However, as can be seen from the results, dystrophic mice on average tended to spend more time in the light segment than in the dark in both the longitudinal and cross-sectional studies. This was an unexpected finding but one possible explanation is that in the absence of rod photoreceptors and in the presence of rudimentary cones, the cones might be the only source of visual stimulus and hence guidance. These dystrophic animals might prefer to stay in the brighter environment than to stay in complete darkness where they have no perception of light. Another possible explanation is that the dystrophic mice were responding to an incidental cue that was sufficiently weak not to be identified in cases when the animal's vision was intact, as in the normal mice. However, since the temperature, background noise and scent of the apparatus were controlled in this arena, we believe that lighting difference was the only denominator in their behaviour. These results are in keeping with previous studies on older mice (more

than 6-week-old) that normal mice have superior performance in vision related tasks than dystrophic rd mice (Lindzey and Winston, 1962; Wimer and Weller, 1965). Another study had shown that younger rd mice (less than 6-week-old) and normal mice of the same age had no difference in their vision related performance (Nagy and McKay, 1972); our study used older animals, and the transplants and related functional tests were performed in rd mice older than 6-week-old and therefore our findings do not contradict other studies.

Following transplantation, there was a change in the behaviour of the grafted dystrophic mice. Their behaviour shifted from spending more time in the light compartment pre-operatively to spending more time in the dark compartment post-transplantation. This pattern was observed in all groups (histology-positive, histology-negative and sham control groups), and this shift was particularly clear in the results from histology-positive group at two weeks post-transplantation (Figure 24). Unfortunately due the small sample size of the experimental groups, the difference between the groups was ***not*** statistically significant. The result for histology-positive group is encouraging but has to be interpreted with cautions and it will require a large study with a larger number of experimental animals to clarify these findings. It is estimated that for the results to reach a statistically significant level of $p< 0.05$, one would require at least 100 experimental and 100 control animals.

In the experiment to measure changes in retinal threshold, three mice showed threshold recovery and histological evidence of a surviving graft but only one animal had its retinal threshold nearly restored to normal. This suggests that some functional recovery is possible, and this recovery could reach a higher centre in the visual

system, namely the SC. However this is indeed a small number of positive results and if one is to repeat the study, it is estimated that one should enroll at least 100 subjects for the threshold test in order to achieve a more representative number of positive results. Our results of some functional recovery in some rd mice following transplantation were confirmed recently by another group that studied light-driven ganglion cell responses in dystrophic rd mice after retinal transplantation (Radner *et al.*, 2001). In their study, they demonstrated the presence of light-driven retinal ganglion cell responses (ON-response only) over the graft 5 weeks after transplanting of normal neonatal retinal tissue into the subretinal space of 3 of 10 rd mice aged 13 days. A light response could not be elicited from any control eye or from non-transplant areas. In addition, they could not elicit light responses from rd mice that received transplants at 8 weeks of age or older. From our study and their study, it seems that rd mouse older than 8 week of age may have less potential for functional recovery after retinal transplantation. A reason for this apparent age-dependent effect may be the higher synaptic plasticity of the developing retinal tissue in younger mice. Foetal retinal tissue has been suggested as a suitable source of donor tissue because of its presumed greater plasticity and lower antigenicity compared with adult retinal tissue. In CNS transplantation experiments, after transplantation of foetal rat retinal tissue over newborn rat superior colliculi, McLoon and Lund (1980) demonstrated survival of retinal transplants and found histochemical evidence for synaptic connections between graft and host tissue. Furthermore, Radel *et al.* (1995) performed functional experiments on newborn rats that had received embryonic neural retinal transplants (E12-15) placed over their midbrains. Although the retinal transplants were organised as rosettes, the transplanted tissue showed preferentially axonal innervation of primary visual centres, such as the pretectum, and photic stimulation of the transplanted tissue caused reproducible PLR. Thus, by using foetal

retinal tissue and a young host, it has demonstrated functional connections between graft and host neural tissue. These observations are consistent with the hypothesis that a younger host retina containing less well-differentiated cells may be more receptive to synaptic contacts, thereby enhancing functional integration with the transplant. Moreover, in older rd mice, the vacated synaptic sites may be blocked by collateral sprouts (as in the CNS) and these sites may not become available again for the new ingrowing connections from grafted cells. However, transplantation in mice is limited by the size of the eye, and for younger mice which have very small eyes, this is a practical limitation. Certainly for experiments in a large animal model of RP, younger donor and younger recipients should be considered.

The results of the LDPT and the retinal threshold measurements do *not* statistically indicate that retinal transplantation restores visual function in dystrophic rd mice, but they are very encouraging, and are suggestive that transplantation may work and indicate that the study should be repeated with a larger normal of animals. Again it is important to interpret these results with extreme caution. Should retinal transplantation work, it is likely to be from reconstructed rod circuitry, firstly because the LDPT used a very low luminance level within the rod photoreceptor range. It is unlikely that the cones can mediate any response at that level of luminance. Secondly, the presence of circuitry between graft and host involved rod-like terminals identified using EM, not seen in parts of the retina where the transplant was absent. Thirdly, the presence of synaptic protein labelling with P84 and 6G3 antibodies at the host-graft interface may be significant. However there are other possible explanations. Firstly Mohand–Said *et al.* (1997) demonstrated an apparent remote protective effect of the transplant on the host retina that led to survival of the remaining photoreceptor cells. They found increased survival of cones in the central quadrants of the retinae after

transplantation in the mid- and far periphery. In our experiments there were still a few cone nuclei remaining in both age-matched control animals and in the 6 to 8-week-old transplant recipients. There was no qualitative difference, however, in the number of cone nuclei between control eyes and the non-transplant areas of the transplant-recipient eyes. Because the donor tissue was not labelled, it was not possible to reliably distinguish between host and transplant cones. Consequently, it was not possible to compare the number of host cones within and outside the transplant area. Nonetheless, no photoreceptor outer segments could be identified in the control eyes or in the non-transplant areas of the transplant-recipients and no recovery of threshold measurement was seen outside the site of the transplant. Thus, if photoreceptor rescue was responsible for the preservation of light responsiveness in these transplant-recipient eyes, the effect was highly localised and regional, in contrast to the remote effect reported by Mohand–Said *et al.* (1997). Secondly, it is also possible that residual host cone cells without outer segments contribute to the observed light response. This is certainly possible in the LDPT as the test is a "global" test for light-dark preference with both eyes opened and it was not easy to determine whether the pattern of behaviour was generated from the remaining cones or from transplanted cells. However, where the retinal threshold measurements have been made, it is clear that the area of lower thresholds corresponds to the region of retina with the transplant (confirmed by histology). If the remaining host cones were to "up-regulate" to the extent where threshold could be reduced, they would have done so in other eyes with no evidence of graft survival. In previous work, grafting embryonic retina into the brain, it was found that such retinae not only sent out axons to visual centres of the host CNS, but that these connections were capable of mediating simple responses, both reflex and learned, to light flashes (Coffey *et al.*, 1989). Thus the principle that transplanted retinae can recreate new functional circuitry has a

precedent. In this context, it is not unreasonable to expect that logical circuit reconstruction might be achievable within the retina itself, but it remains to be seen how much visual function can be regenerated.

There were experimental limitations in this study. Firstly, only relatively short post-operative survivals were studied (up to 6 weeks) and the effect of long-term transplantation is not known. Secondly, although the LDPT was a reasonable basic test of visual function it could be subjected to many external influences and there is a large between subject variability in performance. Thirdly, the visual threshold measurement was a terminal experiment because of Home Office guidelines, and therefore repeated measurements were not possible. The changes with time in individual experimental animals could not be measured. Fourthly, only a small number of histology-positive animals were available for functional tests. This was due to the difficulty in subretinal transplantation in the small mouse eyes and the time constraint in the study period.

Further studies are required to investigate the long-term results of retinal transplantation and its effects on visual function. A larger animal model may be more surgically relevant and more advanced behavioural tests could be used on more intelligent animal models. Furthermore, animal models with slower rates of retinal degeneration could be used to assess the effects of transplantation at earlier stages in the disease process before all the photoreceptors are lost. Results from such studies would have direct relevance for future clinical trials. However, it is important to stress that more animal experiments are needed before one can recommend clinical trials in humans and this will help to ensure patients are not given false expectation.

Future directions in relation to human retinal transplantation

Donor tissue is an issue, both in terms of safety (risk of infectious material) and ethical concern. It is a practical issue as to whether all infectious agents, including prions, can be screened. As retinal disease is not fatal, this makes safety considerations in transplant procedures all the more important. Even though various sources of donor tissue have been tested in transplantation, foetal tissue still appears to be the least immunogenic and has better survival potential. However, controversy arises from the ethics of using foetal tissue per se and the relatively large amounts of material would have to be available if the procedure is to become routine. As induced abortion, for both social or medical reasons, produces healthier foetal tissue than spontaneous abortion, opponents of its use argue that one cannot collect and use human foetal cells without sharing to some degree in the moral responsibility of the abortion that yielded those cells (Gieser, 2001). This and other ethical issues have yet to be resolved. One recent study may point to the way forward. A cultured human RPE cell line has been successfully transplanted into the RCS rat (where the resident RPE cells are unable to phagocytose outer segments and photoreceptors die) and this has led to functional recovery (Lund *et al.*, 2001b). Such findings indicate that cultured and genetically modified human cells can be successfully used in transplantation. Retinal cell culture is technically not as advanced as RPE cell culture, but the emerging use of stem cells, which theoretically can be induced to develop into photoreceptors, is an exciting prospect. Warfvinge *et al.* (2001) have shown retinal integration of grafts of brain-derived precursor cell lines in the subretinal space of normal adult rats. The aim of future research will be to transplant stem cells into the subretinal space of dystrophic eyes and induce their development into photoreceptors.

Apart from using photoreceptor transplantation in retinal dystrophy like RP, there has been suggestion of its use in AMD. The idea surfaced when it was recognised that genetic defects in photoreceptor-specific proteins, such as peripherin/retinal degeneration slow (RDS), may cause phenotypic changes at the level of the RPE and Bruch's membrane, such as drusen and atrophy (Gorin *et al.*, 1995; Wells *et al.*, 1993). Moreover it is now known that a mutation in the gene encoding a rod specific adenosine triphosphate-binding cassette transporter (ABCR) on chromosome 1 can cause a spectrum of retinal diseases including AMD, RP and Stargardt's disease (van Driel *et al.*, 1998). These findings are compelling evidence that certain forms of AMD are due primarily to photoreceptor defects and there may be a role for photoreceptor transplantation with or without RPE transplantation rather than for RPE transplantation alone.

Finally assuming that transplanted photoreceptors can make synapses with host retina in humans, one question still remaining is whether those synapses are appropriate and will provide meaningful signal that can mediate vision function. Patients may expect more than simply light and dark discrimination or other visual reflexes. Whether new visual signals can be transformed into useful visual perception may rely on the plasticity of a mature visual system that has not been utilised possibly for many years after initial visual loss. Recently there has been evidence of such plasticity where vision in amblyopic eyes has improved following disease in the previously better eye (El Mallah *et al.*, 2000; Moseley and Fielder, 2001). Additionally patients with restored monocular central acuity after macular translocation have been able to adapt successfully to the altered perception of the visual environment (Seaber and Machemer, 1997). It is, I believe, not unreasonable to assume that such plasticity may exist for visual processing at the cellular level.

5.0. Learning points

- There is a learning curve with subretinal injection/transplantation technique. It would require practising in at least 20 mice before one can be familiar with it.

- Practicing injection should be performed in dystrophic mice as their retina are thinner, more difficult to handle and has a higher chance of injecting into the vitreal cavity than in normal mice.

- Using a larger animal model may lead to easier transplantation and a higher technical successful rate.

- Animal behaviour tests are inherently and often unpredictable, moreover they can be influenced by many factors, especially environmental and animal learned experience. The simpler the test, the less likely it is to be influenced by these factors.

- Retinal threshold test is a time consuming test. Each test would take a whole day to complete and care must be taken in administrating the correct dosage of anaesthesia in order to achieve the appropriate depth of anaesthesia and not to overdose the animals.

- Retinal microaggregates have been successfully transplanted here but ideally photoreceptor sheets would be a superior choice. However this is not practicable

in the small mouse eyes. Once again a larger animal model would be a better recipient of the sheets of photoreceptors.

- Larger number of animals should be enrolled in order to achieve a statistically significant result.

6.0. Conclusion

A method has been successfully developed to transplant early postnatal syngeneic normal retinal microaggregates into the subretinal space of a rodent model of retinal degeneration. In successful grafts, a continuous layer of cells was formed which consisted predominantly of rod photoreceptors. A substantial area of each graft developed rudimentary outer segments and these aligned regularly if they were in contact with the host RPE. The interface between the graft and the remaining host retina labelled positively for a mouse-specific synaptic protein and typical photoreceptor terminals were seen in this region ultrastructurally, forming characteristic synaptic patterns. This was confirmed with rat to mouse subretinal transplants where a rat-specific synaptic protein was labelled at the host-graft interface. LDPT showed that there was a statistically significant difference in the time spent in the dark compartment between normal and dystrophic animals. The test was used to assess pre- and post-transplantation in dystrophic mouse. The result was suggestive of visual improvement after transplantation but it was *not* statistically significant. It indicates that the study was worth repeating with a larger number of animals. Retinal threshold sensitivity to light stimuli was restored in areas where a successful transplant had been identified in histology-positive rd mice. However it was again a small number of animals and the result should be interpreted with cautions. There are a number of issues yet to be clarified using this model before a clear path can be seen to a clinical therapy. These include the longevity of the subretinal grafts, the possibility of immune rejection and its mechanism, and the differential labelling of donor and host cells as well as producing physiological and behavioural studies directed at defining the quality of vision that can be reconstructed

with such a grafting procedure. There is a need for caution in the transition of such studies from laboratory to clinic (Berson and Jakobiec, 1999).

7.0. References

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8.0. Appendix - Publications from the work presented in this thesis

Kwan, A. S., Wang, S., and Lund, R. D. (1999). Photoreceptor layer reconstruction in a rodent model of retinal degeneration. Exp Neurol 159(1):21-33.

Lund, R. D., Kwan, A. S., Keegan, D. J., Sauve, Y., Coffey, P. J., and Lawrence, J. M. (2001). Cell transplantation as a treatment for retinal disease. Prog Retin Eye Res 20(4):415-449.

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Photoreceptor Layer Reconstruction in a Rodent Model of Retinal Degeneration

A. S. L. Kwan, S. Wang, and R. D. Lund

Neural Transplant Program Department of Pathology, Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, United Kingdom

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We have examined the potential of retinal cell transplantation to dystrophic retinal degeneration mice as a way of replacing photoreceptors lost because of an intrinsic genetic defect. Early postnatal retinae which had been gently dissociated survived for at least 6 weeks after transplantation to the subretinal space. Over a significant area of distribution, transplanted cells formed outer segments which lay in close apposition to the host retinal pigment epithelial cell layer. The grafts integrated with the remaining host retina, sufficient at least to mediate a simple light–dark preference. A new synaptic layer was seen at the graft–host interface, which contained substantial numbers of photoreceptor synapses. This and the fact that the behavior could be elicited at low luminance levels argue for functional circuit reconstruction between grafted cells and host retina. © 1999 Academic Press

Key Words: **retinitis pigmentosa; photoreceptor; transplantation; rd mice; behavior; functional recovery**

INTRODUCTION

Retinitis pigmentosa (RP) and associated diseases of the retina are caused by inherited dystrophy of the photoreceptors with mutations affecting more than 90 genes targeted in most cases at the photoreceptors themselves. RP affects about 1 in 4000 people (4), although local incidence can be increased by inbreeding. Currently these diseases are effectively untreatable. The availability of experimental animals with homologous or analogous defects, occurring either naturally or as a result of transgenic manipulation, has made it possible to explore a number of potential therapies. These include limiting the degenerative process using dietary supplements (6), growth factors (17, 36, 38), gene therapy (2, 11, 33), and transplantation (14, 40). All but the last function by limiting the degenerative process while transplantation has the additional potential of replacing lost photoreceptors.

A series of transplantation studies has explored the potential for replacement of lost photoreceptors in experimental animals, using a variety of donor tissue preparations. These have included microaggregrates (24, 25, 28, 30), cell suspension (15, 22, 23), retinal sheets obtained from Vibratome sectioning (21, 58, 59) or excimer laser preparation (26, 60) of immature or mature retina, and whole or large strips of retinae taken from embryos (1, 3, 20, 55). In previous attempts with microaggregrates or dissociated cells, the main problems were the formation of rosettes and loss of laminar order (30). They also tended to form clumps rather than continuous sheets in the subretinal space (24, 25). The interface with the host has also presented problems; there was evidence of a glial scar that might limit synaptic interactions (25). The use of sheets of retina solves the problem of laminar order and continuity but is difficult to employ in rodents. Whole or large pieces of embryonic retinae provide a better interface with the remaining host retina, although how complete or how normal synaptic integration may be is not yet clear. In none of the studies is there clear evidence that such grafts improve visual responsiveness over preoperative levels. While this work has been extended to patients (13, 31), there is as yet no objective evidence of improved function. These investigations, while providing useful evidence on graft viability, have nevertheless attracted critical comments and argument has been made for the need for more comprehensive study in animal models before contemplating further clinical experiments (5).

In the present work, we have pursued further the possibility of using mechanically dissociated cells and have focused on three issues—the appearance of the graft itself, the interface with the host retina, and the potential for functional restitution after transplantation. These studies have been conducted in an animal model of RP, the retinal degeneration (rd) mouse, in which rod photoreceptors degenerate within the first 3 weeks of life due to a defect in the β-subunit of cGMP phosphodiesterase (9, 39). This mutation has a direct homologue in one of the recessive forms of RP in humans (18, 44, 45).

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METHODS

Animals

All animal care and handling during this study conformed to the requirements of the British Home Office and National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rd mice are bred and maintained in our colony at the Institute of Ophthalmology. Recipient mice were homozygous for the rd locus (C57BL/6J, rd/rd) and donor mice were nondystrophic congenic mice (C57BL/6J, −/−). All animals were reared in cyclic light (12 h on/12 h off) with a room temperature of 21°C from birth.

Donor Tissue Preparation

Donor mice ages 7–9 days received tribromoethanol (230 mg/kg) intraperitoneally as anesthetic prior to decapitation and enucleation. Eyes were collected in Ca^{2+}-free and Mg^{2+}-free Hanks' solution at 4°C. The anterior segments were dissected and neural retina was removed and placed in Ca^{2+}-free and Mg^{2+}-free Hanks' solution at 4°C. The retinae were gently cut into small fragments with microscissors. Gentle trituration was performed using a fine glass pipette (internal diameter of 1 mm) for a few times to further separate the fragments and develop a mixture of microaggregrates and cell suspension. The suspension was allowed to settle at 4°C for 30 min, then excess supernatant was removed. The viability of the tissue was assessed by exclusion of trypan blue vital dye (>95% viability). Cells were kept at 4°C in Hanks' solution until transplantation.

Transplantation

Six- to eight-week-old recipient mice, at a time when practically all the rods have all degenerated, were anesthetized with tribromoethanol (230 mg/kg) intraperitoneally. Both eyes were dilated with 1% Tropicamide (Alcon Laboratories, UK). The head was secured by a nose bar and, to allow easy access, the eye was partially protruded from the orbit by gently tying a releasable suture (6.0 Vicryl) behind the equator of the globe. This allowed good exposure of the intended injection site and stabilized the globe. A cylindrical plastic ring was then placed on the eyelids thus encircling the protruded eye. The ring was filled with 1 ml of 2% sterile hypromellose solution thus submerging the eye. As the viscous hypromellose solution formed a smooth and flat surface, it acted as a contact lens and under the operating microscope the fundus could be seen with ease. A 25-gauge needle was used to dissect away the conjunctiva and to make a superotemporal sclerotomy. Donor material was drawn into a sterile self-drawn fire-polished glass pipette (internal diameter 200 to 250 μm) which was connected to a 10-μl Hamilton syringe via a polyethylene tube. Two microliters of donor suspension containing about 10 to 15 visible retinal microaggregrates in a cell suspension was slowly injected through the sclerotomy into the subretinal space of the eye. A good transplant was indicated by local retinal bleb, which involved about 1/3 of the retina through the fundus view. During the injection, the cornea was punctured (corneal paracentesis) using a 30-gauge needle to release some aqueous fluid and thus reduce the intraocular pressure due to the subretinal bleb. This step, we found, significantly reduced the amount of reflux of cells through the injection site. The releasable suture was removed. The same procedure was repeated on the other eye, and the animal was placed subsequently in an incubator to recover.

Follow-up

No postoperative treatment was given, and no immunosuppression was used. The animals were sacrificed between 2 and 6 weeks after the transplantation.

Light Microscopy and Immunohistochemistry

For light microscopy, animals survived for 2, 4, and 6 weeks postoperatively. They were perfused under tribromoethanol anesthesia with phosphate-buffered saline (PBS) and then 4% periodate/lysine/paraformaldehyde fixative (PLP) (46). A suture was placed at the superior rectus muscle for orientation and the eyes were enucleated. The cornea was perforated and the eye was postfixed in PLP for 1 h and rinsed in PBS solution. The eyes were embedded in low-melting-point wax. Eight-micrometer sections were cut serially and mounted on charged glass slides and stored at 4°C until used. One series of each eye was stained with cresyl violet in order to locate the area of transplant, and selected slides from the other series of the same eyes were labeled using specific antibodies.

Sections were dewaxed and washed with PBS. The slides were incubated in 5% defatted milk (blocking agent). The following primary antibodies were used: rhodopsin (for rod photoreceptor, rho4D2, 1:3000, generous gift from Dr. R. Molday, University of British Columbia, Canada), PKC (for rod bipolar cells, MC5, 1:100, Santa Cruz Biotechnology, Inc., U.S.A.), RT97 (for horizontal cell and retinal ganglion cell axon, monoclonal anti-200-kd neurofilament protein, 1:500, generous gift from Dr. R. Morris, KCL Guy's Hospital, London, UK), GFAP (for astrocytes and reactive Muller cell processes, 1:1000, Sigma-Aldrich Co. Ltd. UK), calbindin (for amacrine cells, 1:200, Sigma-Aldrich Co. Ltd. UK), and P84 (for mouse-specific synaptic protein, 1:1, generous gift from Dr. C. Lagenaur, University of Pittsburgh). Antibodies were applied at the appropriate concentration. Slides were incubated with the primary

antibodies overnight at 4°C. After being washed in PBS for 3 × 10 min, they were incubated with the secondary antibodies diluted in antibody diluent and 1% rat serum for 30 min. They were washed in PBS 3 × 10 min and reacted with an immunohistochemical avidin–biotin–peroxidase complex system (Elite ABC kit standard; Vector Laboratories). After being washed in PBS 3 × 10 min, they were developed in 3,3′-diaminobenzidine solution (Sigma-Aldrich Co. Ltd. UK) and aqueous nickel ammonium sulfate for 3 min, washed in PBS and distilled water, dehydrated through a graded series of alcohols, cleared in xylene, and mounted in DePeX (Merck/BDH, Littleworth, UK).

Electron Microscopy (EM)

Two rd mice at 2.5 weeks posttransplantation and one age-matched dystrophic rd mouse without a transplant were used for this preliminary study. After animals were deeply anesthetized, 0.5 ml of fixative was injected into the posterior chamber of the eye. The fixative consisted of 2.5% paraformaldehyde, 2.5% glutaraldehyde, and 0.01% picric acid in 0.1 M cacodylate buffer (pH 7.4) (49). The dorsal pole of each eye was marked with a suture; the eyes were removed and left in the same fixative for 24 h at 4°C. After being washed in cacodylate buffer, the lenses were removed and the retinae were postfixed in 1% osmium tetroxide for 1 h. After dehydration through a graded series of alcohols to epoxypropane, they were embedded in TAAB embedding resin (TAAB Laboratories, Aldermaston, UK). Semithin sections were stained with 1% toluidine blue in 1% borate buffer, and ultrathin sections were contrasted with uranyl acetate and lead citrate (51). Grids were viewed on a Jeol 1010 electron microscope.

RESULTS

Surgery

A total of 18 rd mice received bilateral transplants (n = 36). Animals were sacrificed at 2 (n = 16), 4 (n = 10), and 6 (n = 6) weeks for light microscopy and at 2.5 weeks (n = 4) for EM. The development of our method of delivering cells into the subretinal space of mice under direct vision has been successful. The introduction of a corneal puncture during the transplant procedure allowed significant lowering of intraocular pressure. This was important as 2 μl of transplant is a sizeable volume in a mouse eye and significant increase in intraocular pressure can lead to reflux of cells from the injection pathway postoperatively. Suture was not used to close the sclerotomy as the sclera in 6-week-old mice eye is very thin and it is likely to damage the underlying choroid and retina. There was minimal trauma to the globe. Four cases (11%) of significant subretinal hemorrhage were seen intraoperatively. In total, 50% (18 of 36 eyes) of the grafts were seen to be positioned correctly in the subretinal space with no obvious damage to the host retina and the rest were seen to have been placed in the vitreous at the time of the surgery.

General Appearance of the Grafts

Histological evidence of grafts was seen in 16 eyes (44.4%); of these grafts were confined to the subretinal space in 10 eyes (27.7% of the total). In the remaining 6 eyes (16.6% of the total), there was leakage of grafted cells into the vitreal cavity. There was no evidence of grafts in the other eyes (55.6%); presumably the grafts had degenerated, effluxed, and, in some cases, been compromised by a persistent retinal detachment. Where present, the subretinal grafts appeared as distinct and continuous sheets (Fig. 1A), occupying usually about one-third of the total retinal area and in some cases as much as one-half. In most cases grafts were in close contact with host retinal pigment epithelia (RPE) and host retinae. They were about four to six cells thick except toward the borders where they were usually reduced to two cells thick. Much of each graft consisted of cells with darkly stained nuclei in cresyl violet-stained sections. No signs of inflammation or rejection were seen. Occasional rosette formations were seen in the grafts with outer segment material in the center, but the outer segment material was irregularly configured. More common was the presence of lengths of retina with outer segments in close apposition to the host RPE (Fig. 1B). These were also clearly evident in rhodopsin antibody-labeled material (Figs. 2A and 2B). Previous studies using whole-retinal and microaggregate grafts suggested that outer segments were more likely to be regularly aligned if they were in contact with the host RPE rather than in rosette formations (25, 56).

Antibody labeling showed that most of the cells within a graft were rhodopsin-positive (Figs. 2A and 2B) and therefore were rods introduced either as postmitotic or as progenitor cells during the transplantation process. This was confirmed by electron microscopy in which the cells in the graft mostly had the typical chromatin pattern of rods. Rhodopsin labeling of age-matched rd retinae failed to show any positive cells and retinae were not labeled beyond the border of the graft (Fig. 2A). Labeling with PKC, calbindin, and RT97 revealed some bipolar, amacrine, and horizontal and ganglion cells in the grafts. These were generally locally and sparsely distributed single cells; in none of the cases studied was there evidence of a systematic laminar distribution of any of these cell types. GFAP antibody showed that reactive glial filaments, presumably in Muller cell processes, were broadly distributed through the grafts (Fig. 3B). This might be expected in such transplants as normal orientation of the donor retina was disrupted. A similar pattern of labeling has

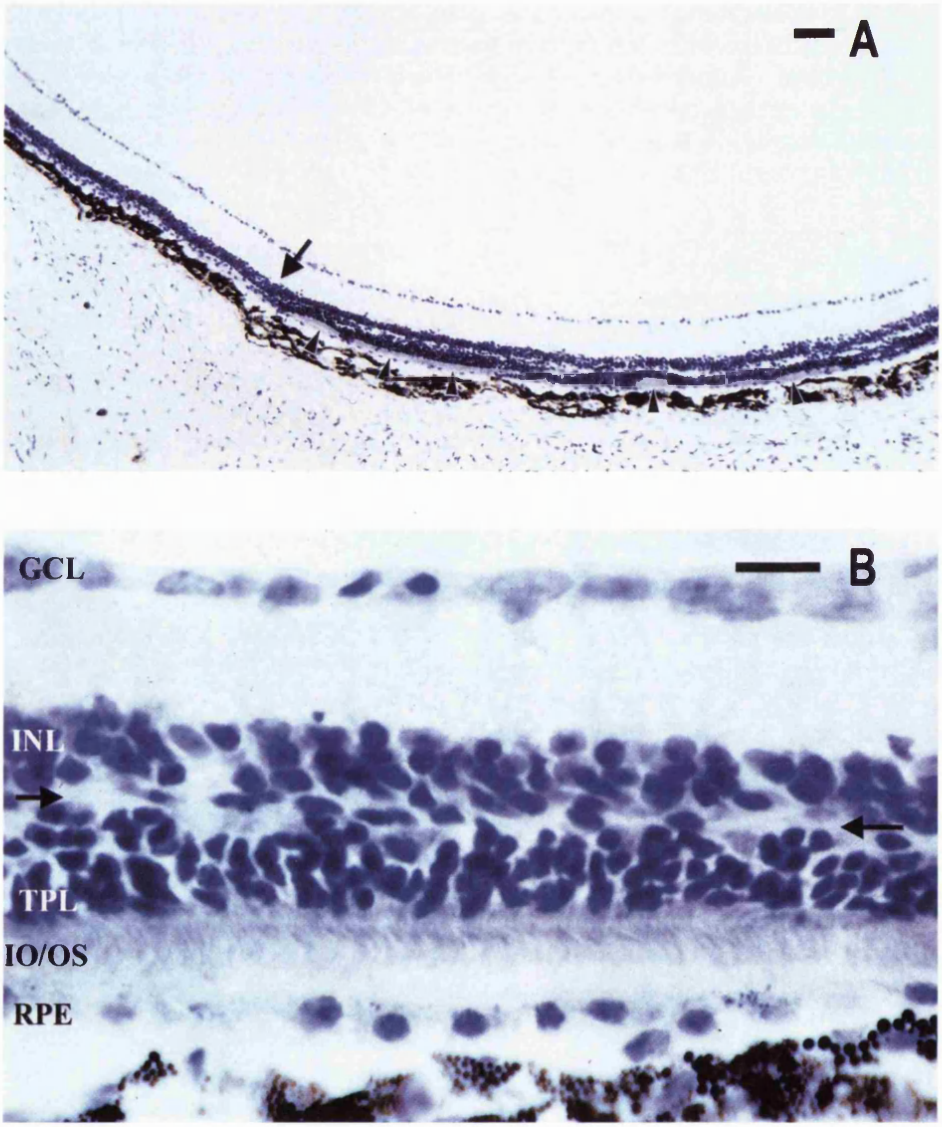

FIG. 1. Cresyl violet staining of a typical subretinal transplant. (A) Note that in the region of retina where there is no transplant (left of the arrow), the outer nuclear layer contains only dispersed cells. The transplanted layer is seen in the subretinal space to the right of the arrow. The widespread presence of pinkish staining of the outer segments is clearly seen (arrowhead). Bar, 0.1 mm. (B) High magnification of the transplanted cells with outer segments in close association with the host RPE. Bar, 0.01 mm. GCL, host ganglion cell layer; INL, host inner nuclear layer; TPL, transplanted cell layer; IO/OS, inner and outer segments of transplanted cells; RPE, host RPE. Arrow, graft–host interface.

been described as a response to injury (27, 54). At the graft–RPE interface, host RPE appeared intact, and RPE cells appeared more full and rounded, especially where outer segments were in contact with them, in contrast to areas where there was no transplant. This might reflect increased metabolic activity associated with the presence of photoreceptors.

Graft–Host Retina Interface

In cresyl violet-stained material of dystrophic rd mice without transplant, there was a single discontinuous layer of loosely distributed cells along the outer border of the retina. These are remaining cones as described in previous work (10, 37). In grafted retinae, these cells could still be identified between graft and host with a cell-free space on either side. One concern was that this space might be filled by a glial barrier situated between graft and host retinae, which would limit synaptic integration. However, in neither GFAP-labeled sections nor retinae examined for electron microscopy was there any evidence for a glial interface. In the rhodopsin-labeled sections, the positively labeled cells were seen up to the interface region (Fig. 2B) but although fine processes were sometimes seen at the border, no examples were seen of processes crossing the border. RT97 labeling of mouse retinae without trans-

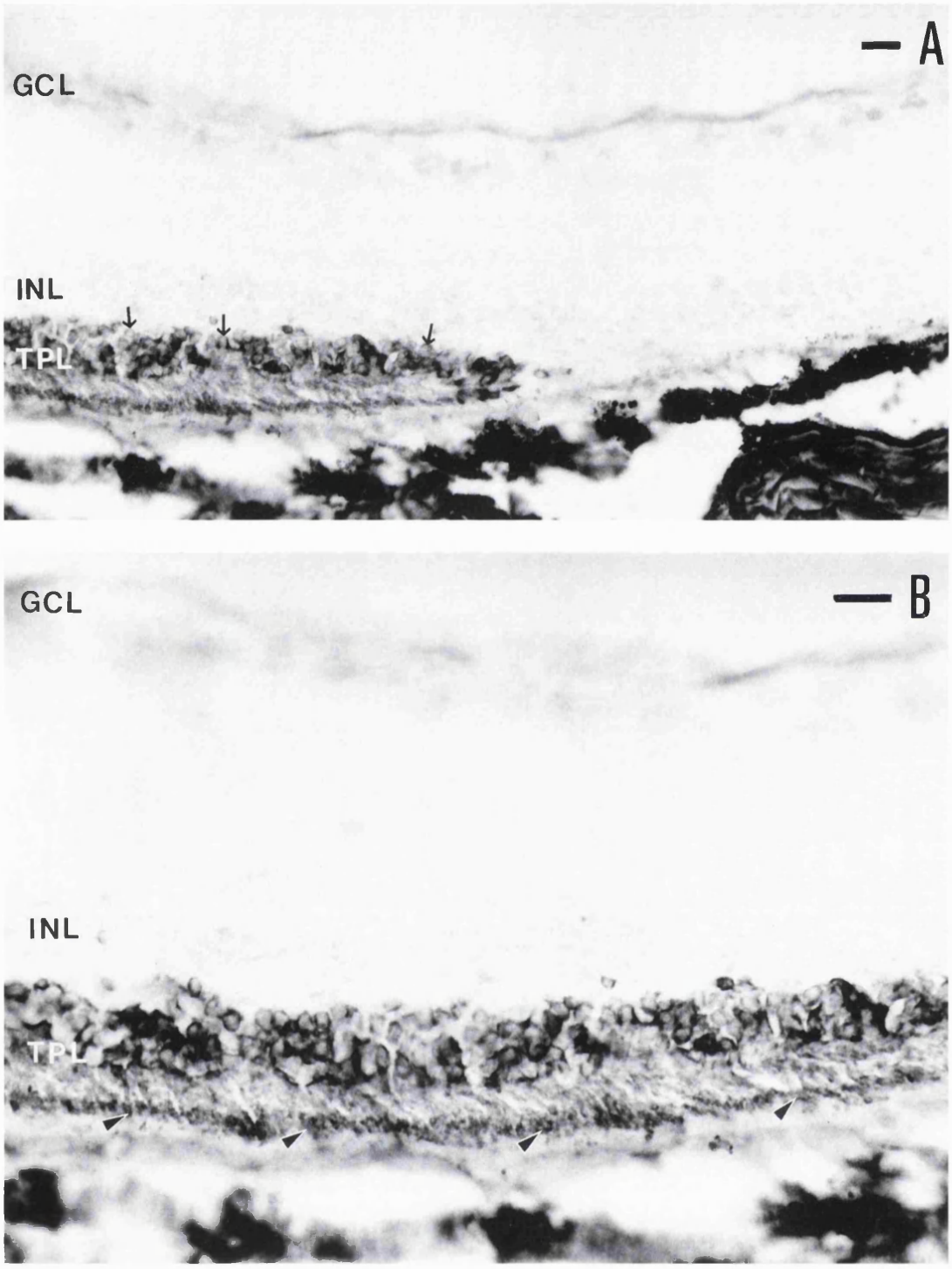

FIG. 2. (A) Rhodopsin labeling at the edge of the subretinal transplant. Note that the cells in the graft are predominantly rhodopsin-positive (arrows), which indicates that they are rod photoreceptor cells. Bar, 0.01 mm. (B) High magnification of rhodopsin-labeled transplanted layer with the outer segments strongly labeled (arrowheads). Bar, 0.01 mm. GCL, host ganglion cell layer; INL, host inner nuclear layer; TPL, transplanted cell layer.

plants showed tangentially running processes of horizontal cells coursing along the outer surface of the retina; a similar appearance was seen in transplanted retinae with no evidence of breaching of the interface (Fig. 3A). In contrast, PKC-labeled rod bipolar cells did show evidence of dendritic growth at the interface. In normal animals (Fig. 4A), PKC antibody labeled a clearly defined and uniform group of bipolar cells. Most of these had cell bodies close to the outer plexiform layer, with occasional ones lying deeper in the inner

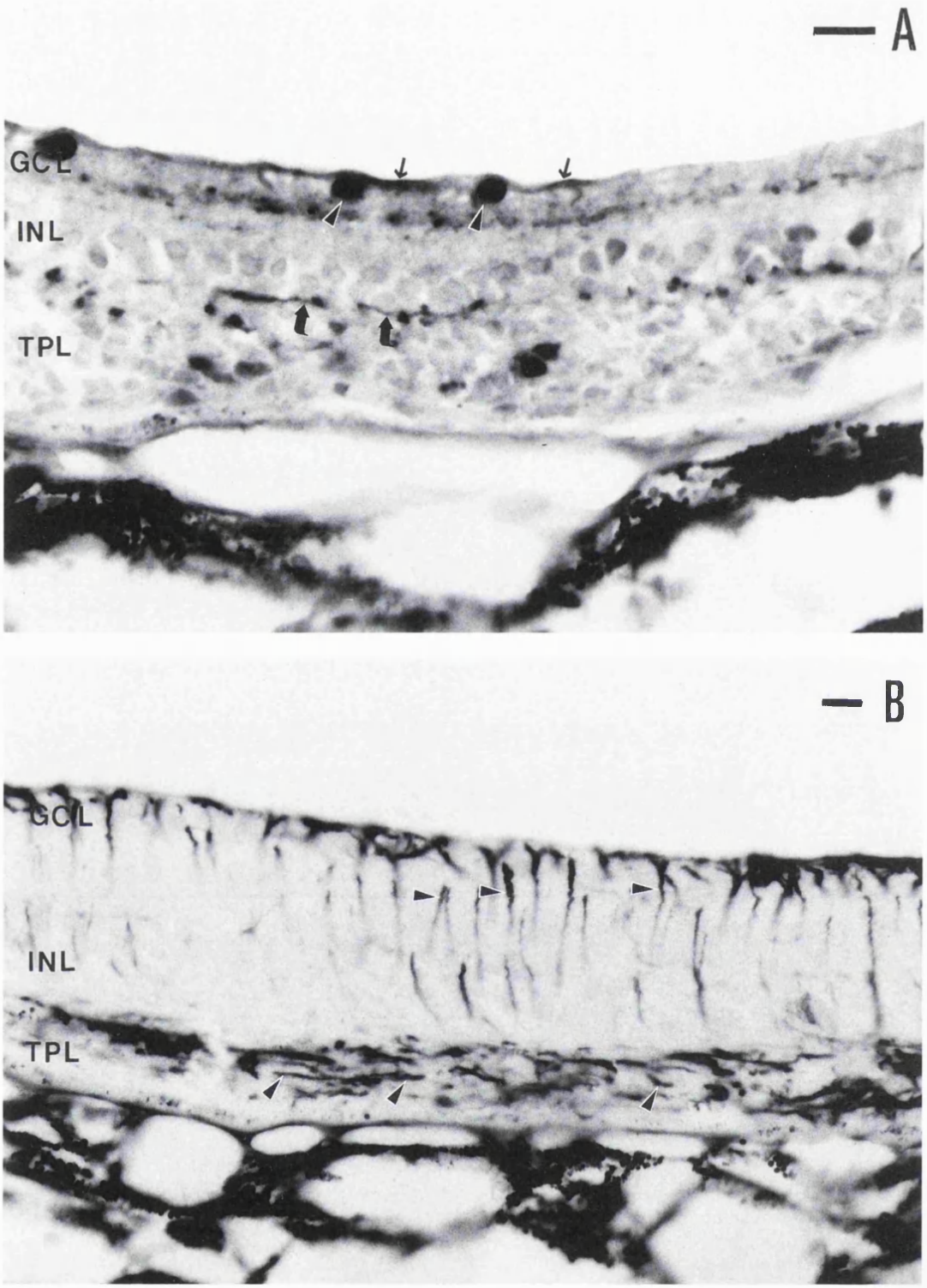

FIG. 3. (A) RT97-labeled section showing positively labeled ganglion cell (arrowheads), their axons (arrows), and horizontal cell processes (curved arrows) in the host retina. A few positively labeled cells are seen in the graft. Bar, 0.01 mm. (B) GFAP labeling shows Muller cell processes in a regular and linear array in the host retina and a more widespread labeling in the graft (arrowheads). Bar, 0.01 mm. GCL, host ganglion cell layer; INL, host inner nuclear layer; TPL, transplanted cell layer.

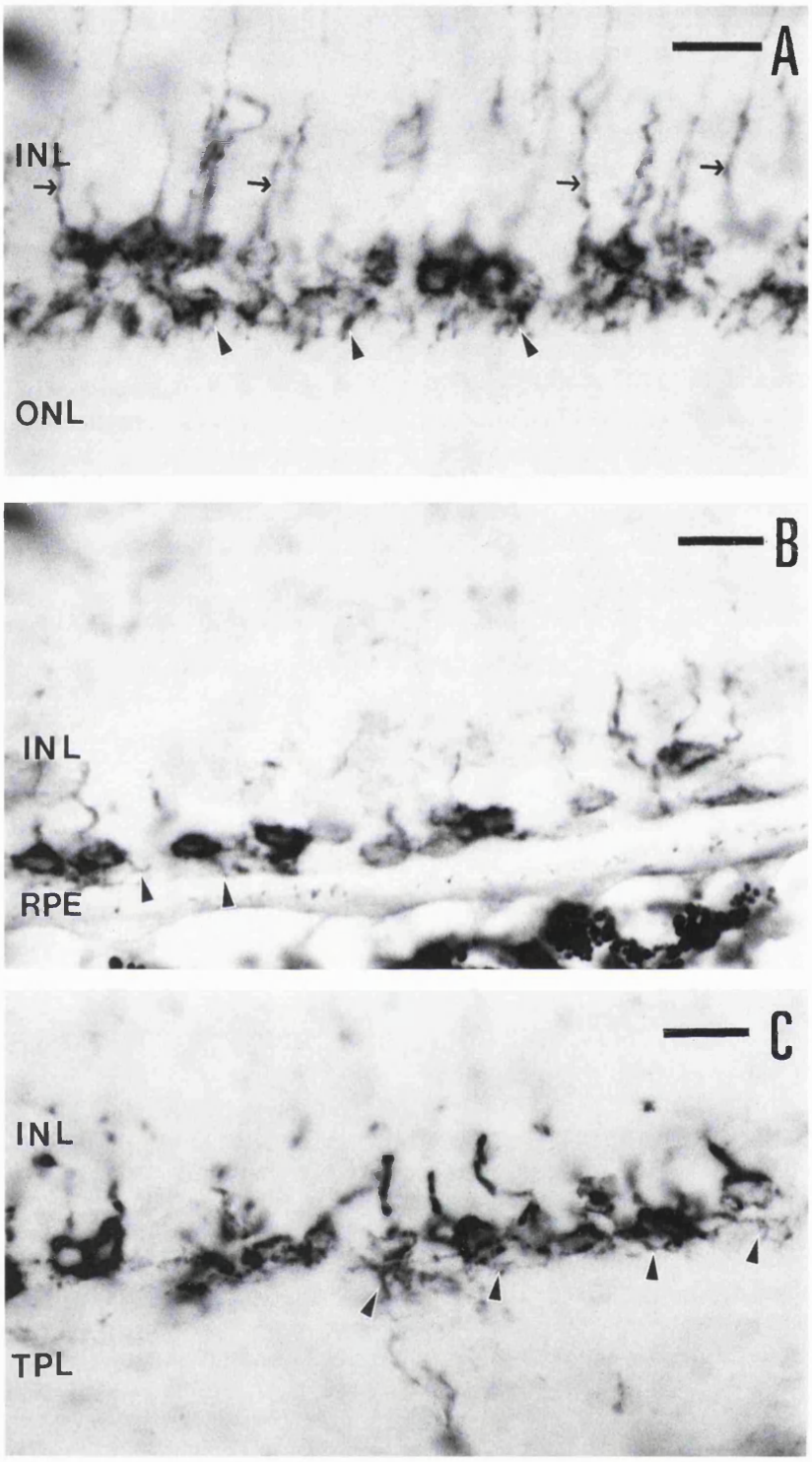

FIG. 4. PKC-labeled retinal sections of (A) normal mouse retina showing axonal processes (arrows) of rod bipolar cells extending into the inner plexiform layer and a large number of dendrites (arrowheads) extending into the outer plexiform layer, (B) dystrophic rd mouse retina showing a reduced number of rod bipolar cells and only a few dendritic processes (arrowheads) next to the RPE, and (C) retina with transplant showing an increase number of PKC-positive cells in the inner nuclear layer and more dendrites (arrowheads) extending into the graft. INL, host inner nuclear layer; ONL, host outer nuclear layer; RPE, host retinal pigment epithelium; TPL, transplanted cell layer. Bar, 0.01 mm.

nuclear layer. Dendritic processes distributed in the outer plexiform layer. They had an axon, which continued unbranched through the retina with dilatations along its course and a large club-like ending in the outer plexiform layer close to the layer of retinal ganglion cells. This is typical of rod bipolar cells, although it is possible that some of the more deeply located somata could be subtypes of cone bipolar cell (16). In dystrophic rd mice (Fig. 4B) there were several differences. First there were very few dendritic branches and these tended to run tangentially at the outer border of the retina. Second, the numbers of bipolar cell bodies appeared reduced and they were discontinuously distributed along the outer border of the retina. Third, the club-like endings in the inner plexiform layer were less evident and they seemed to have smaller arbors than in normal retinae. This appearance was also seen in the retinae of grafted animals away from the area of the transplant. In the region adjacent to the graft (Fig. 4C) there was clear evidence of bushy processes on the apical surface of some of bipolar cell somata. In addition there appeared to be more PKC-positive cells in the outer border of the INL. Whether these were new cells migrated from the graft or host cells that had changed their labeling characteristics was not clear.

The P84 antibody used here was important for examining the distribution of synapses. It is a member of the SIRP (signal-regulatory protein) family (32) and has been identified as a membrane glycoprotein that binds to the tyrosine phosphatases SHP-2 and SHP-1 (12, 29). It is suggested that it may play a key role in intracellular signaling both during synaptogenesis and in mature synapses. In the normal retina, it specifically labels the synaptic regions, namely the inner and outer plexiform layers, and is particularly effective in demonstrating retinal receptor terminals. In the unoperated dystrophic rd mice, a discontinuous line of labeling was seen at the outer border of the retina (Fig. 5A). This tended to lie deep to residual cones and probably reflected the distribution of synapses associated with them. A similar pattern of labeling was also seen in grafted retinae; it did not seem to be more extensive than in controls or in retina adjacent to the graft. Most important was that at the interface between graft and host retinae, an additional continuous line was seen lying outside the cone layer (Fig. 5B). This suggested that there was a separate synaptic interface between graft and host. This could be clearly resolved both in semithin sections and in low-power electron microscopy of adjacent sections. Electron microscopy of this region showed indeed that it contained abundant synapses, some of which were associated with terminals appearing similar to typical rod spherules, suggesting that they might indeed belong to transplanted cells. The terminals contained synaptic ribbons and formed frequent complex synaptic arrays, involving both dyad and triad synapses (Figs. 5C and 5D). Similar synapses were not seen in the comparable location in adjacent retina away from the transplant or in control dystrophic rd mouse retinae (7, 52).

Functional Consequences of Transplantation

In ongoing experiments, light–dark preference behavior, which had previously been used for the assessment of intracerebral retinal graft function (42), was employed as a method of assessing whether the intraretinal grafts could have any effect on visual function. We have found that mice exhibit a light–dark preference behavior and when placed in compartments of different brightness will spend more time in the dark part (35). This behavior has been measured using a computerized monitoring apparatus (TRUSCAN multiparameter activity monitors; Coulbourn Instruments, U.S.A.). As measured by this equipment, a normal mouse spent about 70% of a 30-min test period in the dark segment. Rd mice showed a similar behavior under high-luminance conditions, presumably mediated by the remaining cones, but with lower luminance levels and red light, they showed a random response, spending 50% of the time in each segment. This failure to respond correlates with the loss of rods in these animals. Accordingly, animals for transplantation were pretested for light–dark preference behavior at low-luminance levels. They were then retested 2 weeks posttransplantation. Whereas they showed random behavior before transplantation, in the posttransplantation test they now showed a dark preference (34). This is illustrated in Figs. 6A and 6B for an animal, the histology of which is shown in Fig. 1B.

DISCUSSION

The results show that early postnatal retinal cells which have been gently mechanically dissociated survive for at least 6 weeks after transplantation to the subretinal space. They form outer segments over part of their area, where for the most part they lie adjacent to the host RPE. The graft integrates with the remaining host retina, sufficient at least to mediate a simple visual function. Previous studies using dissociated cells or microaggegates have failed to achieve the large areas of coverage seen here and generally showed frequent rosette formation with only small and discontinuous areas in which outer segments were lined up against the host RPE (24, 25, 30, 57). The differences may be attributable to a number of causes. First, by taking retinal cells late in histogenesis, there is a likely emphasis on photoreceptors being the dominant graft cell. Second, the mild mechanical dissociation not using enzymes gives a better yield of healthy cells and the use of Ca^{2+}- and Mg^{2+}-free medium ensures a greater level

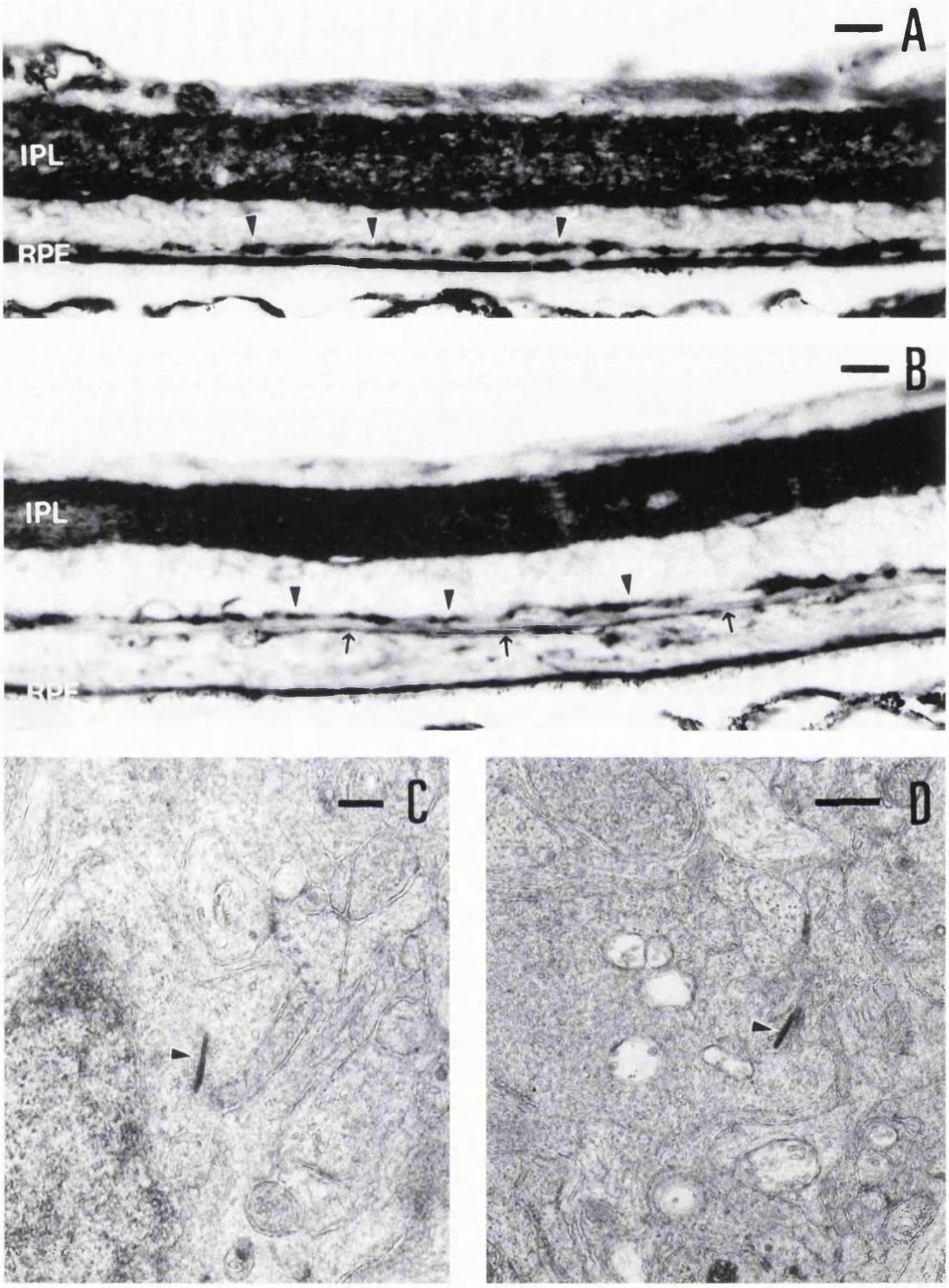

FIG. 5. P84 labeling of (A) dystrophic retina showing the inner plexiform layer and a discontinuous outer plexiform layer (arrowheads) next to the darkly stained and continuous RPE layer and (B) transplanted retina showing the same discontinuous labeling of the host outer plexiform layer (arrowheads) and a new synaptic lamina associated with the graft (arrows). RPE, host retinal pigment epithelium; IPL, inner plexiform layer. Bar, 0.01 mm. (C and D) Electron micrographs showing two typical photoreceptor terminals at the graft–host interface, corresponding to the position of the new synaptic lamina. (C) A perinuclear synapse. Bar, 200 nm. (D) A more typical rod spherule. Bar, 500 nm. Synaptic ribbons can be seen in each (arrowheads).

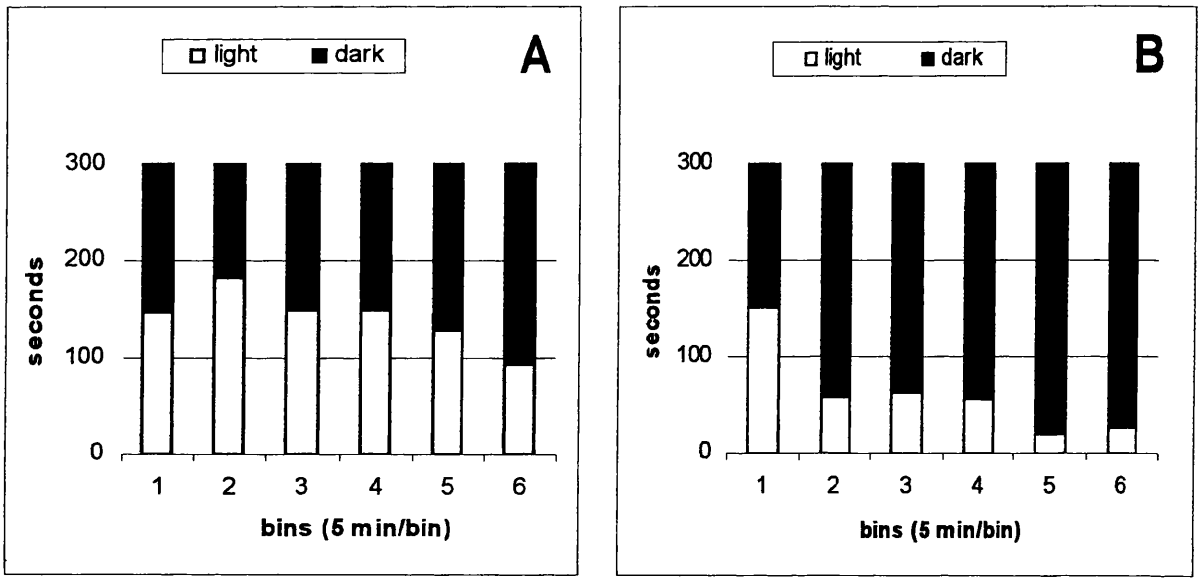

FIG. 6. Records of time spent in the light and dark compartments of the light–dark preference test by a rd mouse (A) prior to transplantation and (B) 2 weeks posttransplantation. (A) A period of over 30 min in which the subject spent an approximately equal amount of time in each compartment is shown, while (B) shows that the subject now had a strong preference for the dark compartment.

of dissociation. Third, by decompressing the globe with a corneal puncture, the amount of cellular efflux from the injection site was significantly reduced and a better coverage of a larger area of retina by the grafted cells was achieved. Furthermore, since the approach used here did not involve a retinotomy to gain access to the subretinal space, the postoperative retinal blebs settled quickly, thus improving the chance of cell survival and integration. A series of preliminary studies varying the transplantation procedure clearly pointed to an improved survival and efficacy using the protocol described here.

Perhaps the most surprising observation is the development of relatively large areas of transplant with outer segments lying against the host RPE. In previous studies this has been achieved only in local areas in a somewhat discontinuous fashion (25, 59). It has been suggested that the integrity of the Muller cells may be necessary for maintaining orientation and graft survival (19), but the present findings of ordered outer segments and disordered Muller cell processes seen with GFAP would suggest that such a relationship is not absolutely obligatory. Some of the previous studies have had difficulty showing unequivocally that there is synaptic integration between graft and host in rd mice. Here there is strong evidence that this does occur. First, the space between graft and host is not completely occupied by a glial scar. Second, and most crucial, is the appearance of a new synaptic lamina, seen in light-microscopic sections labeled with the P84 antibody. This is distinct from the synaptic layer attributable to the remaining cones. It is evident that there are many synapses in the region where the new synaptic lamina is seen in light microscope sections, and many of them appear more like typical rod spherules than cone pedicles. Both the location and the appearance of synapses therefore argue for reconstructed circuitry. There is one concern, however, and that relates to the fact that there appear to be more bipolar cells at the graft–host interface in grafted mice. If some of these have migrated from the graft into the host retina, then it is possible that some of the synapses are between donor photoreceptors and donor bipolar cells. This in itself may not be a major obstacle, because it is still possible that the bipolar cells themselves recreate circuitry downstream. Resolution of this issue must wait for specific donor/host labeling. Fortunately the P84 antibody is mouse-specific and there is a homologous rat-specific antibody so it is possible to identify the compartments using these antibodies in conjunction with cross-species transplantation. Even then, however, evidence for the formation of connections is not on its own sufficient: they must transmit a sensible signal to the host central nervous system. The most compelling evidence for functional integrity comes so far from the fact that a simple response for light–dark preference can be regenerated after transplantation. It has been suggested that one effect of transplantation is to rescue and perhaps enhance the remaining cones (47, 48). While the present study does not contradict that possibility, the fact that circuitry between graft and host involves rod-like terminals, not seen in parts of the retina where the transplant is absent, and the improved light–dark preference response is best seen at low luminance levels, suggests that real circuit reconstruction may have occurred. In previous work grafting

embryonic retina into the brain, it was found not only that such retinae sent out axons to visual centers of the host central nervous system, but also that these connections were suitable to mediate simple responses, both reflex and learned, to light flashes (42). Thus the principle that transplanted retinae can recreate new functional circuitry has a precedent. In this context, it is not unreasonable to expect that logical circuit reconstruction might be achievable within the retina itself, but it remains to be seen how much visual function can be regenerated. In parallel studies, we have been able to show that transplantation of RPE cells limits deterioration of pupillary light reflex, pattern recognition, and other behaviors as well as retaining visual field sensitivity over the area protected by the graft (41, 53, 62). Similar tests should now be applied to the present preparation.

In this study, the transplants were studied over a relatively short time period extending up to 6 weeks. While one study showed that some retinal grafts could survive for a long time in rd mice (25% at 7 months) (25), other work has shown deterioration of grafts with time (28). It was found that 60% of syngeneic retinae grafted into the eyes of rd mice were present 3 days postoperative, but this number gradually dropped to around 10% at 30 days. Studies of the vascular consequences of photoreceptor loss in the rd mouse have found that the deep plexus is differentially lost with time (8, 43). This raises the possibility that if a large graft is added, as in our study, the circulation may not respond sufficiently quickly to accommodate the increased metabolic demands and some level of degeneration may ensue. This is currently under investigation. Moreover, recent studies have shown that there are associated changes in RPE, choroid, ganglional cell and axon, and the presence of vascular formations in rd mice (49, 50, 61): all these may affect the success of retinal transplantation.

In conclusion, we have successfully transplanted early postnatal retinal fragments into a rodent model of retinal degeneration. A clear continuous outer nuclear layer which consisted predominantly of rod photoreceptors was formed. A substantial amount of each graft had outer segments in contact with the host RPE. The interface between the graft and the remaining host retina labeled positively for a mouse-specific synaptic protein and typical photoreceptor terminals were seen in this region in electron microscope material, forming characteristic synaptic patterns. There was evidence that the graft can mediate a simple light–dark preference response. These are encouraging results, but there are a number of issues yet to be clarified using this model before a clear path can be seen to a clinical therapy. These include differential labeling of donor and host cells as well as physiological and behavioral studies directed at defining the quality of vision that can be constructed with such a grafting procedure. The need for caution in the transition of such studies from laboratory to clinic has been effectively argued elsewhere (5).

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Cell Transplantation as a Treatment for Retinal Disease

R.D. Lund[a,1], A.S.L. Kwan[a], D.J. Keegan[a], Y. Sauvé[a,1], P.J. Coffey[b], J.M. Lawrence[a,*]
[a] *Institute of Ophthalmology, Bath Street, London EC1V 9EL, UK*
[b] *Psychology Department, University of Sheffield, Western Bank, Sheffield S10 2TP, UK*

CONTENTS

*Corresponding author. Tel.: +44-20-7608-6947; fax: +44-20-7608-6881; e-mail: jean.lawrence@ucl.ac.uk.
[1] Present address: Moran Eye Center, University of Utah Health Science Center, Salt Lake City, UT 84132, USA.

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Abstract—It has been shown that photoreceptor degeneration can be limited in experimental animals by transplantation of fresh RPE to the subretinal space. There is also evidence that retinal cell transplants can be used to reconstruct retinal circuitry in dystrophic animals. Here we describe and review recent developments that highlight the necessary steps that should be taken prior to embarking on clinical trials in humans.

1. INTRODUCTION

1.1. Retinal diseases

The retinal photoreceptors and adjacent retinal pigment epithelium form a functional unit. Destabilisation of the relationship either as a result of genetic mutations or changes in the overall environment of the outer retina frequently lead to loss of photoreceptors as a result of apoptosis or secondary degeneration. The consequence is a progressive deterioration of vision leading ultimately to blindness. Clinically, a number of disease states fall into this pattern, including age-related macular degeneration (AMD) and retinitis pigmentosa (RP). Within these two groupings there is a spectrum of diseases with different aetiology but similar histopathology (Inglehearn, 1998), and there is an area of overlap between them with RP-like diseases presenting the characteristic symptoms of AMD and vice versa. Together they are the leading cause of blindness in the western world: RP affects 1 in 3500 people (Bundey and Crews, 1984; Bunker *et al.*, 1984; Kaplan *et al.*, 1990) and AMD affects 30% of people over 75 (Sommer *et al.*, 1991; Klein *et al.*, 1992; Mitchell *et al.*, 1995). RP is an inherited disease; the majority of mutations target photoreceptors, but some affect retinal pigment epithelial (RPE) cells directly (Gu *et al.*, 1997; Marlhens *et al.*, 1997; Maw *et al.*, 1997; Petrukhin *et al.*, 1998). Mutations in over 100 genes have been identified which cause photoreceptor loss (Daiger *et al.*, 1996–1999). Their effects are widely distributed involving such processes as movement of materials in cells, movement of molecules between photoreceptors and RPE cells (by targeting transport proteins) and phototransduction (van Soest *et al.*, 1999). In AMD the primary disorder appears to be due to RPE dysfunction and changes in Bruch's membrane such as lipid deposition, protein cross-linking and reduced permeability to nutrients (Green and Enger, 1993; Zarbin 1998). It is apparent that diet and environmental factors play a role in the course of the disease, but there is also evidence of an hereditable component (Yates and Moore, 2000; Allikmets 1999; Gorin *et al.*, 1999; Guymer *et al.*, 1999). Presently, no effective treatments are available for either disease type but there are a number of lines of investigation which suggest potential approaches. One of these is cell transplantation and its potential will be considered in relation to other possible therapies. Since retinal cell transplantation has been the subject of a number of comprehensive reviews in recent years (e.g. Kaplan *et al.*, 1999; Enzmann *et al.*, 1998; Litchfield *et al.*, 1997a; Lund *et al.*, 1997; del Cerro *et al.*, 1997; Gouras and Algvere 1996), this article will concentrate largely on current developments, highlighting the problems still to be resolved. Research has been aided by the availability of a range of animal models with retinal dystrophies (either naturally occurring or engineered) which compare more or less closely with human disorders. This is particularly true for RP, where there is a range of homologous disorders in mice, dogs and cats and more recently these have been augmented by transgenic animals, including mice, rats and pigs, expressing human gene defects for particular forms of RP. While a number of potential transgenic models of AMD are beginning to be generated (e.g. Vinores *et al.*, 2000), all the work involving transplantation that is directed towards finding treatments for AMD has depended on using either normal animals or the retinal degeneration mutant, the Royal College of Surgeons (RCS) rat.

2. GENERAL APPROACHES TO TREATING RETINAL DEGENERATIVE DISEASE

There are two potential treatment strategies that can be adopted: one is to intervene at an early stage of the disease to prevent photoreceptor loss;

the other is to replace lost photoreceptors at a late stage in the disease.

2.1. Treatments directed at preventing photoreceptor loss

2.1.1. *Administration of growth factors*

A number of growth factors (e.g. bFGF, CNTF, GDNF) and, to a lesser extent, their receptors have been identified in the retina or in retinal cultures (e.g. Rakoczy *et al.*, 1993; Gao and Hollyfield, 1995; Jing *et al.*, 1996; Hallböök *et al.*, 1996; Bugra and Hicks, 1997; Carwile *et al.*, 1998; Jomary *et al.*, 1999). There is some variability among the studies, but this is undoubtedly due in part to use of different developmental stages, use of different animals and use of techniques with different sensitivities when studying factor and receptor localisation.

All the above growth factors have been shown to slow photoreceptor loss in a range of rodent models, including light damage (Faktorovich *et al.*, 1992; LaVail *et al.*, 1992; Masuda *et al.*, 1995), the RCS rat (Faktorovich *et al.*, 1990; Perry *et al.*, 1995), the retinal degenerate (rd) (LaVail *et al.*, 1998; Frasson *et al.*, 1999a) and the retinal degenerate slow (rds) mouse (LaVail *et al.*, 1998) as well as in transgenic rats (LaVail *et al.*, 1998). Similar studies are being conducted in larger animals (e.g. CNTF in the Abyssinian cat, Chong *et al.*, 1999). To achieve rescue, these factors have been injected into the vitreous cavity with a single or multiple injection regimen. Since it is apparent that some factors at least (e.g. PEDF, Cayouette *et al.*, 1999) remain in the vitreous for as little as 24 h, other methods of factor delivery need to be devised for the long-term maintenance of photoreceptors. One approach has been to introduce message for growth factors using gene transfection (see below), and the results obtained validate the principle of growth factor delivery as a treatment of potential importance. Functional assessment in growth factor delivery experiments has so far been restricted to electroretinogram (ERG) recordings, which indicate functional responses in some models (GDNF in rd, Frasson *et al.*, 1999a; CNTF in rds, Cayouette *et al.*, 1998) but not in others (bFGF in mi/vitiligo mouse, Smith *et al.*, 1996). This variability suggests, not surprisingly, that individual growth factors may be differentially effective in different forms of RP and that a single factor may not present a cure for all forms of the disease. As yet there is no indication of how much form and pattern vision remains after growth factor treatment. Appropriate dosage protocols (amount and frequency) need to be established for each growth factor, and any side effects assessed. Indeed, Ogilvie *et al.* (2000) have shown that a combination of growth factors will rescue rd mouse photoreceptors in organ culture but each factor presented singly will not.

2.1.2. *Gene therapy*

Several studies have indicated that (a) cells in the retina can be transfected using an adenovirus vector (e.g. Li *et al.*, 1994) and (b) photoreceptor loss may be slowed with appropriate wild-type enzyme transfects (Bennett *et al.*, 1996; Ali *et al.*, 1996,1997, 2000; Jomary *et al.*, 1997). Recent work has shown that growth factor transfects can survive in the retina and reduce photoreceptor loss. Thus, rd mice had more rows of surviving photoreceptors 18 days after Ad-CNTF injection (Cayouette and Gravel, 1997) and improved survival was also seen in the rds mouse for as long as 52 days (Cayouette *et al.*, 1998). Injecting adenovirus-carrying bFGF has improved photoreceptor survival in the RCS rat for 56d compared with untreated rats (Akimoto *et al.*, 1999).

In terms of safety, most of the above studies have reported no detection of virus in the blood or in any other organs, suggesting that infectivity does not spread. However, Dudus *et al.* (1999) found evidence of a persistent transgene product in the CNS after introduction into the eye, raising the possibility that virus might spread beyond the site of injection. Recent fatalities in human gene therapy experiments (Lehrman, 1999; Marshall, 1999) argue for caution in the use of the current vector approaches and for the development of new vectors.

As with the growth factor studies, most of the replication defective adenovirus transfer studies in animals reported a loss of label and loss of photoreceptors after relatively short periods of time. The reasons for this are not known: possibly the transgene is unstable, exposure to the virus is

eventually toxic, or alternatively, the transgenes may persist but become ineffective because other regulatory elements are lost. A further explanation is that the cells themselves are eventually removed by some form of immune rejection (Reichel *et al.*, 1998).

Until safe vectors, producing stable, long-term expression of factors can be identified, this is unlikely to be a suitable treatment, especially since to be maximally effective it should be instituted early in life. In addition, the complex genetics of RP makes it difficult to establish commercially viable treatments when homologous genes are introduced, especially since each patient group will often be quite small (the most common subtype in the USA and Europe, P23 H, represents about 12% of autosomal dominant RP, Gal *et al.*, 1997). For the larger AMD group, since the genetic correlates are presently undefined, a gene therapy treatment would be difficult to develop at this point other than one involving a relatively non-specific approach such as growth factor delivery as described above.

2.1.3. *Cell transplantation*

Introduction of cells into the subretinal space before substantial photoreceptor loss has occurred can limit the disease process and functional deterioration. Donor cells include fresh (Li and Turner 1988a,b, 1991; Lopez *et al.*, 1989), cryo-preserved (Durlu and Tamai, 1997), cultured (Lopez *et al.*, 1987; Sheedlo *et al.*, 1993), or immortalised RPE cells of animal or human origin (Keegan *et al.*, 2000; Coffey *et al.*, 2000), iris pigment epithelial cells (Rezai *et al.*, 1997; Schraermeyer *et al.*, 1999), stem cells (Takahashi *et al.*, 1998; Chacko *et al.*, 2000) and Schwann cells (Lawrence *et al.*, 2000). Retina has also been transplanted to the subretinal space to preserve remaining cones (Mohand-Said *et al.*, 1997).

There have, however, been reports of RPE cell rejection (Jiang *et al.*, 1994, 1995; Gouras and Algvere 1996; Zhang and Bok 1998; Gabrielian *et al.*, 1999), even though the undisturbed subretinal space has been regarded as an immune-privileged site. Nevertheless, some 70 patients have received RPE cell grafts. Results so far have not been encouraging but since most recipients were at an advanced stage of functional loss, little improvement could be expected. Benson *et al.* (1998) noted some limitation of further deterioration, using a series of psychophysical tests: otherwise objective functional assessment after transplantation has not been reported.

In spite of the fact that clinical experiments are in progress, basic research remains essential. New treatments need to be tested, effective screening 'bioassays' must be developed, immune problems must be overcome, alternatives to fresh cell transplantation sought, and a comprehensive assessment of the degree of functional recovery must be made.

2.1.4. *Dietary and drug treatments*

Modification of diet may be useful in some specific forms of retinal dystrophy. Examples include Refsum disease (a phytanic acid deficiency); vitamin A supplementation for a-beta-lipoproteinaemia (Ehlers and Hansen, 1981) and vitamin B6 for gyrate atrophy (Kennaway *et al.*, 1989) but not all patients benefit from the treatment, suggesting disease heterogeneity. Such heterogeneity may also have been a factor in a randomised, controlled, double-blind clinical trial on the use of vitamins A and E in the treatment of RP (Berson *et al.*, 1993), where only a subset of patients appeared to benefit from vitamin A supplementation. Moreover, the beneficial effect was only demonstrated in electroretinograms; there was no treatment effect on visual acuity. In AMD, there is contradictory evidence for the beneficial effects of antioxidants in the diet. Seddon *et al.* (1994) suggested that increasing the consumption of foods rich in certain carotenoids, in particular dark green, leafy vegetables, might decrease the risk of developing advanced or exudative AMD, but both Mares-Perlman *et al.* (1996) and Smith (1999) found no association between AMD and dietary antioxidants.

Recently, D-*cis*-diltiazem, a calcium-channel blocker that also acts on light-sensitive cGMP-gated channels, has been shown to rescue photoreceptors and preserve visual function in the rd mouse (Frasson *et al.*, 1999b), but further studies will be required to assess whether this drug has a wider application.

2.1.5. *Controlling secondary degenerative events*

In humans, most attention has concentrated on controlling the secondary histopathological events in AMD, particularly by treating choroidal vessels that have broken through the weakened Bruch's membrane. Laser photocoagulation has been used to reduce these lesions but there is a risk of visual impairment as a result of the treatment itself, because of thermal damage to adjacent tissue (e.g. Bernstein and Seddon, 1996). Using various light sensitive drugs (photodynamic therapy) it has been possible to limit the collateral damage caused by laser treatment and still prevent the formation of new invasive choroidal blood vessels (e.g. Schmidt-Erfurth, 1999). This treatment does not provide a permanent cure.

In animal studies, the secondary consequences, involving both cell loss in the inner retina and the effects of vascular pathology are also an issue (Villegas-Peréz *et al.*, 1998; Wang *et al.*, 2000) but have received much less attention in experimental interventions (Seaton and Turner 1992; Seaton *et al.*, 1994).

2.2. Treatments directed at replacing lost photoreceptors

Once the photoreceptors are lost, the only option available is to reconstruct the outer retina. This is not an insignificant task, requiring reconstruction of information flow circuitry. Presently, two approaches are being explored—one involving transplantation of new photoreceptors either in cell dissociates or in a retinal sheet and the other to provide a microchip that can take on the phototransduction role of the photoreceptors and relay information to the CNS sufficient for sensory discrimination.

2.2.1. *Cell transplantation*

Early experiments transplanting photoreceptors used embryonic or perinatal tissue grafted into adult rat eyes (Turner *et al.*, 1986). Graft success appeared to be age-dependent, with a marked drop in viability after embryonic day 21 (Li and Turner, 1991). Instead of showing normal tissue orientation, the grafts formed rosettes. Silverman and Hughes (1989a, b) harvested and transplanted photoreceptors as a sheet, a procedure that improved cell orientation. There was also evidence from these pioneer studies that photoreceptors might integrate with host neural structures (del Cerro *et al.*, 1988; Silverman *et al.*, 1992; Gouras *et al.*, 1994b; Adolph *et al.*, 1994). Subsequent work has pursued two directions. In one (Bergstrom, 1994; Aramant *et al.*, 1999; Ghosh and Ehinger, 2000; Sharma, 2000), sheets of embryonic retina have been introduced into the subretinal space using a carrier device that allows them to unfold as a flat sheet. These sheets subsequently integrate with the host retina. In the second, postnatal rodent retina is partially dissociated and introduced as a suspension into the subretinal space (Kwan *et al.*, 1999a,b). After transplantation to rd mice such grafts are capable of forming a well-organised photoreceptor layer, which develops a synaptic interface with host retina. These animals show improved visual function, using photophobia and threshold sensitivity tests (Kwan *et al.*, 1999a,b, 2000).

Clinical retinal transplantation experiments, apart from showing that such grafts appear not to cause additional damage, have produced either no evidence or ambiguous evidence of functional improvement (Kaplan *et al.*, 1997,1998; Das *et al.*, 1999). For systematic application to humans not only is it necessary to identify a source of easily available, tissue-matched, disease-free cells of appropriate age, but neuronal pathways must be reconstructed with sufficient precision to provide an effective substrate for vision. In a pilot attempt to transplant retinal sheets, two blind patients with RP reported some visual sensation (Radtke *et al.*, 1999). Whether such responses were mediated through the transplant or were due to effects on the remaining retina is not presently clear.

2.2.2. *Microelectrode prostheses*

An alternative approach to replacing photoreceptors with cells is to use implanted microphotodiodes to carry a visual signal to the cells of the inner retina (Eckmiller, 1997; Rizzo and Wyatt, 1997; Peachey and Chow, 1999; Zrenner *et al.*, 1999; Humayun and de Juan, e.g. see Majji *et al.*, 1999; Normann *et al.*, 1999). Using various animal models, photodiode arrays have been placed either

subretinally (Peyman *et al.*, 1998) where they would be expected to stimulate bipolar cells or adjacent to the vitreal surface to stimulate cells of the ganglion cell layer (Eckmiller, 1997). These artificial implants appear to remain stable and are biocompatible (Peyman *et al.*, 1998; Zrenner *et al.*, 1999; Majji *et al.*, 1999).

In humans, direct pattern stimulation of the surface of the retina can elicit simple percepts (Humayun *et al.*, 1999), but long-term implantation of functioning photodiode/electrode arrays has yet to be reported.

Much still has to be done to ensure that a significant signal can be presented to cells of the inner retina and that the progressive deterioration of the inner retina (which normally follows outer retinal cell degeneration) does not compromise efficacy. It has yet to be seen how far the CNS can interpret signals delivered through such prostheses to elaborate visual images. Presently, the best that might be expected from such endeavours would likely be a fairly crude image, but this may be sufficient to permit navigation around obstacles.

3. THE IDEAL TRANSPLANT STUDY

In this section, we review the progress that has been made in the various components associated with transplantation. In particular, we consider those issues that should ideally be resolved before transplantation can become a routine clinical therapy.

3.1. Animal models

Most transplantation protocols have been developed using a limited range of animal models of retinal degeneration as well as normal unaffected or light-damaged retinas.

While experiments involving gene therapy strategies generally require models that are homologous to human disease states, transplantation experiments may still be of value even though the model is not necessarily homologous to the human disease. What is perhaps more important is that the primary targets of the disease and the general histopathology are similar.

There are many animal models of retinal degeneration, both natural mutants and transgenics, but most transplantation studies directed at treating retinal dystrophy have been performed using three rodent models: the RCS rat, the rd mouse, and the light-damaged rat.

The RCS rat has a recessive mutation, occurring as a deletion, which disrupts the gene encoding the receptor tyrosine kinase, Mertk (D'Cruz *et al.*, 2000). As a result retinal pigment epithelial cells fail to phagocytose shed rod outer segments effectively and this leads ultimately to photoreceptor cell death and loss of vision (Bourne *et al.*, 1938; Dowling and Sidman, 1962). Some of the secondary histopathological features of the RCS rat, nevertheless, show close similarities with those of RP (Milam *et al.*, 1998). These include the formation of vascular complexes in association with RPE cells, the migration of RPE cells into the retina, the progressive disruption of the inner nuclear layer and the loss of retinal ganglion cells (Villegas-Peréz *et al.*, 1998). From the perspective of transplantation, however, the RCS rat has been used most frequently as a model for AMD because the defect is in the RPE. There are clearly major differences: AMD appears to result, not from a failure of phagocytosis but from a progressive dysfunction of the RPE cell with increased accumulation of intracellular material and abnormal basal laminar deposits (see Zarbin, 1998; Fine *et al.*, 2000). Additionally, there are changes in Bruch's membrane both in terms of increased amounts of lipid deposition, protein cross-linking and reduced permeability to nutrients. In late stages, vessels of the choriocapillaris invade the subretinal space through the weakened membrane. By contrast, the RCS rat RPE is essentially nonfunctional at least with respect to outer segment phagocytosis. Breaching of Bruch's membrane by choroidal vessels does not occur in RCS rats although as in AMD, the inner retinal vessels respond to the altered cell architecture. What the model can show is that it is possible to preserve photoreceptors from degeneration by repopulating with normal RPE cells. What it cannot show is how similar transplants would fare with a defective Bruch's membrane. This is a major concern since the metabolic changes that occur in Bruch's membrane may compromise RPE metabolism.

Furthermore, *in vitro* studies suggest that it is the basal lamina component of Bruch's membrane that is necessary for RPE attachment (Tezel *et al.*, 1999; Tezel and Del Priore, 1999). Failure of attachment occurs on the collagenous and elastin layers of Bruch's membrane and apoptosis results. Lastly, Shirigami *et al.* (1998) have shown that embryonic RPE cells are unable to differentiate on severely damaged Bruch's membrane.

In the rd mouse, a defect in the gene encoding the rod-specific subunit of the phototransduction-associated protein, cGMP-phosphodiesterase (PDE; Bowes *et al.*, 1990; Pittler and Baehr, 1991) results in a massive and rapid loss of incompletely formed rods. This begins around postnatal day 4 and almost all the rods are lost by day 36 (Caley *et al.*, 1972; LaVail and Sidman, 1974; Carter-Dawson *et al.*, 1978). The number of cone photoreceptors decreases at a slower rate, with severe loss by 4 months and about 3% surviving at 18 months of age (Carter-Dawson *et al.*, 1978; Garcia Fernandez *et al.*, 1995). In older mice, surviving cones lack outer segments and have a highly altered morphology (Carter-Dawson *et al.*, 1978). Because the mutation is targeted at the rods, it is unclear why there is also cone degeneration (possibly a loss of trophic support, Mohand-Said *et al.*, 1998) but this is a problem common to many forms of RP for which this animal serves as a model. A direct human homologue of the rd mouse disease has been identified with a mutation involving the same cGMP phosphodiesterase gene (McLaughlin *et al.*, 1993, 1995; Farber *et al.*, 1994; Farber, 1995). Despite the conserved genotype, the disease has a rapid onset in the mouse, compared with the much slower development in humans. However, this early onset makes the rd mouse very suitable not only for studies designed to reconstruct a new photoreceptor layer but also for finding ways of preserving remaining cones and preventing their secondary degeneration.

While rodent models do provide the opportunity to experiment with varying conditions and to establish economically feasible studies that can produce statistically significant numbers of cases, they do have the drawback of having small eyes. This makes them less than ideal when designing surgical procedures that might be useful in the clinic. Accordingly, a number of studies have used rabbit or monkey eyes. These are not retinal dystrophic animals but they are valuable for examining ways of introducing tissue that are more relevant to human surgery. A number of large animal dystrophies that are homologous to forms of RP have been identified and are in the process of characterisation. These include the Abyssinian cat with a primary photoreceptor defect (Narfstrom and Nilsson, 1987); the Briard dog with an RPE 65 gene defect (Veske *et al.*, 1999) and a potential monkey model (Neuringer *et al.*, 1998). Most of these are in short supply at present and therefore cannot be viewed in any other capacity than as pre-clinical models. Presently, transplantation in such animals has been limited to the Abyssinian cat (Ivert *et al.*, 1998).

In general, light-damaged retinas are less satisfactory for transplantation studies because the extent of photoreceptor removal is variable and, since transplantation is also variable, interpretation of results is often confused especially if the outcome is to be measured functionally.

To summarise, while the RCS rat is not homologous to any presently characterised human retinal disease, the results obtained using it for transplantation studies do provide a useful body of information with respect to investigating donor cell properties and the functional consequences of transplantation. As a model for AMD, it cannot be considered complete because the effect of the substrate changes that occur in this disease cannot be mimicked and these may modulate transplant survival and efficacy. Photoreceptor transplantation to diseased retinas has relied largely on rd mice as hosts. The availability of transgenic rats, as well as dogs and cats with clearly defined mutations, will provide a broader range for further study. Larger animal models also provide the opportunity for developing surgical techniques more suitable for the human eye.

3.2. Donor cells

Central to any transplant study is the need to find suitable donor cells. For dissociated cell transplants, the cells must be harvested as a pure population and for clinical use they should also be pathogen free and non-tumourogenic. Bearing in

mind that animal studies should ultimately lead to clinical application, donor tissue should be readily available. Ideally, donor cells to be used in human therapies should first be tested for efficacy in animal models. These requirements argue for a concerted attempt to find alternatives to freshly harvested cells and ideally for the generation of cell/tissue banks suitable for transplantation. While the search for such cell alternatives is underway, a more coherent examination of the optimal conditions for primary cell grafts should also be undertaken to provide a yardstick for cell line studies.

The experimental approaches required to prevent photoreceptor loss are different from those needed for reconstructing the retina and will be described separately.

3.2.1. *Donor cells for the prevention of photoreceptor loss*

The age of the donor tissue is important. For prevention of degeneration caused by RPE defects, transplantation of freshly harvested normal RPE cells can reduce photoreceptor death, provided that surgery occurs at a sufficiently early stage. Although RPE cells from more mature donors can be used, in general, immature cells confer better survival (Li and Turner, 1991). Since the supply of donor tissue is always going to be limited, different methods of storing or engineering cells are being explored. Cells can be stored in optisol (DiLoreto *et al.*, 1996) or cryopreserved (Durlu and Tamai, 1997) for limited periods of time prior to transplantation. Primary cultures of freshly harvested RPE cells can be genetically modified to extend their *in vitro* life-span by transfection with a construct encoding SV40 large T-antigen. These cells can be cloned and screened for the expression of a range of antigens normally produced by RPE cells. In this way specific clones can be identified that most closely resemble normal RPE cells. Such clones can be further tested for chromosomal damage, for abnormal cell division and for potential tumourogenicity. To examine the feasibility of using such a cell line for transplantation, we first identified a rat-derived RPE cell clone (that is effectively immortal) which prevented photoreceptor degeneration and preserved a range of visual behaviours and physiological responses with no obvious deleterious side-effects (Litchfield *et al.*, 1997b). We extended these studies to examine human RPE cells that had been similarly cloned and screened (Winton *et al.*, 2000). In the pilot studies two clones were selected whose antigen profile most closely resembled primary culture cells (Winton *et al.*, 1999). After transplantation into RCS rats, both clones supported photoreceptor survival and limited the deterioration of visual function, but the one that most closely resembled primary cells was the more effective. Safety testing showed that chromosome damage was within acceptable levels and standard tests for tumourogenicity were negative. The immortalisation procedure used, when applied to human tissue, produced cells that had an extended life whereas rat cells were truly immortalised. This adds a further element of safety. Although these findings are extremely encouraging, and large numbers of uniform, carefully screened donor cells can be produced, the safety of genetic constructs remains in question. Other immortalisation procedures may provide even more secure cell lines and spontaneously immortalised human cell lines such as ARPE19 are also effective in limiting the degeneration seen in RCS rats and deserve further attention. The issue of immune incompatibility must still be considered: however, the possibility could be conceived of developing banks of tissue-typed cells suitable for patients with different histocompatibility profiles.

Another approach to avoiding immune problems is to graft cells from the same eye that might take over some at least of the functions of RPE. One such cell is the iris pigment epithelium (IPE). IPE cells and RPE have a common embryonic origin (Hogan *et al.*, 1971; McDonnell 1989) and IPE cells phagocytose photoreceptor outer segments in vitro, although less efficiently than RPE cells (Rezai *et al.*, 1997; Dintelmann *et al.*, 1999). Therefore, they might be expected to mimic RPE cells after transplantation. This is the case, although photoreceptor survival is greater following RPE cell grafts (Rezai *et al.*, 1997, Schraermeyer *et al.*, 1999). RPE and IPE cells synthesise a similar range of cytokines, although the levels differ (Kociok *et al.*, 1998). In clinical terms, the use of such cells would require removing IPE cells from an eye, growing them in culture to increase

the number and at a later time transplanting them back into the subretinal space. Accordingly, a number of AMD patients have received such subretinal IPE grafts and have shown some improvement in visual acuity and in amplitude of ERG response (Abe *et al.*, 1999b). It remains to be shown whether the reduced phagocytic capacity of IPE cells might be an impediment to long-term photoreceptor survival. An alternative transplantation approach, again relying on autografting, is not to use RPE or RPE-like donor cells at all but to select a cell type that acts differently. This approach has been explored using Schwann cells derived from the sciatic nerve. The reason for using these originated from growth factor studies where injected CNTF and bFGF had been shown to slow photoreceptor degeneration (Faktorovich *et al.*, 1990, 1992; Masuda *et al.*, 1995; Perry *et al.*, 1995; LaVail *et al.*, 1992, 1998). Schwann cells also produce CNTF (Sendtner *et al.*, 1992), BDNF (Meyer *et al.*, 1992), GDNF (Hammarberg *et al.*, 1996) and bFGF (Neuberger and de Vries, 1993) as well as other trophic factors known to have retinal activity. It seemed reasonable to explore whether a cell that naturally produces such factors might also be effective in rescuing photoreceptors. This has been shown to be the case: subretinal Schwann cell grafts can limit photoreceptor loss and functional deterioration over periods as long as 9 months in RCS rats (Lawrence *et al.*, 2000). The Schwann cells do not appear to assume the phagocytic role of RPE cells, nor is there strong evidence for any enhanced phagocytosis by the defective host RPE cells as yet. This suggests that their major target is the photoreceptors themselves or the Müller cells. Since autografts could be used, rejection and cross-infectivity problems would not arise. To adapt the approach to a clinically suitable therapy, it would be necessary to take a peripheral nerve biopsy, isolate the Schwann cells from it and grow them *in vitro* prior to transplantation.

Lastly, it has also been suggested that grafts of dissociated retina release neuroprotective factors that are capable of preserving the remaining cones (Mohand-Said *et al.*, 1997). Normally, loss of rods is followed by loss of cones in many forms of RP as well as in the rd mouse, but after transplantation it has been observed that the rd mouse has more cones in the area deep to graft suggesting some trophic support. It is clear that the enhanced cone survival occurs against a variable background, and that the survival effect is not great. Furthermore, there is the remote possibility of cone migration from the graft.

3.2.2. *Donor cells for retinal reconstruction*

In experimental studies dissociated cells, microaggregates, full-sheet embryonic retinas or sectioned photoreceptor layers have been used as donor material. The problem of supplying suitable donor tissue, especially when foetal donor cells would be the most effective source, is a considerable one presenting both ethical and logistic problems. No attempt has yet been made to generate immortalised photoreceptors for transplantation or to use very immature, embryonic retinal cells. An approach that has attracted considerable interest recently is the use of stem cell clones that might differentiate to form photoreceptors. Stem cells are defined as cells which are self-renewing and multipotent. Such cells have been isolated from a number of brain regions (McKay, 1997) and from the retina (Ahmad *et al.*, 1999; Tropepe *et al.*, 2000). Although hippocampal stem cells can migrate into the retina and differentiate into mature cell types they fail to express retinal markers (Takahashi *et al.*, 1998). However, retinal stem cells do develop retinal characteristics when transplanted into the retina (Chacko *et al.*, 2000). These results suggest that many of the cells derived from central neural structures, and expanded *in vitro,* have already been specified to some extent at the time of transplantation, for while they are capable of developing into neurons and glia, they do not express the same antigenic profile as the resident host retinal cells. (Gage, 2000).

To summarise, there is good evidence that cells derived from alternative cell sources can prevent retinal degeneration to the same degree as freshly harvested cells. This makes it possible to avoid the logistic, ethical and rejection problems, as well as the risk of infective agents that are always a problem with fresh donor cell populations. Stem cells are likely to be a valuable alternative to freshly harvested immature photoreceptors and

this emerging technology will clearly be applied to this problem in the immediate future.

3.3. Cell preparation and transplantation procedures

An important part of any transplantation procedure is to identify the optimal conditions for preparing cells prior to transplantation and to develop methods that are effective in ensuring optimal survival of cells placed in the subretinal space. Donor material can be in the form of dissociated cells or pieces of retinal tissue. In both instances the grafts must be introduced into the retina with minimal trauma to themselves and to the host tissue. Additionally, in the case of retinal tissue transplants, it is essential that the graft remains as a sheet and retains proper polarity. This may be better achieved embedding the sheet in a gel or other substrate but this approach has yet to be explored systematically.

In rodent models, early studies concentrated on injecting cell suspensions or aggregates into the subretinal space. These preparations have the advantage of requiring only a small injection tool that causes relatively little damage to the recipient eye. Transcleral and transvitreal techniques have both been tested. Some of the common problems encountered include surgery performed with limited visibility, reflux of donor cells, damage to the recipient ocular structure, ocular haemorrhage, persistent retinal detachment, and vitreous loss. A transvitreal approach is particularly difficult, if not impossible, in small rodent eyes because of the small vitreal volume and the size and curvature of the lens and, for the same reasons, vitrectomy is not practical (Sharma and Ehinger, 1997). Accordingly, this approach has been developed in larger eyes, particularly rabbit (Ghosh and Ehinger, 2000) and monkey (Berglin *et al.*, 1997). In each case because retinal disease mutants are not available, the work has been done in normal animals. In future, it is likely that some of the dog models of retinal disease or the transgenic pig will serve as useful alternatives.

The transcleral technique has the advantage of a direct approach to the subretinal space, but there is nearly always some reflux of donor cells from the scleral entrance site that reduces the size of the graft and the success rate of transplantation. There is also a risk of donor cells breaching Bruch's membrane, and jeopardising the blood–retinal barrier (Al-Amro *et al.*, 1999). Additionally, fibroblasts (derived from the sclera) are likely to be introduced into the retina along with the donor cells. Some modification of the technique has improved the success rate (Lazar and del Cerro, 1992; Sharma and Ehinger, 1997).

In order to conserve some cellular organisation during transplantation, RPE has been cultured and 'patches' of cells have been dissected out prior to grafting (Gouras *et al.*, 1994a; Sheng *et al.*, 1995). In a further development, sheets of RPE, photoreceptors or retina (full- or partial-thickness) have been prepared using either a vibratome or excimer laser (Aramant *et al.*, 1999; Tezel and Kaplan 1998; Huang *et al.*, 1998; Ghosh *et al.*, 1999). These have the advantage of correctly orientating the graft prior to transplantation. Early attempts to transplant sheets of cells had some success in rodents (Silverman and Hughes, 1989b) but with the development of specially designed, patent-pending instruments results have been more encouraging (Seiler and Aramant, 1998; Ghosh *et al.*, 1999). By extending the studies to larger animal models such as rabbits it has been possible, not only to develop techniques, but also to devise surgical approaches appropriate for human treatments. A suitable surgical technique has been devised using pars plana vitrectomy and retinotomy for introduction of the graft (Berglin *et al.*, 1997; Ghosh *et al.*, 1998). Since RPE cells do not survive well without matrix (Tezel and Del Priore, 1997) they have been grown on and transplanted with various substrates such as cryoprecipitated membranes (Farrokh-Siar *et al.*, 1999), anterior lens capsule (Hartmann *et al.*, 1999), cadaver Bruch's membrane (Castellarin *et al.*, 1998), Descemet's membrane (Thumann *et al.*, 1997), synthetic biodegradable polymer films (Giordano *et al.*, 1997), collagen type I (Bhatt *et al.*, 1994) and as microspheres on cross-linked fibrinogen (Oganesian *et al.*, 1999).

In summary, there is still much to done to be define the most suitable and reliable transplantation method for use in humans. Work on larger normal animals does however provide the likely direction that will be needed for therapeutic use.

3.4. Donor cell viability

In any transplant study donor cell viability must be maintained throughout the procedure. Viability should be tested prior to transplantation since surgical excision, mechanical or enzymatic tissue dissociation, maintenance *in vitro* prior to transplantation and injection through a micropipette all have the potential to compromise cell survival. While many studies have examined viability prior to transplantation, it is much more difficult to determine donor cell survival after grafting (see below). This is important to know because there is already some evidence that co-transplantation with growth factors alters cell fate and survival (Gaur *et al.*, 1992; Sheedlo *et al.*, 1998). In addition, where grafts are composed of stem cells or immature cells (possibly removed from the donor during the period of histogenesis) the environment (often mature tissue) in which the cells undergo their terminal mitosis will probably influence the phenotype of the surviving cells. For instance, CNTF supplementation has been shown to alter postmitotic cell determination in the retina (Ezzeddine *et al.*, 1997).

To summarise, we know very little as yet about the conditions which contribute towards optimal cell viability and how they may be enhanced. Acquiring such information depends, to some extent, on better ways of identifying cells post-transplantation and this forms the subject of the next section.

3.5. Identification of donor cells in vitro and in vivo

It is extremely important in all grafting studies to be able to distinguish donor cells particularly after prolonged survival times. Unless they can be identified unequivocally over time, it is always difficult to be sure exactly what role they play in graft survival. Unfortunately, this is not a trivial issue and as a result, cell identification has been missing from many transplant studies. The ideal cell label should be effective for both light and electron microscopy, have a degree of permanence even if the cells divide, and stay within the labelled cells, not leaking into the surrounding tissue for other cells to take up. If donor cells die and are phagocytosed, the label should not become incorporated into the host phagocytes; this is a particular concern when considering RPE cells. The search for suitable labels began when CNS transplantation studies were initiated some 25 years ago (see detailed review by Harvey, 1999). To date no label has achieved all the desirable characteristics outlined above. Nuclear labels include radioactively labelled thymidine and 5-bromo-2-deoxyuridine (BrdU). In the visual system ^{3}H-thymidine has been used to label donor cells transplanted into the retina (Gouras *et al.*, 1985, 1991a; del Cerro *et al.*, 1990; Du *et al.*, 1992) and tectum (Lund and Harvey, 1981). As a nuclear marker it is incorporated into dividing cells, and is particularly useful in labelling embryonic or early postnatal retinal cells. If the labelled cells do not divide more than a few times after transplantation (which results in label dilution) then this is a relatively permanent, non-spreading marker. It has the disadvantage of only labelling donor cells at the S-phase of mitosis and has to be visualised using autoradiography. BrdU, which is also incorporated into dividing cells can be visualized using a specific antibody. It has been used to label both RPE (Ye *et al.*, 1993; Gabrielian *et al.*, 1999) and neuroretina (Seiler and Aramant, 1995, 1998). The one concern with its use lies in the potential for chromosomal damage. An additional nuclear marker, used in CNS transplant studies but not so far in the retina, is the Y-chromosome marker (Harvey *et al.*, 1997). This requires that cells for transplantation are taken only from male donors and that recipients are female. It can be demonstrated using *in situ* hybridisation.

Fluorophores such as Fluoro-Gold (FG) and Fast Blue have been widely used in neurobiology, including the retina (del Cerro *et al.*, 1988, 1989). They are cytoplasmic labels that fluoresce brilliantly. There is a high uptake of the dyes by dissociated retinal cells and FG can identified ultrastructurally by the formation of electron-dense lysosomal and lamellar bodies (del Cerro *et al.*, 1990). Loss of both of these dyes with time has been reported and FG can be toxic. Another fluorophore that has been used is DiI (1,1′-dioctadecyl-3,3,3,3′-tetramethyl-indocarbocyanine perchlorate) which binds to cell membranes. It can be visualised directly or photoconverted to an insoluble precipitate which can then be traced

using electron microscopy (Aramant and Seiler, 1995). However, disadvantages include a reduction in dye intensity with time, risk of dye transfer to surrounding host cells and toxic effects. Furthermore, fluorophores label cell suspensions efficiently, but cells in the centre of aggregates or sheets of tissue are often devoid of label.

Lastly, the fluorescent dye bisbenzimide (Hoechst 33342) has been used to label the nuclei of donor Schwann cells grafted into the retina (Lawrence *et al.*, 2000). Although Hoechst 33342 is rapidly taken up, there is considerable exchange of dye with surrounding host tissues, even though the cells have been thoroughly washed prior to transplantation (also observed e.g. by Harvey, 1999). The dye can be toxic so timing of the labelling process is critical.

When retinal transplantation began in humans, there was a need for a safe marker for donor cells. Fluorescein has been suggested since it already has approval for clinical use and is not known to have any detrimental effects on the retina. However, like the other fluorescent dyes, there is a risk of leakage into adjacent host tissues.

The use of transgenic markers of donor tissue has become more widespread in recent years. Gouras *et al.* (1991b, 1994c) have used retinal tissue from transgenic mice whose photoreceptors express the marker lacZ for retinal transplantation. These transgenic mice have a bovine rhodopsin gene transcription promotor in tandem with an *E. coli* lacZ gene inserted into their genome. This leads to expression of β-galactosidase in 30–40% of the rods. Its presence is revealed by a simple histochemical reaction using X-gal or Blue-gal which produces a characteristic blue reaction product for light microscopy and particles which can be detected by electron microscopy. Alternatively, the gene product can be detected using antibodies. This method has been used to demonstrate synaptic connections in retinal grafts (Gouras *et al.*, 1994b). However, there has been concern over the *E. coli* origin of the label, as it may induce immune responses to the transplanted cells and changes to retinal function (Sakamoto *et al.*, 1998).

Recently another transgene, green fluorescent protein (GFP), derived from the bioluminescent jellyfish, Aequorea victoria, has been used to label mammalian cells, such as RPE (Lai *et al.*, 1999, 2000). In culture the label lasted for at least three months, but seemed to be less stable after transplantation, in part because viral vector induced a rejection response. Recently, the production of GFP transgenic mice may provide a more stable construct for transplantation studies (Okabe *et al.*, 1997).

Transplanted cells may also be distinguished from host cells in experimental studies by grafting between species and using species specific antibody labelling. This was first used for labelling grafts between different strains of mice (Krieger *et al.*, 1982) and then between mouse and rat (Lund *et al.*, 1985). In the case of human cell line to rodent grafts, human-specific cytokeratin and MHC antibodies have been used (Lund *et al.*, 2000). This approach is useful because, like the Y chromosome marker, no extraneous marker has been added to the cells. In addition, internal markers are likely to be more stable than labels that have been added, although there is always the possibility that expression of the specific antigen may down-regulate with time. The principal disadvantage is that because of donor/host disparity, immunosuppression may have to be used and this is not ideal for long survivals.

To summarise, while there are many options for labelling donor cells, there are very few that are stable, long-term markers for cells introduced into a site where phagocytosis is usually very active and therefore re-uptake is a matter of concern. The application of suitable labels to subretinal transplants must be achieved in order to define how the donor cells work and how long they survive and exert their effects.

3.6. Integration of transplanted cells with host retina

The implanted cells must occupy an appropriate location and function normally. For RPE cells this means that they should ideally settle on Bruch's membrane, establish polarity and perform the varied functions normally attributable to RPE cells. None of the present studies has been able to show how many cells are necessary for the preservation of vision. Retinal cell transplants must integrate with the remaining host retina forming sufficient appropriate synaptic connec-

tions to relay signals to the CNS, and provide the necessary substrate for image formation. The role of transplants that prevent photoreceptor degeneration either by replacing supporting cells or improving the environment for photoreceptors and those that construct circuitry are very different. Each will be described separately.

3.6.1. *Prevention of photoreceptor loss*

For RPE cell transplants, despite the fact that it is clear that they do prevent photoreceptor degeneration for a period of time, very little is known about how they dispose after transplantation or exactly how they protect photoreceptors. Do they displace host RPE cells to lie in opposition to Bruch's membrane, do they exhibit polarity, do they produce factors typical of normal RPE cells, how do they relate to the remaining host RPE cells, do they function by upregulating the host dysfunctional RPE cells? If they function autonomously, how many of the functions normally undertaken by RPE cells *in situ* are assumed by these cells and do they establish the intimate relationship with host photoreceptors that is necessary for proper phototransduction? Answers to these questions will partly depend on the availability of stable, long-term markers. With this in mind, we have been using immortalised cell lines and RPE xenografts. Grafted RPE cells can adhere to host Bruch's membrane either by displacing dysfunctional host RPE cells or by replacing RPE cells damaged during the transplantation procedure. These cells also maintain polarity, since appropriately positioned apical and basal end-feet can be identified ultrastructurally. We do not yet know whether the grafted cells assume the usual role of outer segment recycling or whether they produce factors that stimulate the host RPE to function more efficiently.

An alternative approach we have adopted is to graft a non-retinal, growth factor-producing cell type into the subretinal space of dystrophic RCS rats. Schwann cells can be labelled with low affinity nerve growth factor receptor antibody. Ultrastructurally, Schwann cells have the distinct advantage of having a readily identifiable morphology that is quite distinct from that of RPE, photoreceptors or Müller cells. At short survivals, Schwann cells tend to lie between the RPE and photoreceptors, and are surrounded by matrix. At longer survivals (more than 3 months postoperative) they are often intermingled with RPE cells and these regions are associated with good photoreceptor survival (Figs. 1A–C). The mechanism of action of these grafts has still to be confirmed. They do not appear to ingest phagocytosed material, but the growth factors they release may act directly to support photoreceptor survival or indirectly, stimulating either the Müller glia or the RPE cells.

3.6.2. *Retinal reconstruction*

Unlike RPE or Schwann cell transplants, which essentially maintain the *status quo,* neuroretinal transplants must not only replace the photoreceptors but also integrate with the remaining host retinal circuitry to form sufficient appropriate synaptic connections to relay signals to the CNS, capable of image formation. The degree of graft integration with the host retina depends to some extent on the procedure used. In the initial studies in which photoreceptor slices were taken from immature retina, there was evidence of increased numbers of synapses after transplantation to photoreceptor depleted retinas. However, it was not absolutely clear from these studies which were synapses between photoreceptors of donor origin and processes of host inner nuclear layer cells, and which were intra-graft synapses (ie. between photoreceptors and inner nuclear layer cells that had been included in the graft. Using transgenic lacZ markers (Du *et al.*, 1992) this question has been partly resolved. In general, however, transplanted retinal aggregates do not integrate well, with a glial scar often marking the interface between transplant and host. A study using postnatal retina as donor tissue in rd mice has shown good integration between graft and host (Fig. 2A; Kwan *et al.*, 1999b). The interface region is filled not with astrocytic scar tissue, but with a layer which stained specifically with a synaptic antibody (Fig. 2B). Within this layer were mostly rod photoreceptors which were labelled positively with rhodopsin (Fig. 2C).

In another approach (Sharma *et al.*, 1997) using whole immature grafts, there was a progressive

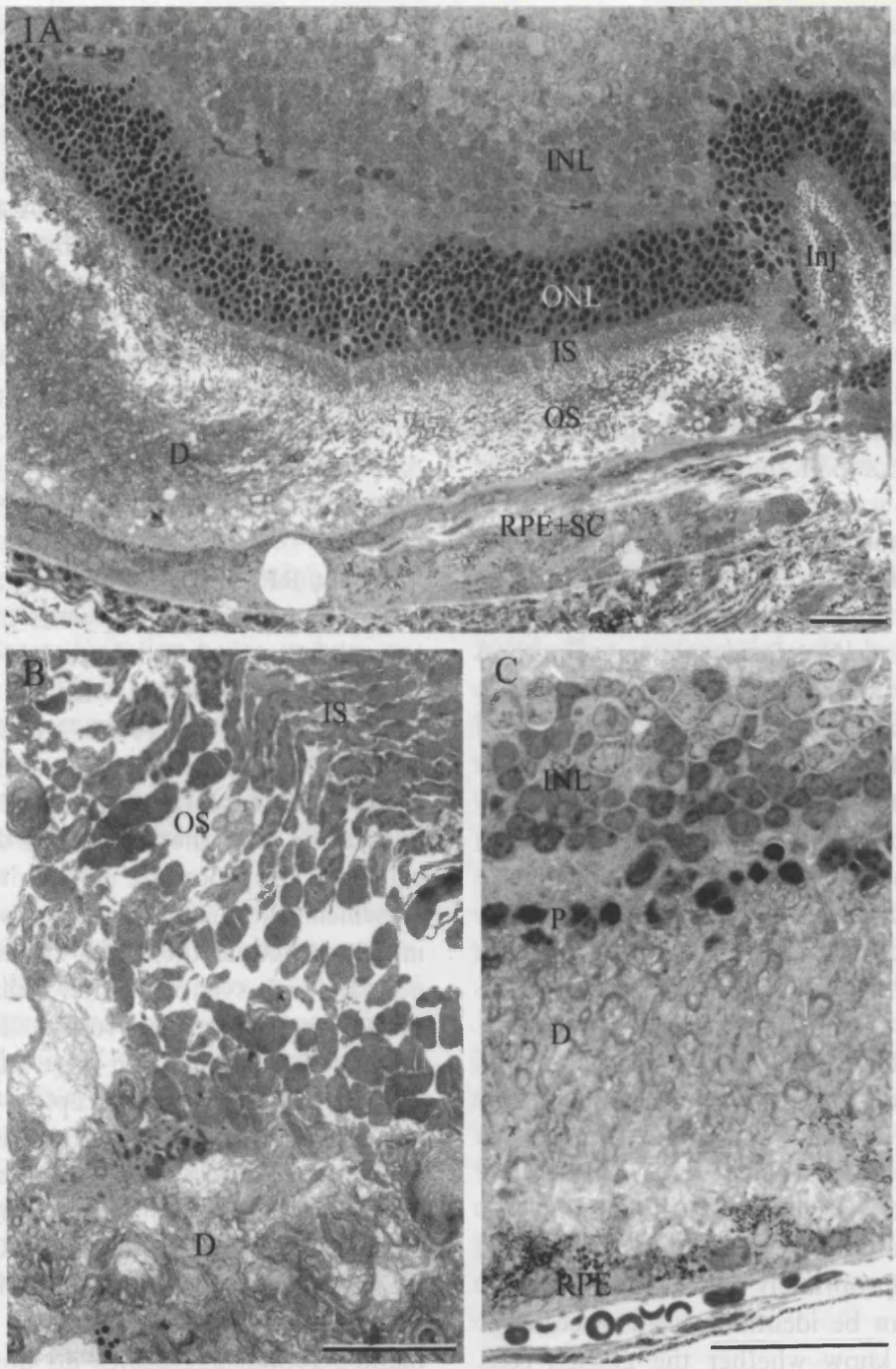

Fig. 1. (A) Semi-thin section from the retina of a 4-month-old dystrophic RCS rat, 3 months after receiving a subretinal neonatal Schwann cell graft. There is an extensive layer of surviving photoreceptors (ONL) with inner (IS) and outer (OS) segments. RPE-SC: retinal pigment epithelial cell layer with Schwann cells; photoreceptor segment debris (D); part of injection site (Inj); inner nuclear layer (INL). Scale bar: 50 μm. (B) Electron micrograph from the same retina shown in A, showing inner segments (IS) and outer segments (OS) of photoreceptors (P). Photoreceptor segment debris (D) adjacent to RPE cell layer. Scale bar: 10 μm. (C) Semi-thin section from the above retina, away from the graft. Few photoreceptors (P) survive, there are no inner and outer photoreceptor segments and the debris zone (D) is extensive. RPE, retinal cell layer. Scale bar: 50 μm.

integration between graft and host. At the electron microscope level, neuronal processes could be observed at, and crossing, the interface (Aramant *et al.*, 1995). However, there was no evidence suggesting that the ordered plexiform layers are reconstructed, or that signals are relayed to the appropriate areas of the CNS for generating visual percepts.

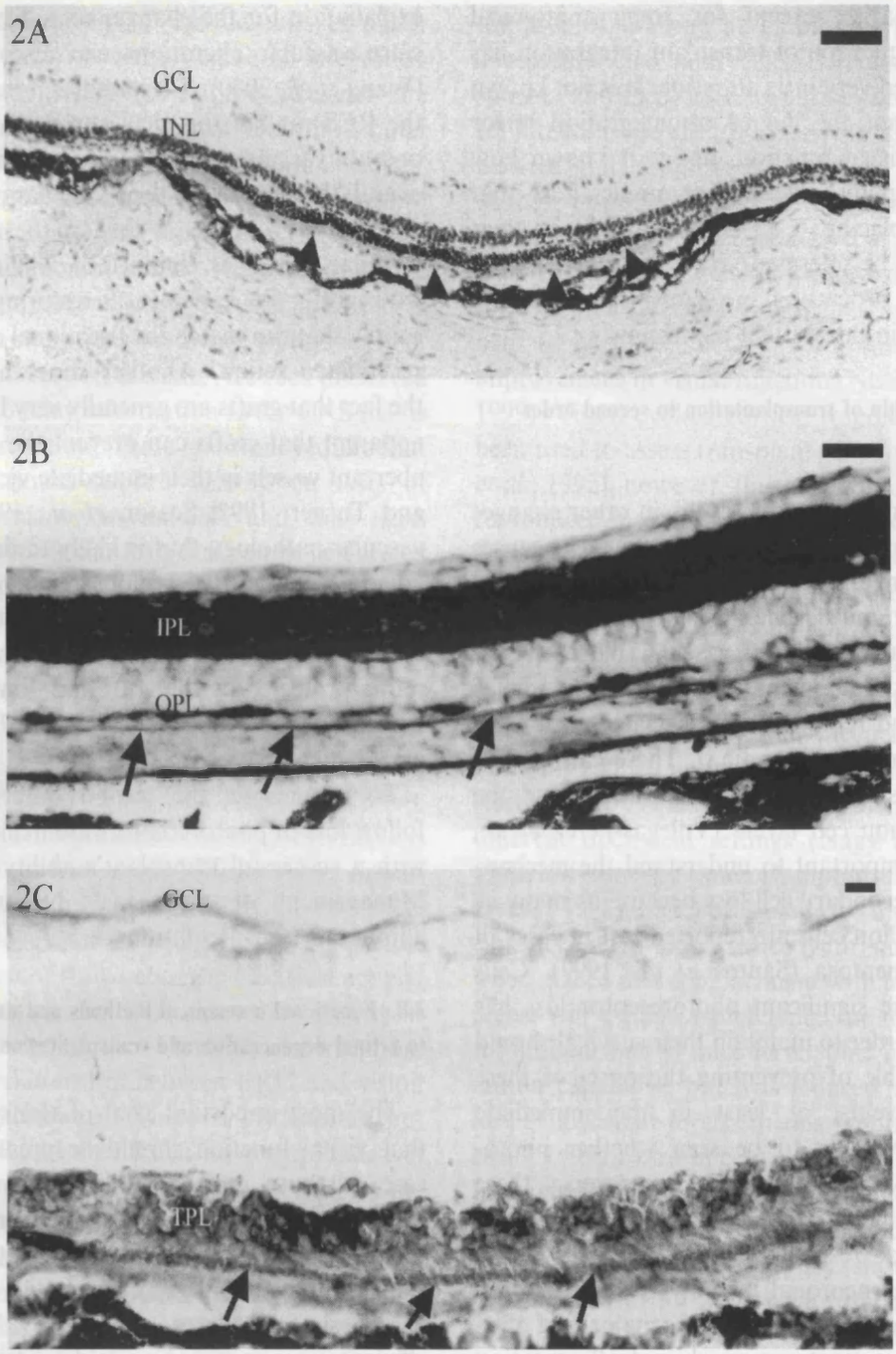

Fig. 2. (A) Polyester wax section from retina of a 2-month-old dystrophic rd mouse, 2 weeks after receiving a subretinal neonatal congenic neuroretinal transplant. An extra layer of transplanted cells is present (arrowheads) in an area where the ganglion cell layer (GCL) and the inner nuclear layer (INL) are normally associated with only a few remaining cone photoreceptors. Scale bar: 100 μm. (B) High magnification of polyester wax section from a 2-month-old rd mouse with neuroretinal transplant. The synaptic layers (outer plexiform layer, OPL and inner plexiform layer, IPL) are labelled with a mouse-specific synaptic protein antibody, P84, obtained from Dr C Lagenaur, University of Pittsburgh. Next to the OPL, an extra lamina (arrows) is seen which corresponds with the newly grafted cells. Scale bar: 50 μm. (C) Polyester wax section showing rhodopsin labelling of the transplanted cells. Note that most of the grafted cells (TPL) are predominantly rhodopsin-positive indicating that they are mostly rod photoreceptor cells and rudimentary outer segments (arrows) can be seen. Scale bar: 10 μm.

To summarise, except for some anatomical studies, the question of transplant integration has so far received very little attention. It is not known how important the degree of integration is for optimising visual function, nor is it known how closely transplanted cells must mimic those that they are replacing. Can some latitude in their performance be tolerated? How far can the CNS information processing mechanisms compensate for disorder in intraretinal processing?

3.7. Relationship of transplantation to second order changes

Loss of photoreceptors results in other changes to the structure of the remaining retina. The causes of these changes may be several fold: transneuronal atrophy and degeneration; the consequences of a significantly reduced vascular plexus in the retina; the development with time of vascular pathology and remodelling (associated with RPE cell migration into the retina). These can lead to neuronal loss in both the inner nuclear and the retinal ganglion cell layers (Villegas-Peréz *et al.*, 1998). It is important to understand the mechanism of this secondary cell loss because as many as 70% of ganglion cells are reported lost in cases of retinitis pigmentosa (Santos *et al.*, 1997). Cells grafted before significant photoreceptor loss has occurred in order to maintain their survival should also be capable of preventing the onset of these secondary events, at least in the immediate vicinity. It remains to be seen whether photoreceptor replacement grafts can reverse these complex changes. As yet we do not know the critical steps responsible for the secondary changes. Transneuronal atrophy or degeneration can occur when a neuron loses the major part of its input and it is most severe when deafferentation occurs in young animals. This might be the mechanism in rd mice where there is an overall loss of retinal ganglion cells (Hurtado-Montalban *et al.*, 2000). It is less clear whether transneuronal degeneration is a factor in the RCS rat with the later onset of photoreceptor loss. Here, ganglion cell loss occurs in a localised fashion, associated with areas of vascular pathology and influx of RPE cells into the retina (Villegas-Peréz *et al.*, 1998). Transneuronal atrophy may not be the only explanation for the changes seen in the rd mouse since vascular alterations can also be observed (Wang *et al.*, 2000). They are less prevalent than in the RCS rat but this may correlate with the late onset of vascular complex formation relative to the overall thinning of the deep vascular plexus. A real concern in rd mice is that if the inner retinal vascular plexus is diminished will these retinas with grafts be capable of restoring a vascular supply that can match the functional demands of a reactivated retina? Another concern stems from the fact that grafts are generally very localised. It is apparent that grafts can prevent the formation of aberrant vessels in their immediate vicinity (Seaton and Turner, 1992; Seaton *et al.*, 1994), but the vascular pathology that is likely to develop in the adjacent non-grafted regions might still compromise graft effects. Furthermore, the vascular changes could encroach on the region of healthy retina protected by the graft and diminish its size. Presently these concerns have not been systematically explored.

To summarise, the second order events that follow loss of photoreceptors are likely to interfere with a successful transplant's ability to function. Management of them may be an important adjunct to transplantation.

3.8. Functional assessment methods and their application to retinal degeneration and transplantation

The most important goal of transplantation is that visual function should be preserved (in the case of those grafts halting or slowing photoreceptor degeneration) or restored (in the case of those grafts replacing lost photoreceptors). Ideally, visual function assessment should be at two levels—simple reflexes, which provide rapid and efficient screening, and more discriminatory tests, such as a-wave amplitude, threshold sensitivity measurements and acuity tests to establish the level of vision that can be preserved or restored by the treatment. In order to achieve accurate assessments of transplant function it is first necessary to have detailed data on the degenerative state and on the effects of sham surgery over time.

The principal measure of treatment success in the retina has often been the thickness of the preserved photoreceptor layer (usually without

specific consideration of the amount of outer segment survival), and the degree of integration with the host. While both these measures are clearly important, little is known about how outer retinal integrity correlates with functional indices. Is there a progressive linear drop-off of vision with thinning of the photoreceptor layer? Can rescued retinas still function normally? These questions are particularly important when rescue is achieved in animals with mutations involving components of the phototransduction cascade. Are such preserved retinas able to relay information to the CNS that is useful for perception? These questions require that assessment should depend heavily on tests of visual performance in animals and that these should relate to similar tests that can be performed in human patients.

3.8.1. *Non-invasive methods of assessing visual function after transplantation*

One of the first methods used to assess function has been the ERG (Jiang and Hamasaki, 1994). This is not an insignificant undertaking: ERG is only valuable when done with extreme rigour. There are also problems in relying on ERG activity alone as an indicator of visual performance. At best, it shows changed electrical activity within the retina but not that this activity is capable of providing the substrates of perception. In fact, the relationship between ERG and vision is not straightforward. There is evidence in humans with RP that the ERG a-wave amplitude can be quite flat with traditional methods and yet the patients still have useful vision (Fishman *et al.*, 1985). Furthermore, recent assessment of ERG in rhodopsin knockout mutants provides confusing and ambiguous results with a minimal a-wave and yet significant b-wave response (Toda *et al.*, 1999). With these shortcomings in mind, which may partly be due to differences in technique, there are still, nevertheless, interesting results from transplantation studies: in two studies that have examined ERG after RPE cell transplantation in young RCS rats, one showed some preservation of ERG with preservation of photoreceptors (Jiang *et al.*, 1994), while Yamamoto *et al.* (1993) were unable to elicit a vitreal ERG but did report weak unitary responses from ganglion cells adjacent to the area of grafting at 11 months post-grafting. Transient 'ON' and 'ON–OFF' spike-like responses and local ERG could be recorded from a rabbit with an electrode on the surface of an isolated graft (Adolph *et al.*, 1994). The presence of these responses suggested that these transplants ensured preservation of some level of retinal neural activity. Recently, a technique has been developed to assess multifocal ERG in the mouse; this should help to correlate the area of transplant to any improvement in visual function (Nusinowitz *et al.*, 1999). Visual evoked cortical responses have also been used to assess transplant function (Silverman *et al.*, 1992); however, this technique has not been reproduced in other transplant studies.

Behavioural tests can also be used to demonstrate visual responses mediated through central neural circuitry. The simplest of these is to perform a basic neurological examination. A three-month-old dystrophic RCS rat will neglect visual cues introduced from the periphery while normally sighted animals orient towards such cues (Hetherington *et al.*, 2000). Light discrimination in different open-field settings (Nagy and McKay, 1972; Mrosovsky and Hampton, 1997; Kwan *et al.*, 1998) has been used to test the visual capacity of rd mice. Mice with normal vision, when placed in a compartment with light and dark areas, will exhibit a preference for the dark. It is recognised that rd mice have some light discrimination capability, but it is reduced with age and lost at low light levels. In this laboratory (Kwan *et al.*, 1999a,b), rd mice were tested for light-dark preference behaviour at a low luminance level (50 lux) in a computerised monitoring apparatus prior to transplantation. They showed random behaviour with no preference for dark compartment. However, after neuroretinal transplantation, they were re-tested at 2 weekly intervals and a dark preference was restored. Other behavioural tests are available that can provide quantitative measures of a variety of visual responses. The first is the pupillary light reflex (PLR), in which the dynamics of pupilloconstriction after exposure to light can be recorded. The amplitude of the response, threshold response, latency and rate of constriction can all be measured. Several studies have shown that in RCS rats, with time, there is reduction in amplitude, increased threshold

response and prolonged latency (Trejo and Cicerone, 1982; Whiteley *et al.*, 1998). There is also a break in the waveform during constriction, possibly reflecting the differential loss of rods over cones and a suggestion of a compensatory mechanism in later stages of degeneration (Whiteley *et al.*, 1998). However, even at one year of age, a response can still be elicited to flashes of bright light, although very few photoreceptors remain at this point. Pupilloconstriction proved to be a very effective test for functional evaluation of retinal transplants placed intracerebrally (Radel *et al.*, 1995a,b), but although it has been used to demonstrate the beneficial effects of intraretinal RPE grafts in RCS rats (Whiteley *et al.*, 1996) particularly for long survivals, it is less valuable for shorter intervals. This is because the pupilloconstrictor response is so sensitive that at early time points, when degeneration is still in progress, transplant- and sham-related effects are often difficult to discriminate from control responses. Other points to be considered are: (1) the response is averaged over the whole retina so that the overall effect is reduced for grafts protecting only small areas of retina; (2) the majority of cells driving the response are located in the ventral retina (Young and Lund, 1998), while in rodents it is more common to place grafts dorsally; (3) it has been suggested that the magnitude of the PLR does not correlate with the number of photoreceptors (Kovalevsky *et al.*, 1995). These caveats indicate that the PLR may not always be an accurate or consistent way of measuring transplant function at least for shorter survivals.

Startle reflex inhibition has also been used to assess retinal transplants in light-blinded rats (del Cerro *et al.*, 1991, 1995). A brief (125 ms), loud (100–120 dB), high frequency (4–14 kHz), white noise tone will cause a startle response in a rat; however, if a light is presented prior to the tone, and the animal detects the light, its startle response will be reduced. This is called pre-pulse inhibition. This reflex is impaired in animals with retinal damage (Wecker and Ison, 1986) or lesions restricted to the superior colliculus (Fendt *et al.*, 1994). Light-blinded animals with grafts appear to show a modest improvement in pre-pulse inhibition dependent upon the time the light is presented prior to the startle stimulus (del Cerro *et al.*, 1991).

A particularly effective reflex test involves measuring an animal's ability to track a moving stimulus. The head-tracking method is based on an optokinetic test devised by Cowey and Franzini (1979). Sighted animals automatically track (Figs. 3A and B), by moving their heads, the movement of a square wave grating of varying spatial frequencies (0.125, 0.25 and 0.5 cycles/deg). By scoring the total time spent tracking the movement, it is possible to establish a measure of visual function. Dystrophic RCS rats lose their head-tracking capability by 8 weeks of age despite the fact that the retina still has a photoreceptor layer several cells thick (Fig. 3B). Sham surgery prolongs the tracking response for some weeks but by 12 weeks post-transplantation only animals with fresh RPE grafts, or grafts of alternative cell types known to preserve photoreceptors (immortalised rat or human RPE cell lines or Schwann cells) continue to respond. In contrast to the PLR, this technique is highly discriminatory at early survival times although it seems less robust with longer survivals in situations in which physiological and anatomical data show transplants are still present. Whether this indicates a progressive loss of area of protected retina with time remains to be seen.

Finally to test acuity, rats can be placed in a two-choice box modified from one described by Cowey *et al.* (1982). The rat's entry into one or other alley is monitored by photocell disruption. Visual stimuli are projected onto opalescent panels at the end of each alley. Rats are first trained on an intensity discrimination task and rewarded for choosing the more brightly illuminated panel. They are then trained to discriminate between horizontal and vertical gratings of 0.15 cycles/deg. Once they have reached a minimum criterion, their visual acuity and contrast sensitivity can be determined. In studies on rats with grafts of RPE cell lines, a level of acuity of better than 0.37 cycles/deg can be sustained, and these figures are comparable to normal perfomance levels (P. Coffey, unpublished observations). Recently, another test of acuity has been used, employing both a water maze and a two-choice box (Prusky *et al.*, 2000). While this test may be a better discriminator of absolute acuity, an animal with only a small region of rescued retina may be unable to locate it when in these relatively stressful conditions. All

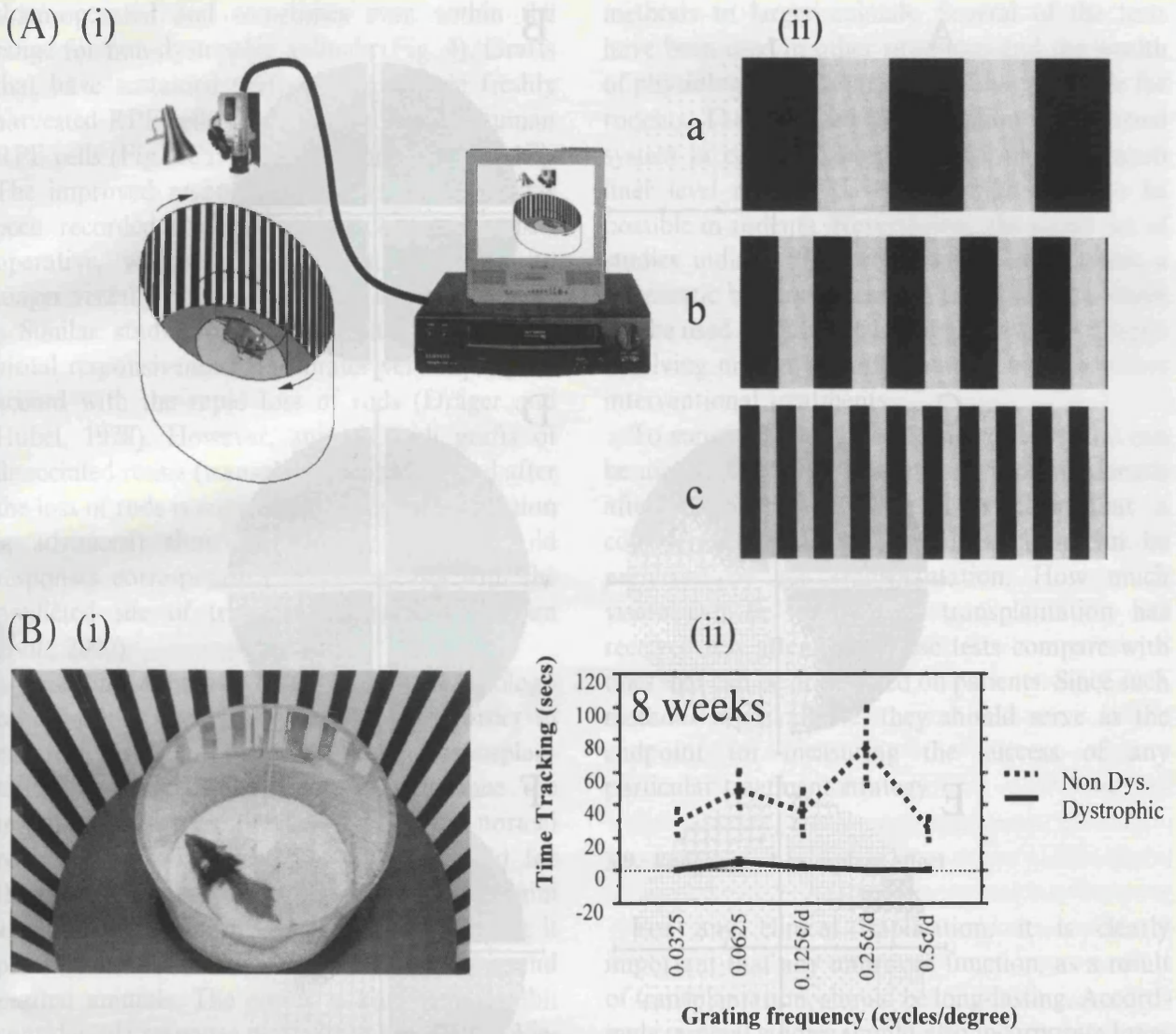

Fig. 3. (A) Optokinetic head tracking as tested in the rat. (i) The rat is placed into a drum onto which a square wave grating is placed. The drum is then slowly rotated clockwise and anticlockwise and the time spent following the gratings noted. (ii) Three gratings of different spatial frequencies are used: (a) 0.125; (b) 0.25; and (c) 0.5 cycles/deg. (B) Optokinetic performance in RCS rats. (i) Picture of rat in the optokinetic apparatus. (ii) The amount of head-tracking observed in 8-week-old pigmented RCS non-dystrophic and dystrophic rats.

the above tests are non-invasive and can be used over a period of time to study the progress of protection or recovery of vision.

3.8.2. *Electrophysiological assessment of transplant function*

Perhaps the most sensitive and accurate measure of transplant function comes from unitary electrophysiological recording from visual centres in the brain. This technique is invasive and time consuming but can provide an in-depth analysis of response properties of cells in regions of the CNS responsible for analysis of visual input. Of these the superior colliculus provides a useful test site because a map of the retina is represented across its surface (Siminoff *et al.*, 1966). It also represents a relatively unfiltered input from the retina and it is likely that a significant part of multiunit responses is due to presynaptic activity. As such it reflects overall ganglion cell output. Recording from points across the superior colliculus allows assessment of the threshold sensitivity responses of the retina to be measured. This can be done for normal (Fig. 4A) and dystrophic (Fig. 4B) animals as well as for sham-operated animals (Figs 4D and

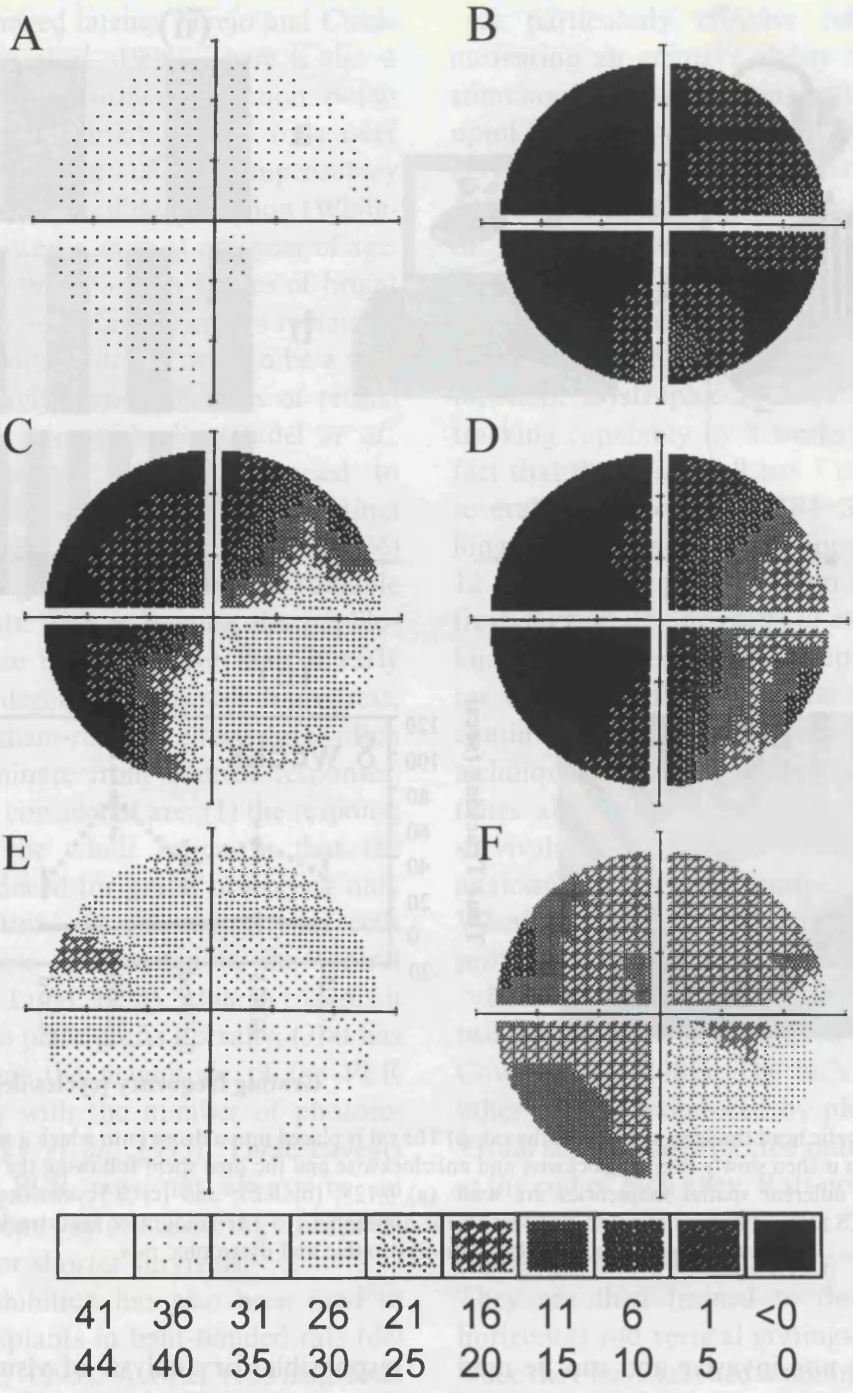

Fig. 4. Threshold light sensitivity maps: Perimetry charts for RCS rats at 6 months of age: (A) congenic; (B) dystrophic; (C) dystrophic with subretinal transplant of human RPE cell-line; (D) dystrophic with subretinal sham injection; and at 4 months of age; (E) dystrophic with subretinal neonatal Schwann cells; (F) dystrophic with subretinal sham injection. The charts are a schematic representation of the dorsal view of the superior colliculus showing visual sensitivity at 76 individual recording sites. The charts are oriented to show the position of the visual receptive fields: the dorsal and ventral visual fields are on the top and bottom of the perimetry charts respectively and the temporal and nasal visual field are on left and right. The subretinal injections were made in the dorso-temporal retina, which corresponds here to the bottom-right of the charts (ventro-nasal field). A log scale of visual sensitivity is shown, measured in candela/m^2.

F) and animals with transplants. Studies have been undertaken on dystrophic RCS rats both over time and after receiving a range of different cell transplants at around 1 month of age. Using this technique it has been possible to show discrete regions (corresponding retinotopically to the transplant location in the retina) with threshold sensitivities several log units better than

sham-operated and sometimes even within the range for non-dystrophic animals (Fig. 4). Grafts that have sustained performance include freshly harvested RPE cells, immortalised rat and human RPE cells (Fig. 4C), and Schwann cells (Fig. 4E). The improved responsiveness in some cases has been recorded for as much as 9 months post-operative, whereas dystrophic animals are no longer visually responsive by this age.

Similar studies on rd mice have shown that visual responsiveness deteriorates very rapidly, in accord with the rapid loss of rods (Dräger and Hubel, 1978). However, animals with grafts of dissociated retina (transplantation performed after the loss of rods is complete and cone degeneration is advanced) show improvement in threshold responses corresponding retinotopically with the predicted site of transplant integration (Kwan *et al.*, 2000).

A second approach involves electrophysiological recording from the visual cortex. In order to examine the effects of degeneration and transplantation it has been necessary to determine the response properties of neurons in the normal rodent cortex. This has now been achieved for the rat and mouse (Girman *et al.*, 1999; Mangini and Pearlman 1980; Métin *et al.*, 1988) making it possible to extend our studies to dystrophic and grafted animals. The cortex is known to exhibit considerable response plasticity (Gilbert and Wiesel, 1992) so an important question yet to be resolved is the degree to which it can adapt to changes in the quality of the input signal. Preliminary studies do however show that in areas of the central visual representation where there are also sustained low threshold sensitivity responses, normal response properties can be elicited from cells in the visual cortex (Girman *et al.*, 2000).

Other approaches that can be used to explore the visual behaviour of rodents include optical imaging of cortical activity and scans (Schuett *et al.*, 2000). These are only just beginning to be applied to the study of retinal degenerative diseases.

The foregoing description has focussed solely on rodent models because these are the only animals for which there is a significant literature on the functional effects of degeneration and repair. It is perfectly feasible, however, to apply many of these methods to larger animals. Several of the tests have been used in other situations and the wealth of physiological data far exceeds that available for rodents. The increased sophistication of the visual system in cats and monkeys also allows a much finer level of analysis than is ever likely to be possible in rodents. Nevertheless, the recent set of studies indicate that it is possible to perform a systematic battery of tests on rats and mice which can be used to show the efficacy of repair strategies involving not only transplantation but also other interventional treatments.

To summarise, there is a range of tests that can be used to examine the status of vision in animals after transplantation and it is clear that a considerable degree of visual function can be *preserved* by cell transplantation. How much vision can be *restored* by transplantation has received less attention. These tests compare with ones that can be or are used on patients. Since such methods are available, they should serve as the endpoint for measuring the success of any particular treatment strategy.

3.9. Longevity of transplant effect

For any clinical application, it is clearly important that any improved function, as a result of transplantation, should be long-lasting. Accordingly, animal studies should also incorporate long-term studies. We have examined RCS rats transplanted with various cell types up to 12 months post-operative. Results so far suggest that there is a progressive loss of preserved photoreceptors with time with likely total loss of photoreceptors by 12 months. Thus, although the grafts can postpone photoreceptor cell death, they may not delay the encroaching effects of dystrophy indefinitely. Nevertheless, the period of preservation represents a significant proportion of the rat's life-span, which if translated to humans could delay the onset of complete blindness significantly. Longevity of effect may be modifiable by many events. The age of the donor cells and their viability during dissociation and transplantation, the natural life span of the donor cell, the host age and the subretinal environment, the availability of a suitable substrate and the immune response of the recipient are all likely to play a part in how

long transplants survive. In addition, the potential immune consequences of mismatched donor and host cells may affect longevity. This is described in more detail in the next section.

3.10. Immune and inflammatory considerations

It is obvious that for successful transplantation, potential immune or inflammatory reactions should be avoided or controlled. While the subretinal space may be considered a partially immunologically privileged site, foreign tissue is not necessarily protected from immune attack. Its immune status is atypical (Jiang and Streilein, 1991; Grisanti *et al.*, 1997) and like the anterior chamber, exhibits a phenomenon now referred to as Anterior Chamber Associated Immune Deviation (ACAID; Streilein *et al.*, 1980; Streilein and Niederkorn, 1985; Ksander and Streilein, 1989). This response to antigen placement in the subretinal space is an active systemic process leading to down-regulation of the normal delayed-type hypersensitivity (DTH) response seen after transplantation of tissue to non-immune privileged sites (Hall, 1991; Piccotti *et al.*, 1997). The mediators of this predictable response are thought to be locally produced cytokines (e.g. TGF-β and IL-10; D'Orazio and Niederkorn, 1998; Takeuchi *et al.*, 1998) and the spleen which divert the immune response from the Th-1 type T-cell population seen in DTH to the Th-2 type (Streilein, 1990, 1995, 1999). Lymphocytes from the spleens of these animals were capable of suppressing DTH when adoptively transferred to naïve individuals (Wenkel and Streilein, 1998). Studies have shown that this process requires an intact blood-retinal barrier (BRB; Wenkel and Streilein, 1998).

Diseased retinas often display leakage and abrogation of the BRB as do dystrophic RCS rat (Villegas-Peréz *et al.*, 1998) and rd mouse (Wang *et al.*, 2000) retinas. Furthermore, one possible consequence of transplantation to the retina will be the breaching of the BRB thus exposing the graft to immune surveillance during this period by lymphocytes which normally circulate in the blood (Wekerle *et al.*, 1987). In both instances the exact mechanisms occurring in these situations have yet to be elucidated. The type, pattern and timing of inflammatory and immuno-modulatory factors (both molecular and cellular) produced in response to surgical injury and placement of the grafted cells must be determined in dystrophic as well as non-dystrophic strains (Abe *et al.*, 1999a). To date no detailed analysis of the inflammatory response to retinal cell transplantation in the immediate post-operative phase has been made in a dystrophic model.

With respect to retinal cell transplantation it is known that the immune privileged status of the sub-retinal space is not absolute (Jiang *et al.*, 1993, 1994, 1995; Wenkel and Streilein, 1998) and allogeneic grafts can still be lost despite immunosuppressive therapy (Cyclosporine-A; Crafoord *et al.*, 2000), indicating possible non-T-cell dependant graft destruction. Non-immune mediated destruction of grafted cells must be considered. It has been recently demonstrated that Fas (CD-95) Fas-ligand (CD-95 L) interactions are important in immune modulation as this interaction leads to the apoptosis of Fas expressing activated T-cells (Griffith *et al.*, 1995; Jorgensen *et al.*, 1998). It has been shown that the RPE cells express Fas-L (Weller *et al.*, 1996; Jorgensen *et al.*, 1998; Winton *et al.*, 2000), and this feature of host RPE cells may be important in down-regulating the activity of activated T-cells in the region (Jorgensen *et al.*, 1998). Moreover, it has also been shown that RPE cells also constituitively express Fas but are resistant to Fas/Fas-L dependent apoptosis under normal conditions. However, in inflammatory conditions such as proliferative vitreo-retinopathy (PVR) this protection is lost (possibly by inhibition of RNA or protein synthesis) and these cells can be apoptosed via the Fas Fas-L pathway (Weller *et al.*, 1996). This may be a mechanism of graft cell loss post-transplantation.

Additionally, it has been shown that RPE cells must attach to undamaged Bruch's membrane (see Section 3.3). Any impediment to grafted RPE cells accessing Bruch's membrane would prejudice against survival and lead to apoptosis. In contemplating such transplantation in humans the graft bed will have to be carefully prepared so that Bruch's membrane is stripped of host cells but remain undamaged.

Another factor to affect the stability of any immune privilege is the loss of vascular integrity since disruption of the blood-retinal barrier will

expose the retina to immune surveillance (Wenkel and Streilein, 1998). This may complicate the use of the RCS rat in transplantation studies unless the grafts are syngeneic because its retinal vessels become fenestrated and 'leaky' as they near the choroid (due to the loss of photoreceptors and RPE migration into the retina). Since vascular integrity is lost in some human retinal diseases, notably RP and neovascular AMD, the potential problems of transplantation into the subretinal space with a persistently compromised BRB cannot be ignored.

The duration of the disease process can affect immune privilege since it is lost in older rd mice (Welge-Lussen *et al.*, 1999). Geneticity of donor and recipient and mismatch of major histocompatibility complex (MHC) haplotypes (especially class I, Zhang and Bok, 1998) can compromise the graft, as does the up-regulation of MHC class II expression in graft cells (Kohen *et al.*, 1997). It is clear from the above evidence and earlier studies in the brain that the question of immune privilege and transplant rejection is a particularly important one for clinical use.

Some neural retinal grafts have been shown to survive for prolonged periods following transplantation to the subretinal space (Gouras *et al.*, 1994b) and a deviant immune response has been described (Jiang *et al.*, 1993). However, an unconventional immune-mediated rejection of the grafts has also been reported (Jiang *et al.*, 1995) which lacks the gross cellular infiltrate that is normally associated with allograft destruction. Neural retinal grafts placed in immune-privileged sites (e.g. anterior chamber) display features of ACAID with a down-regulation of normal delayed-type hypersensitivity, but they eventually succumb to atypical graft rejection (Jiang *et al.*, 1995). However, other authors claim survival of allografts, albeit of foetal tissue, comparable to syngeneic grafts (Larsson *et al.*, 1999).

To provoke graft rejection antigen must be presented to host T-cells in association with MHC II molecules (Van Buskirk *et al.*, 1997) and there is some up-regulation of MHC II expression in subretinal grafts (Ghosh *et al.*, 2000; Larsson *et al.*, 1999). The identity of the antigen-presenting cells in the donor neural retina is not entirely clear but donor microglia (termed passenger leukocytes by Ma and Streilein, 1998) are likely candidates. The observed increase in MHC II expression may be on these microglia. Thus, while neural retina itself may not be immuno-provocative other associated donor cells could be. Their management might provide a focus for control of graft rejection in the future.

In summary, it is clear that mismatched cells introduced into the normal retina are potentially vulnerable to immune surveillance and rejection. Since diseased retinas often have a compromised BRB, the risk of rejection is increased. However, an understanding the basic mechanisms underlying the concept of 'immune privilege' in the retina may help in managing mismatched grafts.

4. TRANSLATION OF ANIMAL STUDIES TO HUMANS

Presently no single experimental animal study has taken into account all the features we have outlined for an 'ideal transplant', namely that it should survive for a prolonged period, be safe, integrate with host cells and work in preserving or reconstructing vision. Nevertheless, in the peer reviewed literature alone, at least 18 people with RP have received either subretinal foetal (Das *et al.*, 1999; Radtke *et al.*, 1999) or adult neuroretinal transplants (Kaplan *et al.*, 1997) and 24 people with either dry or wet AMD have received subretinal RPE cell transplants (Peyman *et al.*, 1991; Gouras and Algvere, 1996; Algvere *et al.*, 1994, 1997, 1999; Weisz *et al.*, 1999). The total number increases to around 100 if one includes abstracts and data presented informally. However, many of the same problems in making the transition from animal models to clinical application have already been encountered in an area where cell transplantation has proven to be of some success clinically—the use of dopamine-secreting cells to alleviate the symptoms of Parkinson's syndrome. This technique was first explored experimentally in the mid-1970s and by the late-1980s convincing evidence of clinical improvement had been obtained. It should be noted that despite this success, the procedure is still far from being a routine treatment for the disease. Because of the parallels with retinal

transplantation and because this area received earlier and more intense interest than retinal transplantation, it is of value to review briefly the history and progress of this research. In Parkinson's syndrome the progressive degeneration of the cells of the substantia nigra in the brainstem deprives the striatum of its dopamine input and this leads to characteristic motor disorders. The symptoms can be partially alleviated by delivering dopamine analogues orally but with time these usually become less effective. The potential of transplantation was first suggested from a less than direct model of the disease in which the nigrostriatal pathway was acutely lesioned unilaterally in rats. This results in an amphetamine-induced rotation, which can be reduced by transplanting new dopamine-producing cells in the striatum itself rather than in their natural site in the substantia nigra (Dunnett *et al.*, 1983a,b). By 1985 (Backlund *et al.*, 1985), the first clinical experiment had been undertaken but without success. However, further attempts followed world-wide, and in some cases extravagant claims of success were made. As many as 1000 patients received transplants using a procedure that was only marginally effective at best. With time, greater success was achieved using foetal substantia nigra cells with a more defined age of donor tissue, with controlled assessment of patients before and after grafting, and with a specific immunosuppression regimen. As a result, some critically ill patients have maintained a level of function that would not otherwise be possible over a period of many years (Lindvall *et al.*, 1992; Zabek *et al.*, 1994; Kordower *et al.*, 1996). A number of points can be highlighted that are germane to retinal transplantation:

(1) It is not always necessary to have an homologous model of the disease to develop a transplant procedure with clinical potential.

(2) It takes a long time from the first clinical experiments to a reliable therapy.

(3) A major difficulty in some of the early clinical studies was a lack of defined and rigorous protocols for patient diagnosis and assessment of functional performance. Once studies were co-ordinated, under the aegis of a central authority, significantly better protocols were devised. Furthermore, in animal models of transplantation it is possible to control for sham surgical effects but this is ethically more difficult in human studies.

(4) The lack of availability of reliable sources of donor cells has been a continuing difficulty. The use of foetal human cells has many logistic and ethical problems associated with it. Various approaches have explored the use of other cell types (e.g. from the adrenal medulla (Backlund *et al.*, 1985) and Sertoli cells of the testis (Sandberg *et al.*, 1997) as well as the potential of using xenografts obtained from such animals as pigs (Kopyov *et al.*, 1992; Deacon *et al.*, 1997, 1999). Immortalised cells and stem cells may also have great potential as donor sources (e.g. Svendsen *et al.*, 1997; Studer *et al.*, 1998; Gray *et al.*, 1999; Schwarz *et al.*, 1999), although the safety of genetically engineered cells remains to be established. Additionally, questions relating to screening of donor tissue for disease and the amount of donor tissue needed to provide symptom relief still need attention.

(5) The age of donor tissue can be very important. Some of the earlier studies used cells derived from a range of foetal ages, probably because of limited tissue availability. In the rodent model of Parkinson's disease the donor age is critical for good survival of grafted neurons (Brundin *et al.*, 1988; Barker *et al.*, 1995) and the same has been shown in a monkey model (Annett *et al.*, 1997). Recently, Bentlage *et al.* (1999) have shown that host age may also play a part, at least in rats.

(6) A large proportion of transplanted neurons die within one week of transplantation, either by necrosis or by apoptosis (see review by Boonman and Isacson, 1998, summarising data from several research groups, showing up to 98% of dopaminergic neurons die after grafting). Some of this loss may be due to damage caused by the preparation of the donor cell suspension (Clarkson *et al.*, 1998) or to the transplantation process, but ischaemia and lack of appropriate growth factors will contribute to the depletion of cell numbers. Accordingly, lazaroids (21-aminosteroids; Nakao *et al.*, 1994; Björklund *et al.*, 1997; Othberg *et al.*, 1997; Karlsson *et al.*, 1999), growth factors (Rosenblad *et al.*, 1996; Zawada *et al.*, 1998) and inhibitors of apoptosis (Schierle *et al.*, 1999) have been used to improve the chemical environment of

the cells before and after grafting. Although it is clear that cell loss is also important in rat, such treatments have yet to be explored.

(7) In early studies one of the obvious difficulties lay in the visualisation of the graft in a living patient, which would allow the discrimination of transplant effects from sham or placebo effects. The advent of positron emission tomography (PET) scanning has revolutionised the assessment of graft function (Wenning *et al.*, 1997; Piccini *et al.*, 1999). For retinal cell transplantation, the increasing sophistication in the design of scanning laser ophthalmoscopes and optical coherence tomography will provide similar opportunities to visualise grafted cells in the eye.

(8) Since the central nervous system lacks a typical lymphatic drainage and is protected by a blood brain barrier it was originally assumed that it was an immune privileged site. However, it was soon realised that MHC incompatible transplants (allografts or xenografts) did not survive indefinitely (e.g. Mason *et al.*, 1986; Widner *et al.*, 1988; Lawrence *et al.*, 1990) and that immunosuppression would be necessary to manage the host immune reaction (e.g. Brundin *et al.*, 1988; Wood *et al.*, 1993; Duan *et al.*, 1996). At present several immunosuppressant regimens have been tested and it will be necessary to establish the safety of each treatment and the duration of dosage needed to prevent transplant rejection. There is some evidence that immuno-suppressants may only have to be administered from just before the time of grafting until the blood–brain barrier has resealed, discontinuing the treatment in the long term. If these procedures eventually transfer to the eye then the concomitant immunosuppressant treatments should not compromise the health of the graft recipients, many of whom are of advanced age.

To summarise, the advances that have already been made in Parkinson's syndrome transplantation provide an important background for similar approaches in the retina.

5. FUTURE DIRECTIONS

Transplantation has been shown to limit or slow photoreceptor loss and concomitant deterioration in visual responsiveness in rodents with retinal diseases that compare with human retinal dystrophies. There is also growing evidence that grafts including photoreceptors, may also serve to reconstruct rod-deficient retinas. Progress is being made in the search for suitable donor cells, in developing reliable transplantation procedures and in establishing proof of principle. There is still much to be done to improve transplant viability. The potential immune problems and strategies for controlling them are beginning to be defined. All this is necessary before these procedures can be transferred to humans as treatments for the retinal degenerative diseases. There remains the very serious question of safety. For the most part, transplantation procedures are not regulated by organisations such as the FDA, but there are considerable hazards associated with introducing foreign cells into the eye, especially if freshly harvested cells are used, which may carry viruses and other potentially infective agents. Unlike many diseases, such as heart disease, for which transplantation is a treatment, deterioration of vision is not in itself fatal, making safety considerations all the more important.

The future evolution of studies towards developing clinical applications will in part depend on the progress of lines of study summarised in this review. They also are likely to depend on a greater integration of studies involving gene therapy and growth factor delivery. To improve the efficacy of a transplanted cell or to ensure that the environment in the subretinal space is permissive of development, it may be desirable to introduce genes into cells prior to transplantation. Furthermore, *ex vivo* gene therapy may also be an effective way of introducing growth factors, necessary for photoreceptor survival into the subretinal space.

Once ideal conditions have been achieved for the transplant there still remains the problem of how the transplant interacts with the degenerating retina and visual system. In pursuing this question useful data can be acquired on a range of basic problems often not easily studied in other situations. For instance, what adaptation occurs centrally as a result of the progressive loss of a sensory signal, and, in turn, how are these adaptive changes affected by the graft? Another question relates to how cell identity is achieved in the

developing retina. It is clear that epigenetic factors play a central role in this, and in any attempt to ensure that transplanted immature or stem cells assume a suitable identity, they must be presented with the appropriate conditions. Such basic considerations as information flow and cell determination need to be understood and, in turn, this knowledge will contribute to optimising conditions for the reconstruction of the damaged retina.

None of these problems are insoluble, nor do they discredit the body of work performed over the past 15 years that has investigated the feasibility of the transplant approach. Indeed many of the issues raised here have emerged from that work.

Once the important obstacles are resolved and avoided there is no reason why transplants should not play an important part in the treatment of retinal disease.

Eventually, as the different retinal disease processes become more clearly understood it should be possible to devise drug therapies or even to reduce possible environmental triggers. However, until then transplantation remains a useful potential therapy. Indeed, once photoreceptors have been lost it is likely to be one of the only useful treatments, the other being microelectrode prostheses.

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