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Studies on Irish Families with Autosomal Dominant
Retinitis Pigmentosa, Congenital Stationary Night
Blindness and on Mouse Models of Inherited
Retinopathies

A Thesis Submitted to the University of Dublin
for the Degree of Doctor of Philosophy

by

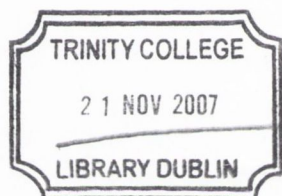
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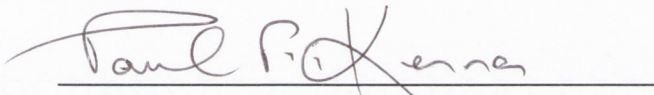
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Declaration

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A handwritten signature in dark ink, reading "Paul F. Kenna", is written over a horizontal line. The signature is stylized, with the first name "Paul" and last name "Kenna" clearly legible, and "F." as a small initial.

Paul F. Kenna M.B., D.O., F.R.C.S.I. (Ophth).

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This thesis is the fruit of more than 15 years of exciting research into the mysteries of Retinitis Pigmentosa at the Ocular Genetics Unit in Trinity College. I was invited to join the group in Trinity at a time when I was planning a full-time career in clinical ophthalmology. I consider it one of the most fortunate events in my life, as it took me on a journey that I had not planned and had not imagined possible.

One of the reasons for my becoming a medical doctor was to ‘make a difference’. I truly believe that the past 15 years spent in the Lab has enabled me to make that difference in a more significant way than had I remained in full-time clinical practice. When the day comes that treatments are available for patients with Retinitis Pigmentosa I know that I, and my colleagues, will have made that day possible by the work described in this thesis and that that endeavour will ‘have made a difference’.

I thank everyone who has helped me on this journey. In particular I thank my parents for their support and understanding. A special debt of gratitude is due to Mr. David Mooney F.R.C.S.I., retired consultant ophthalmologist at the Eye and Ear Hospital Dublin, for his support and encouragement of my decision to enter full-time research.

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Table of Contents

Chapter 1: Irish Autosomal Dominant Families Involved in Molecular Genetic Research

1.1	Introduction	Page 1
1.2	Material and Methods	Page 2
1.2.1	Visual Acuity and Colour Vision Assessment	Page 2
1.2.2	Visual Field Examination	Page 2
1.2.3	Dark Adaptation Assessment	Page 2
1.2.4	Two-colour Dark Adaptometry	Page 3
1.2.5	Electro-oculography	Page 3
1.2.6	Electroretinography	Page 4
1.3	Results	Page 7
1.3.1	Pedigrees Used in Molecular Genetic Analysis	Page 7
1.3.2	Pedigree TCD M1	Page 8
1.3.2.1	Night Vision Difficulties and Visual Acuity Loss	Page 8
1.3.2.2	Pattern of Visual Field Loss	Page 8
1.3.2.3	The Pattern of Electroretinographic Disturbance	Page 8
1.3.2.4	Dark Adapted Thresholds to Blue and Red Targets	Page 9
1.3.3	Pedigrees TCD B and D	Page 9
1.3.4	Pedigree TCD G	Page 10
1.3.4.1	Vision Difficulties and Visual Acuity Loss	Page 10
1.3.4.2	Pattern of Visual Field Loss	Page 10
1.3.4.3	The Pattern of Electroretinographic Disturbance	Page 10
1.3.4.4	Dark Adapted Thresholds to Blue and Red Targets	Page 11
1.3.4.5	Fundus Changes	Page 11
1.4	Discussion	Page 11
1.4.1	Clinical Heterogeneity in adRP	Page 12
	References	Page 16
	Figure 1.1	Page 18
	Figure 1.2	Page 19

Figure 1.3	Page 20
Figure 1.4	Page 21
Figure 1.5	Page 22
Figure 1.6	Page 23
Figure 1.7	Page 24
Figure 1.8	Page 25
Figure 1.9	Page 26
Figure 1.10	Page 27
Figure 1.11	Page 28
Figure 1.12	Page 29
Figure 1.13	Page 30
Figure 1.14	Page 31

Chapter 2: Linkage Study and Mutation Analysis in Family TCD M1

2.1	Linkage Studies on Pedigree TCD M1	Page 32
2.1.1	Chapter Introduction	Page 32
2.1.2	Introduction	Page 33
2.1.3	Materials and Methods	Page 34
2.1.3.1	Southern Analysis	Page 34
2.1.3.2	Data Analysis	Page 34
2.1.4	Results and Discussion	Page 35
Table 2.1		Page 36
Figure 2.1		Page 37
2.2	Identification of a Mutation in the Rhodopsin Gene in TCD M1	Page 38
2.2.1	Introduction	Page 38
2.2.2	Materials and Methods	Page 38
2.2.2.1	PCR/heteroduplex / Direct Sequencing	Page 38
2.2.2.2	Direct Sequencing	Page 39
2.2.2.3	Dot Blot Analysis	Page 39
2.2.3	Results	Page 39
2.2.4	Discussion	Page 40
References		Page 42
Table 2.2A		Page 47
Table 2.2B		Page 47
Figure 2.2		Page 48

Chapter 3: Genotypic and Phenotypic Analyses of Families TCD B and TCD D

3.1	Introduction	Page 49
3.2	Materials and Methods	Page 49
3.2.1	Molecular Genetic Analysis	Page 49
3.2.2	Ophthalmological Assessment of adRP Patients	Page 49
3.3	Results	Page 51
3.3.1	Molecular Genetic Analysis	Page 51
3.3.2	Clinical Characteristics of Affected Individuals	Page 51
3.4	Discussion	Page 53
	References	Page 56
	Figure 3.1	Page 60
	Figure 3.2	Page 61
	Figure 3.3	Page 62
	Figure 3.4	Page 63
	Figure 3.5	Page 64
	Figure 3.6	Page 65
	Figure 3.7	Page 66
	Figure 3.8	Page 67
	Figure 3.9	Page 68
	Table 3.1	Page 69
	Table 3.2	Page 70

Chapter 4: Genotypic and Phenotypic Analyses of a Family with Autosomal
Dominant Congenital Stationary Night Blindness

4.1	Introduction	Page 71
4.2	Materials and Methods	Page 71
4.2.1	Family Study	Page 71
4.2.2	Molecular Genetic Study	Page 72
4.3	Results	Page 73
4.3.1	Clinical Analysis	Page 73
4.3.2	Molecular Genetic Analysis	Page 74
4.4	Discussion	Page 74
	References	Page 80
	Figure 4.1	Page 84
	Figure 4.2	Page 85
	Figure 4.3	Page 86
	Figure 4.4	Page 87
	Figure 4.5	Page 88
	Figure 4.6	Page 89
	Figure 4.7	Page 90

Chapter 5: Rhodopsin, The Nature of Dominance and Possible Treatment
Modalities for Autosomal Dominant Retinopathies

5.1	The Retina	Page 91
	Figure 5.1	Page 91
	Figure 5.2	Page 92
5.2	Rhodopsin	Page 92
	Figure 5.3	Page 94
5.3	Rhodopsin Mutations	Page 94
	Figure 5.4	Page 97
5.4	Possible Modes of Action in adRP	Page 98
5.4.1	Loss of Function	Page 98
5.4.2	Gain of Function	Page 98
5.4.2.1	Aggregation and Inclusion Formation	Page 98
5.4.2.2	Post-Golgi Trafficking	Page 99
5.4.2.3	Endocytosis and Arrestin Binding	Page 100
5.4.2.4	Constitutive Activation	Page 100
5.4.2.5	The Unfolded Protein Response	Page 101
5.5	Dominant-Negative Mechanisms	Page 101
5.6	Possible therapies for adRP	Page 102
5.6.1	Anti-apoptotic Strategies	Page 103
5.6.2	Neurotrophic Factors	Page 104
5.6.3	Treating the Primary Genetic Defect	Page 105
5.6.3.1	Blocking Gene Expression	Page 105
5.6.3.2	Ribozymes	Page 106
5.6.3.3	RNAi	Page 107
5.7	Mutation Independent Strategies for the Treatment of adRP	Page 108
	Conclusion	Page 110
	References	Page 111

Chapter 6: Studies Utilizing the Rho^{-/-} Mouse

6.1.1	Introduction	Page 120
6.1.2	Electroretinography	Page 120
6.1.3	Protocol for Mouse Electroretinography	Page 121
	Figure 6.1	Page 123
	Figure 6.2	Page 124
6.2	Structural and Functional Rescue of Murine Rod Photoreceptors by Human Rhodopsin Transgene	Page 125
6.2.1	Introduction	Page 125
	Figure 6.3	Page 127
6.3	Apoptotic Photoreceptor Death in the Rhodopsin Knockout Mouse in the Presence and Absence of c-fos	Page 128
6.3.1	Introduction	Page 128
6.3.2	Materials and Methods	Page 130
6.3.2.1	Genotyping of Mice by PCR	Page 130
6.3.2.2	Retinal Histology	Page 130
6.3.2.3	Thin sectioning of mouse retinas	Page 131
6.3.2.4	<i>In Situ</i> End Labelling of Fragmented DNA	Page 131
6.3.2.5	DNA Fragmentation Analysis	Page 132
6.3.3	Results and Discussion	Page 132
	Figures 6.4A and 6.4B	Page 135
	Figure 6.5	Page 136
	Figure 6.6	Page 137
	Figure 6.7	Page 138
6.4	Comparative structural and functional analysis of photoreceptor neurons of Rho ^{-/-} mice reveal increased survival on C57BL/6J in comparison to 129Sv genetic background	Page 139
6.4.1	Introduction	Page 139
6.4.2	Materials and Methods	Page 140
6.4.2.1	Generation of Congenic Rho ^{-/-} Mice	Page 140
6.4.2.2	<i>In Situ</i> end-labelling of fragmented DNA	Page 140

6.4.2.3 Quantification of nuclei in retinal sections	Page 141
6.4.2.4 Electroretinographic Analyses	Page 141
6.4.3 Results	Page 142
6.4.4 Discussion	Page 145
References	Page 147
Figure 6.8	Page 153
Figure 6.9	Page 154
Figure 6.10	Page 155

Chapter 7: Studies on Transgenic Mouse Models of Inherited Retinopathies

7.1 Murine Model of Autosomal Dominant Retinitis Pigmentosa Generated by Targeted Deletion at codon 307 of the rds-Peripherin Gene	Page 156
7.1.1 Introduction	Page 156
7.1.2 Methods	Page 157
7.1.2.1 Histology	Page 157
7.1.2.2 Electroretinography	Page 157
7.1.3 Results	Page 158
7.1.3.1 Histopathology of retinas of rds-307 mice	Page 158
7.1.3.2 Electroretinographic analysis	Page 158
7.1.4 Discussion	Page 159
Table 7.1	Page 162
Figure 7.1	Page 164
Figure 7.2	Page 165
Figure 7.3	Page 166
Figure 7.4	Page 167
Figure 7.5	Page 168
Figure 7.6	Page 169
Figure 7.7	Page 170
Figure 7.8	Page 171
7.2 On the molecular pathology of neurodegeneration in IMPDH1-based Retinitis Pigmentosa	Page 172
7.2.1 Introduction	Page 172
7.2.2 Methods	Page 173
7.2.2.1 Mouse Electroretinography	Page 173
7.2.3 Results	Page 174
7.2.3.1 IMPDH and Hprt expression in the murine retina	Page 174
7.2.3.2 Retinal structure and function in IMPDH1 ^{-/-} mice	Page 174
7.2.3.2.1 Electroretinography	Page 174
7.2.3.2.2 Retinal Histology	Page 175

7.2.3.2.3 Functional Analysis and mammalian expression of wild-type and mutant IMPDH1 proteins	Page 176
7.2.3.2.4 Computer protein modelling studies on mutant IMPDH1	Page 177
7.2.4 Discussion	Page 178
References	Page 181
Figure 7.9	Page 188
Figure 7.10	Page 189
Figure 7.11	Page 190
Conclusion	Page 191

Summary

This thesis describes over 17 years of research into the molecular genetics of inherited retinopathies including Retinitis Pigmentosa (RP), the most prevalent form of inherited blindness world-wide, and Congenital Stationary Night Blindness. The work described encompasses research involving families segregating autosomal dominant forms of the conditions, as well as investigations involving a variety of animal models.

The largest human pedigree investigated, TCD MI, manifested a particularly early-onset form of autosomal dominant RP, with symptoms of night blindness and visual field loss evident before the age of 3 years. Family TCDM1 was the subject of extensive molecular genetic research to identify the causative gene, culminating in 1989 in the establishment of linkage to the Chromosome 3 polymorphic marker D3S47 (C17) with a LOD score of 14.7 at a recombination fraction of 0. This linkage, the first described in any form of autosomal dominant RP, pointed to the gene for the rod photoreceptor transduction protein, Rhodopsin, as a key candidate. In 1992 a Met207Arg mutation in this gene was identified in affected members of the TCDM1.

Two other families, TCD B and TCD D, likewise reported symptoms of night vision difficulties and peripheral vision impairment from the age of 10 years. A Tyr178Cys rhodopsin was identified in affected members of these families.

Congenital Stationary Night Blindness (CSNB) is a condition in which patients experience profound impairment of ability to see in low-light situations but retain essentially normal vision in full lighting. Clinical and molecular genetic investigations into a large kindred, segregating an autosomal dominant form of this condition, resulted in the discovery of a Thr94Ile rhodopsin mutation. Protein modeling and *in cellulo* expression studies indicated that this mutation probably acted by constitutive activation of Transducin by the mutant protein.

The dissection of gene function, of necessity, demands that investigations be carried out in the living organism. In this regard mouse models of human inherited retinopathies have proved invaluable.

The rhodopsin knockout animal (*Rho*^{-/-}) has been especially useful in providing insights into the biology of photoreceptor degeneration. I describe work in which a

normal human rhodopsin transgene rescued the pathology in this knockout animal. Ablation of the *c-fos* gene had been shown to protect against light-induced retinal toxicity by other investigators. Investigations which I carried out showed that lack of this gene was not protective in *Rho*^{-/-} mice. Analysis of the rate of retinal degeneration in *Rho*^{-/-} bred onto two different genetic backgrounds, C57 BL/6J and 129Sv, found that the C57Bl/6J background slowed the progression of the retinopathy to a significant extent. This suggested that a modifier gene or genes might exist which could attenuate the phenotype in C57Bl/6J animals.

Mice in which a single base is deleted at codon 307 of the peripherin/rds gene ($\Delta 307$) develop a progressive retinal degeneration. Histological and electrophysiological investigations showed that the retinopathy in mice heterozygous and homozygous for this mutation resulted in a more rapid degeneration than that seen in the naturally occurring null mutant, the rds-knockout animal. These data indicated that the $\Delta 307$ mutation most probably had a dominant-negative mode of action.

IMPDH1 gene mutations account for 2 – 5% of human autosomal dominant RP as well as some forms of Leber's Congenital Amaurosis. Histological and electrophysiological investigations of the homozygous IMPDH1 knockout mouse, described in this thesis, demonstrated that these animals developed a quite mild phenotype, suggesting that IMPDH1 mutations in human patients probably have a dominant-negative mode of action.

The data presented in this thesis, together with the work of other groups worldwide, has enormously expanded our knowledge of the pathophysiology of inherited retinopathies in humans and has brought forward the day when gene-based treatments will be available for conditions which, at present, are inexorably progressive and untreatable.

Chapter 1

Autosomal Dominant Retinitis Pigmentosa Families Utilised for Genetic Linkage Studies

1.1 Introduction:

A number of Irish pedigrees, segregating autosomal dominantly inherited forms of Retinitis Pigmentosa or Congenital Stationary Night Blindness, will be described in this chapter of the thesis. The family structure, together with a description of the clinical investigations carried out on members of each family will be presented.

Two important prerequisites must be present for the localization of a disease gene:

- i) An adequate number of informative markers covering the genome
- ii) Families segregating the disease in which to run these markers.

Initially the markers used in genetic linkage work consisted of such variations as the ABO, Mns and Rhesus blood groups, HLA polymorphisms, and a relatively small number of protein polymorphisms. These so-called classical markers covered a relatively small amount of the entire genome. Recently, DNA polymorphisms covering almost the entire genome have greatly expanded our ability to detect co-segregation of disease genes and marker loci. The information content of these DNA markers, restriction fragment length polymorphisms (RFLPs), variable number tandem repeat minisatellites (VNTRs) and poly-CA repeat microsatellites varies considerably.

The amount of information obtained in a linkage study depends not only on the particular markers used but also on the families in which the markers are run. The size and structure of the families governs their informativeness to a large extent. Not surprisingly, large families are better than small and multi-generation families are better than nuclear. The attraction in using one large multi-generation kindred is further enhanced by the fact that the problem of possible genetic heterogeneity is obviated. In a linkage study of autosomal dominant Retinitis Pigmentosa this point is one of considerable importance as genetic heterogeneity in this condition has been postulated for some time (Berson, *et al.*, 1969; Massof, Finkelstein 1981; Fishman, *et al.*, 1985; Lyness, *et al.*, 1985) and is now well established (<http://www.sph.uth.tmc.edu/Retnet/>).

Taking these considerations into account, at the start of this study a search was made for large, multi-generation families, using the pedigrees collected in the Research Department of the Eye and Ear Hospital, Dublin. Probandes were questioned in order to obtain as detailed a family tree as possible. Those kindreds

suggesting autosomal dominant transmission of the condition were chosen for detailed assessment. Nuclear units of these extended families were asked to attend the Research Department of the Eye and Ear Hospital, Dublin. Efforts were made to assess all individuals in a pedigree, both subjectively affected and normal, using examination modalities outlined below.

1.2 Materials and Methods:

1.2.1 Visual Acuity and Colour Vision Assessment:

Corrected visual acuity was tested using a standard Snellen test type at 6 metres in a well-illuminated room. Colour vision assessment was performed using the Farnsworth-Munsell 100 Hue test. Subjects were requested to arrange 85 coloured caps (4 boxes containing approximately 21 caps each) in order, such that each successive cap was closest in hue to the preceding one. This arranging was done in a cabinet which was illuminated by MacBeth lamps to achieve Northern Standard Daylight. Error scores were calculated for each subject and these were plotted on graph paper to discern if any colour deficit axis was present.

1.2.2 Visual Field Examination

Visual fields were assessed by Goldmann kinetic perimetry. The Goldmann bowl perimeter was standardized according to the manufacturers' (Haag Streit) instructions. Peripheral isopters were routinely measured with the IV4e and I4e targets. If there was any suggestion of the presence of scotomata these were carefully delineated by examination with targets of appropriate size and intensity.

1.2.3 Dark Adaptation Assessment:

A Goldmann-Weekers Dark Adaptometer was used to measure final dark-adapted rod thresholds. In each case the subject's left eye was assessed. Following dilation of the pupil with Tropicamide 1% and Phenylephrine 2.5% the subject was light adapted for 5 minutes by exposure to a light source of intensity 8 lux. The subject then dark-adapted for 30 minutes. The subject was asked to fixate a dim red target positioned so that the 11° test stimulus would fall on the retinal area 15° above fixation. This area was chosen to give a region with a good cone/rod mix. The

intensity of the stimulus was raised until the subject just became aware of it. The subject signaled this by saying “on”. The intensity was then reduced until the subject said “off”, indicating the moment when he/she could no longer appreciate the stimulus. The true absolute threshold was taken as mid-way between the “on” and “off” thresholds.

1.2.4 Two Colour Dark Adaptometry:

Selected individuals in all the kindreds examined were assessed using 2-colour dark-adaptometry. The protocol was based on that described by Sondheimer, *et al.*, (1979). The initial part of the procedure was as described above. Following 30 minutes dark adaptation, blue and orange targets, each of 2° were presented in a manner similar to that described for standard dark adaptation assessment. The blue target was obtained by using a Cokin™ 021 filter and the orange by using a Cokin™ 027 filter. Estimation of the blue and orange thresholds were made at 15°, 30° and 45° above, below, nasal and temporal to fixation. The diligence with which the patient maintained fixation on the dim red target was provided by assessment of the thresholds in the retinal area 15° nasal to fixation. At this retinal locus the stimulus would fall on the optic nerve head or blind spot. As a result, the thresholds in this area would be significantly higher compared to other loci.

1.2.5 Electro-oculography:

Electro-oculography was carried out according to a standard protocol (Marmor, Zrenner 1993). Active electrodes were placed close to each canthus and a ground electrode was placed on the forehead. Subjects were asked to alternate fixation between two red targets arranged so that they subtended a visual angle of 40°. Potentials were recorded after 10 minutes in the dark. Recordings were then made after 10 minutes in the light. The ratio of the dark trough to the light peak was calculated and converted into a percentage. This percentage is referred to as the Arden Index. In our laboratory an index of 160% or less was taken to be abnormal.

1.2.6 Electroretinography:

All individuals who attended the Research Department had electroretinographic assessment. This was the critical method in assigning disease status. The equipment and protocols will be described in some detail.

Initially all individuals were assessed using a protocol based on that of Geoffery Arden and co-workers (1983). Pupillary dilation was achieved by instilling Tropicamide 1% and Phenylephrine 2.5% drops into both eyes. Subjects were dark adapted for 30 minutes. The patient's forehead was cleansed using Omniprep™ solution (D. O. Weaver and Co., Aurora, Colorado, U. S. A.). Under dim red illumination (0.21 cd/m^2 at the position of the patient), ground and reference electrodes were pasted to the forehead with EC 2 electrode cream (Grass Instrument Co.). Gold foil electrodes were then inserted into the lower sulcus of each eye. The flash stimulus source (an OS 5 strobe) was sited behind an opalescent screen approximately 15 centimetres from the patient. Flash intensities were measured using a Gossen Mastersix photometer (Gossen GMBH, Erlangen, Germany).

Rod isolated responses were elicited by a blue flash of $2 \text{ candelas/metre}^2$ (cd/m^2) intensity. The subject was then exposed to flashes of increasing intensity. Mixed rod and cone responses were obtained by stimulation with a flash of maximal intensity. Isolated cone responses were elicited by a 30-Hertz stimulus of intensity 5 cd/m^2 .

It soon became apparent that this protocol had a number of potentials for variability. In particular it was very difficult to precisely control the distance the patient was from the light source. There was also no means of assuring that the patients' eyes were fully open or that the patient was fixating the centre of the diffusing screen. The recording of the 30Hz flicker responses proved difficult due to the natural tendency of the patient to close his/her eyes because of the intensity of the stimulus. The logistics of this arrangement demanded that all responses be elicited in the dark, as there were no facilities for assessing cone function under controlled light adapting conditions which suppressed rod activity. In addition, the amplitudes generated tended to be low in comparison to values reported elsewhere (Berson 1987). These problems presented difficulties in assessing borderline cases, particularly involving children in the presymptomatic age range.

In order to overcome these problems the protocol was altered in the light of the recommendations of the International Standardization Committee (Marmor 1989). The strongest recommendations of this committee were:

- i) The use of full field (Ganzfeld) dome stimulation
- ii) The use of corneal contact lens electrodes incorporating lid specula.

A Ganzfeld bowl was fashioned from a polycarbonate light fitment of 50 centimetres diameter. This bowl was altered so as to incorporate a 10 cm opening for the patient's head and another 10 cm opening to allow for placement of the flash stimulator, above and slightly in front of the patient. The outside of the globe was painted black and the interior was coated with anti-reflecting paint. A chin rest and forehead bar was incorporated in order to standardize the distance of the patient from the back of the bowl. Background illumination of the bowl was provided by a 15-Watt bulb powered by mains electricity and attenuated by a dimmer switch to achieve the desired out-put during light adaptation. A 6 Volt bulb powered by four 1.5V dry cell batteries in series was used to maintain background illumination of the bowl during recording of the cone responses. A variable resistor enabled the out-put to be set at the desired level. This arrangement was devised in order to eliminate mains interference during recording of these responses.

The electroretinogram (ERG) was recorded in the following manner using contact lens electrodes of the Henkes type. The subject was dark adapted for at least 30 minutes following dilation of the pupils with Tropicamide 1% and Phenylephrine 2.5%. At the end of this period of dark adaptation a further drop of Tropicamide was applied to each eye in addition to a drop of the corneal anaesthetic, Benoxinate 1%. Ground and reference electrodes were applied as described above. The concavity of the contact lens electrode was filled with 0.9% saline and the lens was inserted under dim red illumination. Electrical contact with the cornea was maintained by the film of saline. In order to minimize any artifacts, the patient was requested to try and relax as much as possible and, in particular, not to move the eyes or squeeze the eyelids. He/she was asked to gaze at the centre of the back of the bowl.

The maximum out-put of the flash unit using this arrangement was measured at 1.6 cd/m^2 , using the Gossen Mastersix photometer. This was taken as the "standard flash". The rod-isolated response was the first to be elicited, by a single

blue flash of intensity 3.3 Log units below that of the standard flash. Attenuation of the standard flash was achieved by reducing the stimulator out-put to $1/8^{\text{th}}$ of maximum (setting 16 to setting 2 on the OS 5) and interposing a number of neutral density filters and a single blue filter between the flash and the Ganzfeld bowl. Ideally the response to a single flash was recorded. In certain cases, averaging of at most 2 or 3 responses was done if, for some reason, the response to the single flash was unsatisfactory. If averaging was required, the interval between successive flashes was at least 15 seconds in order not to reduce rod activity by partial light adaptation. The second response to be measured was that to a white flash of intensity 3.55 Log units below standard flash intensity. The flash intensity was increased to 2.6 Log units, and 1.8 Log units below that of the standard flash for the next two responses. The maximal combined rod and cone response was elicited by the standard flash. Oscillatory potentials were measured in response to the standard flash after resetting of the high-pass filter to 200Hz.

A background light of intensity 22 cd/m^2 was then switched on in the interior of the Ganzfeld bowl and the patient light adapted for 10 minutes. At the end of this period the cone responses in the presence of the rod suppressing background illumination, were recorded.

Firstly, the responses to a single standard flash were recorded. The repetition rate was then set at 30Hz and cone flicker responses were measured. Intensity setting 8 i.e. $1/2$ the intensity of the standard flash, was used, as the OS 5 stimulator could only generate stimuli of 30Hz at this intensity setting. The first few responses were discarded. The subsequent waveforms were averaged.

In 2000 we purchased a RETIscanTM electrodiagnostic system (Roland Consult, Wiesbaden, Germany). This equipment also adheres to the ISCEV clinical standards and gave equivalent results for a set of normals as our previous equipment.

In accordance with standard practice in analysing electroretinographic responses the first negative waveform was designated the 'a' wave. The first positive waveform was designated the 'b' wave. The b wave amplitude was measured from the trough of the a wave to the peak of the b wave. The implicit times of the waveforms were measured from flash onset to the peak of the wave.

This protocol was standardized by analysis of the results obtained from at least 25 normal individuals (50 eyes). The mean for the various responses as well as the standard deviation were calculated. Abnormal responses were designated as those which fell outside 2 standard deviations from the mean.

1.3 Results: A Clinical Description of the Pedigrees Investigated

Fifteen Retinitis Pigmentosa kindreds, which satisfied criteria for autosomal dominant transmission, were ascertained. Conditions for characterization as probable autosomal dominant type included:

- i) Two or more generations affected,
- ii) Evidence of male-to-male transmission,
- iii) Females as severely affected as males
- iv) Roughly 50% of the offspring of an affected individual inheriting the condition.

1.3.1 Pedigrees Used in Molecular Genetic Analysis

Following the taking of family histories from probands, five kindreds were identified as showing promise for molecular genetic analysis. These pedigrees were designated TCD M1, TCD B, TCD D TCD G and TCD ZMK. It was readily apparent that the retinopathies segregating in these kindreds, while satisfying the criteria for classification as “Retinitis Pigmentosa”, differed in a number of aspects.

The fifth kindred, TCD ZMK, a family in which Retinitis Pigmentosa and a sensorineural form of deafness co-segregated and which was initially thought to show an autosomal dominant mode of inheritance, was the subject of extensive genetic linkage analysis (Kenna *et al.*, 1997) and was later found to have a C-->A transversion at position 12258 in MTTS2, the gene encoding the second mitochondrial serine tRNA (Mansergh *et al.*, 1999).

The results of studies on TCD M1, TCD B, TCD D and TCD G will be presented in this thesis.

1.3.2 Pedigree TCD M1:

1.3.2.1 Night Vision Difficulties and Visual Acuity Loss:

All affected individuals in the kindred TCD M1 (Fig. 1.1), without exception, reported difficulties in dealing with low-light situations for as long as they could remember. This very early onset of night blindness was corroborated by several mothers of affected individuals who spontaneously reported being able to predict which of their offspring would be affected, by observation of the difficulties which they encountered at night-time once they started to crawl. In addition, some of these mothers reported that their affected children also tended to ignore toys which were not directly in front of them, indicating that, even at the early age of 2 to 3 years, affected individuals were possibly suffering from peripheral visual field loss.

No affected individual in TCD M1 over the age of 30 years had corrected visual acuity equal to or better than 6/12. Posterior sub-capsular lens opacities were generally apparent in affected individuals by the third or fourth decades.

Fundus changes indicative of Retinitis Pigmentosa were evident even from a very early age (Figs. 1.2 and 1.3).

1.3.2.2 Pattern of Visual Field Loss:

Visual field examination using Goldman kinetic perimetry and IV4e and I4e targets showed early, concentric loss of peripheral visual field in TCD M1 affecteds (Fig. 1.4). No affected individual in this kindred over the age of 30 years had a field extending more than 15° from fixation.

1.3.2.3 The Pattern of Electroretinographic Disturbance:

Affected individuals as young as 6 years of age in the pedigree TCD M1 showed severely abnormal electroretinogram tracings. In none of the affected individuals was it possible to record a convincing rod-isolated response on electroretinography. The cone-isolated responses were better preserved in the earlier stages of the retinopathy. In those individuals from whom cone-dominated electroretinographic responses were recordable, the responses were typically reduced in amplitude as well as being very significantly delayed in timing. Older individuals

in this kindred showed unrecordable rod and cone responses (Fig. 1.5).

1.3.2.4 Dark Adapted Thresholds to Blue and Red Targets:

Owing to the early onset of field loss in affected individuals in TCD M1 this assessment could only be carried out on the younger members of this kindred. Figure 1.6 shows a typical profile from such an affected child. Cones mediated the stimuli for the whole duration of the assessment, as the threshold for the red stimulus is consistently lower than for the blue. In addition, this pattern is observed in all retinal loci tested.

1.3.3 Pedigrees TCD B and TCD D

These kindreds showed a form of retinitis pigmentosa which was very similar to that described for TCD M1. In both kindreds, affected individuals were affected at an early age, all noting night vision difficulties before the age of 10 years with symptoms referable to peripheral vision impairment being obvious by the early to mid-teens.

On 2-colour dark adaptometry those affected individuals tested showed a pattern similar to TCD M1. Similarly, no rod-isolated electroretinographic responses were ever recorded, even from the youngest affected individual tested, while the cone responses in general were delayed and reduced in amplitude. The phenotypes in these kindreds will be described in greater detail in chapter 3.

1.3.4 Pedigree TCD G

1.3.4.1 Night Vision Difficulties and Visual Acuity Loss:

Most affected individuals in the kindred TCD G (Fig. 1.7) did not report night vision difficulties until their early to middle thirties. Some individuals in their late thirties denied symptoms of night vision impairment, despite showing obvious clinical evidence of being affected.

Many sufferers in TCD G had a best-corrected visual acuity of 6/6 or better into the fifth decade. Individuals over the age of 60 years in TCD G tended to have lenticular opacities, particularly of the posterior sub-capsular variety. One 65 year old affected female in the pedigree TCD G who had reduction of visual acuity to Hand Movements at 1 metre due to a dense lens opacity achieved vision of 6/12 following extracapsular cataract extraction and insertion of a posterior chamber intra-ocular lens.

1.3.4.2 Pattern of Visual Field Loss:

The characteristic pattern of visual field loss in patients from TCD G was of initial loss of superior field (Fig. 1.8), followed by the development of mid-peripheral scotomata (Fig. 1.9). In time this progressed to concentric narrowing of the field with preservation of a temporal island of vision. Subsequent loss of this island resulted in the characteristic “tunnel” visual field. This end stage occurred in the 7th decade in this pedigree.

1.3.4.3 Pattern of Electroretinographic Disturbance:

In pedigree TCD G rod, and to a greater extent cone, responses were detectable in adults as old as 45 years. Initial reduction in amplitudes appeared in the rod-isolated responses. As the patients grow older their rod responses decreased further in amplitude and in addition their cone responses began to diminish (Fig. 1.10). By the 4th decade their rod responses were largely abolished and their cone responses were greatly reduced in amplitude and also prolonged in the time from flash onset to b wave maximum (Fig. 1.11). The only individual aged over 60 years in this pedigree who was tested had an extinguished ERG. She was the only

in this pedigree who was tested had an extinguished ERG. She was the only individual in this pedigree in whom no rod or cone responses could be elicited.

1.3.4.4 Dark Adapted Thresholds to Blue and Red Targets:

Affected individuals from TCD G, showed a markedly different pattern to target detection compared to TCD M1, TCD B and TCD D. As shown in Figure 1.12, detection of the blue stimulus was mediated in some retinal loci by rods and that of the orange target by the cones. Indeed the thresholds for detection approached normal values in some areas. In other retinal loci however, rod and cone thresholds were both elevated.

1.3.4.5 Fundus Changes:

Retinal changes suggestive of Retinitis Pigmentosa develop later in affected individuals in TCD G. The fundus appearance of a 20-year old affected male is shown in Figure 1.13. Apart from some mild thinning of the retinal pigment epithelium there is very little evidence of the retinopathy. The retinal changes are more florid in his 56-year old uncle who manifests all of the classical retinal signs of the condition (Fig. 1.14).

1.4 Discussion:

The term “Retinitis Pigmentosa”, although semantically incorrect, has been accepted for many years as referring to a wide range of clinically different retinopathies characterized by a number of common features. The presence of certain clinical features is widely accepted as justifying the diagnosis. These include:

- i) Bilateral involvement,
- ii) Loss of peripheral vision,
- iii) Evidence of rod photoreceptor dysfunction as revealed by an elevated rod final dark adapted threshold and/or electroretinographic rod responses which are reduced in amplitude and prolonged in implicit time or are non detectable,
- iv) Evidence of progressive loss of photoreceptor function.

Since the latter part of the 19th century it had been recognised that this

condition could be inherited in X-linked, autosomal recessive and autosomal dominant patterns. Heterogeneity in clinical presentation, even within the same genetic type, had been suspected for many years.

1.4.1 Clinical Heterogeneity in Autosomal Dominant Retinitis Pigmentosa

Attempts had been made to classify autosomal dominant Retinitis Pigmentosa using electrophysiological, psychophysical and clinical criteria. Berson, *et al.*, (1968) reported the results of a study of a fully penetrant autosomal dominant kindred. They separated the rod and cone components of the dark-adapted electroretinogram by the use of both ganzfeld stimulation and scotopically balanced lights, i.e. lights which produced equal subjective thresholds in the ganzfeld for normal subjects and which also produced equal rod electroretinographic responses near threshold in normals. In the pedigree studied they showed that rod function deteriorated before cone function over the entire retina. The defective rod function included both a reduction in amplitude and a delay in the b wave implicit time. In the early stages of the disease the cone electroretinogram responses were normal. Berson, *et al.*, (1969) applied similar techniques to the study of a kindred showing autosomal dominant transmission of the retinopathy but with reduced penetrance. In this pedigree the rod responses in the early stages of the disease again showed reduction in b wave amplitude and delay in implicit time. The cone responses however showed delay in implicit time even when the cone amplitudes were normal. The authors felt that this delay in cone implicit time distinguished dominant Retinitis Pigmentosa with reduced penetrance from the fully penetrant form, as, even in the later stages of the fully penetrant form when cone amplitudes were reduced, the cone implicit time was not markedly delayed.

Marmor (1979) also utilized electroretinographic assessment of rod and cone function in the study of patients with Retinitis Pigmentosa. He found that delayed cone b wave implicit time was the rule in the 70 patients, covering all three genetic categories, whom he studied, 58 of them showing delayed timing. Most dominant patients in his study showed cone b wave delay, although pedigree information did not allow confident assignment to fully or partially penetrant categories. His results suggested that patients with a normal implicit time had better visual acuity and a milder form of the condition, which he termed "delimited disease".

Differentiation of dominant Retinitis Pigmentosa patients on the basis of psychophysical criteria has been reported in several studies. Massof and Finkelstein (1981) divided this condition into two forms on the basis of two colour dark-adapted sensitivity profiles. Taking advantage of the difference between rod and cone spectral sensitivities they evaluated rod sensitivity relative to cone sensitivity. Following 45 minutes of dark adaptation, absolute thresholds were measured at the fovea and at 2.5°, 5°, 10°, 15° and from 20° to 80° in 10° steps along the nasal and temporal meridians using a Tubinger perimeter. Two stimuli, each subtending a visual angle of 2°, were used, a short wavelength stimulus of λ_{max} 500 nanometres (nm) and a long wavelength stimulus of λ_{max} 650nm. 25 patients from 13 families were assessed. At each retinal locus tested the absolute thresholds for the two targets were measured. The differences in threshold luminance between the two targets were plotted as a function of field eccentricity. A threshold difference of 2 log units between the two stimuli would indicate that rods mediated detection whereas no difference in threshold would indicate that the cones mediated detection. A threshold difference between 0 and 2 would indicate that the rods detected the 500nm target and the cones the 650nm target. On this basis the patients were divided into two categories.

Type 1 patients had initial diffuse loss of rod sensitivity followed by extensive cone sensitivity loss later in the disease. Type 2 patients had threshold elevations suggesting combined rod and cone sensitivity losses, which followed a regionalized pattern. In this category of dominant Retinitis Pigmentosa the initial losses in sensitivity appeared to occur in the mid-peripheral region. Later in the disease process the central 10° became involved and the losses in the mid-periphery increased. The far periphery appeared to retain some rod sensitivity, even in the oldest patients. Patients with the Type 1 pattern tended to report problems with night vision early in the course of the disease whereas patients in the Type 2 category had adult onset of nyctalopia. The authors compared the electroretinographic amplitudes obtained with full-field stimulation under dark and light adapted conditions in their patients. In the 8 patients from whom rod and cone responses could be elicited, they calculated the ratio of cone to rod amplitudes, each expressed as a percentage of the mean. The Type 1 patients had ratios ranging from 2 to 3 indicating that the rod amplitudes were more affected than the cone, as predicted by the psychophysical data. The four Type 2 patients on the other hand had ratios ranging from 0.8 to 1.7,

three of them with ratios close to 1, again in accordance with the results of the two colour dark-adapted profiles. No correlation was found between rod and cone b wave implicit times and the two sub-types.

Another attempt to classify dominant Retinitis Pigmentosa on the basis of two-colour perimetry was made by Lyness, *et al.*, (1985). They used a modified, computer-controlled Lister perimeter which functioned as an automated static perimeter/adaptometer (Ernst, *et al.*, 1983), to present two 0.9° targets, a green one of $\lambda_{\max}$ 530nm, the other a red target of $\lambda_{\max}$ 660nm, to the dark-adapted eye. Thirty to forty loci were tested in total along the 0° , 60° , 180° and 315° meridians in the right eye. Thresholds were expressed as the logarithm of the ratio of the patient's reading to the normal as defined by the mean obtained from a minimum of 15 normal observers. The difference between the threshold elevations of the green and red targets (the "G-R index") gave an indication as to pattern of photoreceptor dysfunction. An index near 0 indicated that cone function was disturbed to as much as or to a greater extent than rod function. When the index had a significant positive value, rod function was more affected than cone.

Analysing 104 patients from 44 autosomal dominant kindreds, they discerned two distinct patterns of photoreceptor dysfunction. Patients in whom all retinal zones tested showed elevation for the green target of at least 1.5 log units and in whom, in at least one zone, the red target elevation was less than 1 log unit while the G-R index was greater than 1.5, were classified as D type. If, in at least one zone, thresholds for both targets were less than one and, in at least one zone, the elevations for both were greater than 0.5 log units the patient was classified as R type. Thirteen patients from 4 families were placed into the D sub-group, while 28 patients from 13 families were put into the R sub-group. Most of those tested, 68 individuals from 27 families could not be classified. Two patients in the D sub-group who did not fit into the strict criteria for this category were classified on the basis of the prevailing pattern in other family members. Electroretinographic assessment of those patients who could be classified on the psychophysical criteria showed that no member of the D sub-group had evidence of rod generated amplitudes while most had evidence of some, albeit greatly reduced, cone amplitudes of greater than 20 microvolts (μV). The R type patients had rod and cone amplitudes greater than 20 μV .

On the basis of these classifications the TCD M1, TCD B and TCD D pedigrees would fall broadly into the Type 1/Type D categories whereas TCD G

would fall into the Type II/Type R categories.

As will become readily apparent when the results of molecular genetic analysis in these families are presented, these classification systems became somewhat redundant, although the results of two-colour dark-adaptometry retained some value in relation to possible genotype/phenotype correlation.

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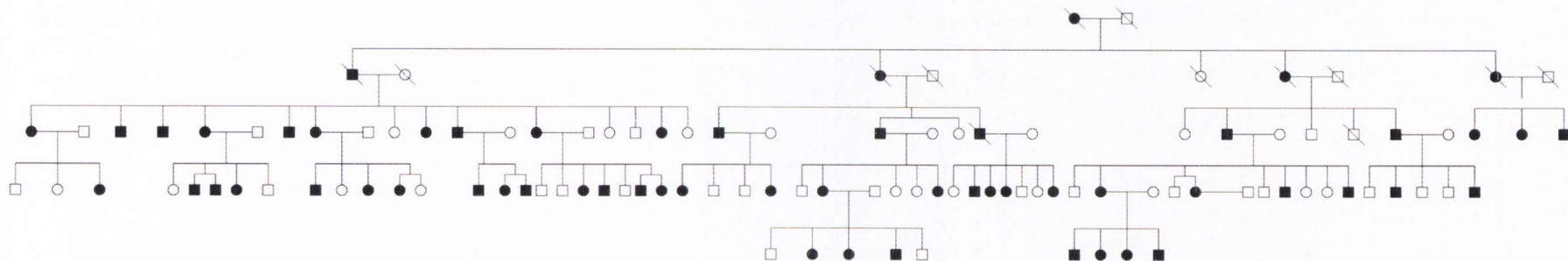


Figure 1.1: TCD M1, a large Irish family segregating Retinitis Pigmentosa in an autosomal dominant fashion. By convention, squares indicate male gender, circles indicate female gender, filled symbols indicate affected, open symbols indicate unaffected status. Symbols bisected by a diagonal line indicate deceased individuals.

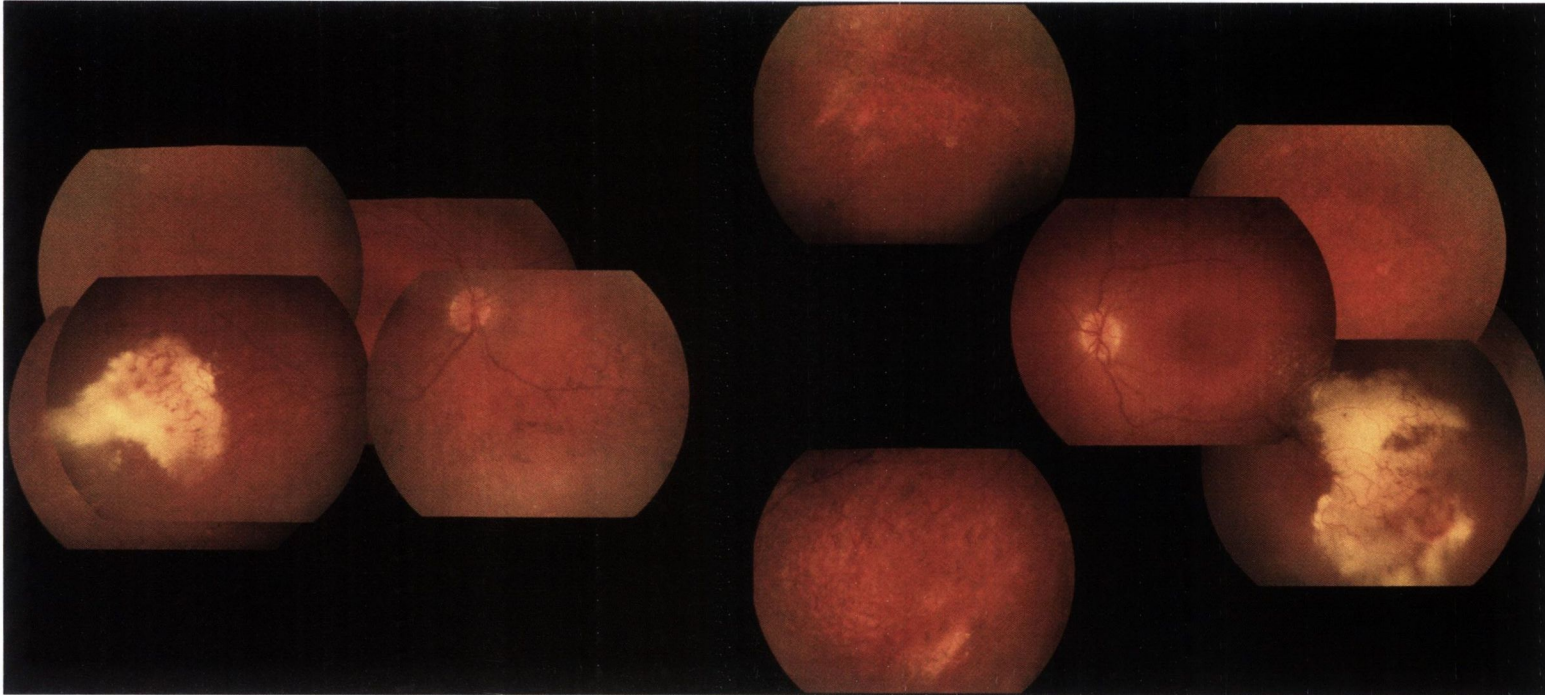


Figure 1.2: Collage of fundus photographs from a 12-year old affected individual in TCD M1. Note the disc pallor, vascular attenuation and peripheral pigmentary disturbance. This patient also shows bilateral Coats' reactions, an unusual finding in Retinitis Pigmentosa.

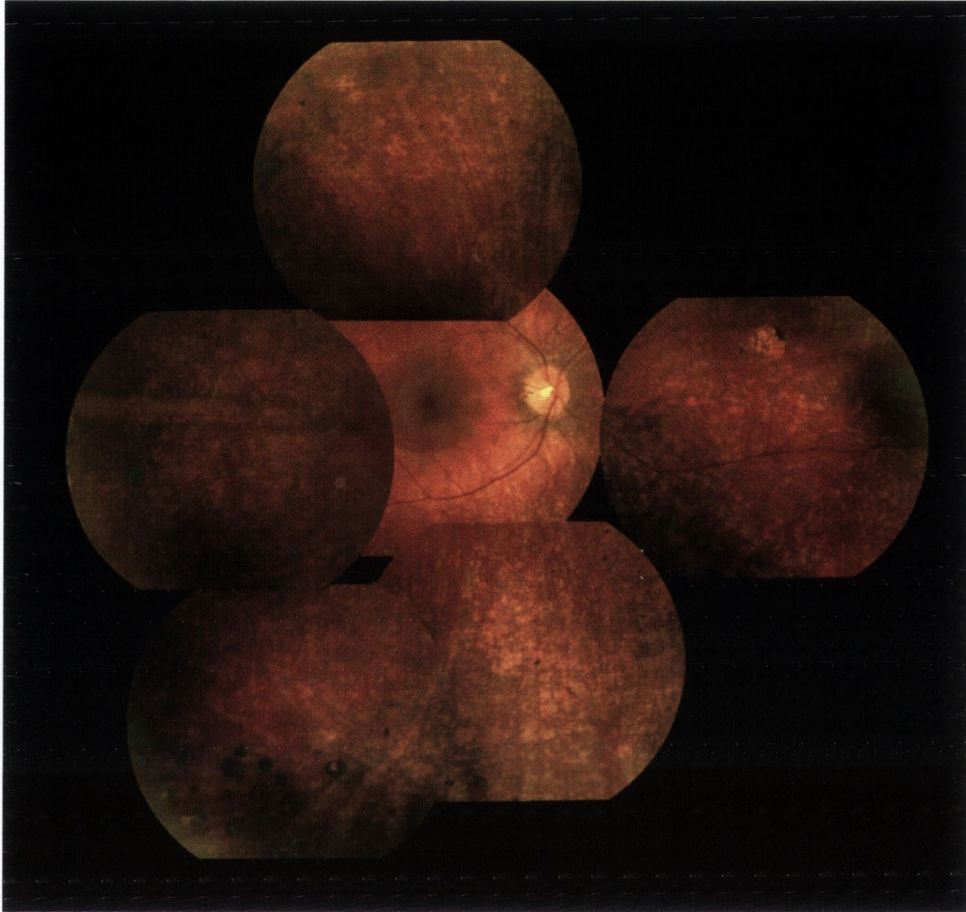


Figure 1.3: Collage of fundus photographs from the right eye of a 10-year old affected individual in TCD M1. Note the disc pallor, vascular attenuation, ‘moth-eaten’ appearance of the retinal mid-peripheries and extensive peripheral pigmentary disturbance.

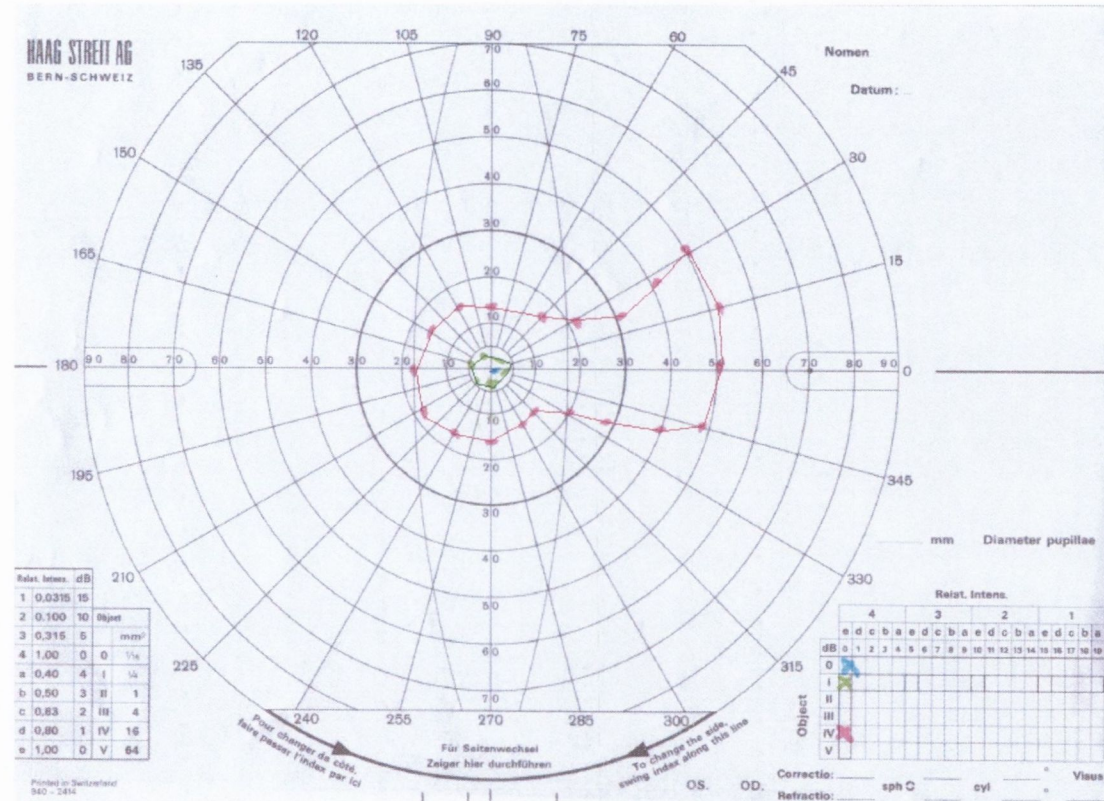


Figure 1.4: Goldmann perimetry of the left eye of a 10-year old affected individual from TCD M1. Noted the marked constriction, even to the large IV4e target.

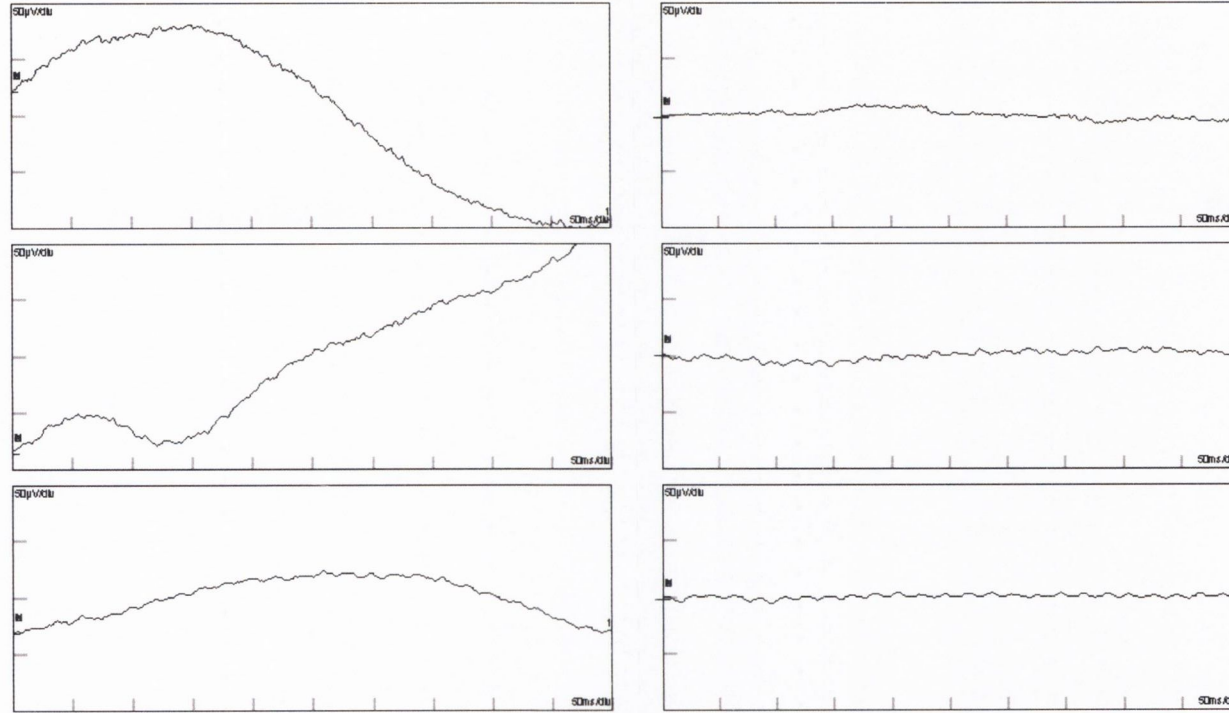


Figure 1.5: Electretinogram tracings from a 45-year old affected individual from TCD M1.

Top Panel: Rod-isolated responses; Middle Panel: Maximal dark-adapted, mixed rod and cone responses; Bottom Panel: Cone-isolated responses
No convincing responses were obtained to any of the flash stimuli, even to the brightest flash. The responses from the right eye are contaminated by blink artifacts.

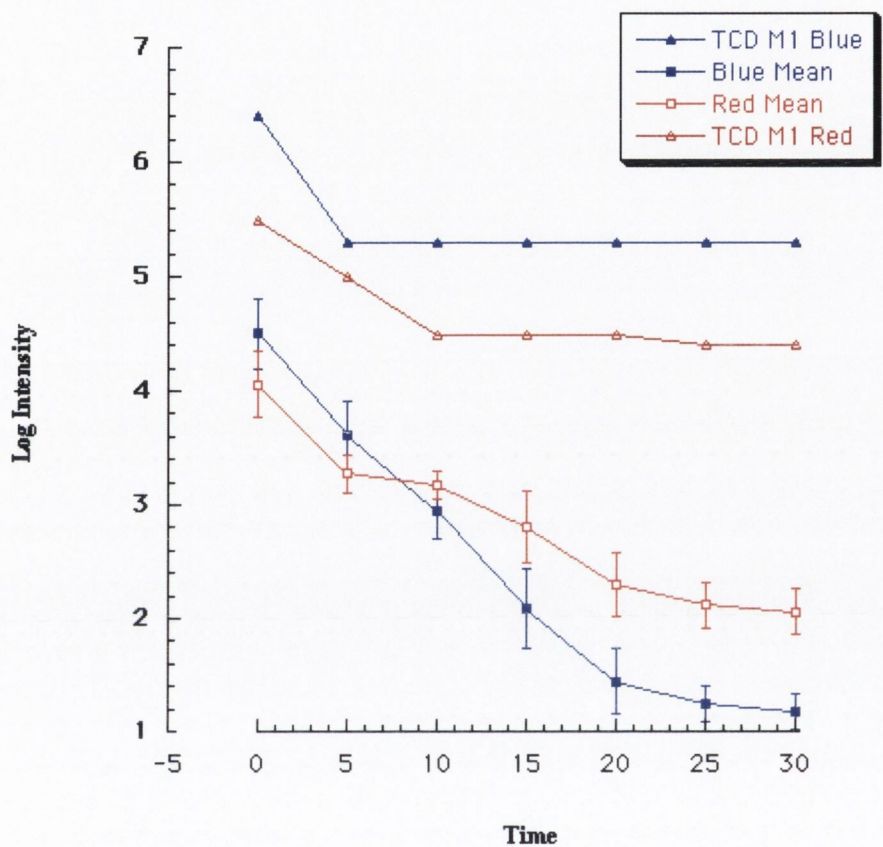


Figure 1.6: 2-colour dark-adaptometry profile of an affected individual from family TCD M1. Note that in the patient the blue and red targets are mediated by the cones, as the red threshold for detection is always lower than the blue. The reverse is seen in the thresholds for the normals (N= 18). Standard error bars are shown for the normals.

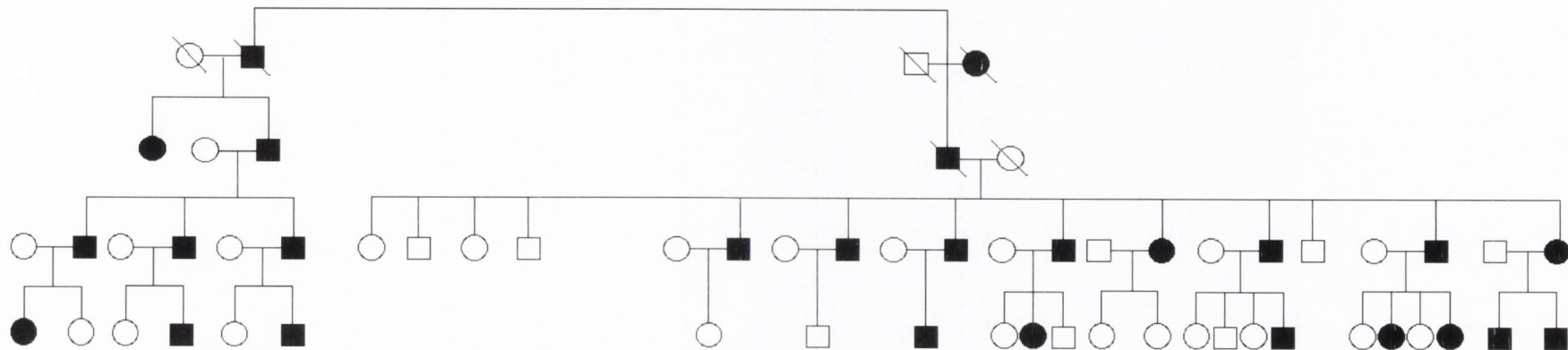


Figure 1.7: Family TCD G, a large Irish family segregating an autosomal dominant form of Retinitis Pigmentosa. This family was the subject of molecular genetic analysis and affected members were found to have an A to G substitution at codon 212, in exon 2 of the Peripherin/rds gene (Farrar *et al.*, 1992).

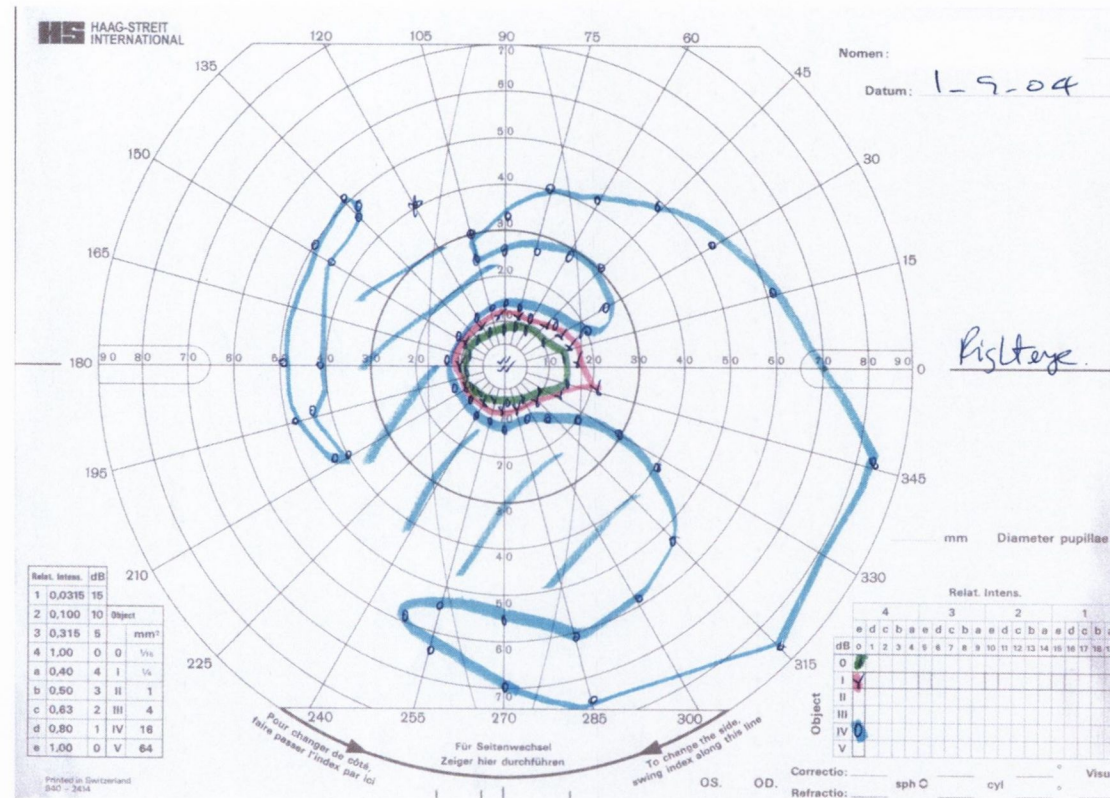


Figure 1.9: Goldmann perimetry from an affected individual from pedigree TCD G, aged 56 years. Note the mid-peripheral scotoma superiorly, nasally and inferiorly and the concentric constriction to the I4e and O4e targets.

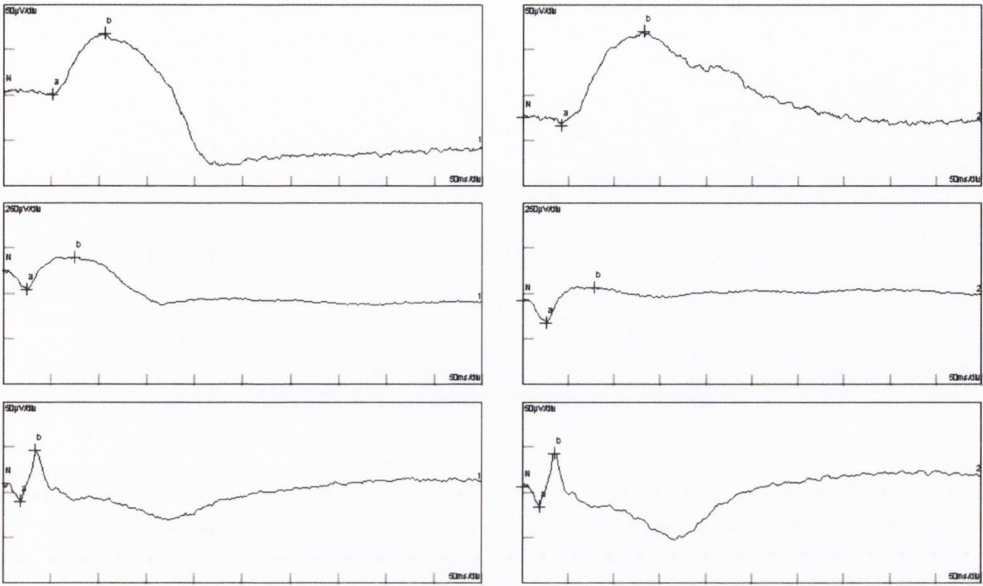


Figure 1.10: Electroretinogram tracings from a 20-year old affected individual from TCD G.

Top Panel: Rod-isolated responses; Middle Panel: Maximal dark-adapted, mixed rod and cone responses; Bottom Panel: Cone-isolated responses

The rod and cone responses are moderately reduced in amplitudes. The cone responses are slightly delayed in timing.

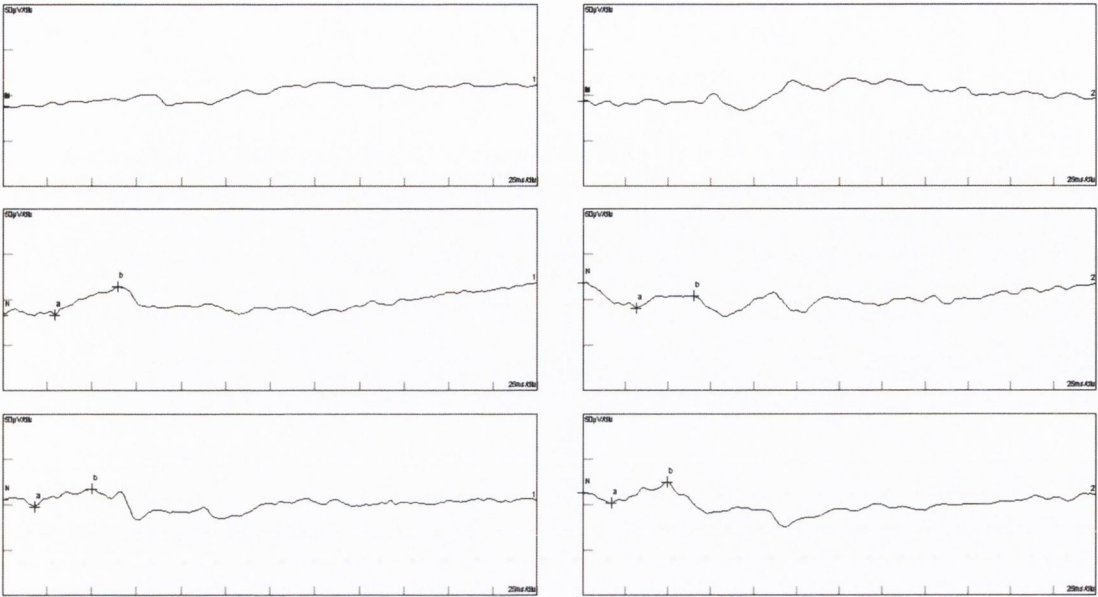


Figure 1.11: Electroretinogram tracings from a 56-year old affected individual from TCD G.

Top Panel: Rod-isolated responses; Middle Panel: Maximal dark-adapted, mixed rod and cone responses; Bottom Panel: Cone-isolated responses

The rod responses are non-recordable. The cone responses are still evident, although very significantly reduced in amplitude. The delay in the cone timing is more marked when compared to Figure 8.

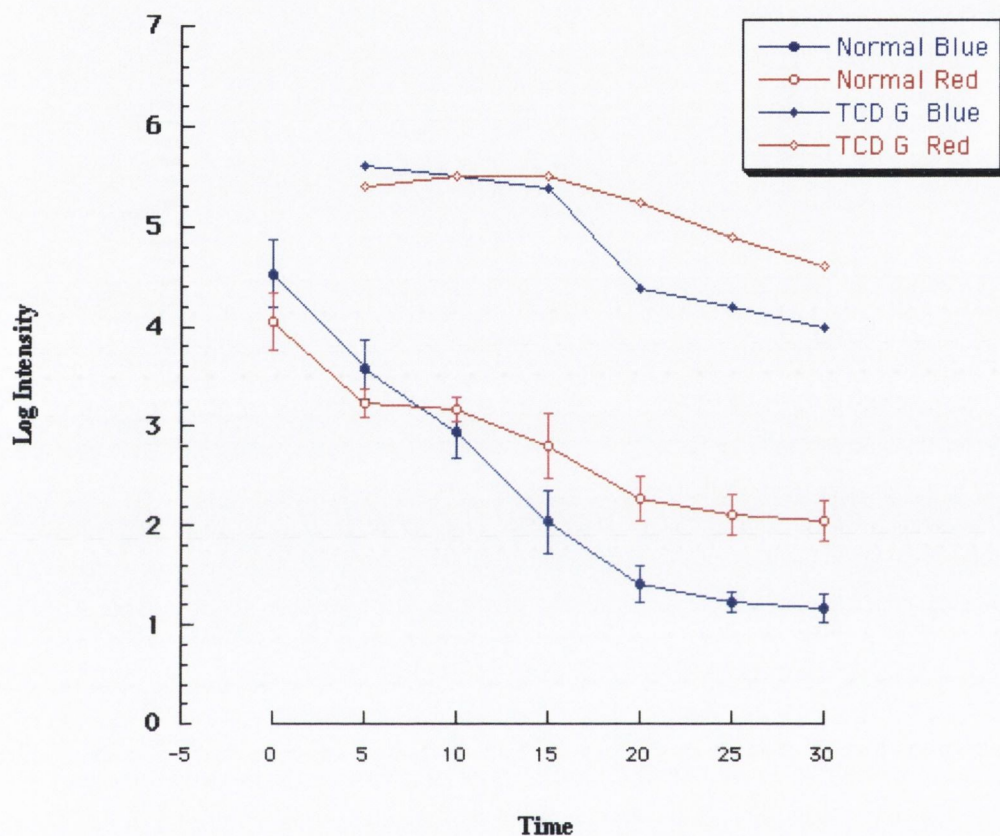


Figure 1.12: 2-colour dark-adaptometry profile of an affected individual from family TCD G. Note that in the patient as rod sensitivity increases the blue and red targets are mediated by the rods as the blue threshold for detection becomes lower than that for red, although the thresholds never reach normal values for either target. The same pattern is seen in the thresholds for the normals (N= 18). Standard error bars are shown for the normals.

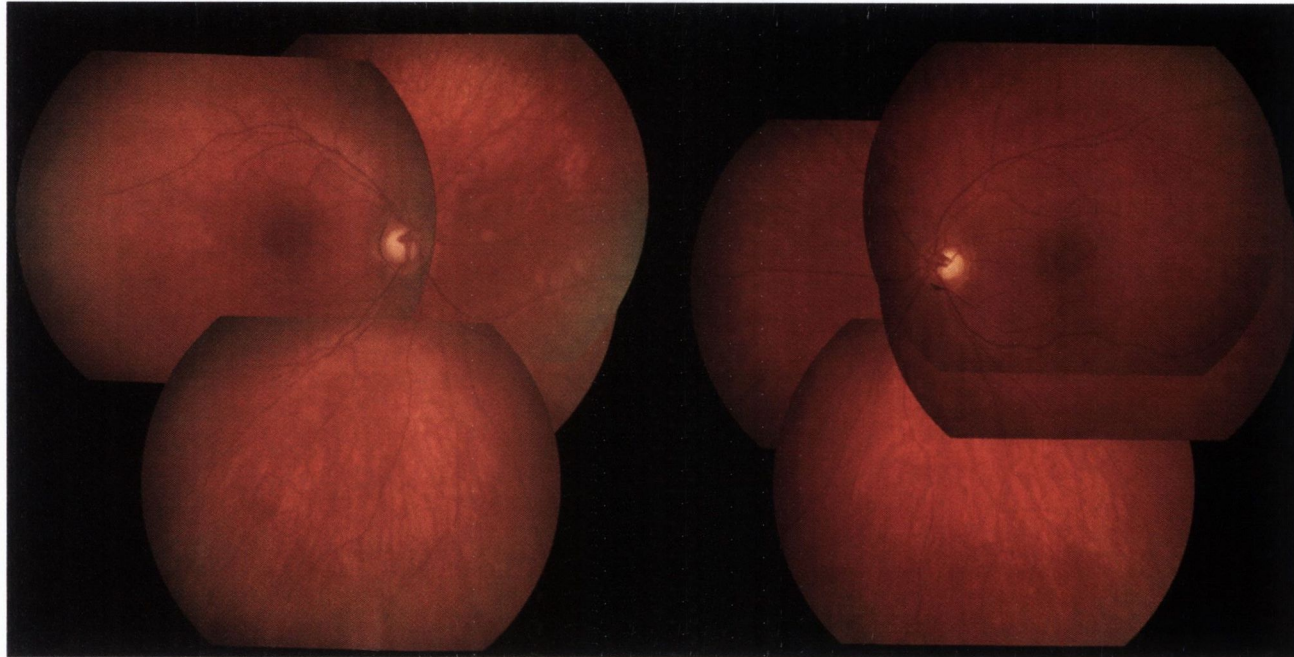


Figure 1.13: Mild fundus changes in a 20-year old affected individual from family TCD G.

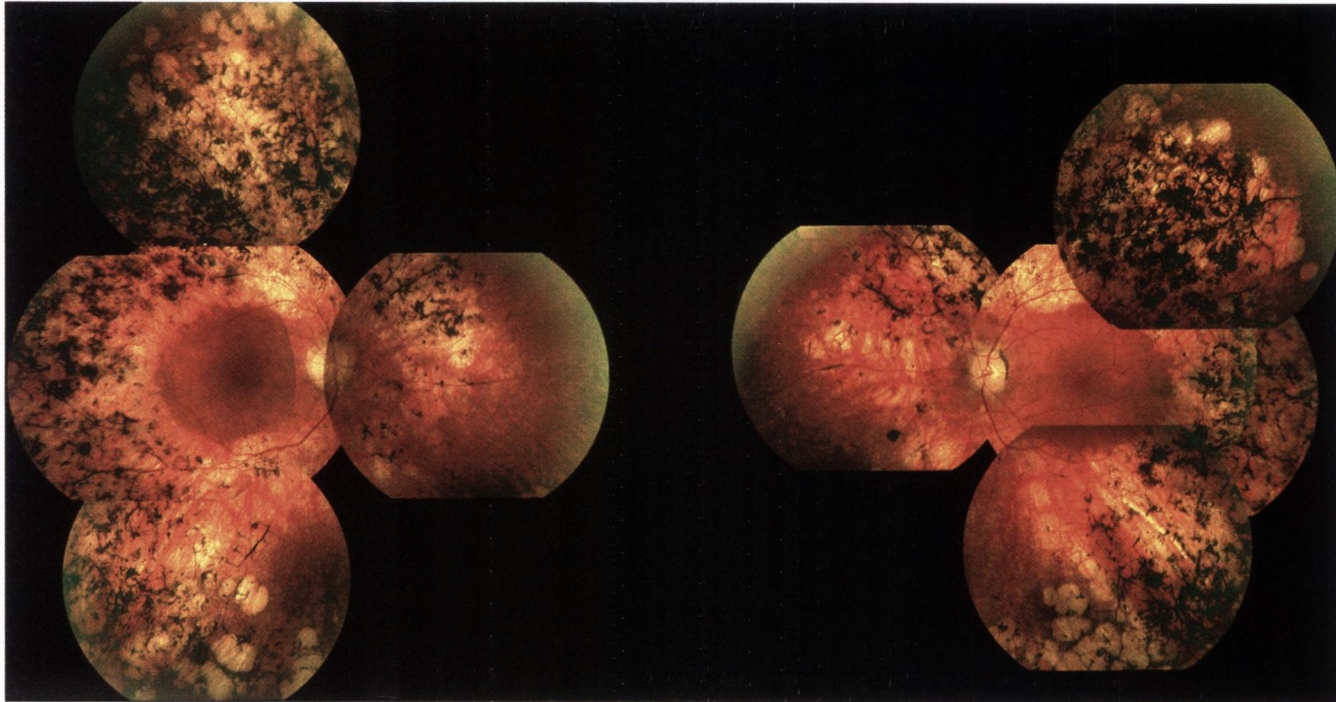


Figure 1.14: Florid retinal signs of Retinitis Pigmentosa found in a 56-year old affected male from TCD G. Both optic discs are pale, there is marked arteriolar attenuation, obvious thinning of the retinal pigment epithelium outside of the macular areas and heavy bone-spicule intra-retinal pigment deposits in all four quadrants.

Chapter 2

Linkage Study and Mutation Analysis in Family TCD M1

2.1 Linkage Studies on Pedigree TCD M1

2.1.1 Chapter Introduction

In this chapter the discovery of the first linkage of any form of autosomal dominant Retinitis Pigmentosa (RP), to the long arm of chromosome 3, and the subsequent discovery of the gene mutation responsible for the disease in the linked family (TCD M1) is described.

Prior to the chromosome 3 linkage report TCD M1 had been the subject of a number of linkage studies which had excluded a significant percentage of the genome. Bradley *et al.*, (1989) reported the results of linkage analysis in family TCD M1 using nine DNA markers on chromosome 1, including the rhesus blood group, which previous papers (Field *et al.*, 1982; Heckenlively *et al.*, 1982 and Daiger *et al.*, 1987) had suggested might be linked to adRP. Close linkage between the adRP locus and any of the marker loci was excluded using two-point analysis. With use of the sex-averaged maps and a strategy of rolling multipoint analyses, a 224-cM region of chromosome 1, including almost the entire short arm, was excluded from harbouring the gene segregating in TCD M1.

Daiger *et al.*, (1989) reported exclusion of an adRP gene from chromosome 4 in two families, including family TCD M1.

A third paper on family TCD M1 (Farrar *et al.*, 1989) reported exclusion of the gene in the family from chromosomes 6, 13, 20, and 21, excluding approximately 500 cM, or 15% of the genome.

Within a year of my having joined the group, by great good fortune, I ran the polymorphic marker D3S47 (also known as C17) in family TCD M1. This resulted in a maximum LOD score of 14.7, the highest LOD score ever reported in a single family. This linkage was reported in 1989 (McWilliam P, Farrar GJ, Kenna P, Bradley DG, Humphries MM, Sharp EM, McConnell DJ, Lawler M, Sheils D, Ryan C, Stevens K, Daiger S and Humphries P. Autosomal Dominant Retinitis Pigmentosa (adRP): Localization of an adRP Gene to the Long Arm of Chromosome 3 Genomics. 1989 Oct;5(3):619-622). This paper is reproduced in sections 2.1.2 to 2.1.4 of this chapter.

2.1.2 Introduction

Retinitis pigmentosa (RP) describes a group of clinically and genetically heterogeneous inherited retinopathies, primarily involving photoreceptor degeneration. Clinical features typically include reduced night and peripheral vision, retinal pigment epithelium degeneration, retinal vessel attenuation and optic disc pallor (Heckenlively, 1988). The prevalence of the disorder is approximately 1 in 4000; indicating that RP is a major cause of blindness in human populations (Bundey and Crews 1984; Bunker *et al.*, 1984; Grondahl 1986). RP can be genetically subclassified on the basis of the mode of inheritance. Preliminary studies in Ireland have shown that the distribution of RP pedigrees is 15% autosomal dominant, 17% X-linked, 30% autosomal recessive and 38% isolated or sporadic cases (Farrar, unpublished). Because subclassification of RP pedigrees is often difficult (Rothberg *et al.*, 1982; Beatty and Boughman, 1986), the development of closely linked markers would enable more accurate categorization of this etiologically heterogeneous group of disorders.

Autosomal dominant retinitis pigmentosa has been clinically subclassified into two groups on the basis of psychophysical testing and age of onset (Massof and Finkelstein, 1981; Farber *et al.*, 1985; Lyness *et al.*, 1985). Type I adRP shows a diffuse loss of rod sensitivity, later loss of cone sensitivity and early onset of night blindness in childhood; while Type II adRP shows a regional loss of both rod and cone sensitivity with a later onset of night blindness in adulthood. The clinical heterogeneity observed in adRP may be paralleled by genetic heterogeneity; therefore the present linkage study has been based on a single large pedigree (TCD M1), exhibiting early onset Type I adRP.

We have previously reported that the adRP gene segregating in TCD M1 has been excluded from approximately 50% of the genome (Bradley *et al.*, 1989; Humphries *et al.*, 1989 and Daiger *et al.*, 1989), including the region around the rhesus locus on 1p, where loose linkage to an adRP gene had been reported (Field *et al.*, 1982; Heckenlively *et al.*, 1982; Daiger *et al.*, 1987; Yijian *et al.*, 1987). We now present data strongly indicating a location for this adRP locus on 3q.

2.1.3 Materials and Methods

The structure of TCD M1 is given in Fig.2.1. The early onset, rod-cone retinopathy in TCD M1 is suggestive of Type I adRP. All affected members exhibited severely reduced or extinguished electroretinograms (ERGs) with greater involvement of the scotopic mechanism, indicating a rod-cone degeneration. Similarly, electro-oculograms (EOGs) of affected individuals resulted in Arden indexes of approximately 100%, again suggestive of a rod-cone degeneration. In addition, affected individuals exhibited fundoscopic disturbances typical of adRP, including pigmentary deposits concentrating in the mid-peripheral retina, thinning of the retinal pigment epithelium, attenuation of retinal vessels, and pallor of the optic disc. Early onset of the disease has been demonstrated by the finding of classical retinal pigmentary changes in fundi of patients of less than 10 years of age. The calculated segregation ratio (0.58) for adRP in TCD M1 did not differ significantly from that expected for an autosomal dominant gene (0.5), suggesting full penetrance of the adRP gene in TCD M1. Notably, the early onset of the disease and full penetrance of the adRP gene allowed confident assignment of the normal phenotype to at-risk individuals shown to be unaffected.

2.1.3.1 Southern Analysis

DNA extracted from peripheral blood was digested with Msp1, size fractionated by agarose gel electrophoresis, and then blotted onto Zetabond filters as previously described (Humphries *et al.*, 1987). Filters were hybridized to the polymorphic probe D3S47 (C17) in the presence of purified, sonicated human DNA to block repeat sequences. The probe was labeled by random priming (Feinberg and Vogelstein, 1983, 1984).

2.1.3.2 Data Analysis

Computations were performed using the data management package LINKSYS (Attwood and Bryant, 1984) in conjunction with the program LIPED (Ott, 1974).

2.1.4 Results and Discussion

On the basis of pairwise analysis, the adRP gene segregating in TCD M1 has been closely linked to the polymorphic marker D3S47 (C17), with a maximum lod score of 14.7 at 0.00 recombination and a 1-lod confidence interval of approximately 0-4.5 cM (Table 2.1). The haplotyped pedigree given in Fig 2.1 indicates that 36 individuals were informative with respect to adRP and D3S47; no recombinants were observed. D3S47 has previously been localized to the long arm of chromosome 3 (Donis-Keller *et al.*, 1987). The significant, tight linkage of adRP to D3S47 enables the confident assignment of an adRP locus (RP1) to 3q.

In the undertaking of systematic linkage studies with a clinically heterogeneous disorder such as adRP, one of the primary concerns must be the possibility of genetic heterogeneity. However, this issue has been avoided in the present study by using only TCD M1, a single large, well-characterized adRP pedigree of Irish origin.

Previous linkage studies have tentatively located an adRP gene (RP1) close to the rhesus (RH) locus on chromosome 1 (Field *et al.*, 1982; Heckenlively *et al.*, 1982; Daiger *et al.*, 1987; Yijian *et al.*, 1987). The initial exclusion of the adRP gene in TCD M1 from the region around RH (Bradley *et al.*, 1989) and now the assignment of an adRP gene to chromosome 3 highlight the possibility of genetic heterogeneity in adRP. The extension of linkage studies to further adRP pedigrees will resolve the issue of genetic heterogeneity in adRP and will provide a means with which to investigate whether clinical heterogeneity in adRP is paralleled by genetic heterogeneity.

Both retinol-binding proteins 1 and 2 have been assigned to chromosome 3q (Colantuoni *et al.*, 1986; Demmer *et al.*, 1987; Nilsson *et al.*, 1989). Retinol binding proteins are involved in the transportation of retinol (lipid alcohol vitamin A) to vitamin A-dependent tissues, including the retinal pigment epithelium (Heckenlively 1988). Similarly, rhodopsin, the major integral protein of the rod outer segments, has been assigned to 3q21-3q24 (Nathans *et al.*, 1986). Moreover, abnormal levels of rhodopsin and retinol-binding proteins have been observed in RP patients (Rahi 1972; Kemp *et al.*, 1983). It is therefore interesting to speculate that rhodopsin and/or retinol-binding protein 1 or 2 may be potential candidate genes for adRP or that possibly there is a cluster of genes with retinal functions on 3q. In this regard, the extension of linkages studies to the other autosomal forms of RP, both primary

autosomal recessive retinitis pigmentosa (arRP) and arRP incorporated into complex syndromes will be of great interest.

The group of diseases that fall into the category of RP are extremely heterogeneous both clinically and genetically. The localization of the first autosomal RP gene now provides a means with which to investigate the etiologies of this group of retinopathies, and thus will enable more accurate methods of subclassification.

Table 2.1
Pairwise LOD Score between adRP and the Chromosome 3 Marker D3S47

	$\Theta \ (\Theta_m = \Theta_r)$										
	0.00	0.001	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45
D3S47	14.7	14.67	13.54	12.32	11.03	9.38	8.24	6.72	5.10	3.38	1.57

Following our report of linkage in TCD M1 to the long arm chromosome 3 and the identification of the gene for rhodopsin as a strong candidate in our family, Dryja *et al.*, (1990) reported the first gene mutation in any form of RP, a proline to histidine (P23H) substitution resulting from a cytosine-to-adenine transversion at codon 23 of the rhodopsin gene. Farrar *et al.*, (1990) reported that this particular mutation was not found in patients with adRP in Europe. Haplotype analysis showed that, most probably, the presence of the P23H mutation at quite high frequency in North American adRP patients was the result of a founder effect.

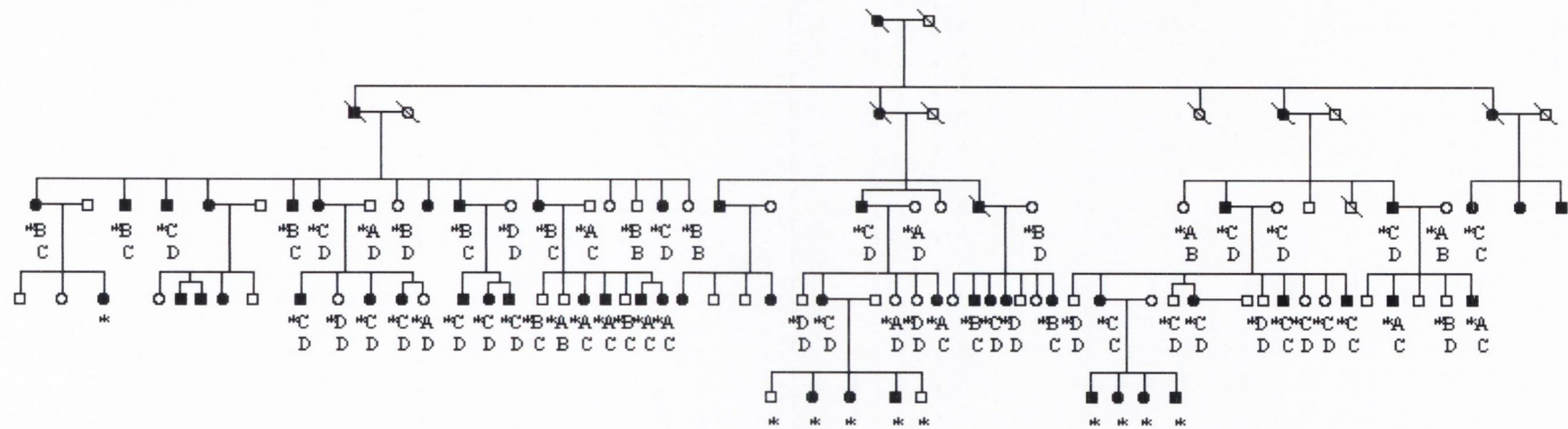


Figure 2.1: TCD M1, a large Irish pedigree segregating autosomal dominant Retinitis Pigmentosa, has been typed with the DNA marker D3S47, which has two independent polymorphic systems, A1 (14.0 kb) and B1 (3.5 kb). Haplotypes for individuals are indicated, where A = A1B1 (0.0638), B = A1B2 (0.2262), C = A2B1 (0.1562), and D = A2B2 (0.5538). The asterisks indicate individuals from whom DNA samples have been obtained.

2.2 Identification of a Mutation in the Rhodopsin gene in TCD M1

2.2.1 Introduction

Using the techniques of heteroduplex detection and direct genomic sequencing, we identified a novel mutation in the rhodopsin gene in Family TCD M1 used to localise an adRP gene to the long arm of chromosome 3. This mutation at codon 207 of the rhodopsin gene results in the replacement of a conserved, neutrally charged, methionine residue with a positively charged arginine residue in the fifth transmembrane domain of the protein. This mutation was reported by us in 1992 (Farrar GJ, Findlay JB, Kumar-Singh R, Kenna P, Humphries MM, Sharpe E, Humphries P. Autosomal dominant retinitis pigmentosa: a novel mutation in the rhodopsin gene in the original 3q linked family. *Hum Mol Genet.* 1992 Dec;1(9):769-71).

2.2.2 Materials and Methods

2.2.2.1 PCR /heteroduplex / Direct Sequencing

The five exons of the rhodopsin gene were PCR-amplified incorporating α^{32} dCTP, using the protocol described elsewhere (Farrar *et al.*, 1991). Amplified products were run on 5% polyacrylamide with 10% urea and 1% glycerol slowly overnight (3 Watts) at room temperature to assess for the presence of heteroduplex DNA (White *et al.*, 1992). Samples were also run on 5% polyacrylamide gels without 10% urea.

2.2.2.2 Direct Sequencing

Amplified DNA fragments were chloroform extracted, treated with Proteinase K and purified using the Promega 'Magic PCR Pres' kit. 250ng of purified DNA was sequenced using 1pmol of sequencing primer end labeled with γ -dATP. Sequencing primers were either the original primers used for amplification or in some instances nested primers within the amplified products.

2.2.2.3 Dot Blot Analysis

γ -dATP end-labeled primers covering the site of the mutation at codon 207 were hybridised to amplified DNA products immobilised on nylon filters (Hybond-N). Membranes were hybridised to the normal and mutant allele specific oligonucleotides (ASOs) and then washed in 2xSSC at 58°C and 62°C respectively.

The ASO sequences were as follows

Normal: 5'TGTCATCTACATGTTTCGTGG3'

Mutant: 5'TGTCATCTACAGGTTTCGTGG3'

2.2.3 Results

The five exons of the rhodopsin gene were PCR-amplified from both affected and unaffected members of the TCD-M1 family. The presence / absence of a potential disease causing mutation was assessed using a number of new techniques for identification of sequence variation, such as single strand conformation polymorphism electrophoresis and heteroduplex analysis (White *et al.*, 1992). In the latter, amplified products are run on non-denaturing polyacrylamide gels to assess for the presence of heteroduplex DNA. Typically, heteroduplex DNA runs more slowly than homoduplex DNA, hence using this method one can identify potential sequence differences between two rhodopsin alleles.

Using this technique, we observed that heteroduplex DNA was present in

affected individuals and absent in unaffected individuals from TCDM1, in the DNA amplified from exon 3 of the rhodopsin gene. After direct genomic sequencing, we established that this sequence alteration was the result of a T --> G base change at codon 207, which results in the substitution of a methionine residue for an arginine residue. Using an ASO based assay, we observed that the disease phenotype and the mutation at codon 207 were co-inherited (Fig 2.2), giving a maximum LOD score of 13.5 at zero recombination between adRP and the mutant rhodopsin gene (Table 2.2A). We tested 100 normal unrelated individuals for the presence of this mutation and found that it was absent in all these controls, thereby greatly reducing the possibility that this is simply a neutral polymorphism present in the normal population.

2.2.4 Discussion

In this study, using the techniques of heteroduplex detection and direct genomic sequencing, we identified a novel mutation at codon 207 of the rhodopsin gene which co-segregates with the disease phenotype in the large Irish family originally used to place the first adRP gene on 3q (McWilliam *et al.*, 1989, Farrar *et al.*, 1990).

Interestingly, the methionine residue at codon 207 is conserved in all animal species sequenced to date (Table 2.2B), suggesting that the methionine at this position may play an important role in the structure and / or functional relationship of the protein. Rhodopsin is a transmembrane glycoprotein that is located in the outer segments of the rod photoreceptor discs. The methionine residue at codon 207 is found tucked into the fifth transmembrane domain of the protein. The introduction of a positively charged residue into this neutrally charged transmembrane domain is likely to destabilise this domain to the extent that it may prevent the rhodopsin protein from folding correctly into the membrane. This may have catastrophic effects on the functioning of the protein and on the integrity of the outer segment

membrane, thereby leading to photoreceptor cell death.

The mutations observed within the rhodopsin gene and implicated in human inherited retinopathies have an extremely broad clinical spectrum. This is clearly demonstrated by recent findings suggesting that rhodopsin mutations may be responsible for three clinically separate disease entities: adRP, arRP and congenital stationary night blindness (CSNB). In excess of 100 different rhodopsin mutations to date have been identified in adRP families (<http://www.sph.uth.tmc.edu/Retnet/>). In this category alone, there is a large variation in age of onset, presentation, progression and clinical severity of the retinopathy. For example patients with the Methionine --> Arginine substitution at codon 207, show particularly early onset of the disease. Mothers of affected patients accurately predicted which infants would be affected by observing difficulties negotiating obstacles while crawling in low light situations. Although the onset of night blindness is congenital, affected children have normal or near normal central visual acuity and none has developed nystagmus, thus differentiating the retinopathy in this kindred from Leber's Congenital Amaurosis.

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Table 2.2 A. Two point LOD scores between the disease gene and the rhodopsin codon 207 mutation segregating in TCDM1,

Θ	0.4	0.3	0.2	0.1	0.05	<u>0.00</u>
2 point LOD scores	3.54	7.72	11.42	14.71	16.23	<u>17.38</u>

Table 2.2 B. Alignment of residues 202-228 in 20 visual pigment sequences. This region of the protein is likely to contain the 5th transmembrane segment.

SFVIF	M	FVVHFIPLIVIFFCYGOLVF	Bovine
SFVIF	M	FVVHFSIPLIVFFCYGQLVF	Ovine
SFVIF	M	FVVHFTIPMIVIFFCYGQLVF	Mouse
SFVIF	M	FVVHFTIPMIIIIFFCYGOLVF	Human
SFVIF	M	FVVHFLVPFVIIFFCYGRLLC	Lamprey
SYMIV	L	MVTCCIIPLAIIXLCYLQVWL	Human Red
SYMVV	L	MVTCCFFPLAIIICYLQVWL	Chicken Red
SYNIV	L	MITCCFIPLGIIILCYIAVWW	Fish Red
SYMVT	L	LLTCCILPLSVIIICYIFVWN	Fish Green 1
SYXIT	L	MLTCCILPLSIIIIICYIFVWS	Fish Green 2
SYTWF	L	FIFCFIVPLSLICFSYTQLLR	Human Blue
SNILC	M	ZIFAFMCPIVVIFFCYFNIVM	Squid
SYLIF	Y	SIFVYYIPLFLICYSYWFIIA	Drosophila-Rh1
SYLIF	Y	SIFVYYLPLFLIVYSYWFIIA	Calliphora-Rh1
SYLIT	Y	SLFVYYTPLFLICYSYWFIIA	Drosophila-Rh2
LFVAC	I	FFFSFVCPTTMITYYYSQIVG	Drosophila-Rh3
LFVGT	I	FFFSFVCPTLMILYYYSQIVG	Drosophila-Rh4

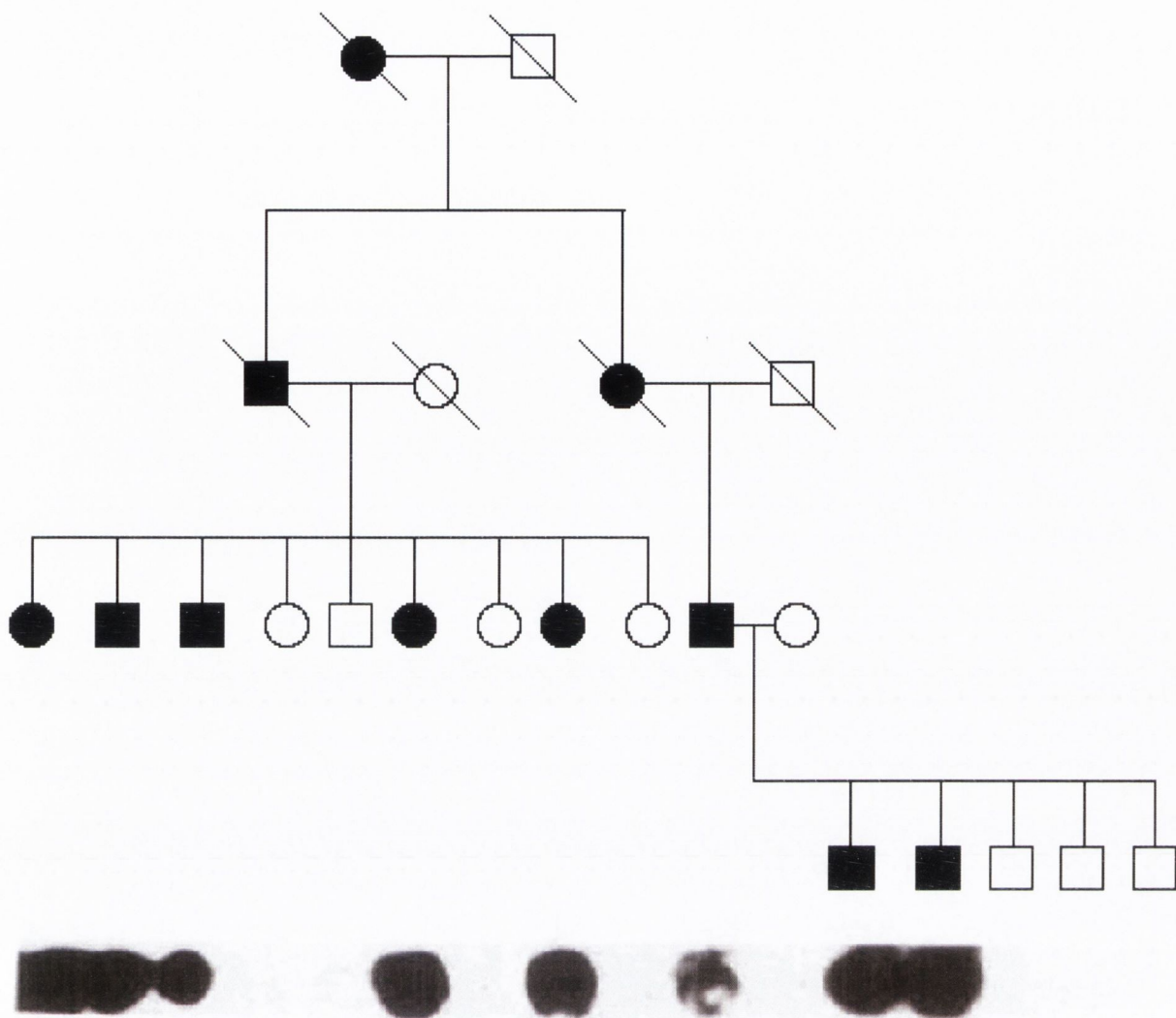


Figure 2.2: Amplified DNA from affected and unaffected individuals from TCD M1 was hybridised to both the normal (5'TGTCATCTACATGTTCGTGG3') and mutant (5'TGTCATCTACAGGTTCGTGG3') allele specific oligonucleotides (ASOs) around codon 207. In this figure for convenience only a part of this large family is shown hybridising to the mutant ASO. Only affected members of the family hybridised to the mutant ASO.

Chapter 3

Genotypic and Phenotypic Analyses of Families TCD B and TCD D

3.1 Introduction:

In this chapter I correlate the clinical and molecular genetic findings in a number of affected individuals from 2 Irish autosomal dominant Retinitis Pigmentosa families in which a mutation at Codon 178 of the Rhodopsin gene was found. We reported this novel mutation in 1991 (Farrar GJ, Kenna P, Redmond R, Shiels D, McWilliam P, Humphries MM, Sharp EM, Jordan S, Kumar-Singh R, Humphries P. Autosomal dominant retinitis pigmentosa: a mutation in codon 178 of the rhodopsin gene in two families of Celtic origin. *Genomics*. 1991 Dec;11(4):1170-1).

3.2 Materials and Methods

3.2.1 Molecular Genetic Analysis

Sixty-one affected individuals from European and American families in which the disease segregated in an autosomal dominant fashion were screened for mutations in the coding regions (exons) of the Rhodopsin gene by the method of single strand conformational gel electrophoresis (SSCPE) (Orita *et al.*, 1989). This technique reveals alterations (mutations) in the base sequence of DNA segments, amplified by means of the Polymerase Chain Reaction (PCR) (Yandell *et al.*, 1989), as changes in mobility during non-denaturing polyacrylamide gel electrophoresis. DNA segments in which mobility shifts were identified were then directly sequenced (Saiki *et al.*, 1988) to identify the base change(s) responsible. All individuals in the extended pedigrees from which the index individuals had been drawn were then examined for the mutation to test for co-segregation of the disease and the mutation. In addition, a panel of 71 unrelated, normal controls was tested for the mutation.

3.2.2 Ophthalmological Assessment of adRP Patients

All individuals were assessed in the Research Department of the Eye and Ear Hospital, Dublin. Every patient had corrected Snellen visual acuity measured. Goldman perimetry using IV 4e and I 4e targets was then performed followed by

assessment of color vision using the Farnsworth Munsell 100 Hue test under standardized conditions.

All individuals had electroretinographic (ERG) assessment using a protocol based on that proposed by the International Standardization Committee (Marmor *et al.*, 1989). Briefly, the patient's pupils were dilated with Tropicamide BP 1% and Phenylephrine BP 2.5%. The subject was then dark adapted for at least 30 minutes. Following corneal anaesthesia induced with Benoxinate BP 0.4%, corneal contact lens electrodes of the Henkes type were inserted under dim red illumination. A Ganzfeld bowl was used to present a series of flashes produced by an OS5 photostimulator located above and slightly behind the subject's head. The intensities of the flashes were altered by a combination of varying the output of the photostimulator and the interposition of neutral density filters. Wave forms were analysed using the Medelec "Mistral" data averaging and display system. The intensity of the flash at the patient's eye was measured using a Gossen "Mastersix" photometer. The standard flash produced by this protocol (setting 16) was 1.5 candelas per metre² per second. Waveforms were analysed using the standard conventions (Einthoven 1908).

The responses of the dark adapted eye to a series of single flashes of increasing intensity, commencing with a dim blue flash 3.3 Log units below that of the standard flash, were used to assess rod function. A light of intensity 22 cd/m² was then used to illuminate the interior of the bowl and the patient was light adapted for 10 minutes. Cone responses to a single flash of intensity 0.76 Log units below that of the standard, and to 30 Hz flicker, at setting 8, in the presence of the background illumination, were then assessed.

Two colour dark adaptometry using a Goldman-Weekers dark adaptometer and red and blue filters was performed on selected individuals (Sondheimer *et al.*, 1979). Following pupillary dilation as described above, the subject's left eye was bleached by exposure to a light of 2650 apostilbs for 5 minutes. Thresholds to two 2° targets, one blue, the other red, at 15° above fixation were measured at 1 to 2 minute intervals for 30 minutes. Flashing targets were presented at the rate of 1 second on, 1 second off. The true threshold was taken as the average of the "on" threshold and the "off" threshold for each testing. Following 30 minutes of dark adaptation, thresholds to the same stimuli were measured at 0°, 10°, 20°, 30°, 40° and 50° in the vertical and horizontal meridians. Finally, the dark-adapted threshold to an 11° white

target at 15° above fixation was measured.

Anterior segment biomicroscopy and applanation tonometry was then carried out, followed by direct and indirect fundoscopy, special attention being paid to the presence of pathology in the macular area. Most individuals had colour retinal photography.

3.3 Results

3.3.1 Molecular Genetic Analysis

SSCPE revealed the presence of a mobility shift in two unrelated individuals. An A>G transversion at position 3815 in codon 178 of exon 3 of the Rhodopsin gene was identified by direct sequencing of the region concerned. This transversion destroyed an Rsa I restriction enzyme site (GTAC > GTGC). Subsequent analysis of the kindreds concerned, using Rsa I digestion of a 283 base pair (bp) PCR amplified product surrounding the mutation site, showed that the mutation segregated perfectly with the disease (Fig. 3.1). The mutation was not found in a panel of 71 unrelated, normal controls, thus indicating that the mutation was unlikely to be a neutral polymorphism.

This transversion results in the substitution of a Cysteine for a Tyrosine residue in the DE loop of the intradiscal domain of the Rhodopsin protein. It is of interest to note that this Tyrosine residue is highly conserved, as its presence in the Rhodopsin protein of such divergent species as man (Nathans *et al.*, 1984), mouse (Baehr *et al.*, 1988), cow (Nathans *et al.*, 1983), fruitfly (Zuker *et al.*, 1985) and octopus (Ovchinnikov *et al.*, 1988) attests.

3.3.2 Clinical Characteristics of Affected Individuals

Pedigree TCD B (Fig. 3.2) is a 5-generation kindred with affected individuals in every generation and evidence of male to male transmission of RP, thus satisfying the criteria for an autosomal dominant hereditary pattern. Pedigree TCD D (Fig. 3.3) is a 3 generation kindred in which the disease also segregates in an autosomal dominant fashion.

Affected individuals in the two families in which this mutation was found were notable for the early age at which symptoms of night blindness became apparent (Table 1). Adult affecteds recalled becoming aware of difficulties in low light situations at approximately 10 years of age. The two young affecteds in TCD B, V 1 aged 12 and V 2 aged 10 years respectively, deny any symptoms referable to nyctalopia, despite having elevation of final dark adapted thresholds to white light of more than 3 Log units.

Loss of peripheral visual field also appears to occur early in this form of RP. The two youngest patients tested, TCD B V 1 and TCD B V 2, demonstrated very significant concentric constriction using the IV 4e target (Fig. 3.4). Preferential loss of superior field was revealed using the III 4e and I 4e targets (Fig. 3.5). By 16 years of age, patient III 1 of pedigree TCD D, showed gross constriction even with the larger target with preservation of a significant temporal isthmus. By 26 years of age, patient IV 2 in TCD B, had tubular fields with an isolated island in the far temporal area.

In keeping with the evidence of early impairment of rod function no rod isolated responses could be detected on electroretinography, even in the youngest affected individuals (Fig. 3.6). In contrast, cone responses could be detected in the younger patients (Fig. 3.6). These were characterized by marked prolongation in implicit times and reduction in amplitudes (Table 3.2). No responses, either rod or cone generated, could be measured in 3 of the patients, aged 62, 41 and 26 years respectively.

The results of two colour dark adaptometry confirmed the severe and early impairment of rod function. The threshold for the blue stimulus was always greater than that for the red, indicating that the thresholds were mediated by the cones. This pattern was present in all retinal loci tested (Fig. 3.7) and was consistent with a Massof and Finkelstein Type 1 classification (Massof and Finkelstein 1981). This pattern was evident even in younger patients.

All patients in these pedigrees showed evidence of significant vitreous gel disturbance, with at least grade 2 cells found in all affected persons. TCD D III 1, a 16 year old, had bilateral posterior vitreous detachments. Two patients, TCD B III 1 and TCD D II 1, developed early posterior subcapsular lens opacities, at 46 and 36 years of age respectively, requiring extraction and intraocular lens implantation. None of the other affected individuals in either pedigree had evidence of lenticular opacities.

The distribution of the intra-retinal pigmentary deposits varied in affected individuals. The youngest patients examined, TCD B V 1 and V 2, displayed subtle changes in the Retinal Pigment Epithelium (RPE). These consisted of a moth-eaten appearance, particularly in the inferior mid-periphery. This was accompanied by mild arteriolar attenuation (Fig. 3.8). The disc appearance was normal. Their mother, TCD B IV 1, had atrophic macular changes and more obvious retinal arteriolar attenuation. She had classical bone spicule pigmentary deposits which were more numerous in the nasal, and temporal mid-peripheries (Fig. 3.9). Patients TCD B IV 2 and TCD III 1 had a somewhat asymmetrical distribution of pigmentary deposits, in as much as they were more dense in the superior mid to far periphery compared to the inferior. In addition there were particularly heavy deposits in the temporal retina. The two oldest affected individuals, TCD B III 1 and TCD D II 1, in addition to atrophic macular changes, had symmetrically distributed pigment deposits. Patient TCD D III 1, a 16 year old, also exhibited a bull's eye maculopathy.

3.4 Discussion

A number of mutations had been discovered in the Rhodopsin gene in adRP patients since the first localization of a gene for this condition to the long arm of Chromosome 3. At the time of publication, clinical descriptions of the type of adRP resulting from specific mutations had been reported for only 2 of these, the mutations in codons 23, (Heckenlively *et al.*, 1991; Berson *et al.*, 1991) and 58 (Fishman *et al.*, 1991). Apart from its intrinsic interest to clinicians, the correlation of gene mutation with clinical findings may help to indicate which mutation is most likely to be present in a given form of ADRP, thus facilitating molecular genetic analysis and the establishment of prognosis.

The clinical picture associated with the first mutation to be reported in this gene, in Codon 23, had recently been described. In general, this mutation appeared to result in a relatively mild form of the condition. In the cases reported by Heckenlively *et al.*, (1991), affected individuals were notable for regional involvement, there being an inequality in the distribution of pigmentary deposits between the upper and lower retinal hemispheres. Onset of nightblindness in the cases reported appeared to be late, only one patient reporting an onset of mild nyctalopia in childhood. The 2 oldest patients, 52 and 62 years of age respectively,

both retained central visual acuity of 6/6.

Berson *et al.*, (1991) also reported that this mutation appeared to result in a relatively mild disease with better visual acuity and electroretinographic amplitudes 2 to 3 times greater in 17 patients with adRP attributed to the codon 23 mutation compared to 131 non-codon 23 adRP patients. 15 of the 29 cases described reported the onset of night blindness in the third decade or later, while only one reported symptoms referable to loss of visual field before the age of 20 years. In contradistinction to the cases reported by Heckenlively *et al.*, (1991) the vast majority of Berson's cases had pigmentary deposits in all 4 quadrants. The severity of symptoms and of electroretinographic impairment varied not only between families but also between affected individuals in the same family.

Fishman *et al.*, (1991) reported the clinical findings associated with a Codon 58 Rhodopsin mutation. All had a regionalized distribution of pigment deposition, predominantly in the inferior hemi-retina with concomitant superior visual field impairment. ERG amplitudes appeared to be relatively preserved and showed a slow deterioration over time. Rod and cone amplitudes, albeit reduced, were recordable in all patients. The rod responses were either normal or prolonged in implicit times, whereas the cone implicit times were prolonged. On the basis of regional differences in rod thresholds these patients could be placed in the Massof and Finkelstein Type 2 category.

Patients with the codon 178 mutation which I describe here had an early onset form of adRP. In contrast to the 2 previously documented Rhodopsin mutations no rod-isolated responses could be elicited, even from young affected individuals with this mutation. The more severe impairment of Rhodopsin function in this mutation was confirmed by the psychophysical profile in which thresholds were mediated exclusively by cones. Again this pattern was evident even in young affected individuals. Cone ERG amplitudes, although prolonged in implicit time in all cases, were better preserved. These however, tended to be lost at a relatively early age.

This mutation was associated in some cases with an early onset of posterior subcapsular lens opacification. Atrophy of the macular area tended to appear in the 3rd to 4th decades, resulting in severe central visual impairment.

While there appeared to be some intra-familial variation in the distribution of pigmentary deposits, as regards age of onset of night blindness and field loss, and deterioration of visual acuity, there was a marked degree of similarity between the

two families.

A Tyrosine to Cysteine substitution, in the intradiscal loop between the 4th and 5th transmembrane helices of Rhodopsin, obviously results in a catastrophic impairment of Rhodopsin function. Doi *et al.*, (1990) deleted a number of different amino acids in Rhodopsin by site specific mutagenesis and studied the characteristics of the proteins produced following expression in Cos-1 cells. Deletion of amino acids 177 and 178 resulted in a mutant which failed to generate a rhodopsin-like chromophore when, after expression, the membranes were incubated with 11-*cis*-retinal. Abnormal glycosylation was also noted. They also speculated that the defective protein produced remained in the endoplasmic reticulum and was not transported to the plasma membrane.

The B-C, D-E, and F-G loops may be cooperatively involved in the formation of a specific folded structure on the intradiscal face of the protein (Doi *et al.*, 1990). The disulphide bond between Cysteines 110 and 187 is particularly important in the correct structure and function of bovine Rhodopsin (Karnik *et al.*, 1988). Substitution of a Serine for each of these Cysteines has been demonstrated to result in proteins which were expressed at lower levels, showed defective glycosylation, failed to generate a chromophore when incubated with 11-*cis*-retinal and did not activate Transducin in the presence of light (Karnik *et al.*, 1988). The presence of a Cysteine residue at position 178 might affect this bonding in a detrimental fashion. These findings strongly suggested that a mutation in this region of the Rhodopsin gene might result in severe dysfunction of the protein which would be manifested clinically at an early age.

The possibility of this particular mutation should be borne in mind in all cases where the disease is characterized by early onset of night blindness, evidence of early and severe impairment of rod function, and reduction in visual acuity at an early age due to atrophic macular changes and /or posterior subcapsular lens opacities.

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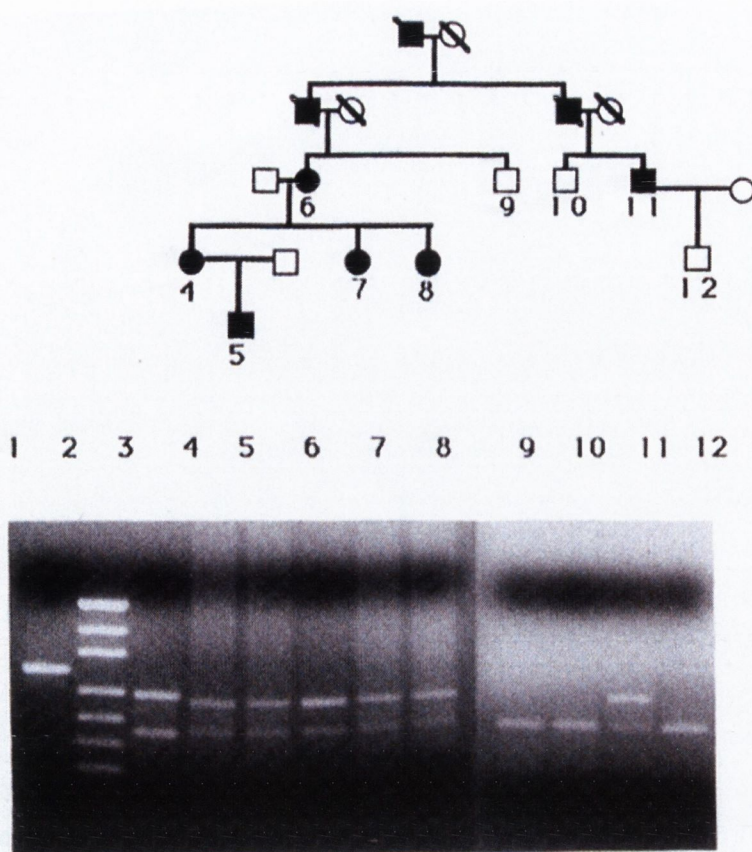


Figure 3.1: The amplified exon 3 fragment is 283 bp in length (lane 1). The codon 178 mutation destroys an *RsaI* site that occurs 52 bps from the 5' end of the fragment. There is a neutral *RsaI* polymorphism which occurs 67 bps from the 3' end, the very much rarer allele being the absence of this site. All affected members of the adRP family tested are homozygous for the presence of the *RsaI* site of the neutral polymorphism and heterozygous for the mutation (lanes 4-8 and 11). The normal allele when cut with *RsaI* gives DNA fragments of 51, 165 and 67 bps and the mutant allele gives fragments of 216 and 67 bps. In contrast, the unaffected members of the family are homozygous for both *RsaI* sites and when cut give fragments of 51, 165 and 67 bps (lanes 9, 10, and 12). A normal control individual heterozygous for the neutral polymorphism and therefore having fragments of 232, 165, and 51 bps has been run to indicate that both the neutral polymorphism and the putative mutation are distinguishable (lane 3). The size standard is *MspI*-cut PUC DNA (lane 2).

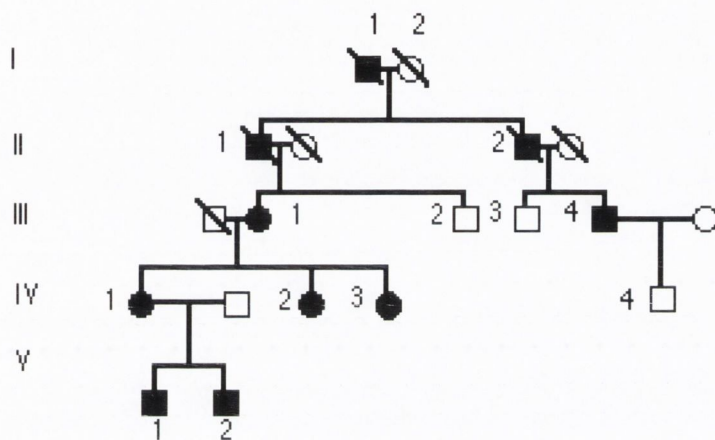


Figure 3.2: TCD B, a 5 generation pedigree segregating adRP. Generations are represented by Roman numerals. Individuals in a generation are represented by Arabic numerals. Affected patients are designated by closed symbols.

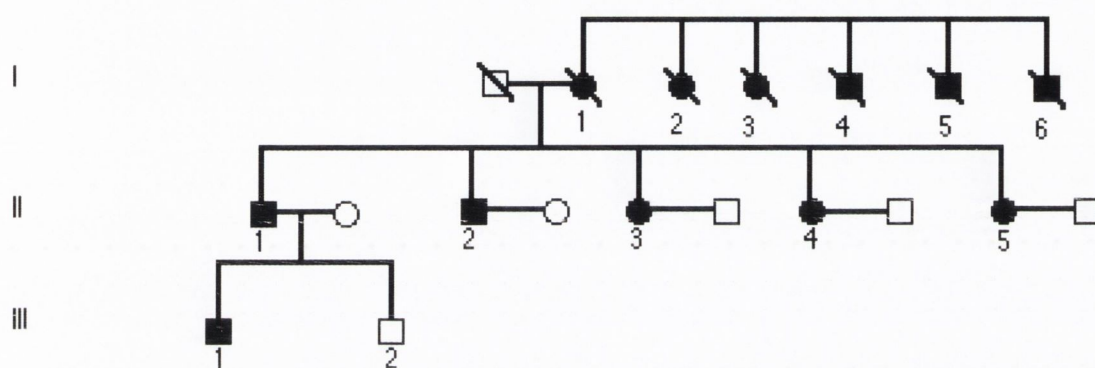
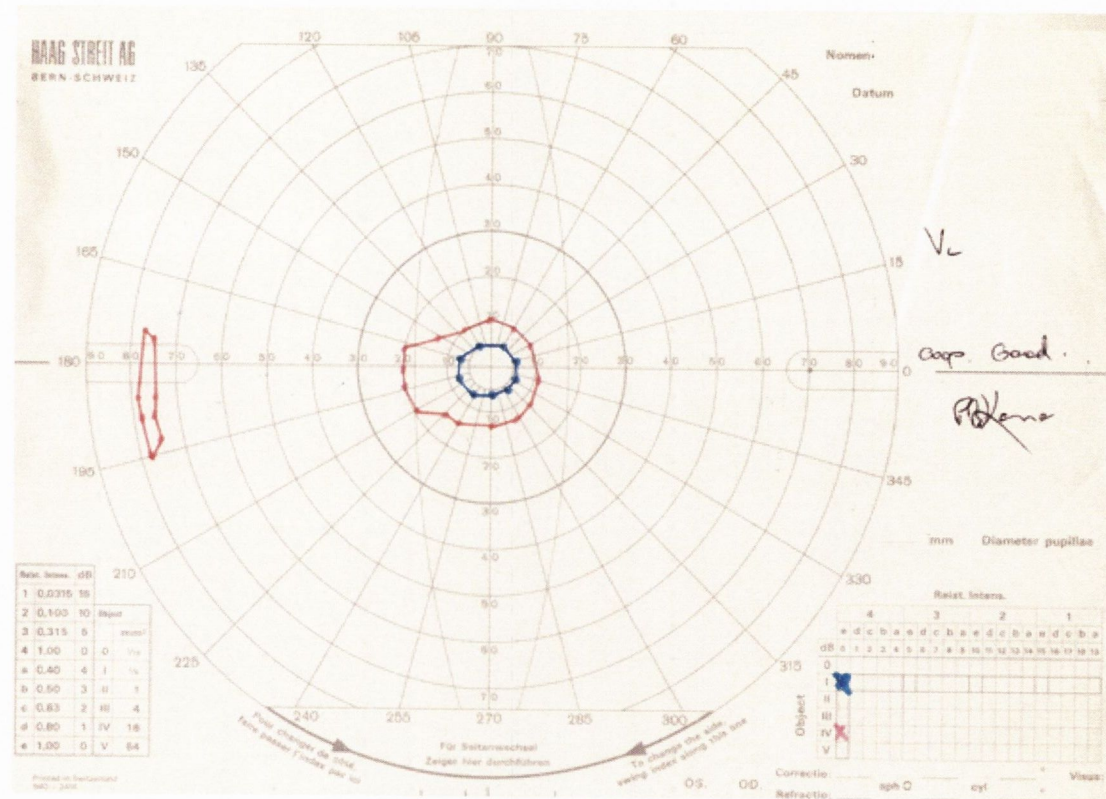


Figure 3.3: TCD D, a 3-generation kindred segregating adRP. Generations are represented by Roman numerals. Individuals in a generation are represented by Arabic numerals. Affected patients are designated by closed symbols.



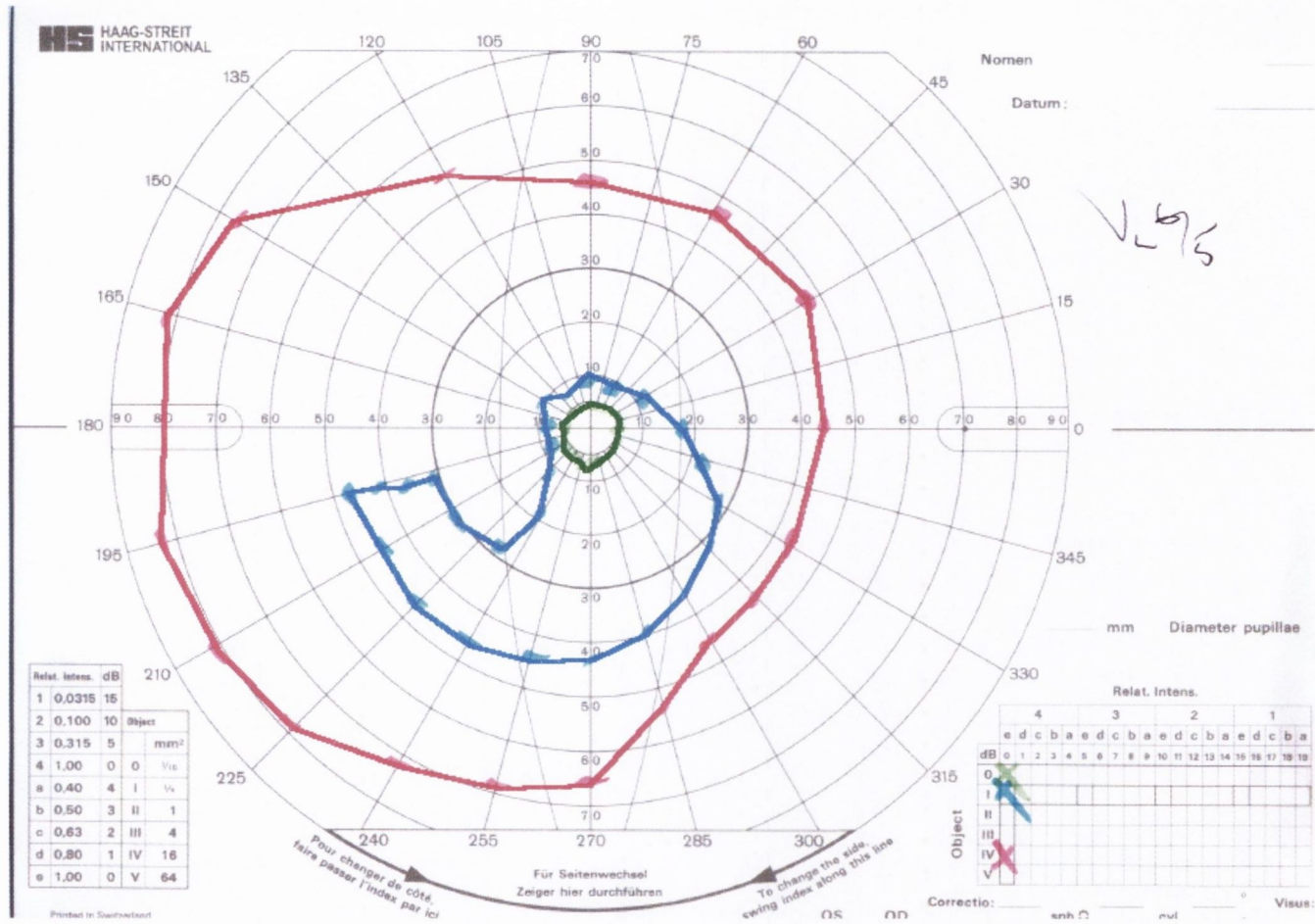


Figure 3.5: Goldmann perimetry of the left eye from a 12-year old affected individual in family TCD B, showing preferential loss of the superior field to the I4e (blue) and O4e (green) targets.

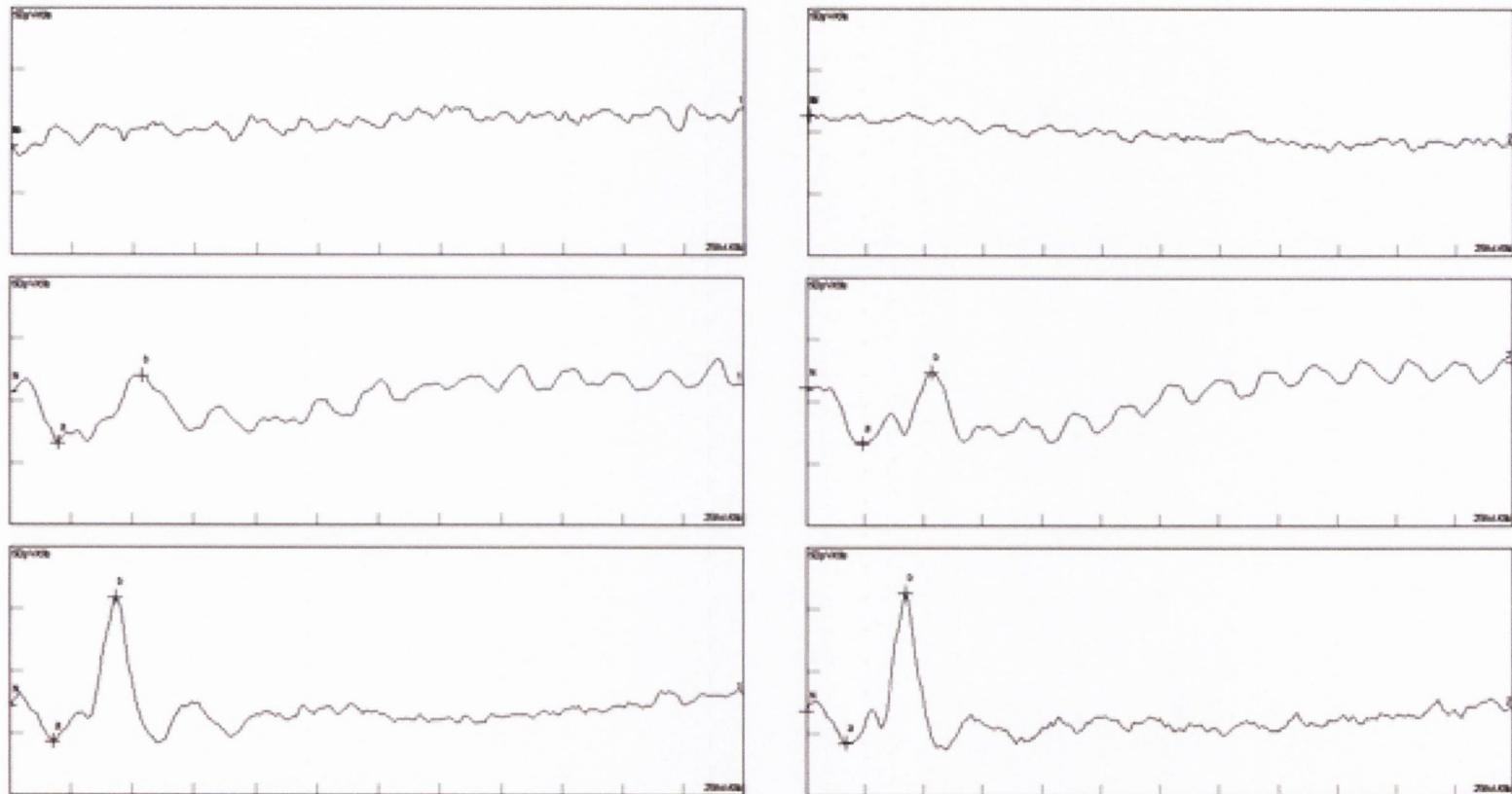


Figure 3.6: Ganzfeld ERG tracings from a 12 year-old member of TCD B. Top panel: rod-isolated responses; middle panel: mixed dark-adapted rod/cone response to maximal intensity flash, and lower panel: cone-isolated responses.

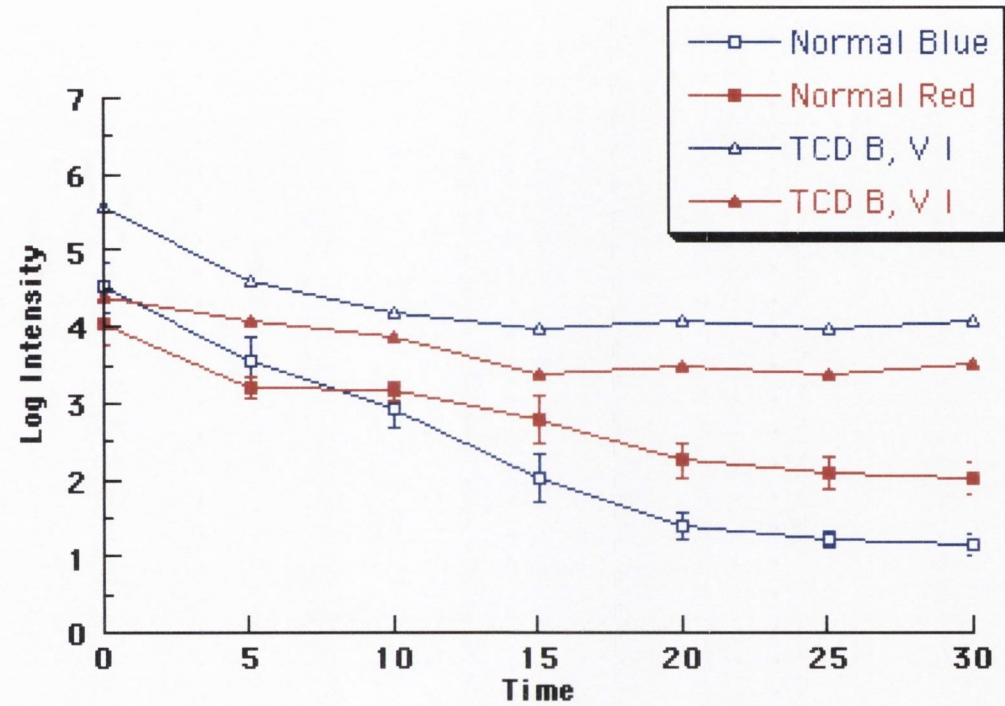


Figure 3.7: 2-colour dark adaptometry profile at 15° above fixation. For patient TCD B, V I, detection of both the red and blue targets is always mediated by the cones as evidenced by the lower threshold of detection to the red target compared to the blue. In contrast, in the normal individual, detection is mediated by the rods once rod adaptation has occurred as evidenced by the lower threshold of detection to the blue target compared to the red.

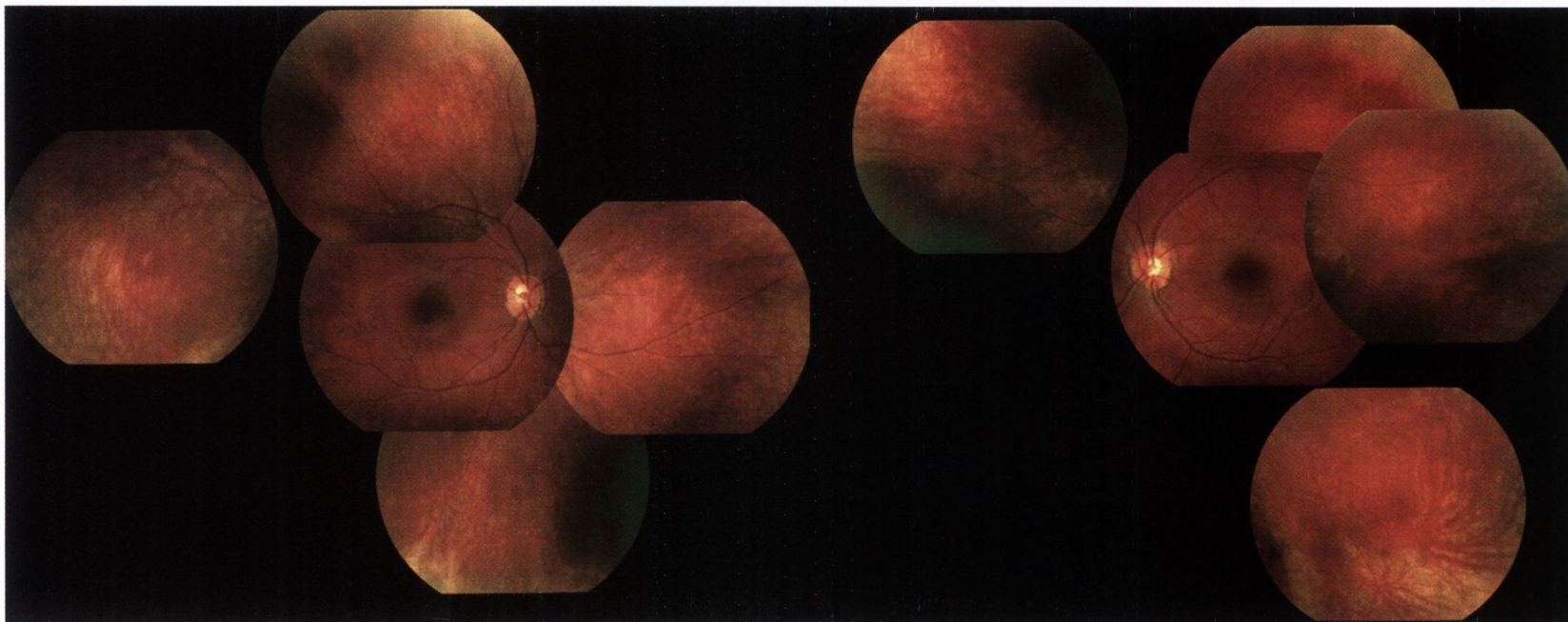


Figure 3.8: Collage of fundus photographs of patient TCD B V 1 showing subtle retinal pigmentary mottling.

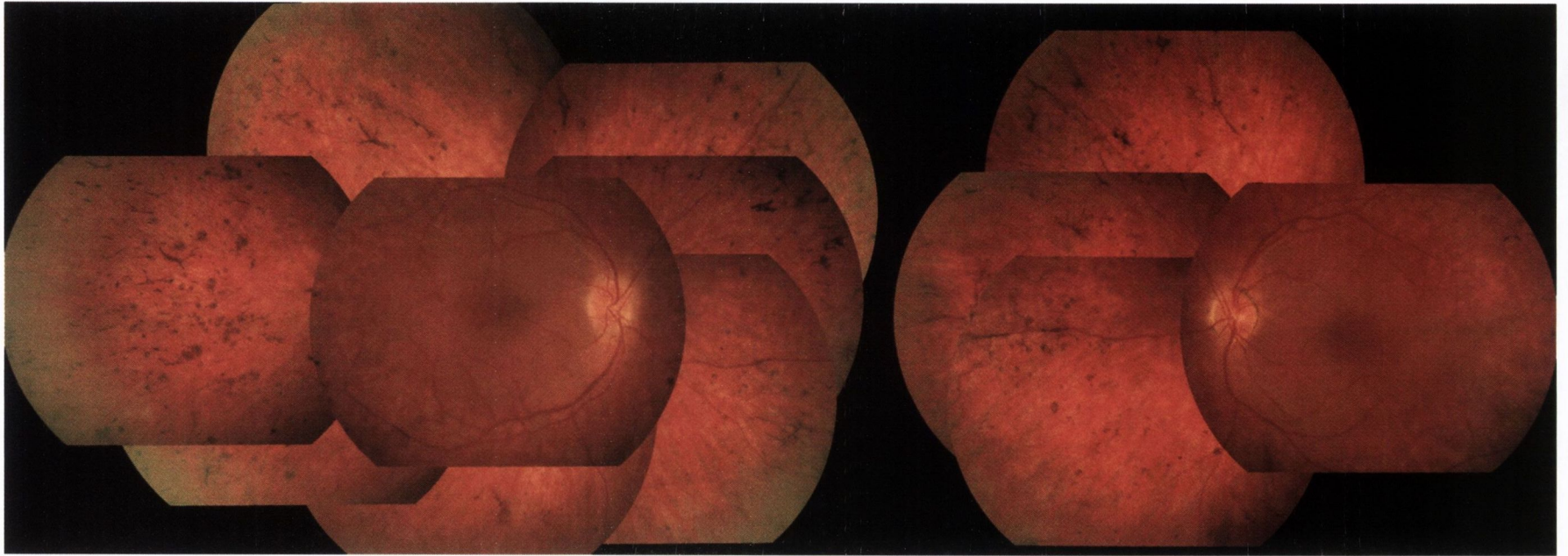


Figure 3.9: Collage of fundus photographs of TCD B, IV 1 showing moderate disc pallor, arteriolar attenuation, atrophic macular changes and extensive intra-retinal pigmentary deposits, particularly in the nasal and temporal mid-peripheries.

Table 1 Age of Onset of Symptoms			
Patient's Pedigree and Number	Age at Assessment	Age of Onset of Night Blindness	Age of Onset of Field Loss
TCD B III 1	62	5-6	Mid Teens
TCD B IV 1	32	10	16
TCD B IV 2	26	12	None
TCD B V 1	12	None	None
TCD B V 2	10	None	None
TCD D II 1	41	8	15
TCD D III 1	16	8	11

Table 3.1: Age of onset of symptoms. Values refer to age in years.

Table 2 Electoretinographic Data												
Patient's Pedigree and Number	Rod b Wave				Cone Responses							
					S F b Wave				30 Hz			
	Amplitude μ V		Implicit Time (ms)		Amplitude μ V		Implicit Time (ms)		Amplitude μ V		Implicit Time (ms)	
	RE	LE	RE	LE	RE	LE	RE	LE	RE	LE	RE	LE
TCD B III 1	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
TCD B IV 1	NR	NR	NR	NR	BR	28.7	BR	49.2	13.3	13.4	45.3	48.4
TCD B IV 2	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
TCD B V 1	NR	NR	NR	NR	66.4	66.4	49.8	49.8	31.4	31.9	45.7	45.7
TCD B V 2	NR	NR	NR	NR	62.5	46.9	43.9	43.9	53.8	54.8	54.8	54.8
TCD D II 1	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
TCD D III 1	NR	NR	NR	NR	18.2	13.0	35.7	35.7	5.37	6.93	33.2	33.2

Table 3.2: ERG values for dark-adapted rod isolated responses, light-adapted single flash (SF) cone responses and light-adapted 35 Hz cone responses. NR = Not recordable; BR = Blink reflex; SD = Standard deviation.

Chapter 4

Genotypic and Phenotypic Analyses of a Family with Autosomal Dominant Congenital Stationary Night Blindness

4.1 Introduction:

Over one hundred mutations within the rhodopsin gene have been shown to be responsible for some forms of retinitis pigmentosa, a progressive retinal degeneration characterized by night blindness and subsequent disturbance of day vision which may eventually result in total blindness. Congenital stationary night blindness (CSNB) is an uncommon, inherited retinal dysfunction in which patients complain of night vision difficulties of a non-progressive nature only and in which generally there is no involvement of day vision. In this chapter I report the results of molecular genetic analysis of an Irish family segregating an autosomal dominant form of CSNB in which a previously unreported threonine to isoleucine substitution at codon 94 in the rhodopsin gene was found to segregate with the disease. Computer modeling suggested that constitutive activation of transducin by the altered rhodopsin protein was a plausible mechanism for disease causation in this family. This mutation was reported in Human Mutation in 1999 (al-Jandal *et al.*).

4.2 Materials and Methods

4.2.1 Family Study

Two nuclear Irish families were independently ascertained and a diagnosis of congenital stationary night blindness made. Subsequent pedigree analysis uncovered a relationship between these two families and a large four generation lineage was established in which the disease segregated in an autosomal dominant fashion (Fig. 4.1).

Individuals, indicated with an asterisk in Figure 4.1, were questioned regarding the age of onset and progression of their night vision problems as well as any daytime visual symptoms. Visual examination included determination of best-corrected Snellen visual acuity and visual field analysis by Goldmann perimetry using IV4e, I4e and O4e targets. Individuals III 5 and IV 1 had colour vision assessed using the Farnsworth-Munsell 100 hue test.

Dark-adaptation studies (Sondheimer *et al.*, 1979) were performed using a Goldmann Weekers dark-adaptometer and two 2° targets, one blue, the other red, presented at 15° above fixation which gave an indication of the relative contributions of the rod and cone photoreceptors to dark adaptation.

Electroretinography (ERG) was performed on all patients using a protocol which met the ISCEV standard for clinical electroretinography (Marmor *et al.*, 1989). Following 30 minutes of dark adaptation the responses to single flashes of -3.5 log units intensity, presented in a Ganzfeld bowl, were used to assess rod function. The mixed rod and cone responses of the dark-adapted eye to a flash of maximal intensity were then measured. A rod suppressing background light of intensity 22 cd/m² was then used to illuminate the interior of the bowl and the patient was light adapted for 10 minutes. Cone responses to a series of maximal intensity flashes, presented singly and at 30 Hz flicker in the presence of the background illumination, were then measured.

All patients underwent slit lamp biomicroscopy and detailed fundus examination using both direct and indirect ophthalmoscopy. Individuals III, 5 and IV, 1 had colour fundus photographs taken.

4.2.2 Molecular Genetic Study

Using DNA from an affected and an unaffected individual from the kindred, all five rhodopsin exons were amplified by polymerase chain reaction (PCR). Direct sequencing of the amplified products was performed using a protocol previously described (Farrar *et al.*, 1990). $\gamma^{32}\text{P}$ end-labelled allele specific oligonucleotides (ASOs) for the normal (5' CACCAGCATCCCCTACACCTC 3') and mutant (5' CACCAGCATCCTCTACACCTC 3') sequences were used for hybridization to 2 μl of PCR-amplified exon 1 samples, dot blotted onto Hybond N nylon membranes, from all individuals who were clinically assessed. The hybridization temperature used was $T_m - 5^\circ\text{C}$ and was calculated for each ASO. Following hybridization, the blots were washed in 2X SSC at the respective hybridization temperatures and subsequently exposed to X-ray film at -70°C .

4.3 Results

4.3.1 Clinical Analysis

All individuals questioned reported night vision difficulties for as long as they could remember. None had noted any subjective deterioration over time. Older individuals in the pedigree felt quite safe driving at night time in well lit urban environments. One patient, living in a rural area, was comfortable driving at night time under full headlights. No one reported any subjective problems with day vision.

All patients achieved best-corrected visual acuity of 20/20 (6/6 in metric notation) or better as measured by Snellen chart. No colour vision abnormalities were noted. A 23-year old affected female had a total error score of 59. The upper limit of normal for age 23 is 78 (Kinnear and Sahraie, 2002).

Goldmann perimetry was entirely normal in all individuals to all three target sizes, even in the two oldest patients, III, 5 and III, 11, aged 59 years and 57 years respectively.

Two-colour dark adaptometry (Fig. 4.2) showed that there was no rod contribution to target detection as the threshold for detection of the red target was consistently lower than for the blue, the reverse of the pattern seen in 18 normal individuals after the rod/cone break. The thresholds for detection were significantly elevated in all affected individuals. In patient IV, 1 (Fig. 4.1) the magnitude of threshold elevation remained unchanged even after 2 hours of dark adaptation.

Electroretinography supported the dark adaptometry findings in that no detectable rod isolated responses could be recorded in any affected individual (Fig. 4.3A). The dark-adapted response to the most intense flash showed an “a” wave which in all affected individuals was significantly reduced in amplitude. The “b” wave was also significantly reduced in amplitude and had a quicker implicit time compared to normal. The “b” wave amplitude in all patients was less than that of the “a” wave, the reverse of that seen in normal individuals. The oscillatory potentials, the ripples visible on the upslope of the “b” wave response of the dark adapted eye to the maximal intensity flash, were well preserved. The cone responses to both single flash and 30Hz flickers were normal in both timing and amplitudes (Fig. 4.3B).

Slit lamp biomicroscopy showed no abnormalities of the anterior segments, lens or vitreous of any of the affected individuals. Fundoscopy showed no significant abnormalities in any individuals examined (Fig. 4.4). In patient III, 5 an inferior

temporal retinal pigment epithelial disturbance was noted in the right eye which may have resulted from a childhood sporting injury.

4.3.2 Molecular Genetic Analysis:

A previously unreported missense mutation (ACC to ATC) in codon 94 in the first exon of the rhodopsin gene was discovered in the affected individual by direct sequencing (Fig. 4.5). The mutation results in a Threonine to Isoleucine substitution. Examination of DNA from all clinically evaluated individuals in the pedigree using allele specific oligonucleotides (ASO) for both the wild type and mutant alleles showed that only affected individuals in the pedigree hybridized with the mutant ASO (Fig. 4.6). DNA from 52 randomly selected, unrelated and normal individuals failed to show hybridization with the mutant ASO.

4.4 Discussion

Both retinitis pigmentosa (RP) and congenital stationary night blindness (CSNB) share the primary symptom of impairment of night vision. In marked contradistinction to most forms of RP, CSNB is a non-progressive condition which never results in impairment of day vision.

CSNB has been classified in the past on the basis of fundoscopic features (normal or abnormal) and electroretinographic characteristics (Carr E. 1996; Schubert and Bornschein 1952; Miyaki *et al.*, 1986).

Carr *et al.* (1966) described a patient with non-recordable rod responses in whom the dark adapted bright flash “a” and “b” waves were greatly reduced in amplitude. This patient also had reduced amplitude cone flicker responses. Peachey *et al.* (1990) described a patient with similar dark-adapted ERG parameters but with normal cone flicker responses. On the basis of these findings it was speculated that defects in rod phototransduction might account for the retinal dysfunction.

The patients described in this paper show electroretinographic features which are similar to those described by Peachey *et al.*, (1990). No rod-isolated responses could be recorded in any of our patients while cone derived ERG responses were entirely normal. The maximal dark-adapted responses recorded in our patients show a similar reduction in “a” wave and “b” wave amplitudes and marked acceleration of the “b” wave implicit times suggesting that these waveforms may be cone derived.

The absence of a rod input is corroborated by the dark-adapted profiles of our patients which show that at all times the stimulus detection is cone mediated.

Only two other rhodopsin mutations have been reported in patients with congenital night blindness. Dryja *et al.*, (1993) reported a heterozygous Ala 292 Glu mutation in a simplex case of CSNB. This mutant opsin was expressed in COS cells and was found to be capable of activating transducin in the absence of 11-*cis*-retinal. These authors speculated that in their patient the constitutively active opsin could saturate the rod electrical responses and mimic the effect of a steady background light in desensitizing the rods. Their patient showed non-recordable rod responses, reduced amplitude responses to white flashes with shortened implicit times and borderline normal 30 Hz flickers. Unavailability of key related individuals precluded the establishment of a definitive inheritance pattern.

Sieving *et al.*, (1992 and 1995) described an autosomal dominant congenitally nightblind kindred in which they detected a Gly 90 Asp mutation in the rhodopsin gene. Rao *et al.*, (1994) expressed this mutant opsin *in vitro* and demonstrated that it too was constitutively active in the absence of retinal. None of their patients showed recordable rod responses. Most of their patients had normal cone electrophysiology, apart from the oldest affected member of their kindred who showed reduced amplitude responses to both single flash and 30 Hz flickers. Fundus reflectometry in the 38-year old proband showed normal rhodopsin density. Two of their patients, aged 38 and 64 years, also demonstrated peripheral retinal pigmentary changes which were associated with visual field loss. In view of this the authors hesitated to classify the condition as “stationary”.

A mutation in the beta subunit of the rod cGMP phosphodiesterase gene (Gal *et al.*, 1994) has also been documented in the Rambusch pedigree, a kindred segregating autosomal dominant CSNB. In this family (Rosenberg *et al.* 1991) rod isolated responses were non-recordable and, as in our patients, the maximal dark-adapted responses showed significant reduction in “a” and “b” wave amplitudes. The “b” wave timing in this response appeared to be shortened as in our patients. The character of this response in the Danish family appeared different to ours in that the “b” wave exceeded the baseline in all the ERG tracings shown whereas in our family the “b” never approached the baseline and in all cases the “b” wave amplitude was less than the “a” wave amplitude.

A Gly 38 Asp mutation in the alpha subunit of rod transducin (Dryja *et al.*, 1996) has been reported in the well known Nougaret CSNB kindred, first described

by Cunier in 1838 and the earliest documented autosomal dominantly inherited disease in man. The mutated gene product has also been postulated to be constitutively active.

Oguchi disease, an autosomal recessive form of CSNB in which there is marked delay in rod adaptation to dark and in which the characteristic Mizuo phenomenon is seen, has been shown to be due to mutations in the arrestin (Fuchs *et al.*, 1996) or the rhodopsin kinase genes (Yamamoto *et al.*, 1997). Absence of functional protein products of either of these two genes is predicted to lead to longer than normal persistence of photo-activated rhodopsin with consequent prolonged desensitization of the rod photoreceptor.

Evidence from protein chemistry and site directed mutagenesis has enabled the generation of a model of the 3-D structure of the rhodopsin protein. Key features of the protein include the lysine residue at codon 296, site of the Schiff base linkage to the retinal chromophore, and the Schiff base counterion - Glu 113. Studies of various substitutions at both of these sites suggest that mutations at either codon can result in constitutive activation of the apo-protein form, possibly due to the disruption of a salt bridge between Lys 296 and Glu 113 (Robinson *et al.*, 1992; Cohen *et al.*, 1993). A computer generated model of the rhodopsin molecule as viewed from above is presented in Figure 4.7A. This demonstrates that the introduction of an Isoleucine at codon 94 has the potential to cause steric hindrance and, probably more seriously, alter a relatively polar environment to a more hydrophobic one. In Figure 4.7B a horizontal view of rhodopsin is presented which shows that the codon 94 Thr side chain lies close to the Lys 296 retinal Schiff base.

These observations led us to speculate that the mechanism of action of this new Thr-94-Ile rhodopsin mutation might be constitutive activation of transducin by the altered rhodopsin protein.

Publication of this paper prompted a number of groups to study the effects of the Thr-94-Ile mutation on the *in vitro* biochemistry of rhodopsin using site directed mutagenesis and expression in COS-1 cells. (Ramon *et al.*, 2003; Gross *et al.*, 2003).

Ramon and co-workers (2003) found that similar elution profiles were obtained for the wild type and the Thr94Ile mutant with most of the protein eluting with pH 6 buffer, with no salt, in the third fraction, and only a very small amount of non-retinal binding fraction eluting with added salt. In contrast, the protein from another mutant Thr94K eluted predominantly at high salt conditions with very little protein being eluted at low salt condition indicating that most of the protein is

misfolded. The authors concluded that the Thr94Ile mutant protein did not undergo misfolding, a phenomenon frequently seen in mutated rhodopsins associated with retinitis pigmentosa (Liu *et al.*, 1996; Garriga *et al.*, 1996; Hwa *et al.*, 1997).

UV-visible absorption spectrum analysis in the dark showed that Thr94Ile formed chromophore like the wild type protein. They found a slightly higher A_{280}/A_{500} ratio in the mutant protein which they attributed to a change in the ϵ of the maximum of the visible absorption band for the mutant protein. The Thr94Ile mutant showed a blue-shifted visible absorption maximum in the visible region at 478 nm when compared to wild-type protein. The observed blue shift in the $\lambda_{\max}$ in the visible absorption bands for Thr94Ile, involving a change from a polar to a hydrophobic side chain, indicated that Thr-94 in rhodopsin is located close to the retinal binding pocket.

Photobleaching studies showed that the behaviour of Thr94Ile was similar to wild type on illumination.

The decay of the photointermediate MetaII formed upon rhodopsin illumination was examined for the wild type and the Thr94Ile mutant by fluorescence spectroscopy. The $t_{1/2}$ values for the MetaII decay for the wild type rhodopsin was typically about 15 minutes under the conditions of the assay. The rate of decay of the Thr94Ile mutant was c.6.5 times slower than wild-type. The authors remarked that this was the slowest decay yet reported for a mutant rhodopsin.

Assays were carried out to measure the chromophore stability in the dark (thermal bleaching) and the accessibility of the protonated Schiff base in the dark (hydroxylamine reactivity). Thr94Ile showed reduced thermal stability with 70% loss of absorption in 5 hours when compared to wild-type. The accessibility of the PSB toward hydroxylamine was also measured in the dark. Wild type rhodopsin was completely stable to NH_2OH reactivity in the dark for 2 hours, and the susceptibility of the mutants T94D and Thr94Ile to hydroxylamine again followed the order they showed for thermal instability. Wild-type rhodopsin was completely stable to NH_2OH reactivity in the dark for 2 hours. In contrast, more than 80% of the mutant Thr94Ile had reacted with hydroxylamine after 2 hours.

The authors studied the binding of all *trans* retinal by the Thr94Ile mutant, showing that it bound all-*trans*-retinal forming a chromophore with its visible absorption band located at 466 nm, close to the 478 nm band observed for the same mutant regenerated with 11-*cis*-retinal. As the $\lambda_{\max}$ for the protonated Schiff base under denaturing conditions is 440 nm, the chromophore for all-*trans*-retinal-

constituted pigment reflects most likely the presence of a protonated Schiff base linkage. The presence of the protonated Schiff base was confirmed by acidification of the sample that resulted in the formation of the 440-nm characteristic absorption.

The results obtained indicated that the dark state conformation of the Thr94Ile mutant must be significantly different from that of wild type rhodopsin. This conclusion was reached from the following results. (i) This purified mutant protein showed reduced thermal stability in the dark, and even at the moderate temperature of 37°C the decay of the chromophoric band was greatly accelerated compared with that of the wild type rhodopsin. (ii) Thr94Ile mutant protein showed reactivity toward hydroxylamine in the dark. This was interpreted as an increase in the accessibility of the protonated Schiff base by the reagent, reflecting structural alterations in the protonated Schiff base environment and in the intradiscal domain, in the dark state. (iii) Thr94Ile mutant was able to regenerate chromophore with all-*trans*-retinal to give a protonated Schiff base.

Using transducin activation assays Gross *et al.*, (2003) found that when assayed in isolated COS cell membranes, the Thr94Ile mutant rhodopsin displayed activity for the light-dependent activation of transducin that was comparable to that of wild-type rhodopsin under the same conditions. The opsin form of the Thr94Ile mutant was different from the wild type in that it displayed a small but significant level of constitutive ability to activate transducin. The activity seen with Thr94Ile was lower than that observed for two other night blindness rhodopsin mutants (G90D and A292E) but greater than the background activity measured in the presence of 11-*cis*-retinal, and also above the activity observed for wild-type opsin. The Thr94Ile mutant was similar to the other two rhodopsin night blindness mutants in being constitutively active.

Rhodopsin in DDM (dodecylmaltose) solution, when exposed to light, converted within milliseconds to that of the active MetaII intermediate with an absorption maximum at 380 nm, characteristic of an unprotonated Schiff base. As in the Ramon study the Thr94Ile mutant showed normal bleaching kinetics compared to wild-type but displayed markedly slow decay of MetaII. Wild-type MetaII decayed with a half-life of c. 2.6 minutes at 25°C. The Thr94Ile MII intermediate decayed with a half-life of c. 20 minutes, approximately 8-times slower than wild-type MetaII. The authors considered that the slower decay of Thr94Ile MetaII most probably did not account for the night blindness symptoms of affected individuals as

decay of wild-type MetaII is not rate-limiting in turn-off of the photoresponse in rod photoreceptor cells (Chen *et al.*, 1995).

In addition, the long-lived MII phenotype is unique to T94I among night blindness mutants; the MII intermediates of the G90D and A292E mutant rhodopsins decay with kinetics similar to those of the wild-type intermediate.

In conclusion, in this chapter I have described pedigree analysis, detailed clinical investigation and molecular genetic studies which revealed the cause of one form of autosomal dominantly inherited congenital stationary night blindness to be a Thr94Ile mutation in the rhodopsin gene. This discovery, together with the identification by other investigators of further mutations in the rhodopsin gene in patients with stationary night blindness, stimulated a number of *in vitro* studies of the biochemistry of the resultant mutant rhodopsin proteins.

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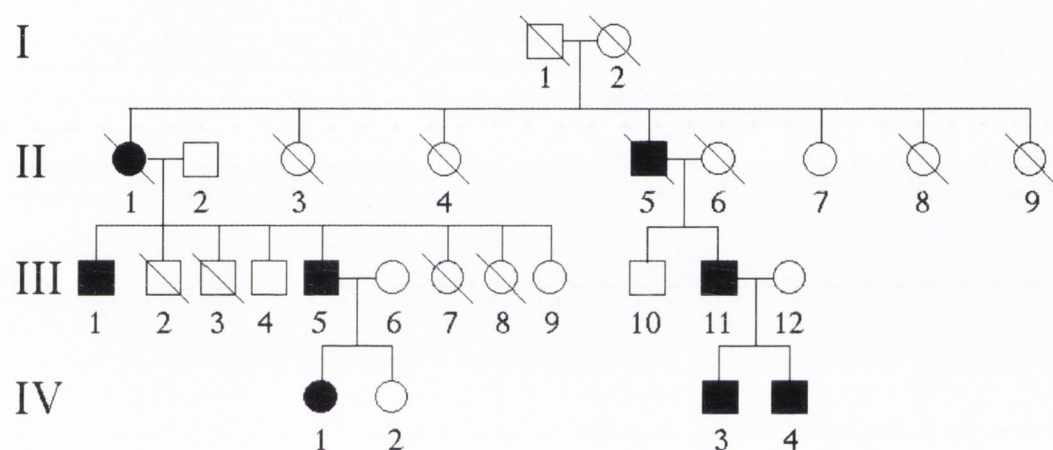


Figure 4.1: Family TCD BC. Filled circle, affected female; filled square, affected male. Diagonal bar, deceased person; asterisk, individual clinically assessed.

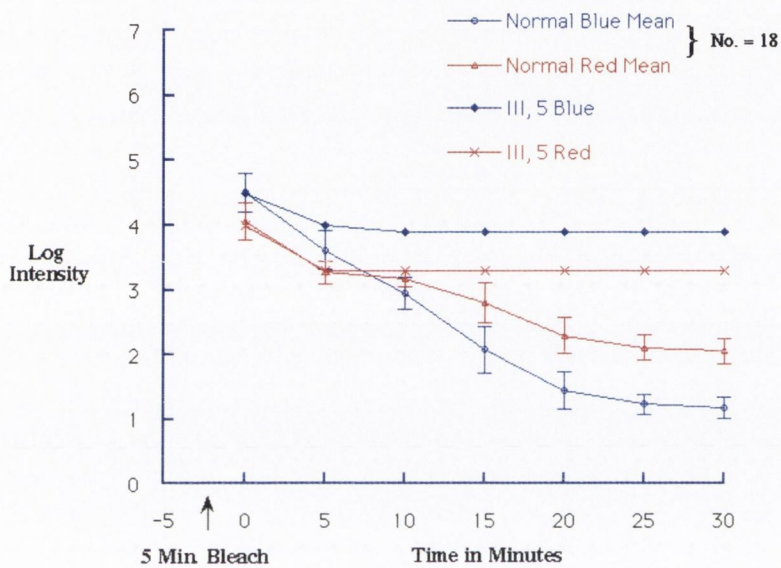


Figure 4.2: Two-colour dark adaptometry profile over 30-min dark adaptation. Before threshold determination, the subject was exposed to a rhodopsin bleaching light of 2,650 apostilbs for 5 min. Note the absence of the normal crossover in patient III, 5 and the reversal of the blue and red thresholds indicating that stimulus detection is mediated exclusively by cones.

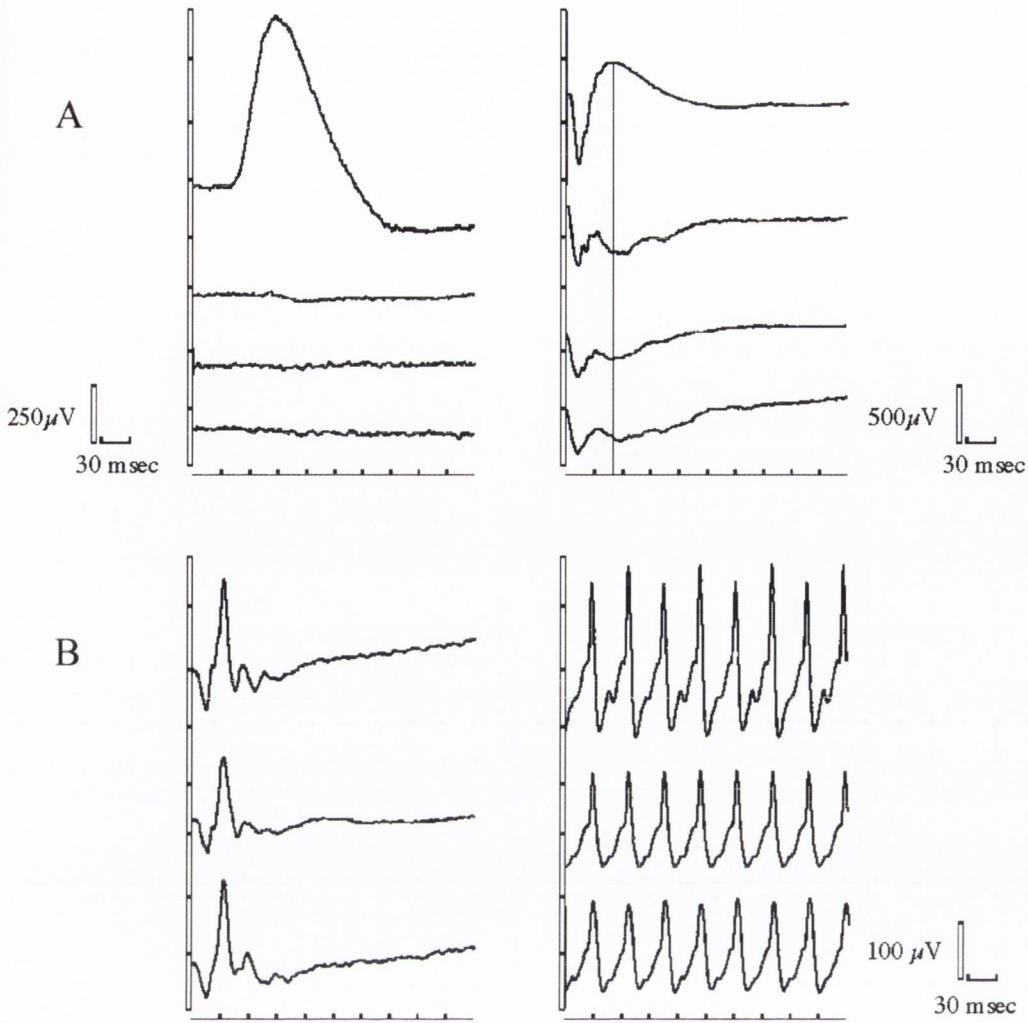


Figure 4.3: A: Dark-adapted electroretinogram (ERG) responses. Left: rod isolated responses. Right: Combined rod and cone responses to maximal intensity flash.

B: Light-adapted ERG responses. Left: Single flash cone responses. Right: 30-Hz flicker cone responses. From top: Normal; III, 5; IV, 1 (Fig. 4.1).

By convention, the “a” wave is designated as the first negative waveform after the stimulus. The “b” wave is designated as the first positive waveform. The “a” and “b” wave implicit times are measured from initiation of the flash to the peak of the relevant waveform component.

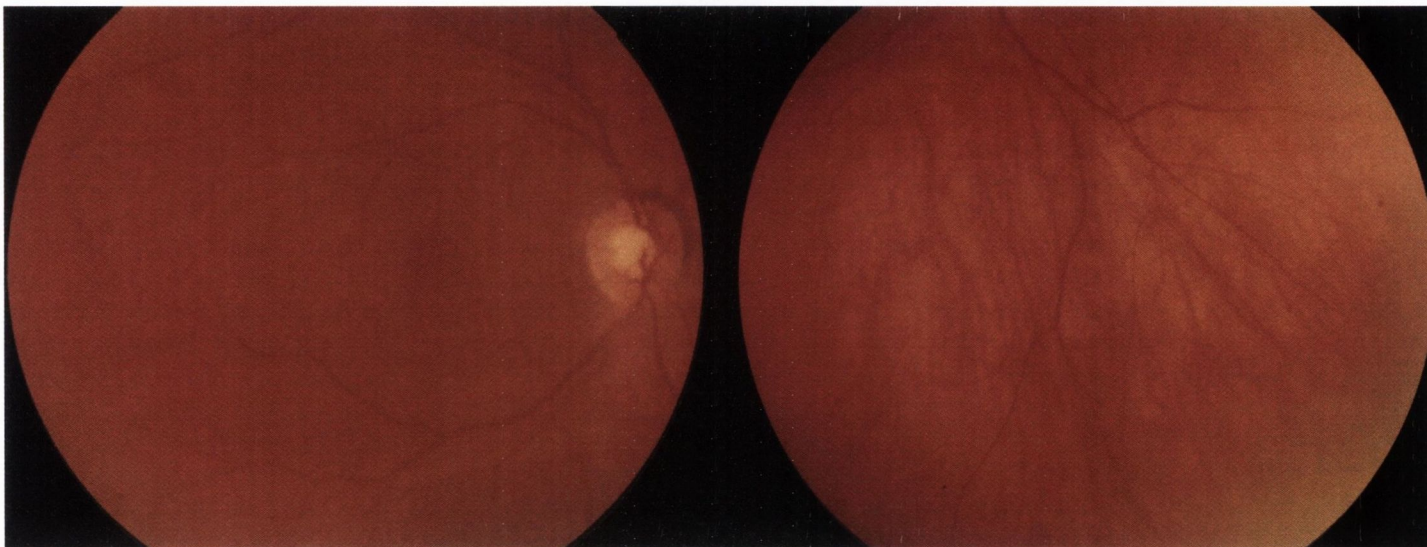


Figure 4.4: Normal fundus appearance of an affected individual in Family TCD BC

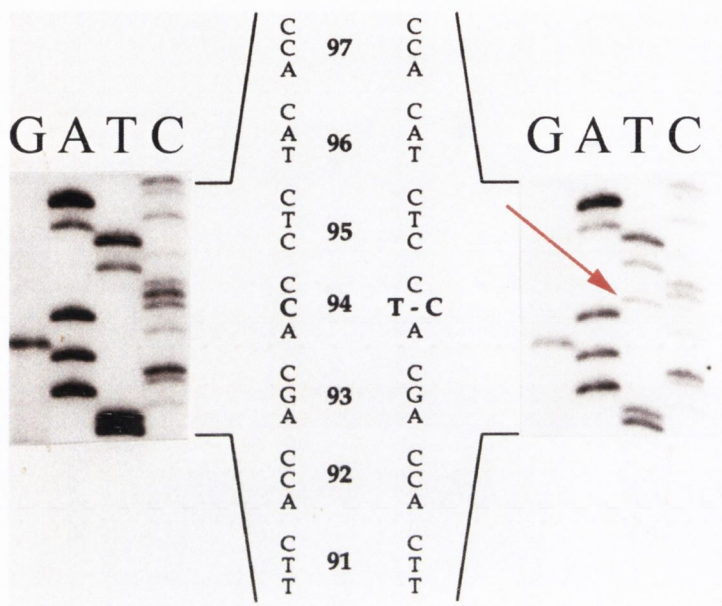


Figure 4.5: Direct sequence of the region surrounding codon 94 of the rhodopsin gene in an affected individual from TCD BC showing the C → T substitution (red arrow).

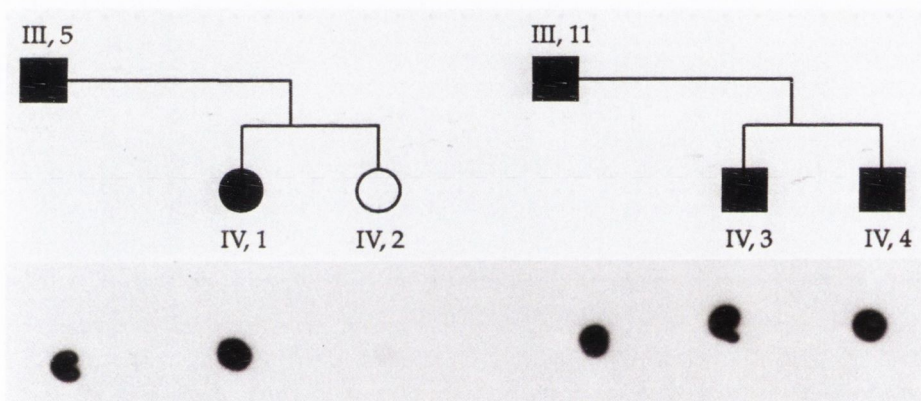


Figure 4.6: $\gamma^{32}\text{P}$ end-labeled allele specific oligonucleotide (ASO) for the mutant (5' CACCAGCATCCTCTACACCTC 3') sequence was used for hybridization to 2 μl of PCR-amplified exon 1 samples, dot blotted onto Hybond N nylon membranes. The mutant ASO hybridizes only to the DNA of affected individuals.

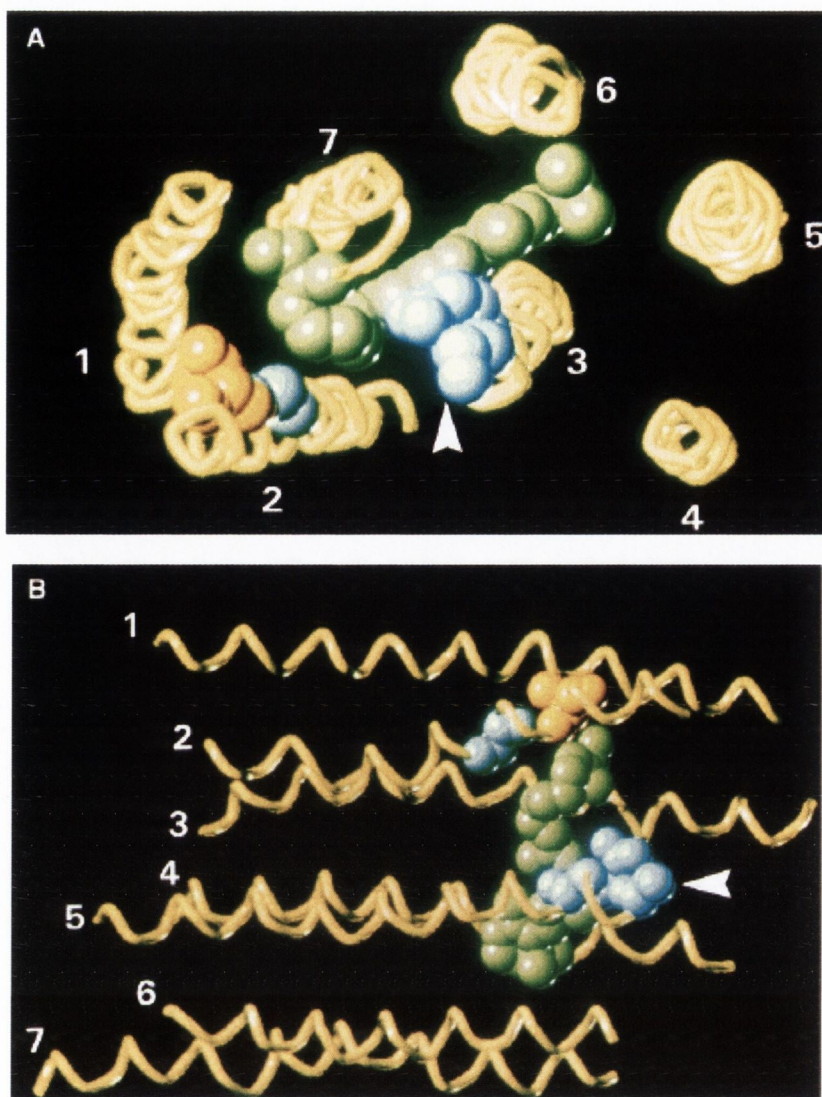


Figure 4.7. **A:** Computer modeling of the rhodopsin molecule, as viewed from above. **B:** Computer modeling of rhodopsin shown from a side-on perspective. Transmembrane domains (*yellow*) are numbered 1–7. The retinal chromophore attached to Lys 296 is shown in *green*. Its counterion, Glu 113, is shown in *blue* (*arrowed*). Thr 94 is shown in *orange*, and below it Gly 90 (*blue*).

Chapter 5

Rhodopsin, The Nature of Dominance and Possible Treatment Modalities for Autosomal Dominant Retinopathies

5.1 The Retina:

The retina, ‘an approachable part of the brain’ (Dowling 1987) was first described by the Irish physician Arthur Jacob in 1819. Classically, it is described as a ten-layered structure (Fig. 5.1). The cells which are most intimately associated with visual transduction are the photoreceptor cells, rods (approximately 125 million) and cones (approximately 6.5 million). The cones are particularly dense in the macular area, reaching their highest density at the fovea, whilst the rods predominate in the peripheral retina.

The outer nuclear layer contains the cell bodies of the rods and cones while the inner nuclear layer contains the cell bodies of the bipolar, horizontal and amacrine cells. The third cellular layer is the ganglion cell layer containing the cell bodies of ganglion cells and displaced amacrine cells. Connecting these nerve cell layers are inner and outer plexiform layers where synaptic contacts are made.

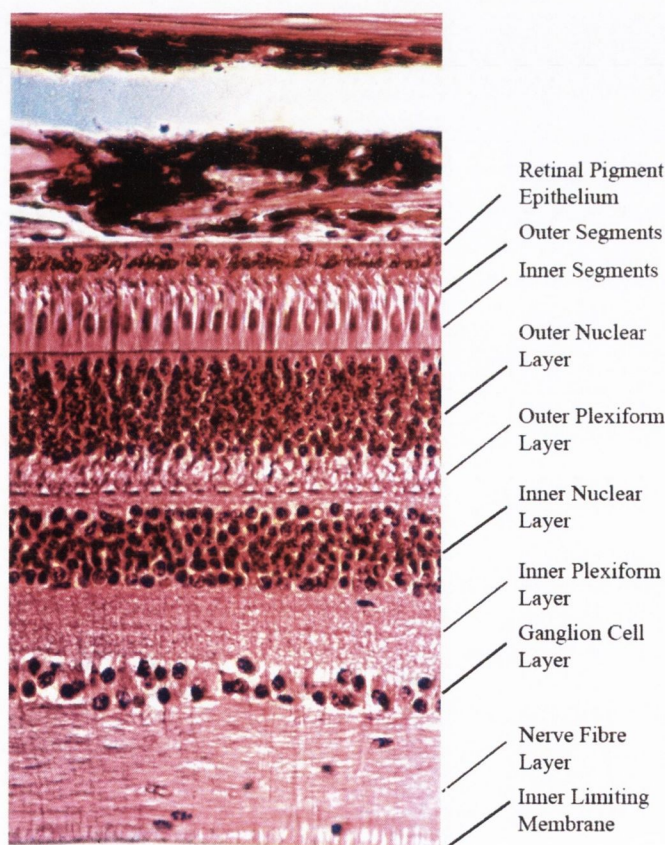


Figure 5.1 The Retina (Adapted from ‘thalamus.wustl.edu/course/eyeret.html’)

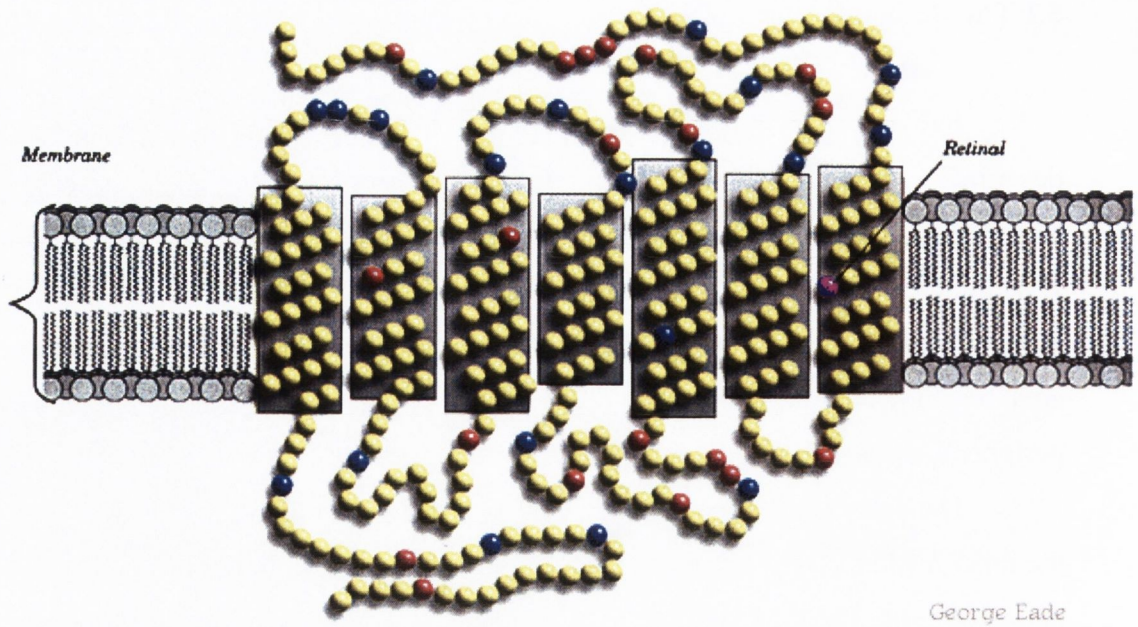


Figure 5.2: Rhodopsin

<http://biology.kenyon.edu/courses/biol114/Chap09/Chap09A.html>

5.2 Rhodopsin

Rhodopsin (Fig. 5.2), the most abundant protein in the rod outer segments and the key element in visual transduction, is a member of the G-protein coupled receptor (GPCR) superfamily. Similar to other members of the family it has a 7 transmembrane helical structure. It is composed of the 348 amino acid residue apoprotein opsin and the 11 *cis*-retinal chromophore covalently bound to Lys-296 in the seventh transmembrane helix, through a protonated Schiff base (PSB) linkage (Hargrave 2001; Pepe 2001; Menon *et al.*, 2001; Sakmar *et al.*, 2002).

Phototransduction (Fig. 5.3) occurs through the capture of light which results in the isomerization of the retinal via the 11–12 C–C bond to the *all-trans* form. This is one of the fastest transformations in biology, occurring in a matter of femtoseconds. The molecule adopts a new shape upon activation. This active form of rhodopsin, metarhodopsin II (MetaII), which has retinal still covalently linked to opsin, activates the trimeric GTP-binding protein transducin. The major hallmarks of activation of the rhodopsin molecule are the outward movement of helices three and six, and the opening of a binding site for transducin between the cytoplasmic loops. MetaII promotes the exchange of bound GDP for GTP at the α -subunit of transducin.

As a result, the α -subunit dissociates from the $\beta\gamma$ -subunits of transducin. The α -subunit of transducin binds to and removes an inhibitory subunit from phosphodiesterase (PDE) resulting in its activation and causing cGMP hydrolysis to GMP. Deactivation of rhodopsin involves multiple phosphorylations of the C-terminal region by rhodopsin kinase and binding by arrestin which competes for transducin binding sites. In order to enter a new activation cycle, rhodopsin must be dephosphorylated, and exchange the used all-*trans* retinal for a fresh 11-*cis* isoform. Deactivation of rhodopsin occurs via Meta II decay to opsin directly or, alternatively, via another intermediate, Meta III.

In the dark, the intracellular concentration of cGMP is high. cGMP binds to ion channels in the plasma membrane of the photoreceptor cell to maintain them in the open configuration allowing the influx of ions into the outer segment. This influx is primarily composed of Na^+ ions, but a small proportion is also accounted for by Ca^{++} ions. At the same time K^+ ions leave the inner segment through K^+ channels. Due to this circulating current along the rod photoreceptor, the so-called dark-current, the membrane potential of the photoreceptor cell is slightly depolarized. This depolarization releases the neurotransmitter glutamate from the synaptic terminal onto postsynaptic cells in the retina, the horizontal and bipolar cells. Hydrolysis of cGMP by activated PDE results in a fall in intracellular cGMP concentration and the consequent closure of the cyclicGMP-gated channels. Since current stops flowing into the cell, the cell hyperpolarizes and glutamate release ceases. This hyperpolarization signal, following processing in the retina and in higher visual centres, is eventually transmitted to the visual cortex.

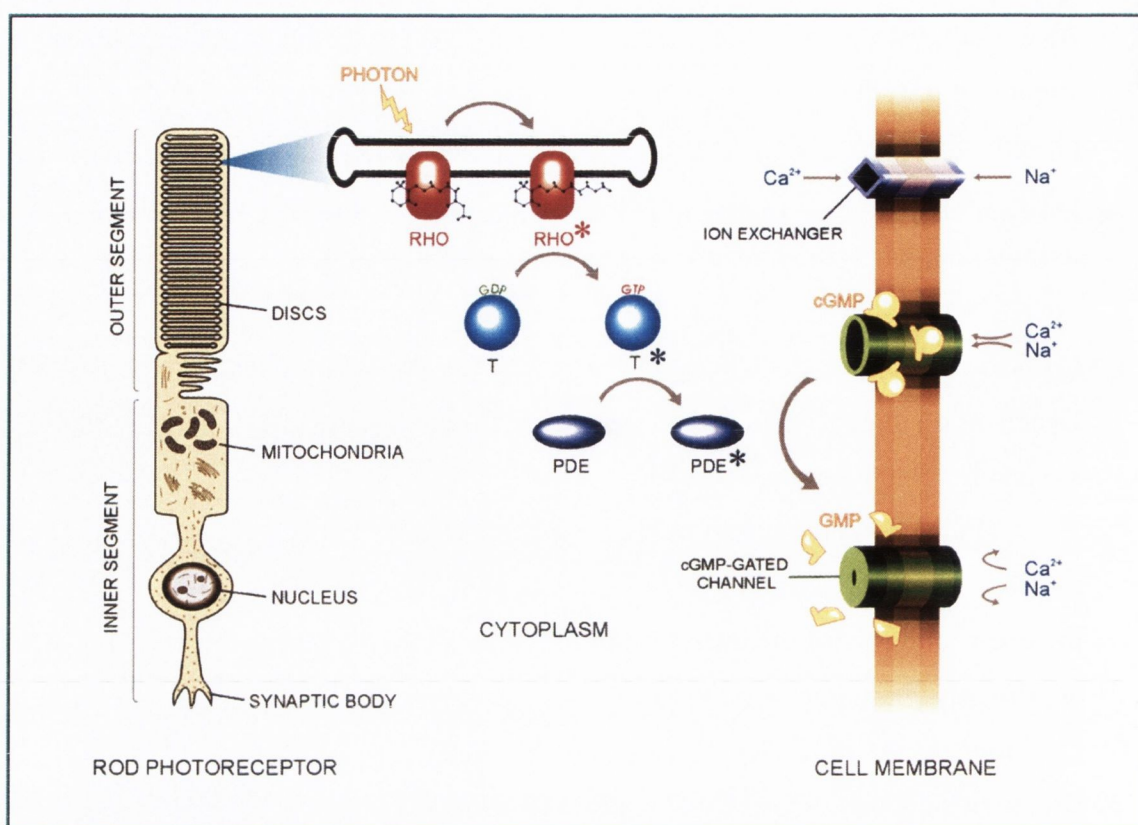


Figure 5.3: The Phototransduction cascade (Illustration courtesy of Dr. A. Kennan)

5.3 Rhodopsin Mutations

Following our report of tight linkage between DS347 and adRP in family TCDM1 and our identification of rhodopsin as a key candidate gene Dryja *et al.*, (1990) reported the first mutation in adRP, a Pro to His change at codon 23. Haplotype analysis indicated that this high frequency results from a founder effect. A study by Farrar *et al.*, (1990) of DNA from adRP families in Europe failed to detect this mutation, suggesting the *de novo* mutation event occurred in North America.

In the past 15 years over 120 different mutations, the vast majority of which are missense point mutations, have been reported in retinitis pigmentosa families, all but two of which have been documented in autosomal dominant kindreds. The latter two mutations have been reported in autosomal recessive forms of the disease (Dryja *et al.*, 1992; Greenberg *et al.*, 2003). The distribution of these mutations is across the entire span of the gene, including the intradiscal, transmembrane and cytoplasmic

domains. This allelic heterogeneity is noteworthy in what is a relatively small gene and would suggest that the structure of the rhodopsin protein is so exquisitely designed that almost any perturbation results in deleterious consequences.

Mutant rhodopsins have been broadly divided into two classes based on their different types of behaviour when expressed in cell culture (Sung *et al.*, 1991; Sung *et al.*, 1993).

Class I mutant rhodopsins resemble the wild-type protein in their plasma membrane localization and ability to complex with 11 *cis*-retinal. Mutations in the C terminal of the protein account for many of these mutants and seem to result in defective post-Golgi trafficking to the outer segments resulting in photoreceptor demise.

Misfolding of the protein characterizes the Class II mutants. This misfolding, by altering the 3-D structure of the protein, may interfere with the ability of the opsin to bind 11 *cis*-retinal. The mutant protein is also liable to be retained in the endoplasmic reticulum (ER).

The Tyr178Cys mutation, which we reported in 2 of our adRP families, was expressed by Sung *et al.*, (1991) in HEK cells. Immunoblot pattern analysis showed that the mutant Tyr178Cys protein accumulated to significantly lower levels when compared to wild-type rhodopsin. When membranes from the transiently infected cells were incubated with an excess of 11 *cis*-retinal, the Tyr178Cys mutant protein failed to produce detectable photopigment. When they examined the localization of the mutant protein they found that it accumulated in the endoplasmic reticulum and was notably absent from the plasma membrane where wild-type rhodopsin localized when similarly expressed in HEK cells. Based on these findings this mutant rhodopsin would fall in to the Class II category.

Mendes *et al.*, (2005) have recently proposed an updated classification of rhodopsin mutants based on behaviour and folding characteristics (Figure 5.4):

Class I mutants fold normally but are not transported to the outer segment.

Class II mutants, as originally described by Sung and co-workers, misfold and are retained in the endoplasmic reticulum.

Class III mutant proteins are folded correctly but affect endocytosis.

Class IV mutant rhodopsins are also correctly folded but affect stability and post-translational modification.

Class V mutants fold correctly but increase transducin activation.

Class VI rhodopsin mutants fold correctly and show constitutive activation in the absence of the chromophore and in the absence of light. The Thr94Ile rhodopsin mutation, which we reported in an Irish family with congenital stationary night blindness, would fall into the category

The ‘unclassified’ mutant category is reserved for those mutants whose folding or behaviour have not yet been elucidated.

The Met207Arg rhodopsin mutation found in TCDM1 has not been studied in expression systems to the same degree as Tyr178Cys. Consequently, according to the revised classification suggested by Mendes *et al.*, this mutant would be ‘unclassified’.

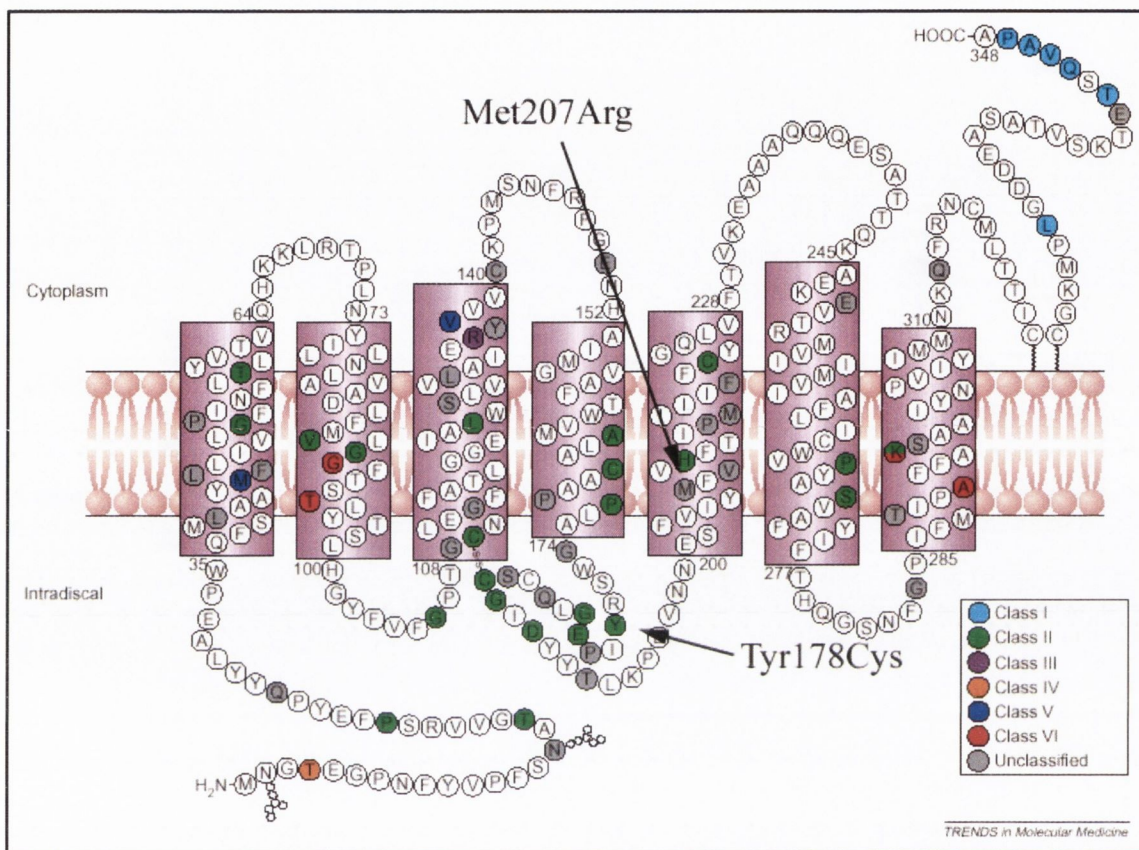


Figure 5.4: A diagram of Rhodopsin, showing the locations of the Met207Arg and Tyr178Cys mutations (Adapted from Mendes *et al.*, 2005)

5.4 Possible Modes of Action in adRP

The mechanisms by which a mutant rhodopsin exerts its pathological effect in the context of a dominant inheritance pattern where both mutant and wild-type protein are both expressed in equal amount are of intrinsic interest but are also of importance in the context of the development of possible therapies.

A number of potential mechanisms have been suggested. These can broadly be divided into 1) loss of function, 2) gain of function or 3) dominant negative.

5.4.1 Loss of Function

Studies of mice which are heterozygous for wild-type rhodopsin show that retinal morphology and function are essentially normal (Humphries *et al.*, 1997). Supporting the hypothesis that insufficient quantities of normal protein are not responsible for the retinal degeneration in adRP patients is the fact that a null mutation in the rhodopsin gene has not yet been identified in any adRP patients.

5.4.2 Gain of Function

It is widely accepted that photoreceptor cell loss in retinitis pigmentosa is most likely the result of programmed cell death or apoptosis (Portera-Cailliau *et al.*, 1994). A number of different mechanisms (Wilson and Wensel 2003; Mendes *et al.*, 2005) have been described by which dominantly acting mutant rhodopsins might act to result in photoreceptor demise.

5.4.2.1 Aggregation and Inclusion Formation

The mutant protein may have a direct toxic effect on the photoreceptor cell. This putative ‘gain of function’ has been supported by the evidence that misfolded mutant rhodopsin, when expressed in cell expression systems, forms cytoplasmic aggregates which become sequestered in aggresomes and that these can interfere with the ubiquitin proteasome system (Saliba *et al.*, 2002). This system has a vital role to play in maintaining cell viability. It is possible that inhibition of this system by mutant opsin might initiate a cascade which results in apoptosis. Recent studies

(Illing *et al.*, 2002; Saliba *et al.*, 2002; Chapple and Cheetham, 2003) have shown that Pro23His mutant rhodopsin, the most frequent RP mutation found in adRP patients in North America, is extremely prone to aggregation. These aggregates accumulate near the centrosomes and form aggresome-like structures. These are subsequently ubiquitinated, recruit cellular chaperones, and disrupt the intermediate filament network.

The accumulation of prefibrillar amyloid aggregates has been shown to be cytotoxic in murine fibroblasts due to activation of Ca^{++} and reactive oxygen species pathways (Bucciantini *et al.*, 2004). A similar process, resulting in photoreceptor cell death, might occur due to accumulation of mutant opsin aggregates.

Mutant opsin aggregates might trap other photoreceptor proteins such as wild-type rhodopsin. Depletion of vital cellular proteins has been postulated as a mechanism for cell death in a number of degenerative neurological diseases. Chen *et al.*, (2004) showed that this mechanism may be the cause for the cone/rod degeneration which is a feature of Spinocerebellar ataxia type 7. In transgenic mice they detected co-precipitation of the ataxin-7 protein and the homeodomain transcription factor Crx. The aggregated proteins functionally interacted by co-occupying the promoter and enhancer regions of Crx-regulated retinal genes *in vivo* resulting in retinal degeneration.

5.4.2.2 Post-Golgi Trafficking

Sung *et al.*, (1994) showed that Class I rhodopsin mutants were not transported properly to the rod outer segments. Incorrectly localized mutant rhodopsin might overwhelm the normal vesicular trafficking mechanisms thus interfering with transport of proteins vital for proper cellular functioning. The large amount of mutant protein in the plasma membrane might disrupt normal synaptic transmission. Alternatively, the metabolic burden of dealing with continuous degradation of mutant protein might initiate the apoptotic cascade.

5.4.2.3 Endocytosis and Arrestin Binding

Chuang *et al.*, (2004) demonstrated structural and functional impairment of endocytic pathways by mutant rhodopsin-arrestin complexes in retinitis pigmentosa. These complexes might interfere with the interplay between different endocytic compartments, resulting in an imbalance in nutrient levels or in failure to remove MetaII from the rod inner segment.

5.4.2.4 Constitutive Activation

Constitutive activation has been postulated as a cause for the night blindness seen in the relatively benign condition termed congenital stationary night blindness (CSNB). In only one form of adRP, namely that due to a point mutation at codon 296 which interferes with 11 *cis*-retinal binding, has this mechanism been postulated. Lem and Fain (2004) hypothesized an intriguing theory to explain why a common mechanism might result in two quite radically different clinical entities. They suggested that it was the continuous nature of the activation which occurs in the 296 adRP mutant which accounted for the retinopathy. In the constitutive activation seen in CSNB, such as in the Gly90Asp, Ala292Glu and Thr94Ile rhodopsin mutations, the activation could be terminated, even if partially or intermittently by binding of the mutant opsin to 11 *cis*-retinal. These mutant proteins are capable of binding to 11 *cis*-retinal albeit at varying rates. The intermittent nature of the activation may be the factor protecting the photoreceptors from degeneration. In Leber's Congenital Amaurosis (LCA) due to mutations in the RPE65 gene 11 *cis*-retinal is not synthesized and as a consequence the opsin cannot bind to the chromophore. Woodruff *et al.*, (2003) suggested that constitutive transducin-mediated signaling by unbound opsin is responsible for the severe retinal degeneration seen in this condition.

5.4.2.5 The Unfolded Protein Response

Correct functioning of the endoplasmic reticulum (ER) is vital for cell homeostasis. As a consequence of cellular stress (e.g hypoxia or the accumulation of misfolded mutant protein) an unfolded-protein response (UPR) is initiated by the ER (Rutkowski and Kaufman, 2004). The UPR can alleviate ER stress by reducing the accumulation of misfolded proteins by induction of ER-localized chaperones, molecules which protect cells by their ability to fold, disaggregate, and refold damaged proteins or by their ability to facilitate degradation of damaged proteins. However, if ER stress cannot be alleviated, the UPR can result in apoptosis via CCAAT/enhancer binding protein (C/EBP), caspase activation, Ca^{++} release and mitochondrial signaling. Rebello *et al.*, (2004) have shown evidence that mutations in the Carbonic Anhydrase IV (CA4) gene, responsible for RP17, may induce apoptosis in the endothelial cells of the choriocapillaris by this mechanism.

5.5 Dominant-Negative Mechanisms

Mutant rhodopsin proteins may interfere in a 'dominant negative' fashion with the function, stability, assembly, folding or trafficking of the wild-type protein. Kurada *et al.*, (1998) showed the mutant rhodopsin proteins in *Drosophila* interfered with post-translational maturation of wild-type rhodopsin.

Rajan and Kopito (2005) demonstrated that when P23H mutant rhodopsin and wild-type rhodopsin were expressed in equal quantities in cell culture, replicating the situation in the photoreceptors of an adRP patient, retention and/or mislocalization of wild-type rhodopsin in the endoplasmic reticulum resulted. They showed that the mutant and wild-type proteins formed oligomeric complexes and that the mutant protein directly interfered with the maturation of the wild-type rhodopsin through the secretory pathway and that as a consequence this complex became ubiquitinated and subjected to proteasomal degradation.

These putative processes are not mutually exclusive and it is possible that the degeneration of photoreceptors in some forms of adRP may be due to two or more of these mechanisms operating concurrently and that different rhodopsin mutants might act differently in producing photoreceptor cell death. Resolving the issue of the

mechanism by which a dominantly active rhodopsin mutant acts has obvious importance in the context of possible therapies.

5.6 Possible therapies for adRP

The retina presents a number of favourable features in the context of gene therapy. The tissue is readily accessible by surgical techniques which have been validated and is contained within a chamber which is immune privileged, reducing the chances of unwanted systemic immune responses. As photoreceptors are terminally differentiated, successful integration of a therapeutic gene construct might offer the prospect of prolonged benefit.

Proof of principle has been achieved in a number of autosomal recessive retinitis pigmentosa animal models (Bennett *et al.*, 1996; Takahashi *et al.*, 1999; Ali *et al.*, 2000; Acland *et al.*, 2001; Pawlyk *et al.*, 2005; Tschernutter *et al.*, 2005). As a result of these advances the first human clinical trials of gene replacement therapy will start shortly in the early onset form of retinitis pigmentosa, Leber's Congenital Amaurosis.

Conceptually, autosomal recessive disease is the most amenable to gene replacement therapy as one is not concerned about the presence of an endogenously produced mutant protein but rather the lack of any effective protein product resulting from the homozygous gene mutation. In this situation, delivery of the normal gene has a high probability of inducing a functional rescue as has been shown in the reports referenced above and indeed in many other reports.

The situation in autosomal dominant forms of retinitis pigmentosa is altogether different. In this instance, it is the presence of the mutated protein which determines the disease pathology despite the presence of an equal quantity of the normal gene product.

The genetic heterogeneity, which has become obvious over the past 15 years since our identification of the rhodopsin gene as a candidate in adRP, would be challenge enough in designing gene-based treatments but this problem is compounded by the allelic heterogeneity demonstrated not only in rhodopsin-linked adRP alone, but replicated in other dominantly acting forms of RP such as those due to peripherin/rds, IMPDH1 and Oxygen Regulated Protein 1 (RP1). Although, in theory, a technology which was developed to treat one dominantly acting mutation

could be adapted to treat every disease-causing mutation in the same gene, the economic burden of developing such therapies would be overwhelming. The treatment of dominantly-acting gene mutations therefore requires novel strategies where the individual disease-causing mutation is not targeted for treatment (in practice there are too many) but a therapy is devised which deals globally with all potential disease-causing mutations within a single gene, enabling all mutations in the same gene to be treated by the same therapeutic.

A number of different strategies suggest themselves in this regard. The final common pathway, programmed cell death, could be targeted in which case, not only could allelic heterogeneity be overcome but genetic heterogeneity would also be less of an issue. Neurotrophic factors could be delivered in an attempt to prolong the life of degenerating photoreceptors. Of particular interest are a number of strategies in which gene therapies might deal with the primary pathology yet still overcome the enormous problems of allelic heterogeneity.

5.6.1 Anti-apoptotic Strategies

There has been some success in this regard. A number of studies have provided preliminary evidence that inhibition of important elements of the apoptotic pathway, such as the caspase family of aspartate cysteine-directed proteases, may provide protection against photoreceptor cell suicide. These proteins are synthesized as inactive zymogens and are activated by cleavage at specific aspartic acids. Once activated, they activate other caspases in a protease cascade, eventually leading to the cleavage of specific target proteins and programmed cell death.

The irreversible caspase-3 inhibitor N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone partially protected photoreceptors from degeneration when injected intravitreally into rhodopsin S334Ter transgenic rats. (Liu *et al.*, 1999).

The anti-apoptotic baculoviral gene p35 has also been shown to protect photoreceptors against apoptosis. Davidson and Steller (1998) demonstrated functional rescue of degenerating photoreceptors when the broad-spectrum caspase-inhibitory cell survival protein baculovirus p35 was expressed in the eyes of *ninaE* mutant *Drosophila*. *NinaE* is the gene responsible for rhodopsin production in *Drosophila*. When flies expressing p35 were crossed with flies carrying the *rdgC*³⁰⁶

or the *ninaE* mutations the offspring which carried either of the mutations and also expressed p35 showed no signs of retinal degeneration when compared with mutant flies not expressing the caspase inhibitor. In addition, the anti-apoptotic effect appeared to last for the entire life-span of the fly.

Bennett *et al.*, (1998) subretinally injected a recombinant replication-defective adenovirus containing the human cDNA for bcl-2, an anti-apoptotic protein, under the control of a 2.5 kb human rhodopsin upstream fragment into the rd/rd mouse in which the retinal degeneration is due to a homogyous mutation in the β PDE gene. This construct resulted in histologically detectable retinal rescue lasting 6 weeks after birth, although the rescue was not as great as that achieved by subretinal injection of an adenoviral construct containing the wild-type β PDE cDNA.

5.6.2 Neurotrophic Factors

Numerous studies have shown that a variety of neurotrophic factors can slow the rate of photoreceptor loss in animal models of retinitis pigmentosa.

Lau *et al.*, (2000) reported slowing of the retinal degeneration in the transgenic rat model carrying an S344Ter-4 opsin mutation following intraocular injection with AAV2 vector delivered fibroblast growth factor 2 (FGF-2). Curiously, while the rescue of ERG responses was greater in the FGF treated eye compared to untreated fellow eyes, they appeared no different to the responses recorded from fellow eyes treated with an AAV2 vector carrying an eGFP reporter gene, suggesting that the sub-retinal surgery itself might have had some part to play in the rescue.

McGee Sanftner *et al.*, (2001), in the same animal model, showed that subretinal injection of an AAV2 vector carrying the cDNA for glial cell line derived neurotrophic factor (GDNF) could stabilize the rod inner and outer segments and increase photoreceptor survival time with corresponding improvement in the electroretinographic responses compared to the control. Green *et al.*, (2001) demonstrated that fibroblast growth factor 5 (FGF-5) and fibroblast growth factor 18 (FGF-18) could also significantly rescue the degeneration in transgenic rats carrying either the Pro23His or the S344Ter-4 mutations. The rescue however did not result in increased electroretinographic responses.

Ciliary neurotrophic factor (CNTF) has been shown to be neuroprotective in rds and P216L transgenic mice. High levels of expression of the growth factor were noted to have adverse consequences for the electroretinographic responses indicating that levels of growth factors might need to be controlled for a beneficial rather than a deleterious effect to be achieved (Liang *et al.*, 2001, Schlichtenbrede *et al.*, 2003). CNTF is the first agent to have entered Phase I clinic trial for the treatment of retinitis pigmentosa (http://www.neurotech.fr/news_press_100803.asp).

5.6.3 Treating the Primary Genetic Defect

Intuitively, directing a therapeutic to the primary cause of the pathology would appear to offer the best prospect of sustained treatment. Since the first discovery of a disease-associated mutation in adRP considerable progress has been made in this regard and the prospects for gene-based therapies reaching the bed-side within the medium term future appear promising.

5.6.3.1 Blocking Gene Expression

Triplex-forming oligodeoxyribonucleotides (TFOs) can block gene expression by binding to transcription factor binding sites in the promoter (Postel *et al.*, 1991), by inhibiting transcription initiation (Duval-Valentin *et al.*, 1992) or by blocking progression of RNA polymerase through the coding region of a gene (Scaggiante *et al.*, 1994).

Intody *et al.*, (2000) used psoralen-modified TFOs and UVA irradiation to form photoadducts at two target sites in a plasmid expressing a rhodopsin–EGFP fusion, which was then transfected into HT1080 cells. Each TFO reduced rhodopsin–GFP expression by 70 to 80%. Treatment with both simultaneously reduced expression by 90%. They also showed that genes lacking the rhodopsin target sequences were unaffected and a psoralen–TFO not directed against any rhodopsin sequences was without effect suggesting the triple-helix approach has high specificity.

5.6.3.2 Ribozymes

Ribozymes are RNA enzymes which catalyze a variety of reactions within cells. They can be used to reduce the expression of a disease-causing gene by digesting the mRNA which it encodes (Haseloff and Gerlach, 1988; Pley *et al.*, 1994).

Ribozymes have specific requirements for a particular cleavage site. Hammerhead ribozymes for example, will cleave mRNA only at NUX sites (N, any nucleoside; U, uracil; X, any nucleoside other than G). Hairpin ribozymes have a larger catalytic core of 34 nucleotides and the targeting domains require more specificity (Earnshaw and Gait, 1997). However, being inherently more stable hairpin ribozymes function better than hammerhead ribozymes under physiological conditions. Bearing this specificity in mind only mutations causing a sequence variation at ribozyme cleavage sites are targetable by these agents if a mutation specific approach is desired.

The ability of ribozymes to effectively suppress gene expression has been successfully demonstrated *in vivo* by a number of groups. Lewin *et al.*, (1998) showed that injection of 2 microlitres of an rAAV vector, carrying either a hairpin or hammerhead ribozyme targeting P23H mutant opsin mRNA, into the subretinal space of a rat with a single copy of the Pro23His transgene, resulted in a reduction in the amount of P23H transgene mRNA and a slowing of the rate of photoreceptor degeneration for at least 3 months. Subsequently, LaVail *et al.*, (2000) showed that the rescue was prolonged for at least 8 months. More recently Liu *et al.*, (2005) reported that two different hammerhead ribozymes, targeting the mRNA of the gamma subunit of photodiesterase and delivered by subretinal injection into wild-type mice, resulted in a loss of photoreceptors and in a significant reduction in electroretinographic amplitudes.

Ribozymes, while attractive agents for suppression of mutated transcripts, are limited to targets in open loop structures of transcripts. Such limitations, considering a requirement to suppress >100 dominant mutations even within the rhodopsin gene alone, would appear to exclude such agents as tools for suppression of the majority of mutations.

5.6.3.3 RNAi

RNA interference (RNAi), a new technology that has emerged rapidly over the past four years enables potent suppression of gene expression in a sequence-specific manner. RNAi was initially identified in 1998 by Fire *et al.*, (1998) who discovered that double-stranded RNA (dsRNA) could potentially induce gene silencing in the nematode *Caenorhabditis elegans*. Short interfering RNA (siRNA) was first detected in plant systems by Hamilton and Baulcombe (1999), and subsequently in 2001 was found to operate in mammals (Elbashir *et al.*, 2001).

The RNAi pathway is a two step process. In the first step, dsRNA is processed into an siRNA by the RNaseIII enzymes Dicer and Drosha. In the second step, siRNAs are loaded into the effector complex RNA-induced silencing complex (RISC). The siRNA is unwound in a strand specific manner during RISC assembly. This single-stranded siRNA locates mRNA targets by Watson–Crick base pairing. Gene silencing is a result of the nucleolytic degradation of the targeted mRNA by the RNaseH enzyme Slicer (Argonaute).

The RNA polymerase III (Pol III) promoter-based short hairpin (sh)RNA expression systems (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002) allow long-term endogenous suppression of mRNAs. In terms of ocular disease genes, vascular endothelial growth factor (VEGF) (Reich *et al.*, 2003), which is associated with choroidal neovascularization in the “wet” form of age-related macular degeneration (AMD); transforming growth factor-beta type II receptor (TbetaRII) (Nakamura *et al.*, 2004), which is implicated in corneal haze and subconjunctival scarring after laser and glaucoma surgery; and rho/RHO (Kiang *et al.*, 2005; Cashman *et al.*, 2005) and rds-peripherin (Palfi *et al.*, 2005) which are associated with retinal degenerations (including RP) have been successfully targeted by RNAi. Moreover, shRNA vectors have been electroporated into mouse retinæ and shown to knock down expression of cone-rod homeobox and neural retina leucine zipper genes (*nrl*) (Matsuda and Cepko, 2004).

Compared to ribozyme technology, RNAi is at least as potent, is less dependent on RNA secondary structure, and, in principle, does not require any particular sequence motif. These molecules are showing promise for the down-regulation of mutated genes in dominantly acting diseases and in cancers (Miller *et al.*, 2003; Martinez *et al.*, 2002).

5.7 Mutation Independent Strategies for the Treatment of adRP

Since the identification of the first gene mutation in rhodopsin following our localization of the gene in TCDM1 to chromosome 3q, the degree of genetic and allelic heterogeneity which has come to light has been astonishing to say the least. Autosomal dominant retinitis pigmentosa may well be the most heterogeneous of all genetic diseases described to date. The extent of allelic heterogeneity is an enormous problem in the context of therapies for adRP. The economic burden of developing a therapy for each of the approximately 150 individual mutations already described in the rhodopsin gene alone would be insupportable, not to mention the regulatory nightmare involved in validating each and every mutation-dependent therapy. Apart from the P23H mutation, most of the other mutations occur at a very low frequency. While an economic case might possibly be made for development of a gene-based treatment for P23H associated adRP, none could be made for the vast majority of the others. As outlined above, the design constraints associated with ribozymes and siRNAs would also contribute to the difficulties.

With these difficulties in mind, strategies have been developed in our laboratory which are independent of the mutation and where, theoretically, one or a small number of therapeutics could treat all cases of adRP associated with a particular gene. These strategies have been validated in a number of studies utilizing ribozymes and siRNA constructs targeting transcripts derived from the rhodopsin, RDS-peripherin, collagen 1A1 and 1A2 genes (Millington-Ward *et al.*, 1997, 1999; O'Neill *et al.*, 2000; Kiang *et al.*, 2005; Palfi *et al.*, 2006), genes in which the issue of allelic heterogeneity is abundantly evident.

In the first strategy both the mutant and wild-type alleles are suppressed – the cell is thus effectively converted into a null mutant. At the same time, a replacement gene is delivered to the cell which has been engineered so that it escapes suppression. This could be achieved by targeting the 3' or 5' untranslated region (UTR) of both mutant and wild-type alleles and modifying the UTR of the replacement gene so that it escapes suppression.

The second strategy uses the fact that an amino acid may be coded for by more than one codon (e.g., both the triplets GAA and GAG code for the amino acid glutamic acid). In this instance suppression is targeted to degenerate sites in mRNA. As in the first strategy both the mutant and wild-type transcripts are targeted for

suppression and a replacement gene, modified using the degeneracy of the genetic code such that its mRNA escapes suppression but encodes for the wild-type protein, is delivered simultaneously.

The third strategem does not depend for success on engineering a suppression-resistant replacement gene but suppressing the mutant allele only. Rather than targeting the mutation itself a more common non-pathological genetic variation (polymorphism), located in close proximity to the gene mutation is used as the site for suppression. These genetic polymorphisms are much more frequent than the rare disease-causing mutation and targeting a common polymorphism offers greater scope for suppression than targeting the mutation itself.

These strategies have been evaluated *in vitro*, in cell culture and in retinal explant systems. A validation of the suppression portion of these strategies in a living animal was provided by Gorbatyuk *et al.*, (2005) who showed that a ribozyme targeting rhodopsin mRNA resulted in at least 50% reduction in mRNA and protein when subretinally injected into wild-type mice.

Subsequent to demonstration of the 'generic' nature of such approaches in animals (a mouse model expressing normal and mutant human rhodopsins on a murine null-opsin background has been generated; Olsson *et al.*, 1992; McNally *et al.*, 1999), there would be a clear rationale to put in place the elements required to proceed from animal studies toward phase 1 human clinical trials. It is of interest to note that, in this model, wild-type human rhodopsin is sufficiently conserved between mouse and humans to rescue the disease pathology present in $\text{rho}^{-/-}$ mice, therefore validating the use of $\text{rho}^{-/-}$ mice to test the future delivery of the human gene (McNally *et al.*, 1999). While described in the context of inherited retinopathies, such approaches are applicable to targeting any mutation with a dominant-negative effect. Some 1500 human autosomal dominant Mendelian diseases have been described. Also, many dominantly acting mutations underlie the cause of multifactorial diseases such as p53- or Ras-related cancers. Therapeutic intervention at the level of the primary genetic defect in diseases displaying such extensive genetic heterogeneity is thus becoming a realistic prospect. It is also notable that the strategies outlined above are not limited to hammerhead ribozymes, but could involve the use of many of the molecular tools for suppression now becoming available. Such suppression tools include antisense DNA/RNA, peptide nucleic acids, other forms of ribozymes including hairpin ribozymes, trans-splicing

ribozymes, minizymes and maxizymes, and triple helix DNA, to name a few (Sullenger and Cech, 1994; Sioud *et al.*, 1997; Kuwabara *et al.*, 1998; Praseuth *et al.*, 1999; Phylactou, 2000).

Conclusion

Despite the high degree of genetic heterogeneity inherent in disorders such as dominantly inherited RP, there are novel therapeutic approaches in research and development which may effectively circumvent such heterogeneity and thereby provide rationale therapeutics at both the biological and economic levels for this group of debilitating conditions.

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Chapter 6

Studies Utilizing the Rho^{-/-} Mouse

6.1.1 Introduction

A vital prerequisite in the study of gene-based therapies for any form of retinitis pigmentosa is the availability of suitable animal models. A number of naturally occurring animal models (Chang *et al.*, 2002) have been available for many years such as the rd mouse (Keeler *et al.*, 1924; Bruckner 1951 [quoted in Pittler *et al.*, 1993]) and the RCS rat (Dowling *et al.*, 1962) and indeed one of these, the rds mouse, in which a retinal degeneration develops due to the insertion into exon II of the rds gene of a 9.2-kb repetitive genomic element that is very similar to the t haplotype-specific element in the H-2 complex (Ma *et al.*, 1995), was of seminal value to us in our identification of mutations in the equivalent gene in humans as causative of adRP (Farrar *et al.*, 1991; Farrar *et al.*, 1991; Jordan *et al.*, 1992; Jordan *et al.*, 1992).

Since the first identification of a retinopathy-associated gene in humans a large number of transgenic models have been generated (Dalke and Graw 2005). A number of these models are available in our laboratory.

In this chapter and the following chapter, work which I have carried out on a number of mouse models of inherited retinopathies will be described.

6.1.2 Electroretinography

The electroretinogram (ERG) is of immense value in objectively assessing retinal function and is widely used in clinical practice. It is a mass retinal potential which is recorded from the corneal surface of the eye. The waveform generated by a brief flash is in fact a complex amalgam of waveforms generated by various portions of the retina.

Three components of the waveform can typically be differentiated (Fig 6.1 normal ERG). Following flash stimulation there is an initial negative deflection termed the a wave. The early portion of this a wave is due to photo-transduction activity in the rod and cone photoreceptors and the later portion reflects activity in inner retinal components. The a-wave is followed by a larger, cornea-positive deflection termed the b wave. This component of the electroretinogram reflects activity in cells post-synaptic to the photoreceptors. A third component is occasionally observable in mice. This is c wave, a cornea-positive deflection with a

longer time course than the b wave. This reflects the interaction between the response of Müller cells and the hyperpolarization of the apical membrane of the RPE that occurs in response to a decrease in potassium concentration in the subretinal space (Steinberg *et al.*, 1983).

6.1.3 Protocol for Mouse Electroretinography

The protocol for rodent Ganzfeld electroretinography is as follows: The animal is dark adapted for 12 hours and prepared for electroretinography under dim red light produced by means of a Petzl Tactikka Plus™ head-mounted torch.

Pupillary dilatation is achieved by instillation of Cyclopentolate 1% and Phenylephrine HCL 2.5% which are administered topically to each eye 5 minutes prior to anaesthesia. The subject is anaesthetized by means of Ketamine (80mg per kg) and Xylazine (16mg per kg). The anaesthetics are diluted in 0.9% saline and administered by intraperitoneal injection. The electroretinogram is performed 10 minutes following administration of the anaesthetics so that a steady state of anaesthesia is achieved. This delay also allows the topical mydriatics to achieve maximal pupillary dilation.

The subject is placed on a TC 1000 Temperature Controller (CWE Inc, Ardmore, PA 19003 US) heating pad to maintain a constant body temperature of 37°C +/- 0.5°C.

The ERG responses are recorded simultaneously from both eyes by means of small contact lens electrodes specifically designed for rodents (Medical Workshop BV, Groningen, Netherlands) and placed on the corneas, using Amethocaine 1% as topical anaesthesia and Vidisic™ gel (Dr. Mann Pharma, Berlin, Germany) to maintain corneal hydration (Figure 6.2).

Standardized flashes of light are presented to the mouse in a Ganzfeld bowl to ensure uniform retinal illumination. A gold reference electrode is positioned subcutaneously through each cheek and the ground electrode is inserted subcutaneously near the tail. The responses are analysed using RetiScan RetiPort electrophysiology equipment (Roland Consulting GmbH, Brnadenburg, Germany).

In the standard protocol (based on that approved by the International Clinical Standards Committee for human electroretinography) rod-isolated responses are recorded using a dim blue flash presented in the dark-adapted state. The maximal

combined rod/cone response to the maximal intensity flash is then recorded. Following light adaptation for 10 minutes to a background light of 30 candelas per m^2 presented in the Ganzfeld bowl the cone-isolated responses are recorded. The a-wave is measured from the baseline to the trough and the b-wave from the baseline (in the case of rod-isolated responses) or from the a-wave trough.

This protocol is the same as that used for electroretinography on human patients. In the case of human subjects general anaesthesia is not required, the procedure being conducted entirely under topical corneal anaesthesia.

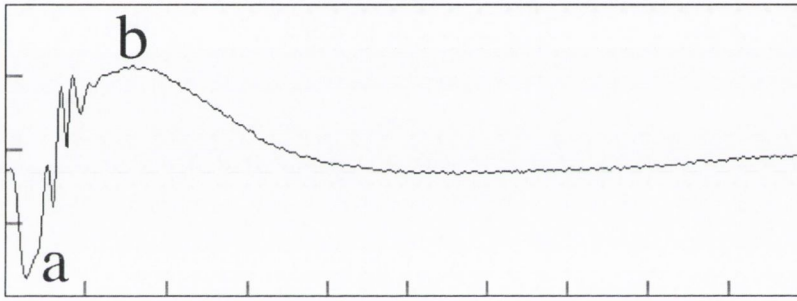
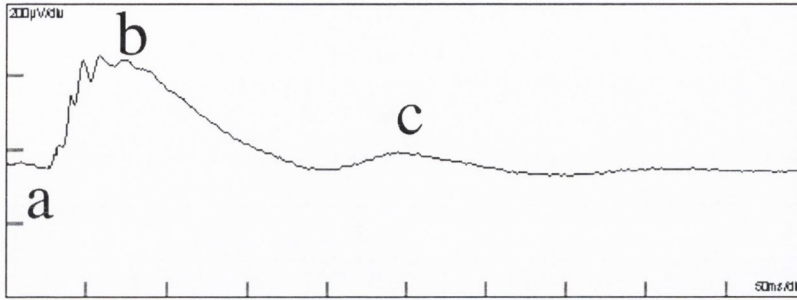


Figure 6.1: Top Panel: A wild-type murine rod-isolated response showing the prominent b wave and the c wave.
 Bottom Panel: A wild-type murine dark-adapted mixed rod and cone response showing prominent a and b waves.



Figure 6.2: A mouse set up for the recording of an electroretinogram. Note the contact lens electrodes *in situ*.

6.2 Structural and Functional Rescue of Murine Rod Photoreceptors by Human Rhodopsin Transgene

The work described in this section was published in Human Molecular Genetics in 1999 (McNally N, Kenna P, Humphries MM, Hobson AH, Khan NW, Bush RA, Sieving PA, Humphries P, Farrar GJ. Hum Mol Genet. 1999 Jul;8(7):1309-12).

6.2.1 Introduction

In the context of gene-based treatments for autosomal dominant forms of RP due to mutations in the rhodopsin gene, curiously, to the best of my knowledge, a naturally occurring mutant has never been described in rodents. Recently, a naturally occurring canine model of adRP due to a mutation in rhodopsin has been described (Kijas *et al.*, 2002).

In order to better understand the functional and structural role of rhodopsin in the normal retina and in the pathogenesis of retinal disease and to provide a model to assess the therapeutic potential of re-introducing functional rhodopsin genes into degenerating retinal tissues, investigators in our laboratory, by means of homologous recombination, introduced a neomycin cassette at codon 135 in exon II of the mouse rhodopsin gene, thus disrupting rhodopsin gene function (Humphries *et al.*, 1997). Homozygous $Rho^{-/-}$ mice were found not to elaborate rod outer segments, losing their photoreceptors gradually over a 3 month period. At 90 days the outer nuclear layer in these animals is reduced to a single layer of nuclei, presumably surviving cone photoreceptors. No rod derived electroretinographic responses could be recorded from the homozygous animals. Heterozygous $Rho^{+/-}$ animals retained the majority of their photoreceptors although the inner and outer segments of these cells displayed some structural disorganization, the outer segments becoming shorter in older mice.

As a prime utility of this knockout animal would be as a resource in the construction of animal models of human autosomal dominant retinopathies it was of interest, and of some importance, to determine if the human rhodopsin gene could rescue the phenotype in the knockout mouse. With this in mind, we crossed homozygous $Rho^{-/-}$ mice with mice expressing a wild-type human rhodopsin

transgene at levels approximating those of the endogenous protein (Olsson *et al.*, 1992). This mouse was kindly provided by Dr.T. J. Dryja. Electroretinographic analysis by Paul Sieving and Ron Bush at the Kellogg Eye Institute, University of Michigan, Ann Arbor USA showed that the rescued mice had 75% normal maximal amplitudes and b wave thresholds within 0.5 log unit of normal wild-type at 20 weeks of age. Immunocytochemical analysis of cryosectioned retinas using a fluorescent anti-rhodopsin antibody showed that rhodopsin was detectable in the wild-type and human transgene rescued animals by post-natal day 3. Immunofluorescence was confined to the rod outer segments and the results appeared similar in both wild-type and transgene rescued mice.

Histological examination of 1 μ m retinal sections from wild-type and transgene rescued animals showed outer nuclear layers that were virtually indistinguishable one from the other as regards thickness and nuclei counts. The rod photoreceptor inner and outer segments were remarkably similar in both types of mouse (Fig 6.3).

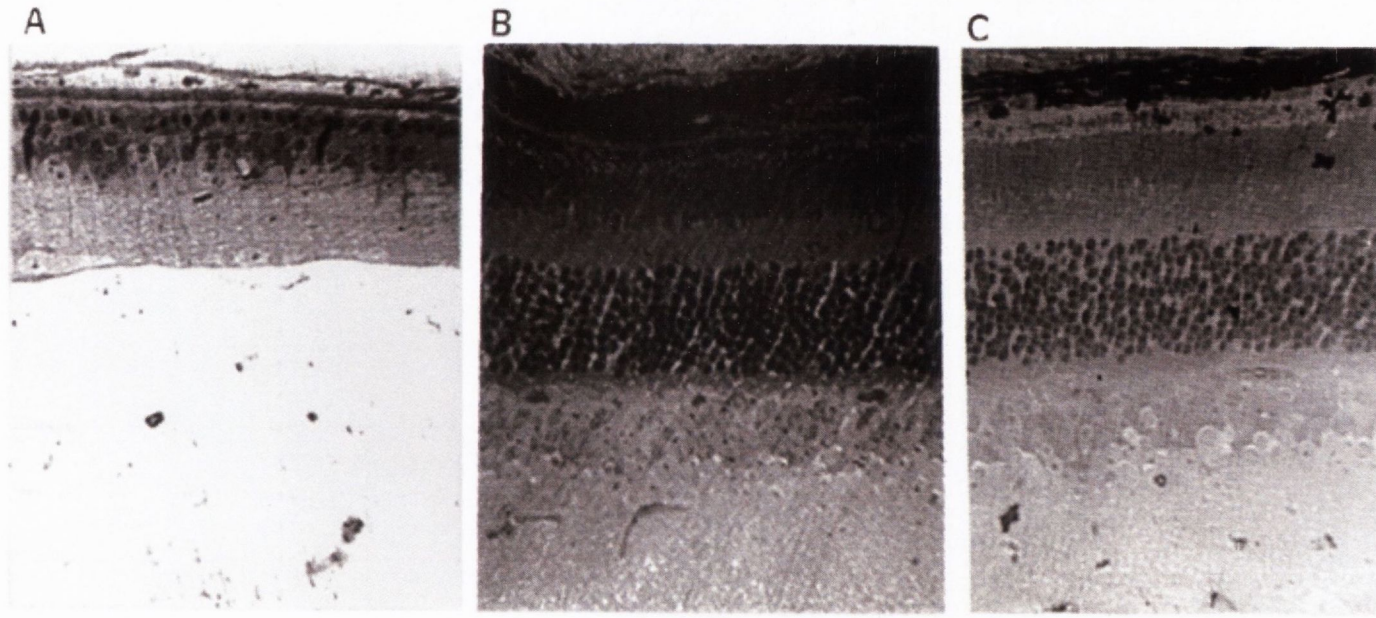


Figure 6.3: Resin-embedded sections of the retinas of 3-month-old $Rho^{-/-}$ (A) and wild-type mice (B), and of retinas from $Rho^{-/-}$ mice rescued by human transgene (C).

6.3 Apoptotic Photoreceptor Death in the Rhodopsin Knockout Mouse in the Presence and Absence of *c-fos*.

The work described in this section was published in Experimental Eye Research in 2000 (Hobson AH, Donovan M, Humphries MM, Tuohy G, McNally N, Carmody R, Cotter T, Farrar GJ, Kenna PF, Humphries P. Exp Eye Res. 2000 Sep;71(3):247-54.)

6.3.1 Introduction:

Apoptosis is a mode of programmed cell death that allows a cell to partake in its own demise. As a genetically regulated process it offers unique possibilities for therapeutic intervention in a genetically heterogeneous disorder such as retinitis pigmentosa. Apoptosis in retinal neurons has been reported in a number of settings (Portera-Cailliau *et al.*, 1994, Lolley *et al.*, 1994; Hafezi *et al.*, 1998). The normal development of the mammalian brain requires apoptotic death of a significant fraction of newly generated neurons. The retina, “an approachable piece of the brain”, is no exception and developmental death in the retina is clearly evident in the studies of several researchers (Young *et al.*, 1984; Penfold and Provis 1986).

Independent studies of cell death in the retina all lead to the same conclusion: apoptosis is the “final common pathway” of death of photoreceptor cells in mature retinas that are perturbed either by certain mutations (Portera-Cailliau *et al.*, 1994) or, by a variety of external stimuli, e.g., excessive light, hypoxia or lead (Chang *et al.*, 1995; Cook *et al.*, 1995; Fox *et al.*, 1997 and Shahinfar *et al.*, 1991).

The main symptom of any retinopathy is visual disturbance without pain, which is implicative of the absence of inflammation despite cellular loss. Apoptotic cell death does not provoke an associated inflammatory response since dying or dead cells are rapidly phagocytosed, either by neighboring healthy cells or macrophages. Delaying the apoptotic process may provide a broader window of opportunity in which to implement a gene-based therapy directed at the primary insult or may in itself be sufficient to result in a significant extension in cone cell viability.

In the naturally occurring rodent model of degenerative retinal disease, the rd mouse, carrying a mutation within the β -subunit of rod photoreceptor cyclic GMP phosphodiesterase, and in the rds mouse, carrying a mutation in the peripherin gene,

apoptosis has been shown to be the mode of death in photoreceptor neurons (Lolley 1994; Portera-Cailliau *et al.*, 1994). Studies of a human rhodopsin mutation causing RP, threonine-17-methionine, have shown that in a majority of individuals carrying this mutation, the retinal degeneration is for the most part confined to the inferior hemi-retina. In the superior retina, rods and cones survive and function normally. Thus patients experience loss of the superior visual field whilst the inferior field is preserved. Studies of a patient's retina at post mortem have directly provided the first evidence of apoptosis in the human eye (Li *et al.*, 1994). Clearly, rod photoreceptors carrying the threonine-17-methionine mutation can survive for several decades if cellular death can be prevented.

In recent years, many important regulators of apoptosis have been identified, including the baculoviral gene product p35. p35 appears to interact with and inhibit the action of key cysteine proteases (caspases) involved in the execution of the apoptotic cell death cascade (Clem and Miller 1994). *Drosophila* homologues of the baculovirus p35 gene were observed to block cell death (Hay *et al.*, 1995). Significantly, Davidson and Steller, (1998) have been successful in producing a transgenic *Drosophila* carrying the p35 gene under the control of an eye-specific promoter. Crossing the p35 transgenic fly onto age-related models of retinal degeneration achieved repression of apoptotic death in these systems.

Manipulation of apoptotic regulatory components has proven successful in the retardation or prevention of retinal degeneration in several experimental systems. Anti-apoptotic genes and factors tested in such systems include over-expression of *bcl2* (Chen *et al.*, 1996), lack of *c-fos* (Hafezi *et al.*, 1997), absence of p53 (Ali *et al.*, 1998), peptide inhibition of caspase activation (Davidson and Steller 1998), and injection of α FGF (fibroblast growth factor), and β -FGF (LaVail *et al.*, 1992). In view of the fact that the *Rho*^{-/-} mouse is likely to be of substantial value in explorations of methods for genetic intervention in RP in man, it is necessary to know more about the time course of apoptosis in these animals. In light of the fact that in light-induced mouse model systems apoptosis was essentially completely inhibited on a *c-fos*^{-/-} genetic background (Hafezi *et al.*, 1997), we were interested to know whether there is any difference in the progression of photoreceptor degeneration in *Rho*^{-/-} *c-fos*^{+/+} and *Rho*^{-/-} *c-fos*^{-/-} animals.

6.3.2 Materials and Methods

Homozygote $Rho^{-/-}$ mice (Humphries *et al.*, 1997) were crossed with heterozygous $c-fos$ mice ($c-fos^{+/-}$, C57BL/6J background: Jackson Laboratory, Bar Harbor, ME) (Johnson *et al.*, 1992). F1 mice with the genotype $Rho^{+/-} c-fos^{+/-}$ were crossed with each other. F2 mice of the genotype $Rho^{-/-} c-fos^{-/-}$ and $Rho^{-/-} c-fos^{+/-}$ were used in this study. $Rho^{+/-} c-fos^{+/-}$ mice were used as age-matched controls.

6.3.2.1 Genotyping of Mice by PCR

Genomic DNA was isolated from mouse tails and the rhodopsin and $c-fos$ alleles were identified by polymerase chain reaction (PCR). Genotypic analysis of mice for the rhodopsin mutation was carried out as previously described (Humphries *et al.*, 1997). PCR amplification conditions for the $c-fos$ genotype were 1 minute at 94°C, 1 minute at 62°C and 1.2 minutes at 72°C for 35 cycles. Primers used to amplify the intact $c-fos$ gene were 5'-CAACGCCGACTACGAGGCGTCAT 3' and 5' - CAAGTGTGCACGCGCTCAGACAA-3' and resulted in a 299 base pair product. Primers used to amplify the targeted $c-fos$ neomycin locus were 5' TAAACGCACGGGTGTTGGGT 3' and 5' CCCCTGCGAGTCACACCCCAG 3' and resulted in a 190 base pair product.

6.3.2.2 Retinal Histology

Animals were euthanased under CO₂. The eye was gently proptosed by placing a curved forceps behind the eye. A small cautery mark was made on the cornea of each eye at the 12 o'clock position using a Solan low temperature cautery (Xomed Surgical Instruments Inc, Jacksonville, USA). Each eye was fixed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde overnight at 4°C. Following overnight fixation the eyes were trimmed of the extraocular muscles and bisected through the optic nerve and the cautery mark. A wedge-shaped incision was made in the cornea of each hemi-globe, in the region of the cautery mark, so that the superior-inferior orientation of subsequent sections could be confidently identified. The hemi-cups were washed six times for 10 minutes in 0.1M phosphate buffered

saline (PBS) pH7.2. The tissue was soaked in 1% Osmium Tetroxide in 0.1M PBS pH7.2 for 30 minutes. Serial washes for 1 hour in 50%, 75%, 95% Ethanol were followed by three washes in 100 % Ethanol for 30, 45 and 45 minutes respectively. The tissue was then washed three times in propylene oxide for 30, 45 and 45 minutes respectively. The eye cups were then soaked in a solution containing 50% Propylene oxide and 50% Agar 100 resin (Agar Scientific Ltd. Cambridge UK) for 2 hours. This was followed by incubation in full strength resin for 4 hours. The hemi-cup was mounted in a BEEM capsule with the conical end removed; the cut surface of the specimen being positioned flush with the cap. The capsule was then filled with fresh resin that was subsequently polymerized at 65°C for 24 hours.

6.3.2.3 Thin sectioning of mouse retinas

0.5µm sections were obtained using a Reichert-Jung Ultracut microtome. Sections were stained with Toluidine Blue (1% sodium Borate (aqueous), 1% Sodium Azure, 1% Toluidine Blue). The orientation of the section was determined by identifying full-length Müller cell processes.

6.3.2.4 *In Situ* End-Labeling of Fragmented DNA (carried out by Dr. Mary Ann Donovan and Tom Cotter)

DNA strand breaks in photoreceptor nuclei were detected by *in situ* end labelling (TUNEL). Enucleated eyes were fixed in 10% buffered formalin for 24 hours, dehydrated, processed and embedded in paraffin. 5µm sections were incubated in 50µl of reaction buffer containing 2.5mM CoCl₂, 0.1U/ml terminal deoxynucleotidyl transferase (TdT) in a 0.1M Na Cacodylate (pH 7.0) buffer and 0.75nM Fluorescein-12-dUTP (Boehringer Mannheim, Germany). These sections were incubated at 37°C for 1 hour in a humidified chamber. Following several washes in PBS, the sections were mounted in Mowiol (Calbiochem) and viewed under a fluorescent microscope (Nikon Eclipse E600) using a fluorescein isothiocyanate (FITC) filter. Three animals of the genotype Rho^{-/-} were used for each time point at 14, 21, 28, 35, and 49 days post birth.

6.3.2.5 DNA Fragmentation Analysis

Enucleated eyes were placed in PBS and retinal dissection was carried out using a watchmaker's forceps. The choroid, sclera and pigmented epithelium were removed and the retina was then separated from the vitreous and lens. Retinal DNA was isolated following phenol/chloroform extraction. Briefly, retinas were placed in 150 μ l of lysis buffer (20mM EDTA, 100mM Tris and 0.8% Sodium Lauryl Sarcosinate) containing Proteinase K (20 μ g/ml) and incubated at 50°C for 18 hours, vortexing occasionally. The DNA was then extracted with phenol/chloroform and chloroform/isoamyl alcohol (vol/vol 24:1). DNA was precipitated with ethanol and the pellet dissolved in Tris EDTA. Total RNase-treated DNA was visualised by including ethidium bromide (0.5 μ g/ml) in the agarose and observed by illuminating on a 302nm UV transilluminator. Three Rho^{-/-} mice were analysed at the 14 and 28 day time point.

6.3.3 Results and Discussion

Rho^{-/-} mice have no recordable rod electroretinogram and lose all of their rod photoreceptors over a period of approximately three months.

The eyes of C57/BL6 (Figure 6.4A) and Rho^{-/-} mice (Figure 6.4B) at 8, 14, 21, 24, 28, 35, and 49 days post birth were paraffin embedded, sectioned, and subjected to *in situ* nick-end (TUNEL) labelling. A clear peak of TUNEL activity was observed in the inner nuclear layer (INL) of the retinas of C57/BL6 wild type (Figure 6.4A (h)) and Rho^{-/-} mice at 8 days post-partum (Figure 6.4B (h)). The main peak of apoptosis within the outer nuclear layer (ONL) of the retinas of Rho^{-/-} mice was observed at 24 days post birth (Figure 6.4B (k)). The peak of apoptotic activity coincided with the presence of internucleosomal DNA laddering in retinal tissues of Rho^{-/-} mice (Figure 6.5). The appearance of the DNA ladder is very weak, probably due to the fact that in the rhodopsin model only a limited number of photoreceptors are undergoing apoptosis at any one time, and these are likely to be rapidly phagocytosed by neighboring cells. In contrast, in the light-induced model (Hafezi *et al.*, 1997) the proportion of photoreceptors undergoing apoptosis at one timepoint is much greater.

We crossed $Rho^{-/-}$ mice with those heterozygous for a targeted disruption of the *c-fos* gene (*c-fos*^{-/-} mice are unable to reproduce). F1 mice of genotype $Rho^{+/-} c-fos^{+/-}$ were crossed, and those animals of genotype $Rho^{-/-} c-fos^{-/-}$ and $Rho^{-/-} c-fos^{+/+}$ were used in the current investigations. *c-fos*^{-/-} mice have limited viability, and in our hands, die at variable ages beyond day 24 post birth. Retinal sections, representative of retinas from 24 day old $Rho^{+/+} c-fos^{+/+}$, $Rho^{-/-} c-fos^{+/+}$ and $Rho^{-/-} c-fos^{-/-}$ animals were examined (Figure 6.6). At this stage the ONL of the retinas of $Rho^{-/-} c-fos^{+/+}$ mice are about two thirds of the thickness of wild-type retinas. Twenty measurements were taken of the ONL thickness in ten mice of each genotype in the region superior to the optic nerve head. The average thickness of the ONLs in $Rho^{+/+} c-fos^{+/+}$, $Rho^{-/-} c-fos^{+/+}$ and $Rho^{-/-} c-fos^{-/-}$ mice at post natal day 24 was calculated to be 57μm, 42μm and 44μm respectively, indicating that there is little or no variation in the thickness of the ONL in $Rho^{-/-} c-fos^{+/+}$ and $Rho^{-/-} c-fos^{-/-}$ animals.

The numbers of photoreceptor cells undergoing apoptosis in $Rho^{-/-} c-fos^{+/+}$ and $Rho^{-/-} c-fos^{-/-}$ animals at postnatal day 24 were examined using the TUNEL assay (Figure 6.7). Analysis of DNA strand breaks in the retinas of $Rho^{-/-} c-fos^{+/+}$ (Figure 6.7(c)) and the $Rho^{-/-} c-fos^{-/-}$ mice (Figure 6.7(d)) indicated that a similar level of labelling of photoreceptor nuclei was observable at day 24 post birth. TUNEL analysis of C57/BL6 mice at 24 days is shown in Figure 6.4A(k), where it was observed that nuclei of the outer nuclear layer were not undergoing apoptosis at this time point. These observations suggest that in double mutant mice, the absence of the *c-fos* gene does not confer any major protective effect against apoptosis.

These data extended previous observations made on the naturally occurring *rd* and *rd*s mice, where the ONL had been shown to degenerate by a process of apoptosis (Lolley, 1994), Portera-Cailliau *et al.*, 1994). *c-fos* is induced in a variety of apoptotic systems (Smeyne *et al.*, 1993); Marti *et al.*, 1994). In view of the fact that the presence of *c-fos* is an absolute requirement for apoptotic photoreceptor cell death in light-induced mouse models, a highly important question is whether mutation-induced apoptosis, as occurs in natural disease, as opposed to light-induced apoptosis in animal models, is suppressible by the absence of *c-fos*. If so, suppressors of transcripts derived from the *c-fos* cellular proto-oncogene could be targeted to retinal tissues in attempts to slow down the degenerative process.

Hafezi *et al.*, (1998) compared the ONL of $rd^{-/-}$ $c-fos^{-/-}$ and $rd^{-/-}$ $c-fos^{+/+}$ mice according to methods similar to those described here and have found no diminution in ONL thickness in $c-fos^{-/-}$ mice. Ours was the second paper reporting similar findings, this time in a model for degenerative retinal disease based on the targeted disruption of the rhodopsin gene. The retinopathy is substantially slower in $Rho^{-/-}$ mice than in $rd^{-/-}$ mice. Therefore, while it was readily possible in $rd^{-/-}$ mice to follow the apoptotic process to the complete degeneration of the outer nuclear layer (21 days), it was not possible in $Rho^{-/-}$ mice, owing to the compromised viability of $c-fos^{-/-}$ animals. In the case of the $Rho^{-/-}$ mouse, complete degeneration of the ONL takes at least 90 days. However, our observations were made either at, or beyond the peak phase of apoptotic death of the photoreceptor cells. Therefore, these data suggest that, as a therapeutic strategy, suppression of $c-fos$ transcription *per se* in degenerating retinas may have limited or no neuroprotective effect.

Days Post
Natal

8

14

21

24

28

A

B

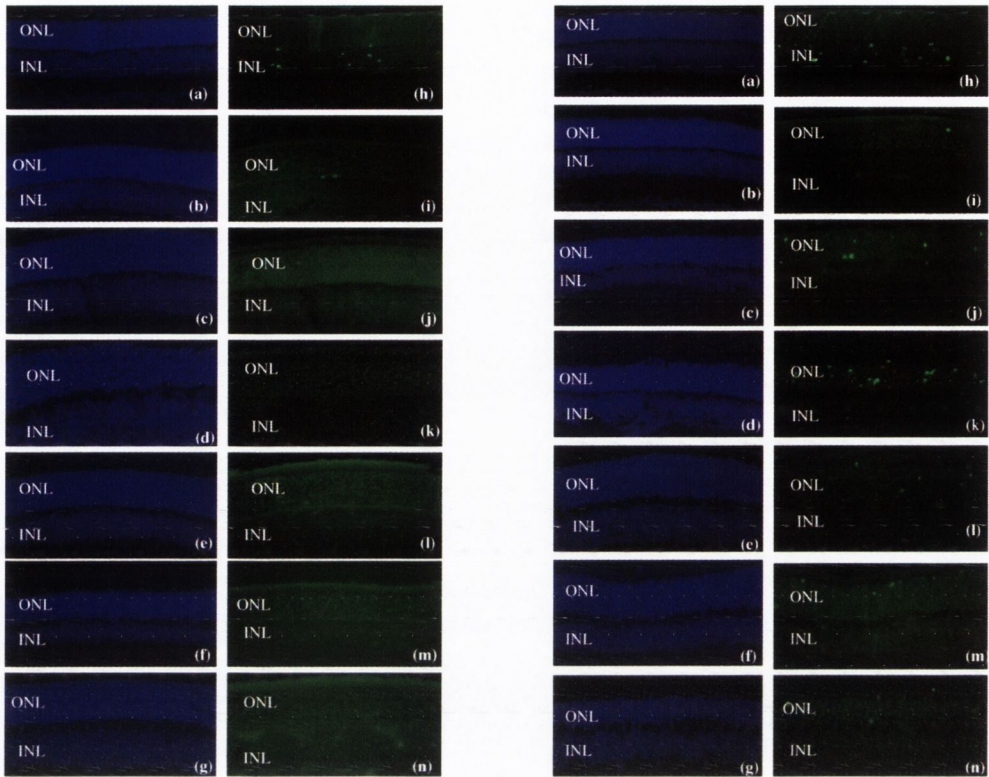


Figure 6.4A and 6.4B.

Examination of DNA strand breakage in photoreceptor nuclei of $Rho^{+/+}$ (A) and of $Rho^{-/-}$ (B) mice. Panels (a)-(g) show Hoescht staining outlining the morphology of retinal sections at day 8, 14, 21, 24, 28, 35 and 49 days. Panels (h)-(n) show *in situ* TdT-dUTP terminal nick-end labelling analysis of retinal sections at the same time points. Retinas of $Rho^{+/+}$ and $Rho^{-/-}$ mice at 8 days post birth show labelling in the INL. This corresponds to the apoptosis observed during retinal development by Penfold and Provis (1986) and Young (12). Retinas of $Rho^{+/+}$ mice at post natal day 14 and older show no labelling. Retinas of $Rho^{-/-}$ mice at days 21 to 28 show labelling in the ONL with a decrease observed at days 35 and 49. Hoescht stained slides were photographed under a DAPI filter (4', 6-diamidino-2-phenylindole, dihydrochloride). Magnification X 400

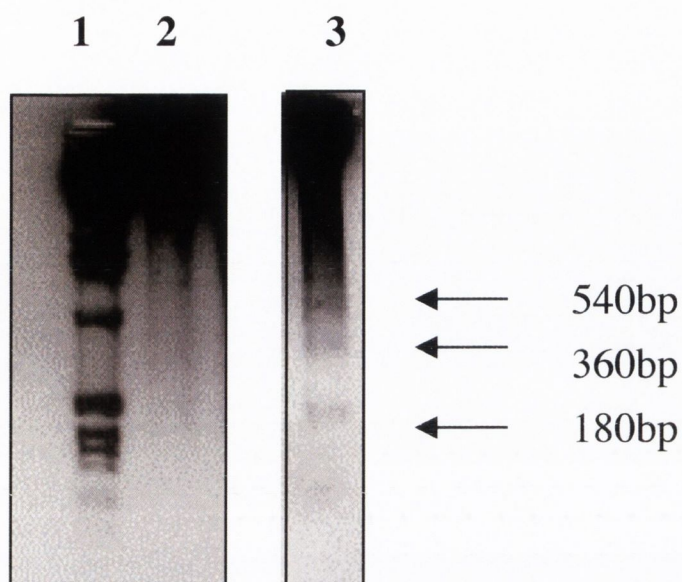


Figure 6.5. DNA fragmentation analysis. Total retinal DNA was prepared from two $\text{Rho}^{-/-}$ mice at 14 days (lane 2) and two $\text{Rho}^{-/-}$ mice at 28 days (lane 3). Mice at 14 days do not yet show signs of internucleosomal fragmentation. Internucleosomal DNA fragments that are multiples of approximately 180bp are evident in mice at 28 days. Lane 1, 100bp ladder.

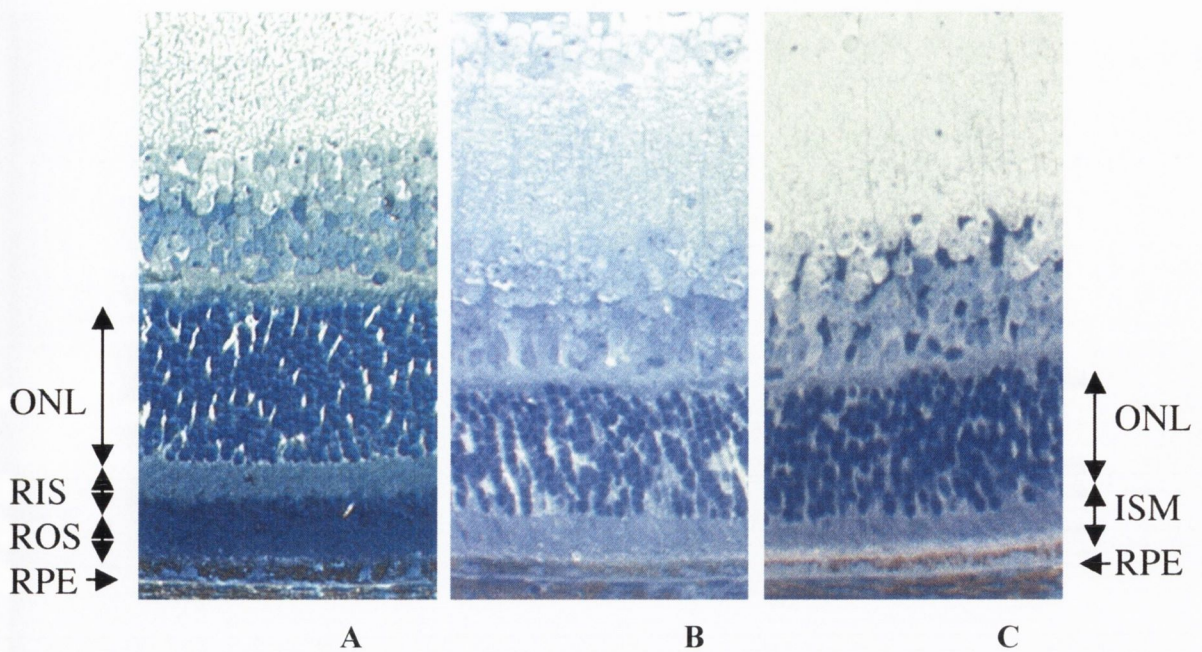


Figure 6.6.

Light microscopy analysis of photoreceptor morphology. All the sections are of epoxy resin embedded mouse retinas taken at 24 days post birth along the vertical meridian (0.5 μ m thickness).

A: Wild-type, C57/BL6 mouse retina ($Rho^{+/+} c-fos^{+/+}$) with 12-13 rows of photoreceptor nuclei in the outer nuclear layer (ONL), Normal rod inner segments (RIS), rod outer segments (ROS) and retinal pigment epithelium (RPE)

B: $Rho^{-/-} c-fos^{+/+}$ mice; **C:** $Rho^{-/-} c-fos^{-/-}$ mice. Both $Rho^{-/-} c-fos^{+/+}$ and $Rho^{-/-} c-fos^{-/-}$ mice show a reduction in the outer nuclear layer thickness to approximately two thirds of that seen in $Rho^{+/+} c-fos^{+/+}$ mice. Inner segment material (ISM) is clearly visible in sections of the $Rho^{-/-} c-fos^{+/+}$ and $Rho^{-/-} c-fos^{-/-}$. Magnification X 200

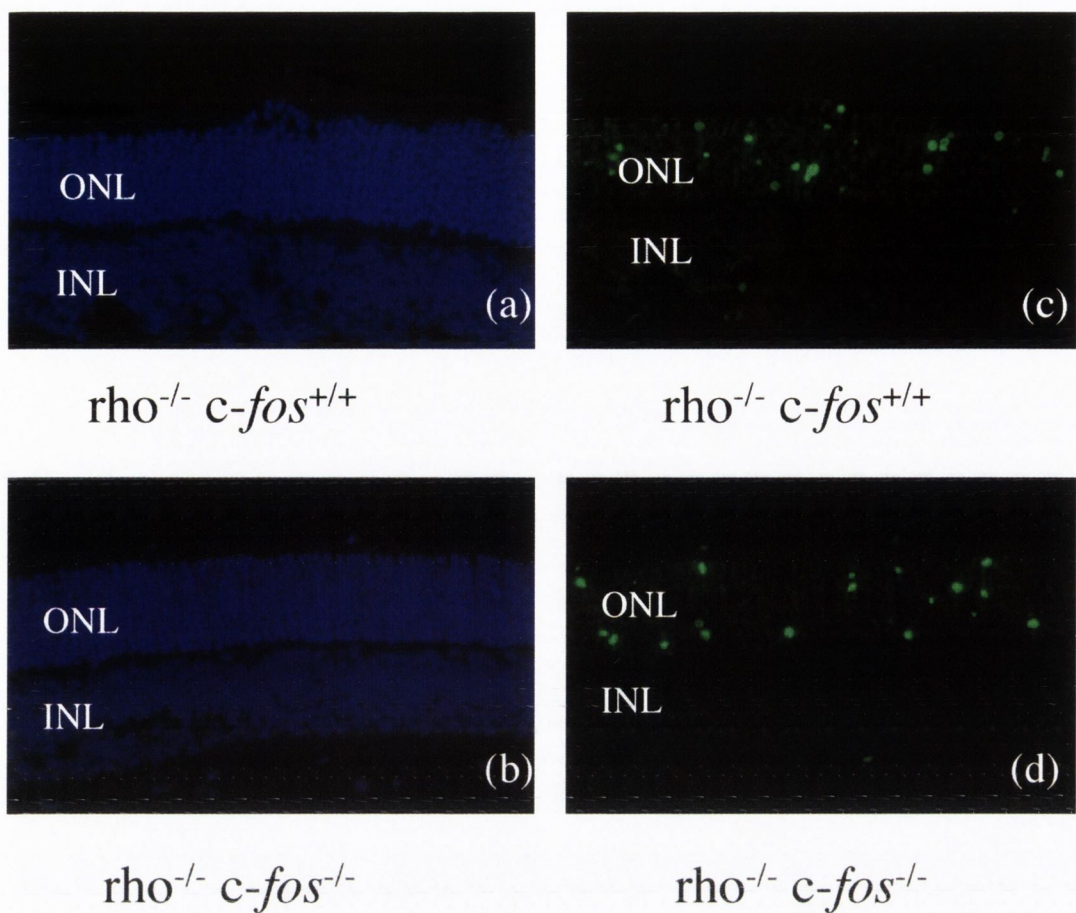


Figure 6.7.

Examination of DNA strand breakage in photoreceptor nuclei of $\rho^{-/-} c-fos^{+/+}$ and $\rho^{-/-} c-fos^{-/-}$ mice by *in situ* TdT-dUTP terminal nick-end labelling (TUNEL) analysis at day 24 post birth. (a) and (b) show Hoescht staining of $\rho^{-/-} c-fos^{+/+}$ and $\rho^{-/-} c-fos^{-/-}$ sections respectively. (c) and (d) show *in situ* TdT-dUTP terminal nick-end labelling analysis of $\rho^{-/-} c-fos^{+/+}$ and $\rho^{-/-} c-fos^{-/-}$ respectively. Similar numbers of nuclei were undergoing apoptosis in the ONL of both genotypes.

Magnification X 400

6.4 Comparative structural and functional analysis of photoreceptor neurons of Rho^{-/-} mice reveal increased survival on C57BL/6J in comparison to 129Sv genetic background.

The work in this section was published in Visual Neuroscience in 2001 (Humphries MM, Kiang S, McNally N, Donovan MA, Sieving PA, Bush RA, Machida S, Cotter T, Hobson A, Farrar J, Humphries P, Kenna P. *Vis Neurosci.* 2001 May-Jun;18(3):437-43.)

6.4.1 Introduction.

Mice carrying a targeted disruption of the rod-opsin gene (Rho^{-/-}) initially develop normal numbers of photoreceptor nuclei (Humphries *et al.*, 1997; Lem *et al.*, 1999).

However, rod photoreceptors do not elaborate outer segments, the membranous discs of which normally contain the photoreactive pigment, rhodopsin. Furthermore, on the original mixed genetic background the outer nuclear layers of the retinas of Rho^{-/-} mice were reduced to a single row of cone photoreceptor nuclei over periods of approximately three months. In the dark-adapted electroretinogram (rod ERG), responses were reduced >50,000-fold, indicative of complete functional knockout of the rod photoreceptor cells (Toda *et al.*, 1999). The light-adapted (cone) ERG was normal in young Rho^{-/-} mice, indicating the presence of functional cone photoreceptors. However, over the three month period cone ERG amplitudes were reduced to 1-2 percent of normal, indicating progressive and extensive cone photoreceptor dysfunction (Toda *et al.*, 1999). Thus, while the retinas of mice and humans differ in relative numbers of rod and cone cells, the retinopathies observed in human RP and in the Rho^{-/-} mouse are broadly similar, each being characterised by loss of ONL, lack of rod function and progressive loss of cone photoreceptor viability.

It is of note that in the naturally occurring *rd* and *rd*s retinopathies in mice, and in a single post mortem analysis of human RP retina, the photoreceptors have been shown to die by apoptosis (Lolley 1999; Portera-Cailliau *et al.*, 1994). Inhibition of the process of apoptosis in degenerating photoreceptors may represent a viable approach to suppression of cell loss. A variety of experimental systems have

been, and are being explored in this regard, including the absence of *c-fos* or *p53* (Hafezi *et al.*, 1997; Ali *et al.*, 1998), use of peptide inhibitors of caspases (Davidson and Stellar, H., 1998), over-expression of *bcl2* or α or β FGF (Chen *et al.*, 1996; La Vail *et al.*, 1992) or expression within the photoreceptor cells of ribozyme suppressors of pro-apoptotic genes (Millington-Ward *et al.*, 1997; O'Neill *et al.*, 2000). More generally, variable expression of phenotype is a characteristic of many hereditary disorders, including RP (Skinner 1990; al-Magthteh *et al.*, 1994), a probable manifestation of the effect of genetic modifier loci (QTLs) on photoreceptor survival. The results reported in this section indicate that on a C57BL6J background the cone photoreceptors of the $Rho^{-/-}$ mouse live and retain function for substantially longer periods than those in mice on a pure 129Sv background.

6.4.2 Materials and Methods.

6.4.2.1 Generation of Congenic $Rho^{-/-}$ mice

$Rho^{-/-}$ mice (Humphries *et al.*, 1997) originally on a mixed C56Bl/6J-129Sv genetic background were backcrossed through twelve successive generations onto pure C57Bl/6J and 129 Sv strains (supplied by Harlan U.K. Ltd). Such mice were intercrossed for use in the investigations to be described.

6.4.2.2 *In situ* end-labelling of fragmented DNA (Carried out by Drs. Mary Anne Donovan and Tom Cotter)

DNA strand breaks in photoreceptor nuclei were detected by *in situ* end-labelling (TUNEL). Enucleated eyes were fixed in 10% buffered formalin for 24 hours, dehydrated, processed and embedded in paraffin. 5 μ M sections were incubated in 50 μ l of reaction buffer containing 2.5mM $CoCl_2$, 0.1U/ml terminal deoxynucleotidyl transferase (TdT) in a 0.1M Na cacodylate (pH7.0) buffer and 0.75nM fluorescein-12-dUTP (Boehringer Mannheim, Germany). Sections were incubated at 37°C for 1 hour in a humidified chamber. After several washes in phosphate-buffered saline (PBS), sections were mounted in Mowiol (Calbiochem)

and viewed under a fluorescent microscope using a fluorescein isothiocyanate (FITC) filter.

6.4.2.3 Quantification of nuclei in retinal sections

Bisected resin-embedded eyes were sectioned along the vertical axis at a thickness of $0.5\mu\text{m}$ using a Reichert-Jung Stereostar Ultracut microtome. Sections were stained with toluidine blue and viewed under a Zeiss Axioplan 2 microscope. Photographs were taken at magnification x200 using an integrated JVC 3-CCCD colour video camera. For 1, 2, 3 and 4 month time points a minimum of 10 retinas each from animals on both the C57BL6J and 129Sv Rho^{-/-} backgrounds and 6 animals from the corresponding wild type backgrounds were examined (over 130 eyes). The number of nuclei in a 100 μm length of the mid periphery in the superior retina were counted. Results, expressed as nuclei per unit length (100 μm) of retina, were calculated using both manual counting and a macro-computer program designed at this laboratory (Figure 6.8b). Results were analysed statistically using the Datadesk-6 package and means and standard errors calculated. The retinas of four additional Rho^{-/-} and 2 wild type animals of each strain were also examined by counting the numbers of nuclei in the ONL along a 100 μm retinal length at twelve equidistant sites for each section (200-400 μm apart). These latter results concurred with the initial study (data not shown).

6.4.2.4 Electroretinographic analyses (Carried out by Drs. Shigeki Machida, Ron Bush and Paul Sieving)

For electroretinographic analysis mice were maintained under 12 hour cyclic light of approximately 400 lux at cage level. Breeding pairs and litters received food and water *ad libitum*. All procedures conformed to the principles of the Society for Neuroscience. Electroretinograms (ERGs) were recorded from 5 Rho^{-/-} mice on the C57BL6J and 129 background at 40, 45, 60 and 80 days of age. Animals were kept in total darkness for at least twelve hours before recordings and were prepared under red dim light. The animals were anaesthetised with a loading dose of xylazine (1.5mg/kg) and ketamine (75mg/kg) given intramuscularly, and maintained on subcutaneous infusion of the same mixture of xylazine and ketamine via pump

(Razel Instruments, Stanford, CT) during the recordings. The pupils were dilated with 0.1% atropine and 0.1% phenylephrine HCL. The animals were held steady with a bite bar and nose clamp. A heating pad maintained body temperature at 37°C. Dark adapted ERGs were recorded on all animals using a chlorided silver wire loop on the center of the cornea, with 1% tetracaine topical anaesthesia and a drop of methyl cellulose to maintain corneal hydration. A chlorided silver reference electrode was positioned at the temporal sclera. A ground electrode was attached to the left ear lobe. Responses were amplified at 10,000 gain from 0.1 to 1,000 Hz, filtered to remove 60-Hz noise, and digitised at a rate of 10 Hz. Up to 40 responses were averaged depending on the signal to noise ratio. The stimulus was a white (2800K) 50 msec flash with an intensity of 2.2 log cd/m² presented in a Ganzfeld bowl.

6.4.3 Results

In order to accurately quantify the extent of photoreceptor degeneration on each genetic background, numbers of nuclei in the ONL of Rho^{-/-} C57 and 129 mice from one to four months of age were counted. Since only about 3% of the photoreceptors in the mouse are cones (Jeon *et al.*, 1998) the majority of cells counted in the ONL were rods. Representative retinal sections are illustrated in Figure 6.8 Top Panel. There were no statistically significant differences in numbers of nuclei in wild type animals from the C57 and 129 backgrounds over the four month period ($p = 0.4741$). However, by three months of age there was a significant difference in numbers of ONL nuclei between the C57 and 129 Rho^{-/-} strains (Figure 6.8 Bottom Panel). This difference was produced by a slower rate of cell loss in C57 mice in the first 3 months as indicated by the slopes of the linear regression lines for the individual data ($p = 0.013$, $n = 9-16$ per time point).

The difference in cell numbers at 3 and 4 months was confirmed by more extensive measurements (see Methods) on four Rho^{-/-} mice of each strain ($p = 0.033$ at 3 months and $p = 0.0001$ at 4 months). These results indicate that there was a higher sustained rate of cell death in Rho^{-/-} mice on the 129 background. This conclusion is supported by direct observation of the apoptotic process in the retinas of C57 and 129 Rho^{-/-} mice using the technique of terminal nick-end labelling, a

procedure enabling apoptosing nuclei to be visualised directly in retinal sections by *in-situ* end labelling of fragmented DNA by terminal deoxynucleotidyl transferase. (Fig. 6.9)

In the naturally occurring *rd* (retinal degeneration) and *rd_s* (retinal degeneration-slow) mice, carrying recessive mutations within genes encoding the β subunit of rod cyclic GMP phosphodiesterase (β PDE) and peripherin respectively, the photoreceptors die by apoptotic mechanisms (Lolley, 1994; Portera-Cailliau *et al.*, 1994).

We compared the features of apoptosis in the retinas of four $\text{Rho}^{-/-}$ mice on C57BL6J and four $\text{Rho}^{-/-}$ mice on 129Sv genetic backgrounds for periods of up to 63 days post birth using *in situ* end labeling of retinal sections (TUNEL analysis). Representative results are shown in Figure 6.9. Retinas of C57 and 129 mice at postnatal days 8 (P8) showed labeling of photoreceptors in the inner nuclear layer of the retina (INL), similar to the apoptosis observed by Penfold and Povis (1986), and Young (1984) during retinal development. Retinas of C57 mice had scattered labeling of photoreceptor nuclei within the outer nuclear layer (ONL) at P21. By P24 labeling was very much more intense and a decrease in labeling was observed from P28 through P63. In contrast, analysis of retinas of 129 mice at P21 and P24 showed sparse labeling in the ONL with an increase by P28. From P35 to P63, there still appeared to be more TUNEL-positive labeling in the ONL in comparison to the retinas of C57 mice at the same time points (Figure 6.9). These data show, as expected from previous studies of apoptosis in the *rd* and *rd_s* mouse, that the photoreceptors of $\text{Rho}^{-/-}$ mice also die by apoptosis. Also, from this limited sampling of the retina it appears that the apoptotic death process is slower in starting in 129s, but is increased over the long term, resulting in a more devastating effect.

The initial study of the ERG of the $\text{Rho}^{-/-}$ mouse showed a steady decline in cone responses after about 45 days (Toda *et al.*, 1999). The histological observations in the present study suggest that cone photoreceptor function might be better preserved in $\text{Rho}^{-/-}$ C57BL/6J, in comparison to $\text{Rho}^{-/-}$ 129Sv mice. Therefore, cone function was measured using the ERG in $\text{Rho}^{-/-}$ mice of each strain at 40, 45, 60 and 80 days of age. Figure 6.10A shows representative ERG waveform series for mice at 40 and 80 days. At 40 days the threshold and amplitude of the cone ERG- b-wave in the $\text{Rho}^{-/-}$ mice of the two background strains were similar. The waveforms of the 129Sv mice however, were broader than C57BL/6J mice and did not show the small

a-wave at high intensity. These differences were also observed in wild-type mice (data not shown), indicating they may be inherent to the two strains. By 80 days, the b-wave threshold was elevated and the amplitude drastically reduced in both strains of $\text{Rho}^{-/-}$ mice. As shown in Fig. 6.10A, however, the C57BL/6J mice retained a much larger b-wave amplitude and lower threshold (greater sensitivity) at this age. The responses shown in Fig. 6.10A indicate that the 80-day cone b-wave of the C57BL/6K mouse at maximum intensity was reduced by about 2/3, but retained the overall notches and peaks, as well as the initial a-wave. It resembled the responses seen at 1 log cd/m^2 in the 40-day old mouse. The maximum b-wave of the 129Sv mouse, however, resembled the threshold response at 40 days, that is, at almost 2 log cd/m^2 dimmer intensity. Thus, there appeared to be almost a one log unit difference in the response characteristics of these two $\text{Rho}^{-/-}$ mice by 80 days.

The average changes in the b-wave amplitude between 40 and 80 days (Fig. 6.10B) in part mirrored the differences between the two strains in rate of cell loss between 1 and 3 months. The 129Sv $\text{Rho}^{-/-}$ mice had a constant rate of decline in the average b-wave amplitude throughout the 40-day period, while the decline was delayed in the C57BL/6J $\text{Rho}^{-/-}$ mice. Both strains had similar amplitudes at 40 days of age, but this was already significantly decreased at 45 days in the 129Sv mice ($p = 0.05$). By contrast, there was not a significant decline in the C57BL/6J b-wave response until at least 60 days, after which the b-wave amplitude declined at a rate similar to that in the 129Sv mice. The b-wave amplitude of 129Sv $\text{Rho}^{-/-}$ mice was significantly lower than C57BL/6J $\text{Rho}^{-/-}$ mice at 45 days ($p < 0.05$), at 60 days ($p < 0.001$), and 80 days ($p < 0.01$). Thus the ERG in C57BL/6J mice did not show a significant loss of cone function until the cell number was almost 50% of its one-month value (Fig. 6.8B), but the cone responses in the 129Sv strain were significantly reduced when the cell number fell below 80% of this value. Since the ERG in $\text{Rho}^{-/-}$ mice originates solely from cones (Toda *et al.*, 1999), these data indicate that cones and cone function in $\text{Rho}^{-/-}$ mice with the C57BL/6J background are much less affected than cones in 129Sv $\text{Rho}^{-/-}$ mice. It suggests that the cones in the former strain are better able to survive the loss of neighbouring rods.

6.4.4 Discussion

The data obtained from this study thus indicate that the C57BL6J genetic background favours the survival of rod and cone photoreceptor neurones in comparison to that of the 129Sv in $Rho^{-/-}$ animals. Whether or not genes on the C57 background directly influence apoptotic mechanisms remains to be established. It is of interest to note that the ocular phenotypes of mice carrying a targeted disruption of the P53 tumour suppressor gene have been shown to be radically different on C57BL6J and 129Sv genetic backgrounds, C57s exhibiting vitreal opacities, retinal folds and vitreal vascularisation, features that might possibly be a result of aberrations of apoptotic developmental regression of the embryonic hyaloid vascular system, although, as pointed out by these authors, angiogenic factors could also be involved in modulation of the P53 phenotype (Ikeada *et al.*, 1999).

It has been shown that different strains of albino mice show large differences in susceptibility to light-induced retinal degeneration (Rapp and Williams 1980; LaVail *et al.*, 1987). The C57BL6J-c21 was one of the most resistant strains but its susceptibility compared to 129Sv is not known.

Studies by Danciger *et al.*, (2000) have resulted in the localisation of a quantitative trait locus on chromosome 3 of pure-bred wild type C57BL6J mice that contributes to protection of the photoreceptor cells against damage induced by constant light. The C57 mice used in the current study have the same haplotype for genetic markers over the 3q interval as those used in the study by Danciger *et al.*, (not shown). Most available evidence points to rhodopsin bleaching as the initial step in light damage by low and moderate levels of visible light (Williams and Howell 1983; Noell *et al.*, 1996) and even damage by UV-A light (Rapp and Ghalayini 1999), though other chromophores may be involved in retinal damage by blue light and UV light (Organisciak and Winkler 1994). The remarkable difference in phenotypes observed for the $Rho^{-/-}$ mouse on the two distinct backgrounds investigated in this study opens up the possibility of comparing quantitative trait loci in both light-induced and in mutation-induced retinopathies. The clinical significance of such observations lies in the eventual understanding of molecular mechanisms underlying phenotype variation in families with RP, where “outliers” manifesting a different stage of degeneration compared to the bulk of other family members can often be identified. Genetic factors are likely to play a significant role

in such phenotypic variation. A delay of 2-3 month in mice could add up to 10% vision span for at least some types of human retinal degeneration.

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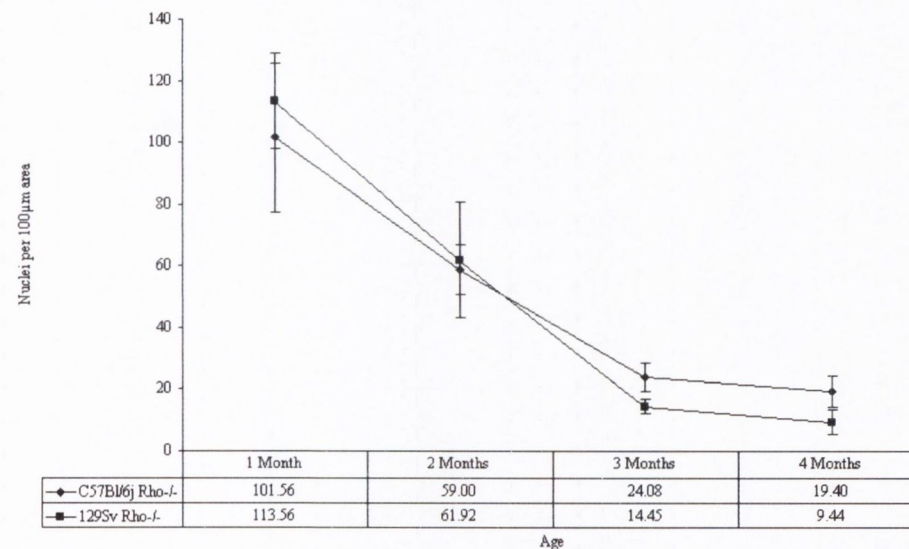
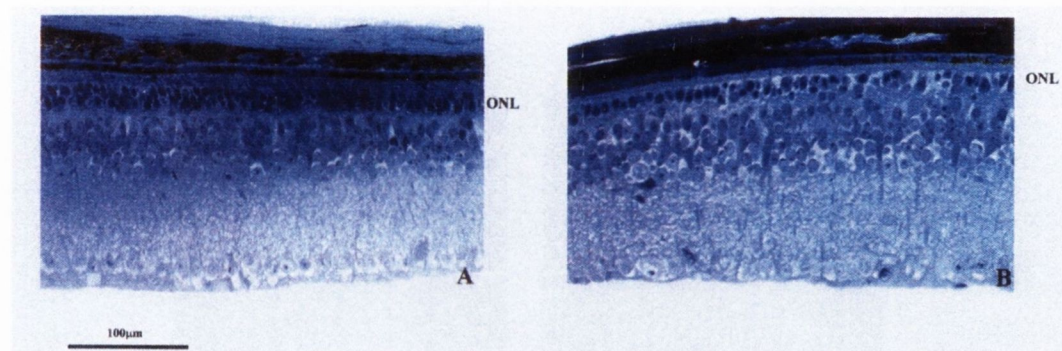


Figure 6.8: Top Panel: Representative retinal sections from C57BL/6J (A) and 129Sv (B) Rho^{-/-} mice at 3 months of age. Bottom Panel: Graphical representation ONL nuclei numbers (mean of at least 10 eyes) at 1 month, 2 months, 3 months and 4 month of age, with standard error bars.

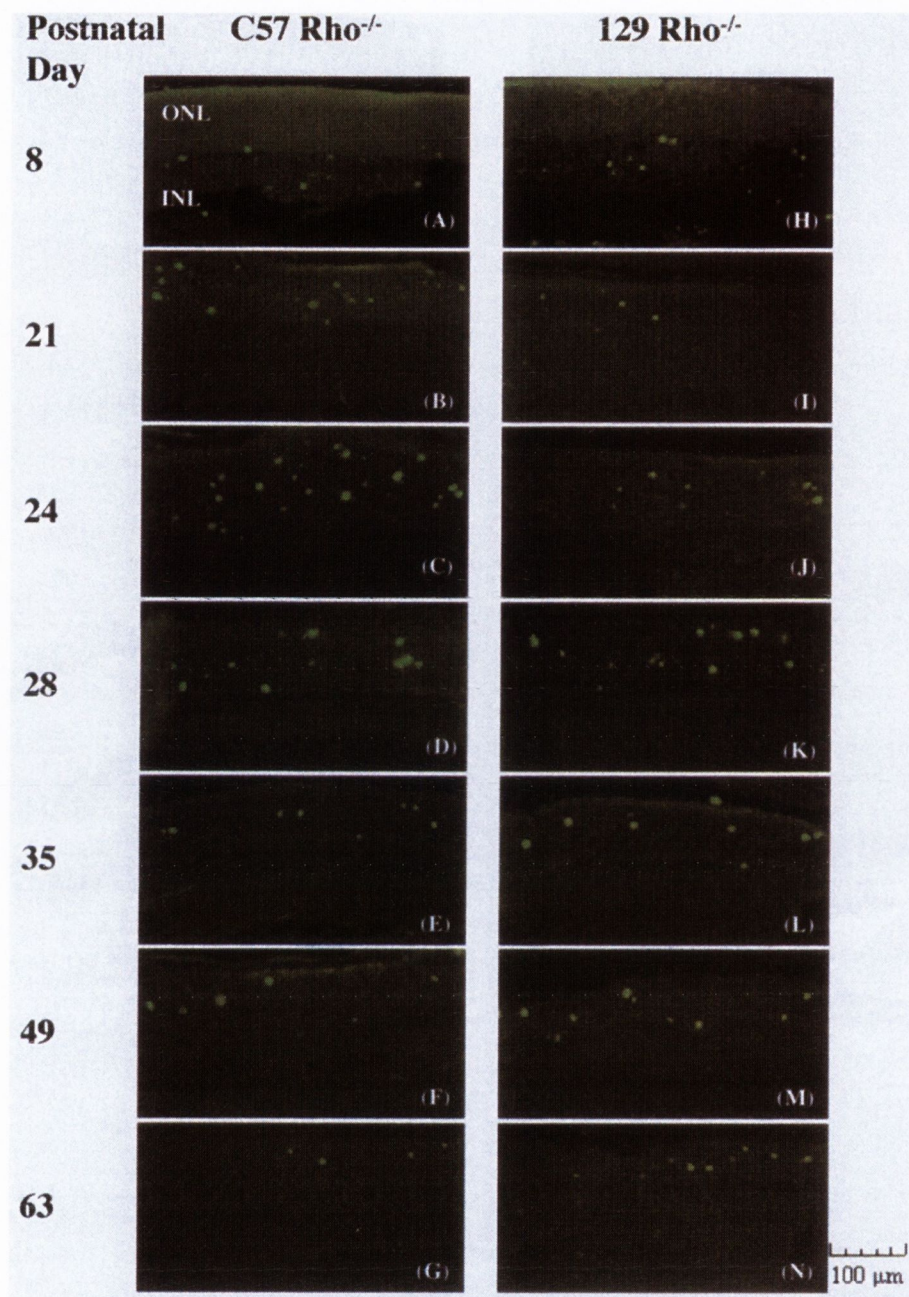


Figure 6.9: Representative analysis of retinal sections of C57Bl/6J and 129Sv Rho^{-/-} mice by *in situ* labeling.

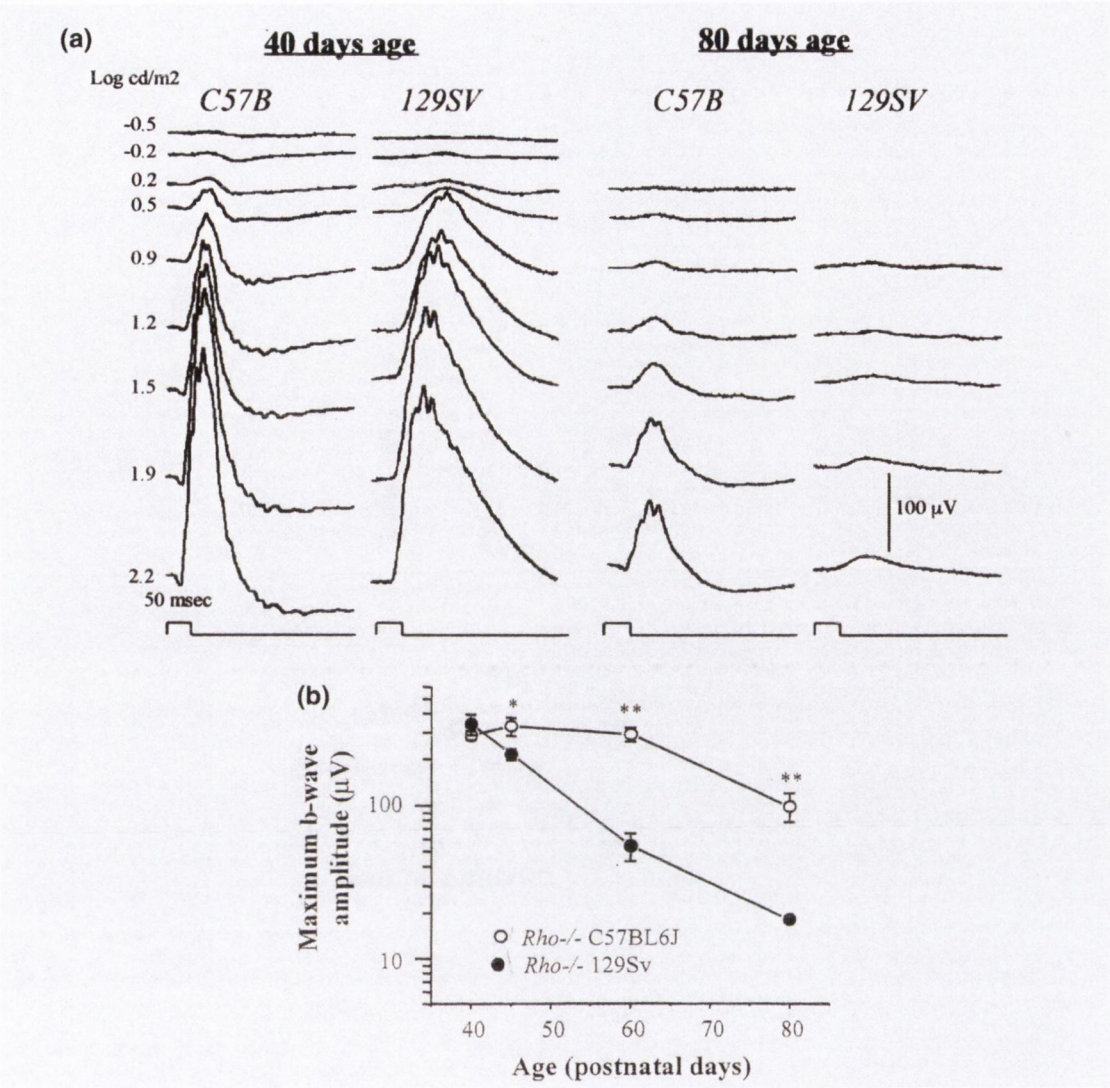


Figure 6.10: (a) Dark adapted ERG waveforms from *Rho*^{-/-} mice on C57BL/6J and 129Sv background at 40 and 80 days of age. The stimulus intensity is indicated on the left of the figure.

(b) Average dark-adapted ERG b wave amplitudes of the same two strains of *Rho*^{-/-} mice at 40 – 80 days of age. The stimulus was a 50 ms white 2.2 log cd/m² flash.

N = 5 per time point. * p < 0.05, **p < 0.01. Error bars = standard error.

Chapter 7

Studies on Transgenic Mouse Models of Human Inherited Retinopathies

7.1 Murine Model of Autosomal Dominant Retinitis Pigmentosa Generated by Targeted Deletion at codon 307 of the rds–Peripherin Gene

This work in this section was published in Human Molecular Genetics in 2002 (McNally N, Kenna PF, Rancourt D, Ahmed T, Stitt A, Colledge WH, Lloyd DG, Palfi A, O'Neill B, Humphries MM, Humphries P, Farrar GJ. Hum Mol Genet. 2002 May 1;11(9):1005-16.)

7.1.1 Introduction:

Peripherin is a transmembrane protein of the outer segment disc membranes of photoreceptor cells, and is located towards the periphery of the discs – hence its name. In rod photoreceptors, peripherin can interact with Rom1 (rod outer segment protein 1) to form heterodimers providing disc stability (Arikawara *et al.*, 1992; Moritz and Molday 1996; Goldberg and Molday 2000). A naturally occurring rds–peripherin null mutation in the mouse causes a retinal degeneration termed ‘retinal degeneration slow’, or rds (Sanyal *et al.*, 1980; Sanyal and Jansen 1981; Connell *et al.*, 1991).

In this study, a single base pair deletion at codon 307 of the rds–peripherin gene was introduced into mice by homologous recombination in ES cells. In humans, the RDS-307 mutation results in a slowly progressive form of autosomal dominant RP (Apfelstedt-Sylla *et al.*, 1995). The human mutation causes a frameshift and as a result creates a stop codon after a further 16 triplets. The expected protein is therefore 26 amino acids shorter than human wild-type RDS–peripherin. The clinical profile of the human disease involves a decline in visual acuity, loss of peripheral vision in the fifth decade of life and onset of night blindness in the sixth decade. Thus, the disease caused by the 307 mutation is relatively mild. The frameshift brought about in the mouse genome by the 1 bp deletion at codon 307 is predicted to result in alteration of the last 40 amino acids of the C terminus of the protein and the addition of an extra 11 amino acids.

7.1.2 Methods

A 1 bp deletion at codon 307 was introduced into exon III of the gene by PCR mutagenesis. Linearized DNA was electroporated into R1 embryonic stem (ES) cells and positive/negative selection was used to enrich for targeted ES cell clones. Selected ES colonies were injected into blastocysts of C57 Bl/6J mice to produce germline chimeras, which transmitted the RDS-307 mutation. Targeted RDS-307 mice were identified by PCR, sequencing and Southern blot analysis by Dr. McNally. Mice that were heterozygous and homozygous for the RDS-307 gene mutation, were generated through inbreeding of germline-transmitting mutants, wild-type litter mates were used as controls. The rds mice used throughout this study were Balb/c -rds/rds mice kindly supplied by Dr Richard L. Sidman.

PCR and sequencing techniques were used to identify mouse genotypes resulting from this breeding protocol (McNally *et al.*, 2002).

7.1.2.1 Histology

Retinal histology was carried out as described in Chapter 6.3.2.2.

7.1.2.2 Electroretinography

Animals were dark-adapted overnight and prepared for ERG under dim red light. Animals were anesthetized by means of ketamine and xylazine. Pupillary dilatation was achieved by instillation of atropine 1% and phenylephrine HCl 2.5%. Standardized flashes of light were presented to the mouse in a Ganzfeld bowl to ensure uniform retinal illumination. The ERG responses were recorded simultaneously from both eyes by means of gold wire loops on the corneas, using amethocaine 1% as topical anaesthesia and methylcellulose to maintain corneal hydration. A gold reference electrode was positioned subcutaneously approximately 1 mm from the temporal canthus and the ground electrode was placed subcutaneously anterior to the tail. The responses were analyzed using RetiScan RetiPort electrophysiology equipment (Roland Consulting GmbH). The protocol used was based on that approved by the International Clinical Standards Committee for human electroretinography. Rod-isolated responses were recorded using a dim white flash (-25 dB maximal intensity) presented in the dark-adapted state. The maximal

combined rod/cone response to the maximal-intensity flash ($3 \text{ cd m}^{-2} \text{ s}^{-1}$) was then recorded. Following light adaptation for 10 min to a background illumination of 30 cd m^{-2} presented in the Ganzfeld bowl, the cone-isolated responses were recorded to both single-flash and 10 Hz maximal-intensity flashes. a-waves were measured from the baseline to the trough and b-waves from the baseline (in the case of rod-isolated responses) or from the a-wave trough.

7.1.3 Results

7.1.3.1 Histopathology of retinas of rds-307 mice

Retinas from rds-307 heterozygous mice were studied by light microscopy at 2, 6, 10 and 12 months of age (Fig. 7.1). Nine or ten rows of photoreceptor nuclei were present in the central retina at 2 months, four or five rows at 6 months, three or four rows at 10 months and two or three rows at 12 months. The degeneration in rds-307 heterozygotes therefore appears to be more rapid than that observed in $\text{rds}^{+/-}$ heterozygotes, which retained six or seven rows of nuclei at 9 months (Kedzierski *et al.*, 2001) and maintained approximately half the normal photoreceptor cell population at 9–10 months (Jansen and Sanyal 1992). Photoreceptor degeneration at 1, 2, 3 and 4 months among wild-type control mice, homozygous rds-307 mice and $\text{rds}^{-/-}$ mice was also compared by counting rows of photoreceptor nuclei in the outer nuclear layer. Homozygous rds-307 mice show relatively rapid photoreceptor degeneration, with only four or five rows of nuclei remaining at 1 month and essentially a single row at 4 months (Fig. 7.2). The $\text{rds}^{-/-}$ mouse retinas analyzed retained eight or nine rows of photoreceptor nuclei at 1 month and three or four rows at 4 months (Fig. 7.2). The findings in $\text{rds}^{-/-}$ homozygotes in the current study are consistent with previously published observations (Sanyal *et al.*, 1980).

7.1.3.2 Electroretinographic analysis

No convincing rod-isolated responses could be elicited from homozygous rds-307 mice even at 1 month of age. Homozygous rds-307 mice showed very significant attenuation of maximal-intensity b-wave amplitudes from an early age. The responses recorded from 2-, 3- and 4-month old mice are shown in Figure 7.3. The corresponding amplitudes from age-matched $\text{rds}^{-/-}$ mice are shown in the same

figure, demonstrating that the phenotype in rds-307 mice is noticeably more severe. Whilst a small-amplitude b-wave could still be recorded from a 4-month-old rds^{-/-} animal, no such peak could be recorded from a 4-month-old rds-307 homozygote.

Figure 7.4 illustrates the ERG responses recorded from 6- and 10-month-old rds-307 heterozygotes. The rod-isolated responses generated by a 6-month-old rds-307 heterozygote compare well with those of a wild-type mouse. The mixed rod/cone response of the dark-adapted eye of this animal to a maximal-intensity flash presented in the dark-adapted state showed significant reduction in both a-wave and b-wave amplitudes, as did the cone-isolated responses to single flash. By 10 months of age, there had been a significant deterioration in rod-isolated b-wave amplitudes, as well as in maximal dark-adapted responses and cone-isolated responses. The time course of the ERG amplitude deterioration mirrored that seen in histology.

7.1.4 Discussion

In designing a therapeutic strategy for a disease such as autosomal dominant RP it is important to establish whether the disease pathology is caused by haploinsufficiency of the wild-type gene product or a dominant-negative effect(s) of mutant protein or alternatively a combination of both mechanisms. To simulate the human condition in an animal model, a targeting approach in mouse ES cells has been adopted, enabling introduction of a subtle mutation (a 1 bp deletion at codon 307) into the endogenous mouse rds-peripherin gene. The pathology observed in the resulting rds-307 mice suggests that disease pathology is due to the presence of mutant peripherin protein and not simply an absence of wild-type protein, since the disease appears to be more severe in homozygous rds-307 mice than in homozygous rds^{-/-} null mice (see Figs 7.1 and 7.2).

It should be noted that at the time these data were derived the $\Delta 307$ animals were on a mixed genetic background. At the time of writing this thesis the animals have just been bred on to pure C57Bl/6J and on to pure 129Sv backgrounds. Phenotypic analysis of these pure-background animals commenced in April 2006.

It is of interest to speculate on possible mechanisms by which the codon 307 mutation in the rds-peripherin gene may exert a dominant-negative effect. It has been previously well established that peripherin protein is involved in maintaining the integrity of the structure of photoreceptor disc membranes (Arikawara *et al.*, 1992; Molday 1994). In this regard, it is worth noting that evidence has been

obtained to suggest that the C terminus of the peripherin protein may be involved in ROS membrane fusion. Studies *in vitro* have suggested that a 15-amino-acid sequence within the C terminus of the protein (at positions 313–327) is involved in aiding ROS membrane fusion (Boesze-Battaglia *et al.*, 1998). Notably, this amino acid sequence is predicted to be altered in the mutant rds-307 protein, and hence it is possible that the ability of the mutant protein to promote membrane fusion may be compromised. In addition, studies carried out in *Xenopus laevis* suggest that the C-terminus of the peripherin protein promotes outer segment localization (Tam *et al.*, 2001; Tam *et al.*, 2004) and therefore a similar situation might pertain in the case of mouse peripherin. A role for peripherin in maintaining the flattened vesicle morphology characteristic of photoreceptor outer segments has been suggested, and two rds–peripherin mutants (P216L and C156Y) have been shown to be unable to flatten membrane vesicles *in vitro* (Wrigley *et al.*, 2000).

It is notable that the predicted C-terminus of the mutant mouse rds-307 protein possesses a significantly higher ratio of basic amino acids and a lower number of acidic amino acids than the wild-type protein, which has a direct effect on pI (8.32 versus 9.34) and hence protein folding *in vivo* (Fig. 7.5). Other factors that may be of significance are the presence of and the relative positions of proline residues in the mutant protein: in general, such residues introduce a 45° kink in the amino acid backbone. In addition, the increased number of non-polar amino acids would induce a larger bulk volume. The introduction of an additional cysteine residue in the mutant may possibly facilitate aberrant bond formation with cysteine residues present in the large intradiscal domain of the protein. To investigate possible divergence in the secondary structure of the mutant protein from the wild-type, both wild-type and mutant sequences were submitted to the Jpred secondary structure prediction utility at EBI (Cuff *et al.*, 1998). Notably, differences in the predicted helical structures for the C terminus of the mutant and wild-type proteins were obtained (Figs 7.6 and 7.7). It is of interest to note that predicted changes in mutant as opposed to normal proteins in the mouse are also likely to be present in mutant human peripherin, specifically, a higher ratio of basic amino acids, pI (8.4 versus 9.16) the presence of proline residues and an additional cysteine residue. Therefore, pathological disease mechanisms, at the molecular level, may be similar in both murine and human retinas.

The development of therapeutics for autosomal dominant RP caused by mutations within the RDS–peripherin gene is severely complicated by the extensive

genetic heterogeneity inherent in this group of conditions. The complexity and fragility of photoreceptor neurons is further highlighted by the observation that some RDS–peripherin mutants cause rod photoreceptors to degenerate while other mutations bring about the degeneration of cone photoreceptor cells (Jacobson *et al.*, 1996). The presence of approximately 50 different mutations within the RDS–peripherin gene alone has stimulated the exploration of methods of generating therapeutic solutions that are independent of specific mutations. Such solutions can be targeted to the primary genetic defect, in this case the RDS–peripherin gene, or alternatively to a secondary effect associated with the pathology, such as apoptosis (programmed cell death). In this regard, it is of note that the rds-307 animal model generated in the current study will be useful for both purposes. Preliminary histological analyses of rds-307 mouse retinas suggest that while the primary stimulus for the disease is mutant peripherin, photoreceptors in rds-307 mice die by a process of apoptosis (Fig. 7.8D). Therefore this new animal model should be of value in the evaluation of various anti-apoptotic or neuroprotective approaches for therapy for inherited retinopathies (Nir *et al.*, 2000; Gruning *et al.*, 1994; Kennan *et al.*, 2002).

However, an alternative approach (or one that may be used in conjunction with the former) is to target the primary genetic defect, that is, the RDS–peripherin mutant in a mutation-independent manner. Technologies exploiting inherent features of the genome such as the degeneracy of the genetic code enable the development of such mutation-independent therapeutics (Millington-Ward *et al.*, 1997; Millington-Ward *et al.*, 1999; O'Neill *et al.*, 2000; Farrar *et al.*, 2002) while still targeting the primary genetic defect. These approaches involve suppression of the mutant and wild-type alleles of the RDS–peripherin gene and in parallel the supply of a replacement RDS–peripherin gene that is protected from suppression but encodes wild-type protein (Farrar *et al.*, 2002). The novel mouse model (rds-307) described here, given the dominant-negative effect of the mutant, should be valuable in the exploration of such mutation-independent strategies (Table 7.1).

Table 7.1. Comparative analyses of human RDS–peripherin and mouse rds–peripherin sequence illustrating co-targetable ribozyme cleavage sites.

Position of cleavage site		Nucleotide sequence around ribozyme cleavage site
Human	306–308	5'-CCCAAGGG CT CTGGCTCATG-3'
Mouse	270–272	5'-CCCAGGGG CT CTGGCT <u>T</u> ATG-3'
Human	582–584	5'-AACATCATC CT CTTCCTTGTG-3'
Mouse	546–548	5'-AACGTCATC CT CTTCCTG <u>G</u> TG-3'
Human	975–977	5'-ACGGAGGAG CT CAACCTGTGG-3'
Mouse	939–941	5'-ACTGAGGAG CT CAACCTCTGG-3'
Human	1050–1052	5'-ATGGGTGTC G TCACGCTCCTC-3'
Mouse	1014–1016	5'-ATGGGCGTC G TCAC <u>A</u> CTTCTC-3'
Human	1047–1049	5'-TCCATGGGT G TCGTCACGCTC-3'
Mouse	1011–1013	5'-TCCATGGG C GTCGTCAC <u>A</u> CTT-3'

Ribozymes have been designed so that they can simultaneously be used to cleave either human or mouse peripherin transcripts. Furthermore, each ribozyme cleavage site occurs at a degenerate position in human and mouse peripherin sequences so that mutation-independent suppression and replacement may be achieved. In addition, ribozyme cleavage sites are located in open-loop structures in both transcripts (as predicted from RNA secondary structures generated by RNA PlotFold). NUX ribozyme cleavage sites are highlighted in bold; mismatches between human and mouse peripherin sequences are underlined.

A comparison between human RDS–peripherin and mouse rds–peripherin nucleotide sequences reveals that there are regions of mouse rds–peripherin sequences for which suppression agents such as hammerhead ribozymes could be designed and that would in principle be of relevance to the human disease, since the sequences at the ribozyme target site are almost identical between mouse and humans. Notably, five areas of such sequence homology exist in predicted open-loop structures of human RDS–peripherin and mouse rds–peripherin transcripts that could serve as targets for ribozymes that could be tested in rds-307 mice but in principle would also be applicable to suppression of the human gene (Table 7.1). Other agents such as small interfering RNSs (siRNAs), discussed in section 5.6.6, may also be of value as potent drivers of suppression.

Hence, in summary, the newly generated rds-307 mouse model described here will be of value in the evaluation of a variety of therapeutic strategies of the sort described above, irrespective of whether the therapy targets the primary genetic defect or secondary effects associated with the disease pathology. In conclusion, in the current study, the first targeted mouse model of a peripherin-linked retinal degeneration has been generated. It has been clearly demonstrated that the resulting mouse has a significant photoreceptor degeneration. Notably, this new mouse model will be of value both in deciphering mechanisms of disease pathology and in exploring novel therapies for this group of retinal conditions.

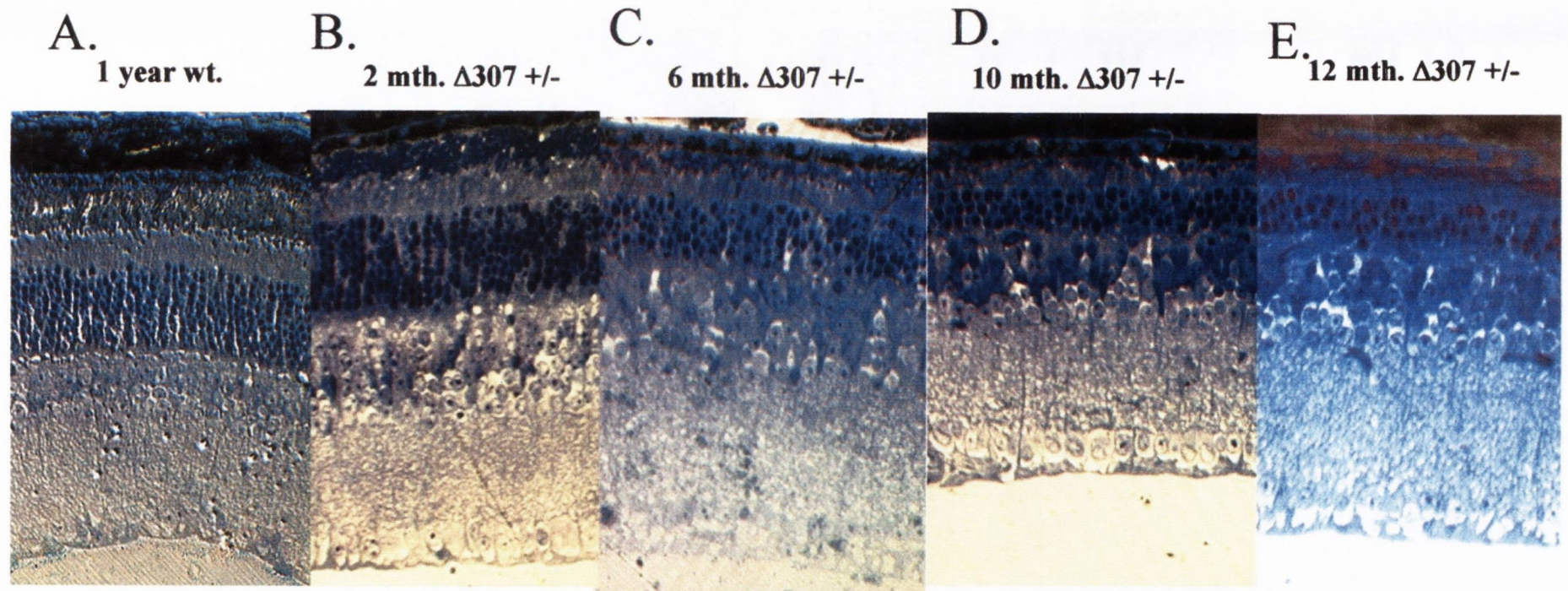


Figure 7.1: Light micrographs of retinal sections of 1-year-old wild-type mouse (A) and 2-, 6-, 10- and 12-month-old heterozygotes for the *rds-307* mutation (B–E), respectively. Magnification 400x.

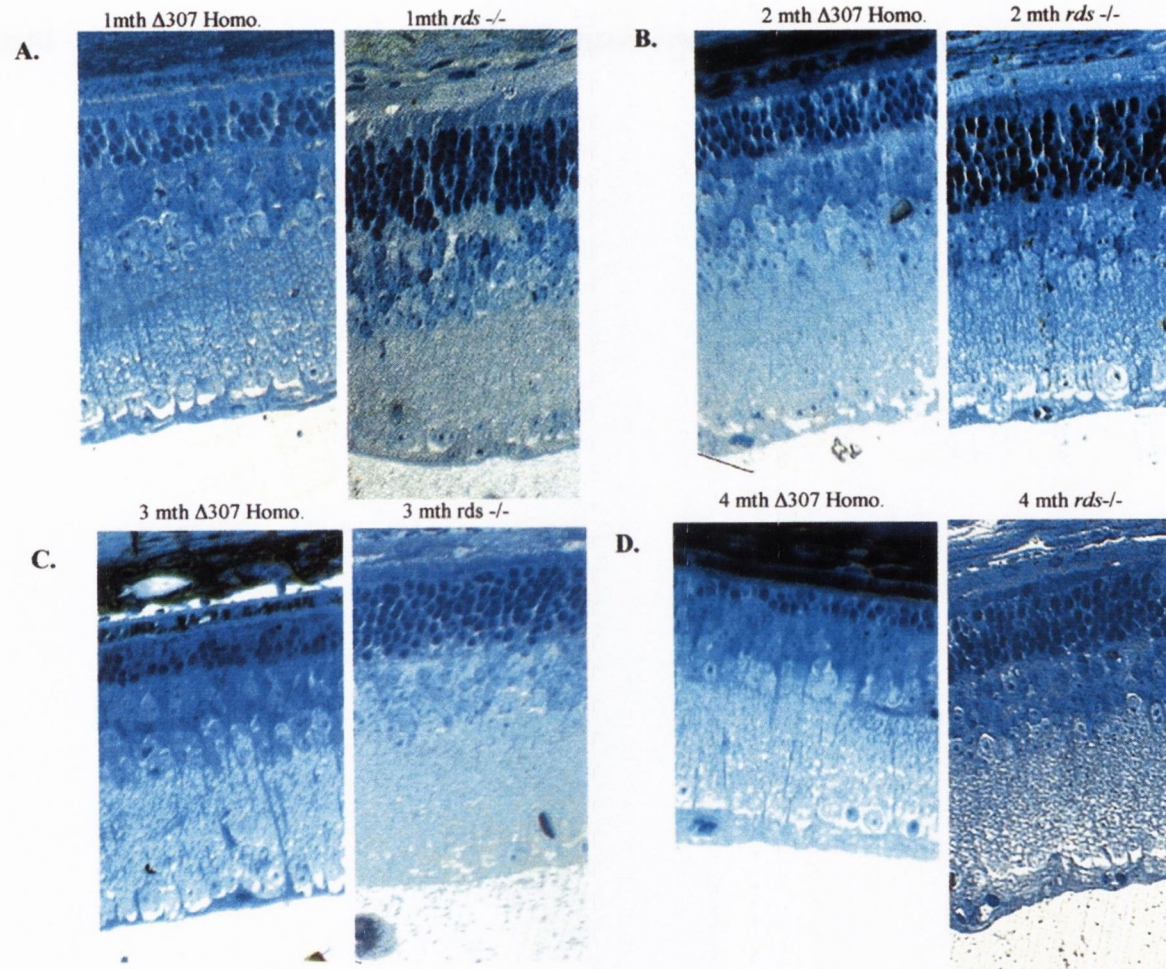


Figure 7.2: Comparison of light micrographs of retinal sections from *rds*-307 homozygotes and *rds*^{-/-} homozygotes at 1, 2, 3 and 4 months of age, respectively. Magnification 400x.

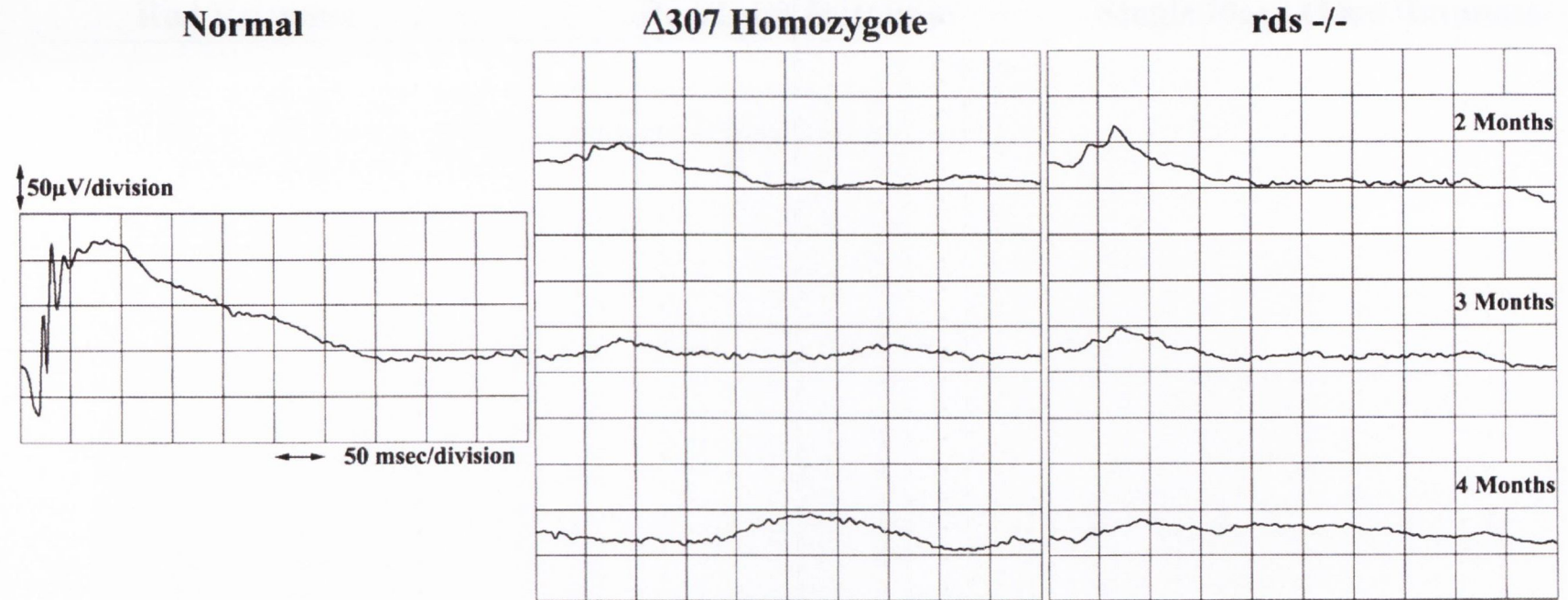


Figure 7.3: Mixed rod/cone electroretinographic responses of 2-, 3- and 4-month-old dark-adapted mice to a maximal-intensity flash. Note the gross attenuation of the b-wave in both $\Delta 307$ homozygotes and *rds*^{-/-} mice, the attenuation being greater in $\Delta 307$ animals.

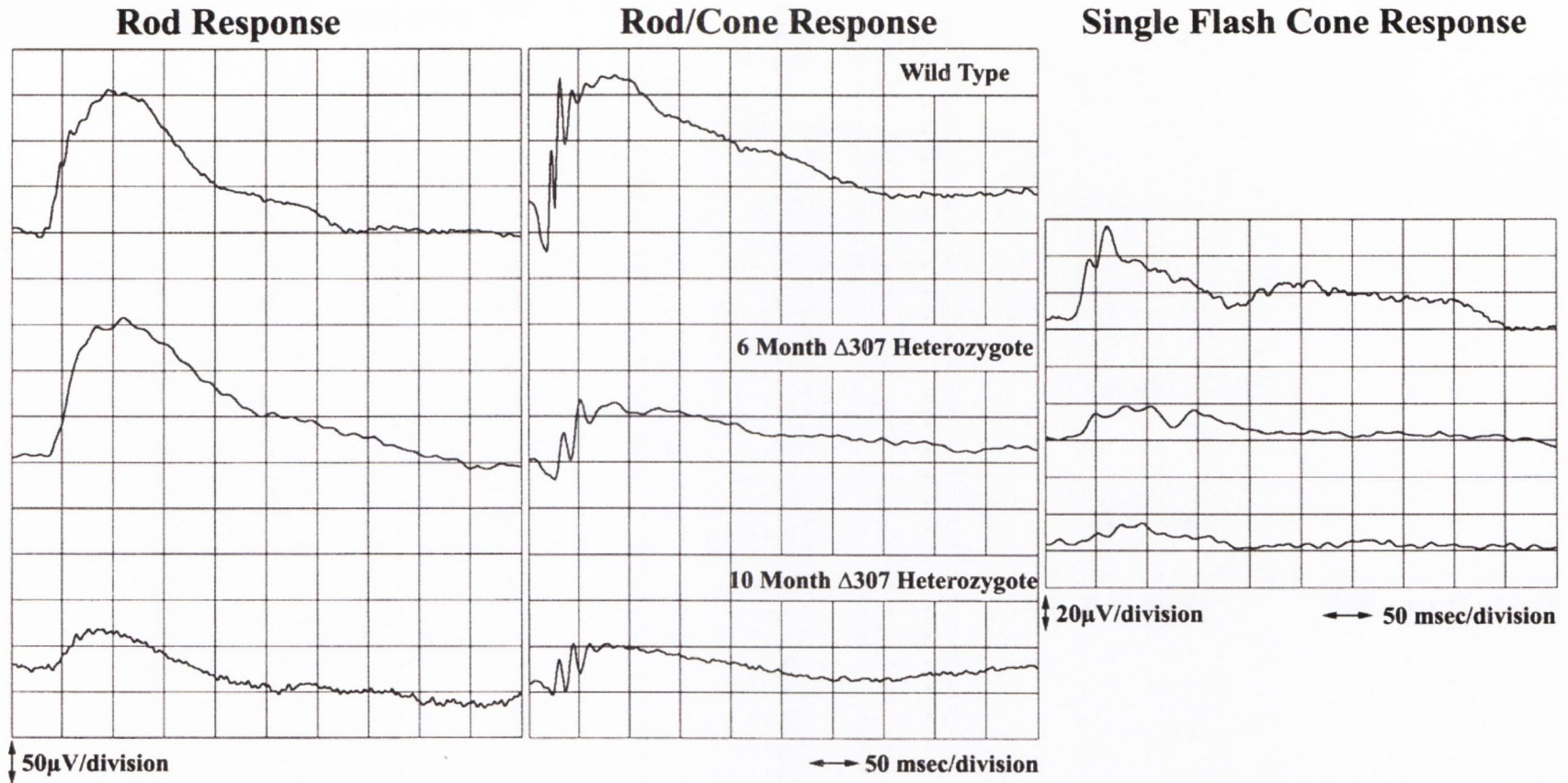


Figure 7.4: Rod-isolated, mixed rod/cone and pure cone responses from 6- and 10-month-old *rds-307* heterozygotes. Relatively normal rod responses persist up to 6 months in heterozygotes, but diminish significantly by 10 months. Mixed rod/cone responses are attenuated at 6 months, as are the pure cone amplitudes, with further deterioration noted by 10 months.

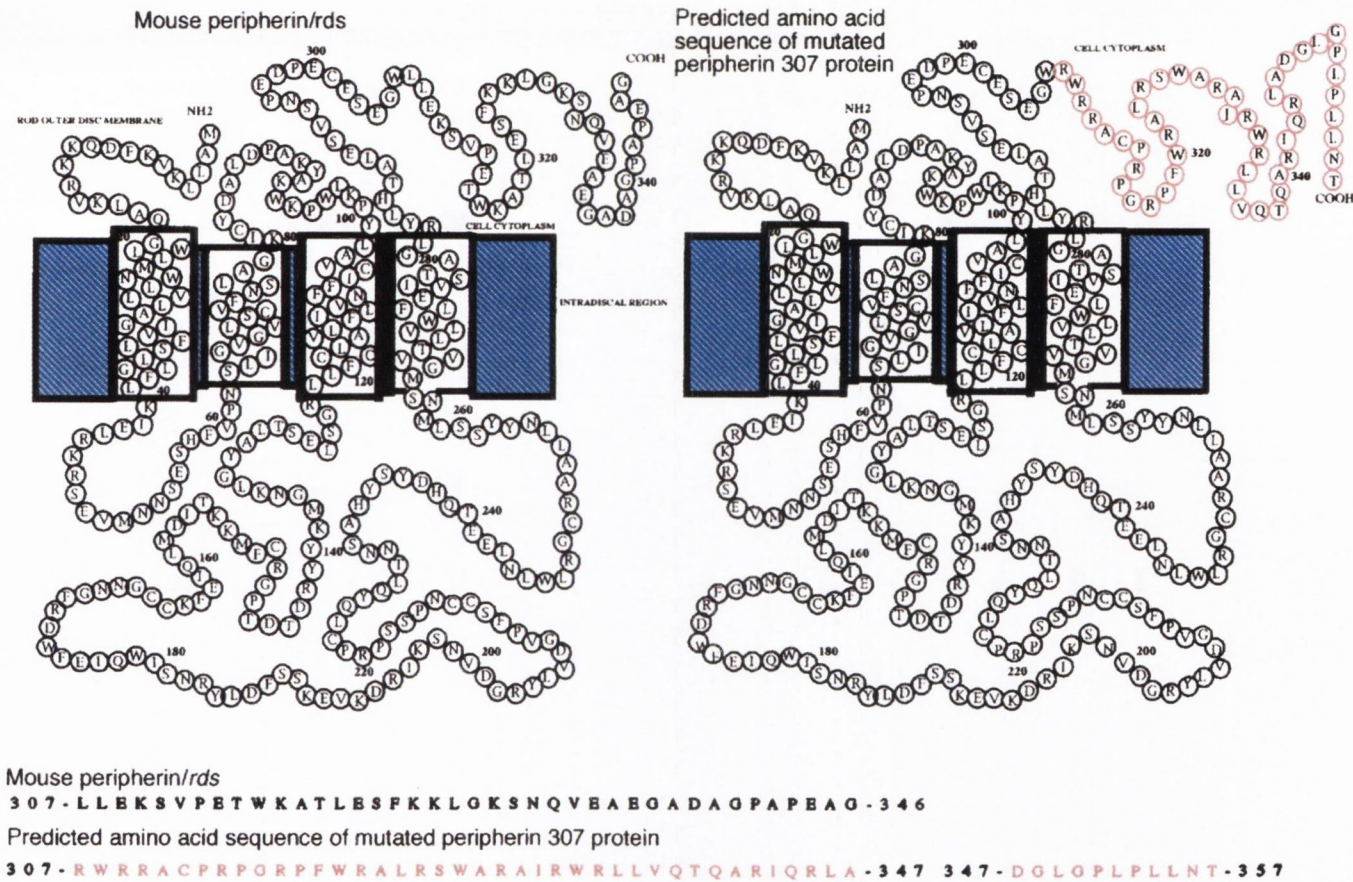


Figure 7.5: Topological model of normal rds–peripherin and mutated $\Delta 307$ rds–peripherin proteins based on findings published by Connell *et al.*, 1991; Bascom *et al.*, 1992 and Molday 1994). The predicted amino acid sequence of the mutated protein is highlighted in red.


```

301-----311-----321-----331-----341---- :
CESEGWLLEKSVPETWKATLESFKKLGKSNQVEAEGADAGPAPEAG : OrigSeq

-----HHHHHHHHHHHHHHHHHHHH-----EEE----- : dsc
-----HHH-----HHHHHHHHHHHH-----E----- : jalign
-----EEE-----HHHHHHHHHHHH-----HHHHHH----- : jfreq
-----HHHHHHHHHHHHHHHHHHHH----- : jhmm
-----E-----HHHHHHHHHHHH----- : jnet
-----HHHHHHHHHHHHHHHHHHHH----- : jpssm
H-----HHHH-----HH--H-----HHHH----- : mul
-----HHHHE-----HHHHHHHHHHHHHHHHHHHH----- : phd
-----EEE-----HHHHHHHHHHHHHHHHHHHH----- : pred
-----HHHHHH----- : zpred

-----E-----HHHHHHHHHHHHHHHHHHHH----- : Jpred

301-----311-----321-----331-----341-----351---- :
CESEGWRWRACPRPGRPFWRALRSWARAIRWRLLVQTQARIQRLADGLGPLLLNY : OrigSeq

-----HHH-----HHHHHHHHHHHH----- : jalign
-----EE-----HHHHHHHHHHHH-----HEE-E----- : jfreq
-----EE-----HHHHHHHHHHHHHHHHHHHH----- : jhmm
-----EE-----HHHHHHHHHHHHHHHHHHHH-----EE-----HHHHHH----- : jnet
-----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHH-----EEEEEEHHHHHHHHHHHH----- : jpssm
H-----HHH-----HHHHHH----- : mul
-----EEE-----HHHHHHHHHHHHHHHHHHHH----- : phd
-----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----- : pred
----- : zpred

-----EE-----HHHHHHHHHHHHHHHHHHHH----- : Jpred

```

Figure 7.6: Jpred output for C-terminal divergent sequences. This figure focuses on the key region of divergence in amino acid sequences at the C termini from residue 301 in both proteins, and illustrates the Jpred output of predicted secondary structure for both proteins (H, helix; E, extended; -, loop). It can be clearly seen that for the mutant protein, 4 of the 10 prediction engines queried report a higher helical (or more highly structured) content for the mutant over the wild-type in the C-terminal region. To verify this trend, the C-terminal sequences for the divergent regions (301-) were submitted to the agadir helical content prediction engine at EMBL. The percentage prediction of helical content for the mutant terminal fragment is 8.07%, compared with a 1.31% helical content predicted for the wild-type protein with this method. Following this prediction, the same sequence fragments were submitted to the ProtScale conformational analysis engine for comparison (Chou and Fasman, 1978; Muñoz and Serrano 1994; Muñoz and Serrano 1994; Muñoz and Serrano 1994; Muñoz and Serrano 1997 and Lacroix *et al.*, 1998).

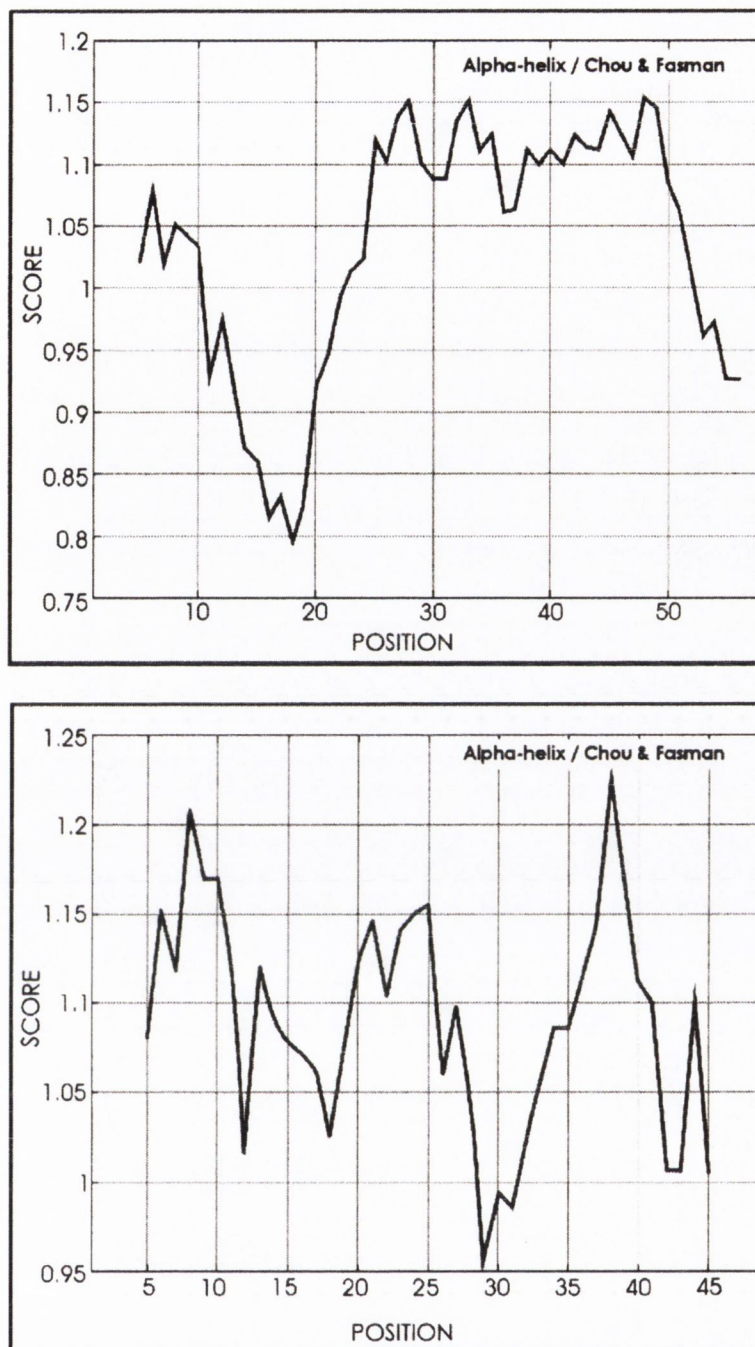


Figure 7.7: ProtScale conformational analysis for C-terminal divergent regions. This figure illustrates the ProtScale output for the wild-type (right) and mutant (left) terminal sequences. It can be seen from these graphs that the murine mutant terminus again has a higher predicted helical content (more structured) over the predicted helical content for the murine wild-type protein in this C-terminal region (8.07% versus 1.31%). Taken together, these predictive techniques indicate that the mutant protein produced with its amino acid variation from the RDS wild-type, has a more highly structured C-terminal domain than the wild-type. Attempts to generate homology structures using both the SwissProt homology engine and Imperial Cancer Pssm threading engines were unsuccessful because of the absence of available structural homologues for the RDS protein.

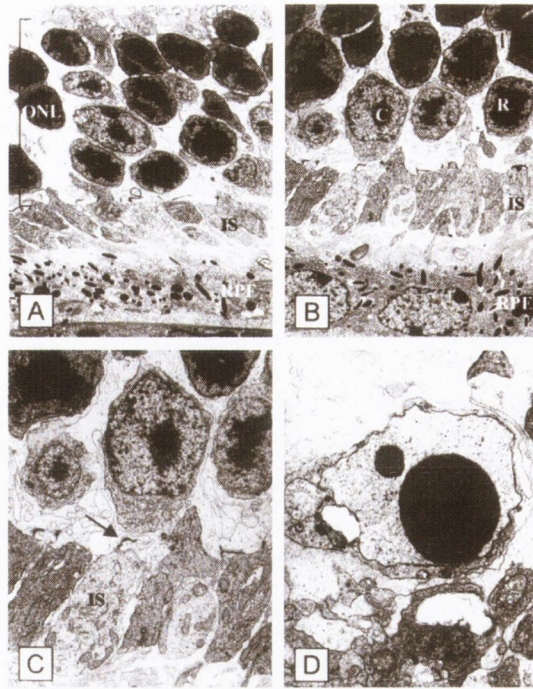


Figure 7.8: Ultrastructural assessment of the retina in mice homozygous for the *rds*-307 mutation at 5 weeks. (A) Comprehensive and widespread loss of photoreceptors, with ONL three or four nuclei in thickness. Photoreceptors have no outer segments, and the photoreceptor inner segments (IS) are adjacent to apical plasma membrane of the RPE. (B) A mixture of rods (R) and cones (C) remain, although it would appear that the cones have survived preferentially in these retinas. The RPE appears normal, although there is little evidence of recent phagosomes in their cytoplasm. (C) The cell shows typical cone photoreceptor morphology, with slightly larger, more electron-lucent nuclei in comparison with rod cells. The cone IS appear normal, although no outer segments are visible. Desmosomes forming the external limiting membrane are also obvious (arrow). (D) Apoptotic cell death is an ongoing process in these eyes, as indicated by numerous apoptotic bodies in the region of the ONL. This dying cell shows marked condensation of nuclear chromatin a hallmark of programmed cell death. Magnification: (A) x2500; (B) x2500; (C) x4000; (D) x8000.

7.2 On the molecular pathology of neurodegeneration in IMPDH1-based retinitis pigmentosa.

The work in this section was published in Human Molecular Genetics in 2004 (Aherne A, Kennan A, Kenna PF, McNally N, Lloyd DG, Alberts IL, Kiang AS, Humphries MM, Ayuso C, Engel PC, Gu JJ, Mitchell BS, Farrar GJ, Humphries P. Hum Mol Genet. 2004 Mar 15;13(6):641-50.)

7.2.1 Introduction:

Mutations within the inosine monophosphate dehydrogenase 1 (IMPDH1) gene cause the RP10 form of autosomal dominant RP, those families identified to date exhibiting an early onset, severe retinal degeneration (Kennan *et al.*, 2002; Bowne *et al.*, 2002; Jordan *et al.*, 1993). Very recently, Bowne *et al.*, (2006) have also reported mutations in the IMPDH1 gene in isolated cases of Leber's Congenital Amaurosis (LCA).

IMPDH1 functions as a tetramer, composed of four identical subunits, each of 55 kDa, and is the rate-limiting enzyme of the *de novo* pathway of guanine nucleotide biosynthesis. It converts inosine monophosphate into xanthosine monophosphate, which is then converted into guanosine diphosphate (GDP) and triphosphate (GTP). The functionally related enzyme, IMPDH2, performs a similar role, the two proteins sharing 84% identity at the amino acid level (Natsumeda *et al.*, 1990). Guanine nucleotides may also be synthesized in the so-called salvage pathway by the enzyme hypoxanthine phosphoribosyl transferase (HPRT). Both IMPDH isoforms exhibit widespread expression patterns but their regulation and levels of expression differ considerably (Senda and Natsumeda 1994). Expression of IMPDH1 has previously been shown to be tissue-specific, with high levels of expression, for example, in the lung, thymus and brain, but barely detectable levels of expression in liver and testis (Gu *et al.*, 2003). In mice, loss of both alleles of the *Impdh* type 2 gene results in very early embryonic lethality (Gu *et al.*, 2000). A mouse deficient in the *Impdh* type 1 gene has recently been generated using standard gene targeting techniques (Gu *et al.*, 2003). In contrast to the *Impdh*2 knockout, it was found that *Impdh* type I enzymatic activity is not essential for normal mouse development or fertility.

7.2.2 Methods

7.2.2.1 Mouse electroretinography

Mouse ERGs were recorded at 6 weeks of age (two animals), 5 months (six animals), 8 months (four animals), 11 months (five animals) and 13 months (three animals). Animals were dark-adapted overnight and prepared for electroretinography under dim red light. Pupillary dilatation was achieved by instillation of cyclopentolate 1% and phenylephrine HCl 10%. The subjects were anaesthetized by means of ketamine (2.08 mg per 15 g body weight) and xylazine (0.21 mg per 15 g body weight) injected intraperitoneally. Standardized flashes of light were presented to the mouse in a Ganzfeld bowl to ensure uniform retinal illumination. The subjects were placed on a TC 1000 Temperature Controller (CWE Inc, Ardmore, PA 19003 US) heating pad to maintain a constant body temperature of $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

The ERG responses were recorded simultaneously from both eyes by means of contact lens electrodes (Medical Workshop, Groningen, Netherlands) using amethocaine 1% as topical anaesthesia and Vidisic (Dr Mann Pharma, Germany) as a conducting agent and to maintain corneal hydration. A reference electrode was positioned subcutaneously approximately 1 mm from the temporal canthus and the ground electrode placed subcutaneously anterior to the tail. The responses were analysed using RetiScan RetiPort electrophysiology equipment (Roland Consulting GmbH). The protocol used was based on that approved by the International Clinical Standards Committee for human electroretinography. Rod-isolated responses were recorded using a dim white flash (-25 dB maximal intensity) presented in the dark-adapted state. The maximal combined rod-cone response to the maximal intensity flash was then recorded. Following light adaptation for 10 min to a background illumination of 30 candelas per m^2 presented in Ganzfeld bowl the cone-isolated responses were recorded to the maximal intensity flash (3 candelas per m^2 per second) presented as a single flash and 10 Hz flickers. a-waves were measured from the baseline to the trough and b-waves from the baseline (in the case of rod-isolated responses) or from the a-wave trough.

7.2.3 Results

7.2.3.1 Impdh and Hprt expression in the murine retina

In order to assess expression patterns of IMPDH1, IMPDH2 and HPRT in retinal tissues, *in situ* hybridizations were carried out using riboprobes specific for transcripts derived from these genes on mouse retinal cryosections. EST data obtained for the IMPDH2 gene (UniGene database; www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) indicate that this isoenzyme is expressed in retinal pigment epithelium (RPE) libraries, hence *Impdh2 in situ* hybridizations were performed on retinal sections from CD1 mice, which do not possess pigment in the RPE. The results of this analysis indicate that *Impdh1* is preferentially and strongly expressed within photoreceptor cells (Fig. 7.9A). Conversely, little or no *Impdh2* expression is detectable in photoreceptors and only low levels of *Hprt* transcript are observed (Fig. 7.9B and C). These results thus indicate that IMPDH1 is largely responsible for the production of guanine nucleotides within photoreceptor cells. In mice lacking the *Impdh1* gene, it has previously been reported that there is no elevation in expression levels of *Impdh* type 2 or HPRT transcripts detectable in response to a deficiency of *Impdh* type 1, in a number of tissues examined (Gu *et al.*, 2003). It is very likely, therefore, that in the case of a defective or absent IMPDH1 protein in the retina, IMPDH2 and HPRT may not be able to substitute for the loss of IMPDH1 activity. This is particularly relevant given that the retina is one of the most energy-demanding tissues of the body and that photoreceptor cells have a high requirement for GTP within the visual transduction cycle (La Cour 2002).

7.2.3.2 Retinal structure and function in *Impdh1*^{-/-} mice

7.2.3.2.1 Electroretinography

We evaluated photoreceptor activity in the retinas of *Impdh1*^{-/-} mice using electroretinography (ERG). Rod-isolated responses (Fig. 7.10A), light-adapted cone responses to single flash (Fig. 7.10B) and maximal, dark-adapted, combined rod and cone responses (Fig. 7.10C) were recorded at 6 weeks of age and 5, 8, 11 and 13 months. At 11 months of age all responses showed a significant reduction in a- and b-

wave amplitudes (Fig. 7.10A–C) and, in the case of the cone responses, significant delay in b-wave timing (Fig. 7.10B). Further deterioration was noted by 13 months. In the case of the maximal, dark-adapted, combined rod and cone responses, progressive reduction in wave amplitudes was noted from 5 months of age, indicating disturbance of phototransduction. This may be explained by disturbance in cGMP-dependent cation channel function, which requires a constant turnover of cGMP, which must be synthesized from GTP. A reduction in ERG amplitude, brought about by membrane hyperpolarization and closing of these channels, may be due to the disturbance in guanine biosynthesis consequent to lack of IMPDH1 activity.

It is notable that the electroretinographic disturbances observed in *Impdh1*^{-/-} mice appear to be much milder than those recorded in humans with dominant IMPDH1 mutations, where generally all ERG responses are unrecordable by the end of the second decade. The response in an affected 17-year-old male from a large RP10 family (Jordan *et al.*, 1993; Kennan *et al.*, 2002) to the maximal intensity flash presented in the dark-adapted state, which normally elicits a mixed rod–cone response, indicates that there is no recordable photoreceptor activity (Fig. 7.10D). The *Impdh1*^{-/-} mouse model at 4 months of age, which we estimate to be approximately equivalent to a teenage human subject in terms of relative lifespan, displays no detectable structural or functional degeneration of the retina.

7.2.3.2 Retinal Histology

Photoreceptor dysfunction in the *Impdh1*^{-/-} mouse is not the result of loss of photoreceptor cell viability, since the outer nuclear layer thickness of the retinas in these mice is similar to wild-type retinas up to 10 months of age (Fig. 7.11A–C), and even by 13 months of age there is only marginal loss of nuclei from the outer nuclear layer of the retina (data not shown). Preliminary evidence suggests some degree of disorganization of photoreceptor outer segments in older animals which is akin to a mild RP-like retinopathy. Thus, IMPDH1 does not appear to be essential for normal retinal development or early visual function. In the light of such observations, it appears unlikely that the autosomal dominant segregation pattern which is characteristic of the RP10 form of retinitis pigmentosa, is caused as a result of ‘haploinsufficiency’ for the normal IMPDH1 gene product. Rather, disease pathology is probably caused by a dominant-negative phenotypic effect exerted by mutant protein.

7.2.3.2.3 Functional analysis and mammalian expression of wild-type and mutant IMPDH1 proteins

Two amino acid substitutions, Arg224Pro and Asp226Asn, originally identified in RP10 families (Kennan *et al.*, 2002; Bowne *et al.*, 2002), lie in the second CBS domain that forms part of a smaller flanking subdomain of the IMPDH enzyme (Colby *et al.*, 1999). This region lies adjacent to the catalytic domain and is not thought to be required for activity (Sintchak *et al.*, 1996; Sintchak and Nimmesgern, 2000). In order to assess possible effects of these two established IMPDH1 mutations on the functional activity of the expressed enzyme, the wild-type and two mutant human IMPDH1 proteins were expressed in a bacterial expression system with incorporation of a His-tag for purification purposes. Protein fractions were purified from soluble cell lysates and enzymatic activity was determined by measuring the absorbance increase caused by reduction of NAD⁺ (Carr *et al.*, 1993). Specific enzyme activities for wild-type, Arg224Pro and Asp226Asn IMPDH1 enzymes were averaged at 1.22, 1.30 and 1.28 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively. Enzyme activities were measured on soluble protein only and all three enzymes showed similar levels of activity for up to 7 days. Therefore, no significant differences in specific enzyme activity were detectable between the wild-type and mutant IMPDH1 proteins. Calculated activities were also comparable to previously published reports of IMPDH1 specific enzyme activity levels (Hager *et al.*, 1995). A dramatic reduction in enzyme activity would be expected if the disease-causing effects of the mutations were primarily attributable to diminished activity under physiological conditions.

Mutant IMPDH1 proteins were found to exhibit marked decreased solubility in comparison to wild-type when expressed at a temperature of 37°C in bacterial cells. In order to investigate this observation further, human embryonic kidney (HEK) 293T cells were transfected with mammalian expression constructs containing wild-type, Arg224Pro or Asp226Asn mutant IMPDH1 sequences, with incorporation of a His-tag for protein detection purposes. After harvesting, cells were separated into total cell protein, soluble cytosolic, nuclear and insoluble pelleted fractions, which were subsequently analysed by SDS-PAGE electrophoresis and western blotting. Wild-type and mutant IMPDH1 proteins were expressed at similarly high levels in whole cell extracts with a band clearly visible at 55 kDa. No protein band of this size was evident in cells transfected with a construct that did not contain an IMPDH1

cDNA insert. Following analysis of cellular fractions obtained after further purification steps, wild-type IMPDH1 protein was shown to localize in the soluble cytosolic fraction, while no mutant protein was detectable in this fraction. Both mutant proteins exhibited considerably decreased solubility and as a result almost all of the mutant protein localized in the final pellet, which was re-suspendable in strong denaturant. These observations suggested that such mutant IMPDH1 proteins might be mis-folded, resulting in the formation of insoluble aggregates within the cell cytosol.

7.2.3.2.4 Computer protein modeling studies on mutant IMPDH1

To further investigate the existence of possible tertiary structural perturbation in mutant IMPDH1, we undertook a series of molecular modelling computational simulations for the Arg224Pro mutation. Proline residues located within α -helices have a tendency to distort the standard helical arrangement by causing the structure to kink about the position of the proline residue (Carr *et al.*, 1993). To test the hypothesis that a mutation from Arg to Pro could result in a mutant protein with perturbed structure correlating to impaired *in vitro* and *in vivo* function, structural homology models were constructed from both Arg-wild-type and Pro-mutant IMPDH1 sequences, building from available crystal structures of human (Colby *et al.*, 1999) and non-human (*Cricetulus griseus*) (Sintchak *et al.*, 1996) IMPDH which exhibited high homology to our sequences of interest. The resultant wild-type and mutant structures were subjected to an extensive computational molecular dynamics simulation to challenge and refine the models (Flohil *et al.*, 2002). No significant deviation in structure was noted for the wild-type protein post-dynamics, but significant structural perturbation was noted in the mutant structure around the area of point mutation (Arg224 to Pro224) by the end of the simulation run. Such structural perturbation would undoubtedly disrupt the formation of the biological homotetramer subunit and correlates to the observed physical and functional data for the mutant protein.

7.2.4 Discussion:

The RP10 gene was initially localized on chromosome 7q as a result of a systematic genetic linkage study mounted on DNA from a large Spanish family (Jordan *et al.*, 1993). It is estimated, based on the number of pedigrees so far identified with the RP10 form of retinopathy, that this genetic sub-type of disease accounts for 2–5% of autosomal dominant cases of RP (Sohocki *et al.*, 2001). The *in situ* hybridization data presented here clearly demonstrate high levels of *Impdh1* expression within the photoreceptor cell layer of mouse retinas, in comparison to levels of transcript derived from the *Impdh2* and *Hprt* genes. The retina has the highest metabolic rate of any tissue of the body, photoreceptors having a particularly high requirement for GTP in visual transduction processes (La Cour 2002). Therefore, depletion of the GTP pool over time is the most likely explanation for the reduction in ERG amplitudes seen in the retinas of older *Impdh1*^{-/-} mice and is the cause of the mild retinopathy that such animals develop. In addition, the human and mouse IMPDH1 sequences share 98% identity at the protein level which strongly suggests that IMPDH1 function in the retina is highly conserved amongst species.

The *Impdh1*^{-/-} mouse represents the first reported model carrying a targeted deletion of a ubiquitously expressed dominant RP-associated gene and has been shown to display no detectable structural or functional degeneration of the retina up to 5 months of age. In contrast, previously reported murine models in which there is a targeted deletion of a dominant RP-associated gene, including the rhodopsin, *rds*-peripherin, *Rpl* and *Crx* knockout mouse models, have resulted in phenotypes in which there is significant degeneration of photoreceptor cells and in many cases, completely diminished ERG amplitudes by 5 months of age (Humphries *et al.*, 1997); Sanyal *et al.*, 1980; Gao *et al.*, 2002; Furukawa *et al.*, 1999). While it will not be possible to directly compare the molecular pathologies associated with autosomal dominant and recessive (represented by the *Impdh1*^{-/-} null mouse) forms of IMPDH1-based disease unless human subjects with the latter form of disease are identified, the data presented nevertheless strongly support the concept that the retinopathy in human subjects carrying dominantly acting mutations is very much more severe than the disease state which has been shown to occur as a result of a targeted disruption of the *Impdh1* gene in mice.

The studies presented on mutant IMPDH1 proteins show that there are no detectable differences in enzyme activity when compared with wild-type, but that a

substantial difference in solubility between wild-type and mutant proteins is observable in both bacterial and mammalian cells. In this regard Mortimer and Hedstrom (2005) have recently reported that they found no difference in the solubility of mutant and wild-type proteins. Such observations, along with distinct perturbations in protein structure predicted by computer modelling of mutant IMPDH1 structure, point to a probable dominant-negative molecular pathology for RP10, as a result of alterations in the folding and solubility of the mutant protein. It is of note that similar differences in solubility between wild-type and mutant proteins have been demonstrated in relation to the RP-associated splicing factor PRPF31 (Deery *et al.*, 2002), and protein folding defects associated with rhodopsin mutations that lead to aggresome formation have also been described (Liu *et al.*, 2002; Illing *et al.*, 2002). Hence, in RP10, mutant IMPDH1 protein misfolding and subsequent aggregation may be a central event in the initiation of neuronal cell death, as it is in a range of other hereditary or multifactorial neurodegenerative diseases including Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis (Sherman and Goldberg 2001; Kopito, 2000; Bence *et al.*, 2001).

Notwithstanding recent successes in restoration of visual function or preservation of the retina in autosomal recessive forms of degenerative retinopathy, including the Briard dog model of Leber congenital amaurosis (Acland *et al.*, 2001) and the RCS rat following AAV-mediated delivery of therapeutic genes to retinal tissues (Smith *et al.*, 2003), the extensive intragenic heterogeneity associated with autosomal dominant forms of retinal degeneration is a major impediment to the development of successful and economically viable therapeutics. For example, in excess of 200 different mutations have been encountered within the rhodopsin and RDS-peripherin genes alone (Retina International Database; www.retina-international.org/sci-news/rhomut.htm). While the IMPDH1 gene was implicated in disease etiology only within the last 4 years, the number of mutations identified within the gene in patients with RP is already rising (Bowne *et al.*, 2003). Hence suppression strategies targeting individual mutations are unlikely to be viable in economic terms. While a series of so-called mutation-independent suppression-replacement strategies has been described, such approaches involve two major steps, the simultaneous suppression of transcripts derived from both normal and mutant alleles, together with the introduction of a replacement gene, altered, for example at third-base degenerate sites, such as to escape suppression but nevertheless encoding functional protein (Millington-Ward *et al.*, 1997; Millington-Ward *et al.*, 1999;

O'Neill *et al.*, 2000). Insights we have gained into the nature of the molecular pathology of retinal degeneration in RP10, combined with studies on the autosomal recessive form of disease in the *Impdh1*^{-/-} mouse, suggest that RP10 may be a particularly attractive potential therapeutic target for two reasons. Firstly, only a single suppression step, involving simultaneous silencing of both normal and mutant alleles of the IMPDH1 gene would, in principle, be sufficient to remove the major dominant negative pathological effect of mutant IMPDH1 protein, leaving a situation in which photoreceptor neurons continue to survive and remain sufficiently functional such as to provide useful vision, possibly well into adult life. Secondly, while gene silencing using RNA interference is now an established technique in mammalian systems (McManus and Sharp 2002), discrimination between normal and mutant transcripts at the level of single nucleotides has so far met with only partial success (Miller *et al.*, 2003). In the case of the therapeutic approach outlined here, such constraints do not apply, since there is not a requirement to target single base changes.

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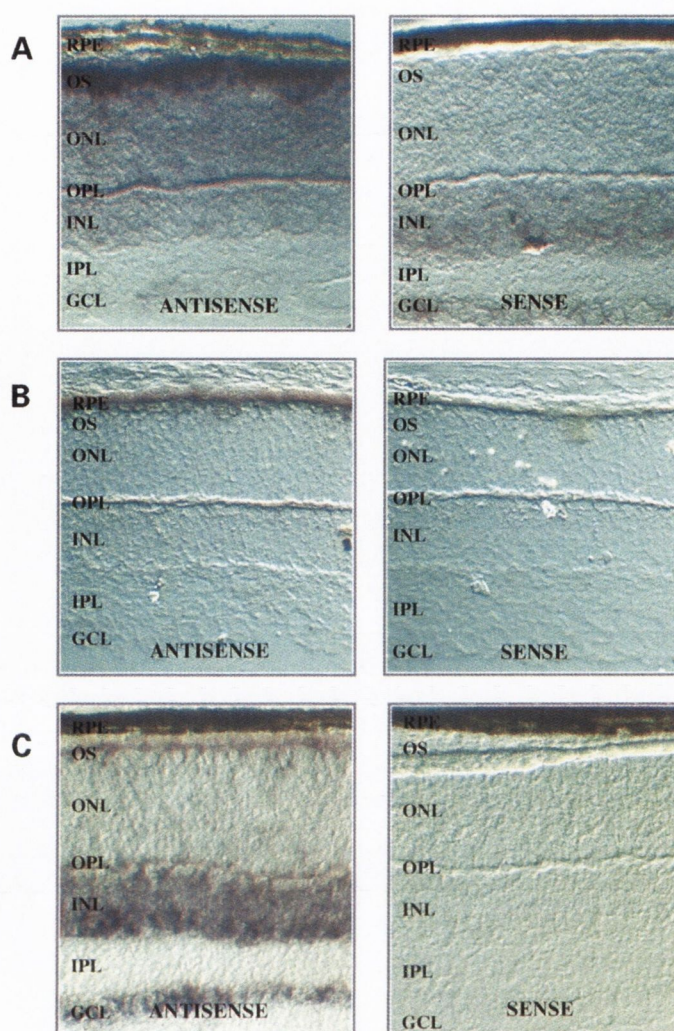


Figure 7.9. *In situ* hybridization studies on mouse retinal cryosections. Retinal sections with antisense and sense DIG-labelled riboprobes for (A) *Impdh1*, (B) *Impdh2* and (C) *Hprt* are shown. The *Impdh2 in situs* are performed on CD1 mouse retinas, which lack the pigment normally found in the RPE, while the *Impdh1* and *Hprt in situs* are performed on wild-type pigmented retinas. Strong expression of *Impdh1* is detected in the photoreceptor neurons. *Impdh2* expression appears to be restricted to the RPE, with possibly very low level staining in the photoreceptor outer segments. Analysis of *Hprt* reveals expression in the inner nuclear and ganglion cell layers along with a lower level of expression in the photoreceptors. (RPE) retinal pigment epithelium, (OS) outer segments, (ONL) outer nuclear layer, (OPL) outer plexiform layer, (INL) inner nuclear layer, (IPL) inner plexiform layer, (GCL) ganglion cell layer. 200x magnification.

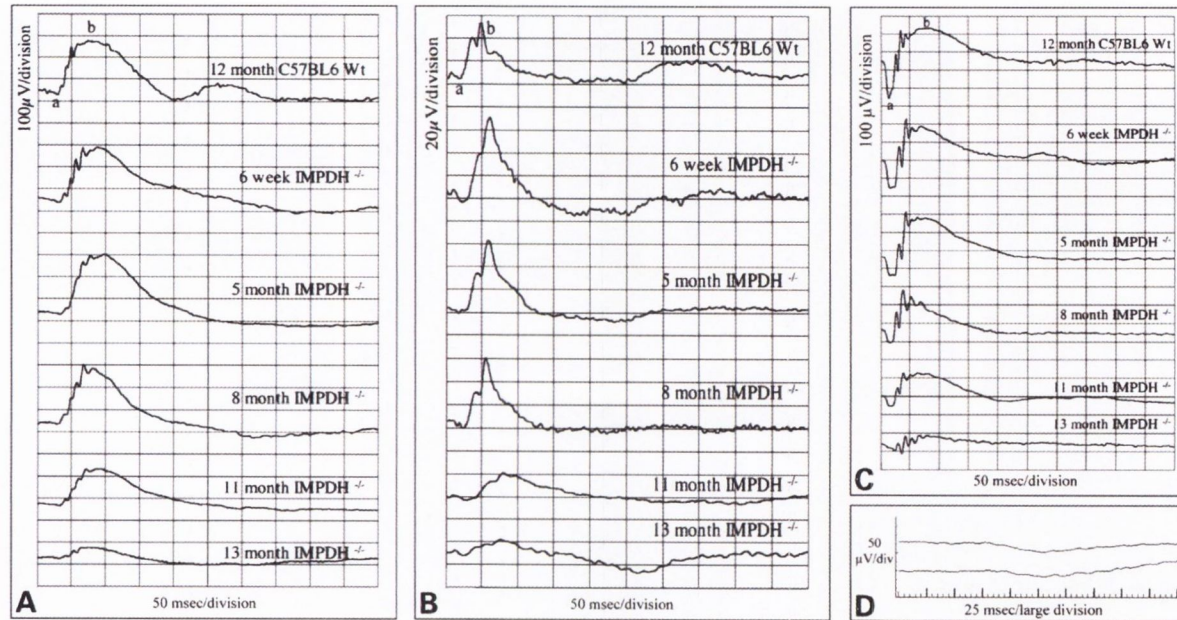


Figure 7.10. ERG analysis in *Impdh1*^{-/-} mice. (A) Rod-isolated responses, (B) light-adapted cone responses to single flash and (C) maximal, dark-adapted, combined rod and cone responses were recorded in a 12-month C57 wild-type animal, and *Impdh1*^{-/-} animals at 6 weeks of age, 5 months, 8 months, 11 months and 13 months. At 11 months of age all responses showed a significant reduction in a- and b-wave amplitudes and, in the case of the cone responses, significant delay in b-wave timing. Further deterioration was noted by 13 months. In the case of the maximal, dark-adapted, combined rod and cone responses progressive reduction in a-wave amplitude was noted from 5 months of age indicating disturbance of phototransduction. (D) ERG analysis of combined rod and cone response in a 17-year-old affected patient harbouring an Arg224Pro IMPDH1 mutation shows completely diminished responses.

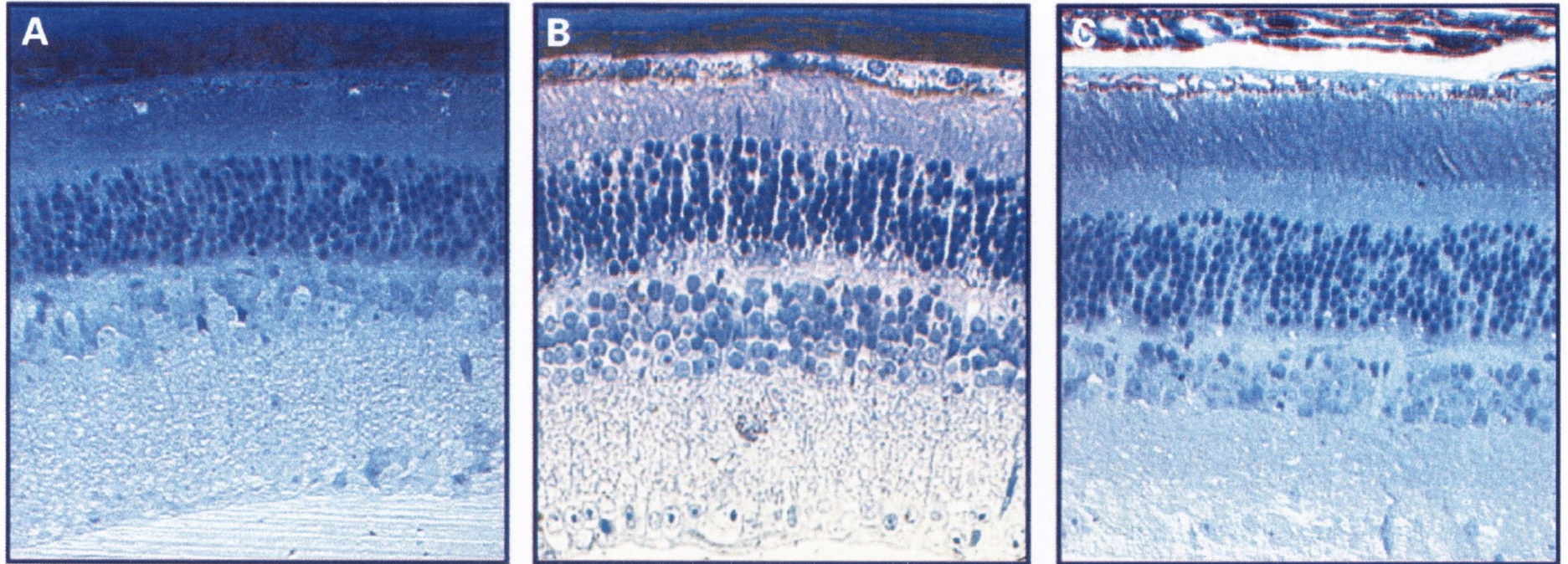


Figure 7.11. Light micrographs of retinal sections from *Impdh1*^{-/-} mice. Sections shown are (A) 6-month *Impdh1*^{-/-}, (B) 10-month *Impdh1*^{-/-}, (C) 10-month wild-type C57/BL6. The outer nuclear layer thickness of *Impdh1*^{-/-} retina is virtually indistinguishable from that of wild-type up to 10 months of age.

Conclusion

Inherited retinopathies, at present, are untreatable. The consequences in terms of human suffering, not to mention in economic terms, of this situation are of major significance. As a clinician who has had to impart the diagnosis of Retinitis Pigmentosa to many patients, this devastation has been all too evident. The inability to offer even the prospect of slowing down the inexorable progression of the condition has been a frustration for clinicians and a tragedy for patients since the condition was first described by von Graefe in the 1850s following the invention of the ophthalmoscope by von Helmholtz in 1851.

Until the molecular genetic revolution of the 1970s and 1980s investigators could only speculate as to the cause of these blinding conditions. The strategy of genetic linkage analysis enabled disease-causing genes to be identified in the absence of any knowledge about the pathophysiology of the condition. This 'reverse genetics' allowed the mysteries of inherited conditions to be revealed.

The research described in this thesis is a classic example of the success of this strategy. A major gene, mutations in which are now known to be responsible for a significant proportion of autosomal dominant Retinitis Pigmentosa, was identified by utilizing this strategy in our family TCD M1.

Gene function, of its nature, can only be observed in all its complexity in a living organism. Animal models of inherited retinopathies, both naturally occurring and genetically manipulated, have been of immense value in the detailed study of the pathophysiology of degenerative retinopathies and are an essential tool in the investigation of possible therapeutic options. The utility of these models is, I hope, well illustrated in the work on the $Rho^{-/-}$, rds-Peripherin codon 307 deletion, and the IMPDH1 $^{-/-}$ mice described in this volume.

It is the most earnest desire of anyone suffering from this condition that a treatment be found. As a result of this work, and the work of many other investigators world-wide, the prospects of treatments for patients with Retinitis Pigmentosa are becoming more realistic.

I am now optimistic that this goal will be achieved in my working life-time and count myself fortunate to have been able to contribute in a small way to realization of this goal.