Retinal Degeneration: Models and Therapies

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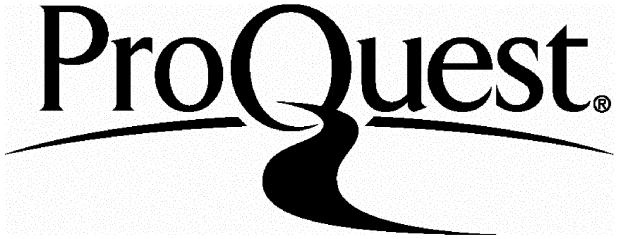

Abstract

Retinal degeneration is one of the leading causes of blindness in the developed world. There is currently no effective therapy. In order to improve our understanding of these conditions, we characterised three animal models of retinal degeneration, namely pigmented retinal degeneration slow (rds) mouse, retinal dystrophy (Rdy) cats and the miniature longhaired dachshund dog (MLHD). We then carried out a series of treatment trials using both inhibitors of caspases and neurotrophic factors in these animals.

Using immunohistochemical techniques, there was mis-localisation of opsin, reduction of synaptophysin in the outer plexiform layer and increased expression of glial fibrillary acidic protein in Muller cells in all of these animal models. Apoptosis was found to be the main mode of photoreceptor cell death.

Photoreceptors in the pigmented rds mouse were found to degenerate more slowly than the albino rds mouse. The Rdy cats had opsin labelled neurites similar to those described in human retinal degeneration. The MLHD dog was demonstrated to have a cone-rod dystrophy (CORD) based on electrophysiological studies. It represents the first animal model of CORD.

As apoptosis is the main mode of cell death, we attempted to either block the apoptotic pathway by caspases inhibitors or prevent photoreceptors entering the apoptotic pathway using neurotrophic factors.

It appears that DEVD, a caspase-3 inhibitor, might slow photoreceptor cell loss in the pigmented rds mice, but z-VAD and YVAD did not. We have also found that Axokine, a ciliary neurotrophic factor analogue, might prolong photoreceptor cell survival in the Rdy cats, but brain derived neurotrophic factor had no effect. Furthermore, Axokine appears to preserve retinal function in the MLHD dogs as

measured by electroretinography for a short period of time after an initial suppression.

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INTRODUCTION

A1: Retina and retinal degeneration

1.1 Study Aims

Hereditary retinal degeneration is characterized by poor night vision and restricted visual field (Bird, 1995). It affects about 1 in 3500 people (Bunker et al., 1984). There is currently no effective treatment and it is one of the leading causes of working age blindness in the developed world (Evans et al., 1996). It has becoming clear that the genetic defects can indirectly lead to cell death. It is of interest that in these, and other retinal degenerations, photoreceptor cell death occurs by apoptosis (Chang et al., 1995; Chang et al., 1993; Cook et al., 1995; Li and Milam, 1995; Tso et al., 1994; Xu et al., 1996).

In vitro studies have suggested that cells can be prevented from entering the apoptotic cascades by neurotrophic factors and apoptosis can be halted or delayed by caspases inhibitors . These observations imply that therapeutic modification of the process leading to apoptosis might modify the course of these disorders. In this thesis, we investigated the effectiveness of these reagents in a number of animal models of genetically determined retinal degeneration.

1.2 Retina - Anatomy and Physiology

Retina is the innermost layer of the eye. It is a multi-layered structure consists of cells mostly from neuronal or glial origin. There is also a vascular bed. It has been described as an approachable part of the brain (Dowling, 1987). Its main function is to capture visual signals. After some local processing, the information is passed on to the central nervous system. In both animals and human, genetic mutations, trauma, infection, inflammation, vascular disease and neoplasia can affect this layer.

From an anatomical standpoint, it can be divided into the inner retina and the outer retina. In the inner retina, the ganglion cells are often the disease-targeted cells, in which glaucoma and optic nerve diseases are the most common cause of ganglion cell dysfunction. In the outer retina, the photoreceptors are the main target in chronic processes. In this thesis, photoreceptor degeneration will be the main focus with a bias towards genetically determined diseases.

The retina is traditionally divided into 10 layers (Fig 1.2a):

1. Retinal pigment epithelium
2. Inner and outer segments of rods and cones
3. Outer limiting membrane
4. Outer nuclear layer
5. Outer plexiform layer
6. Inner nuclear layer
7. Inner plexiform layer
8. Ganglion cell layer
9. Nerve fibre layer
10. Inner limiting membrane

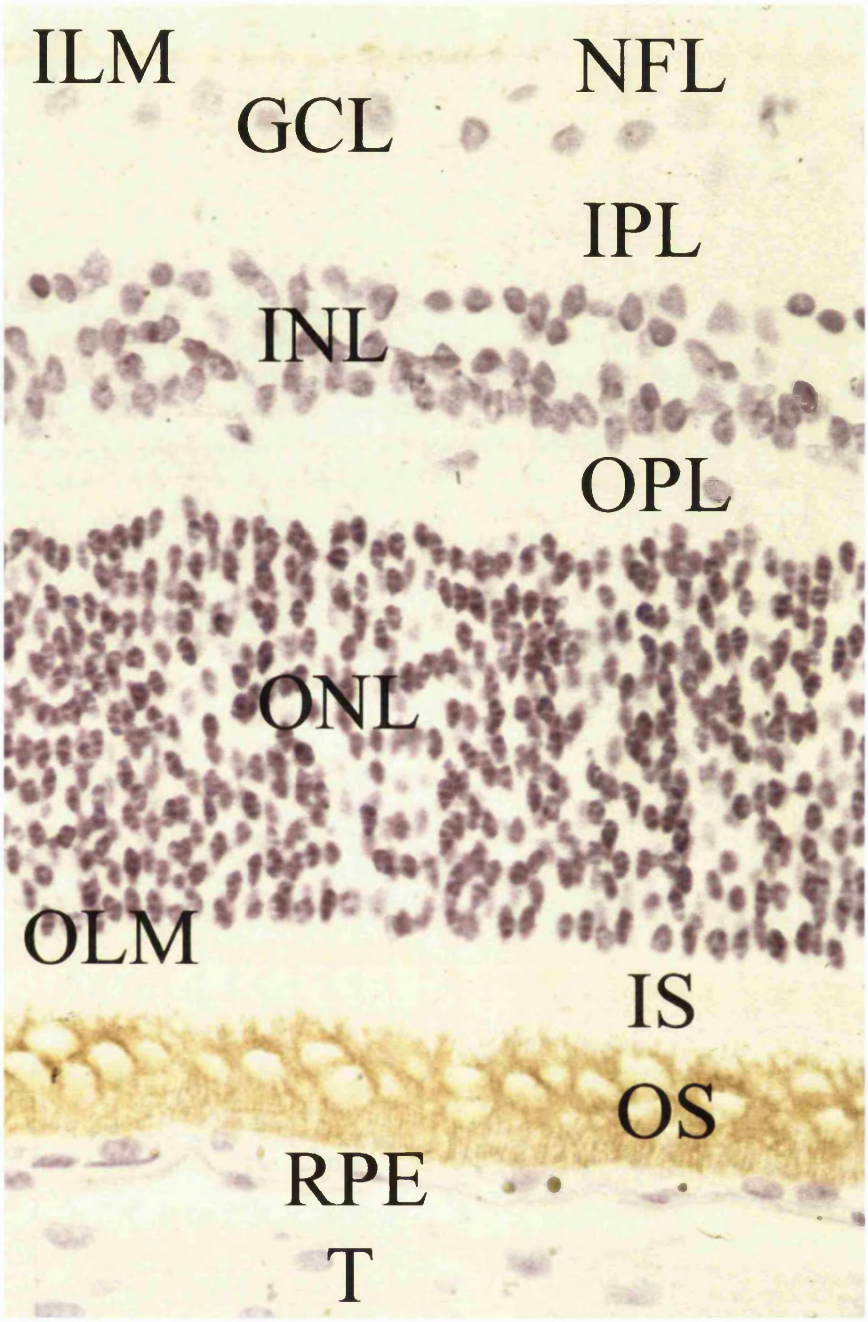

Figure 1.2a: Light microscopy of normal feline retina overlying tapetum (x200)

T = tapetum, RPE = retinal pigment epithelium (non-pigmented in this area), OS = outer segment (highlighted brown by opsin immunohistochemistry), IS = inner segments, OLM = outer limiting membrane, ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer and ILM = inner limiting membrane.

Retinal pigment epithelium (RPE)

The RPE is a single layer of epithelial cells located between the neurosensory retina and the choroid. They are multifunctional in nature and have been compared to macrophages, epithelium, glial cells, melanocytes and hepatocytes. The unique structure of the RPE allows the establishment of a metabolically active interface between retina and choroid.

The RPE is a polarised epithelium with the basal epithelial surface orientated towards the outside of the eye and the apical surface towards the vitreous. At its apical surface, the RPE is in contact with the interphotoreceptor matrix and outer segments of the photoreceptor cells. There is an intricate intimate relationship between the RPE and photoreceptors. Apical protrusions from the RPE form anastamosing ridges and microvilli that lie between the outer segments of the photoreceptors. Shorter microvilli ridges surround the distal one third of the outer segment.

The apical membrane contains recognition molecules for endocytosis, ion channels, transport molecules and the Na+ / K+ ATPase enzyme. The lateral borders of adjacent cells are joined together by a tight junction, a zonula adherens and desmosomes (Hogan et al., 1971). The basal surface rests on the basement membrane that has multiple infoldings (Burns and Feeney-Burns, 1980). The RPE serves not only as a physical barrier for free diffusion between retina and the choroidal circulation, it also aids in the adhesion of the neurosensory retina to its apical processes by both its interdigitations with the outer segments of the photoreceptors, by means of the interphotoreceptor matrix and also through transport pumps. The RPE also functions to improve the optics of the eye by absorbing light energy via its melanin granules thus decreasing light scattering.

Other important functions of the RPE include vitamin A esterification, isomerisation and transport, synthesis of extracellular matrix for both the interphotoreceptor matrix and for its basement membrane, selective delivery of lipids and production of growth factors.

Photoreceptors

Rods and cones are specialised neurons of the vertebrate retina that initiate the primary events of vision (Molday, 1998). Both receptors are elongated cells consisting of several morphologically and functionally distinct regions. However rods and cones differ in their metabolic and morphological characteristics and these differences are reflected in their different specific response kinetics and sensitivity.

The rods contain a visual pigment called rhodopsin, are particularly suited to respond under scotopic conditions, and are most sensitive to blue-green light with a peak absorption at a wavelength of 500 nm. Cones contain cone opsins as their visual pigments and, depending on the actual structure of the opsin molecule, are maximally sensitive to either long wavelength light (red) or middle wavelength light (green light) or short wavelength (blue) (Anhelt, 1998). The basis of colour perception relies on cones of different wavelength sensitivity and the connecting pathways to deliver the signal to the brain. Some primates and humans are trichromatic. Cones are less sensitive, function in bright light and subserve detailed vision and colour perception. Animals that are nocturnal have high densities of rods, whereas those with large cone populations tend to be diurnal.

In human, the cone density is highest in the foveal pit, with a rod free zone of about one degree. This falls rapidly outside the fovea to a fairly even density in the peripheral retina. There is a peak of the rod photoreceptors in a ring around the fovea at about 20 degrees from the foveal pit (Osterberg, 1935).

Differences of Rods and Cones in human

	Rods	Cones
Numbers	120 millions per eye	7 millions per eye
Distribution	Uniformly disturbed throughout the eye except fovea	Red and green sensitive cones concentrated in fovea, blue sensitive cones near fovea. Less concentrated elsewhere
Sensitivity to light	High sensitivity	Lower sensitivity
Outer Segments	Longer, closed discs	Shorter, open discs
Photopigment	More, in membranous discs inside the outer segment	Less, incorporated in folds of the outer segment membrane
Function	Night vision	Daytime and colour vision
Connections	Many rods link to each retinal ganglion cells	One cone to one ganglion cell in fovea
Receptive fields	Larger	Smaller and hence better acuity
Directional sensitivity	Sensitive to light rays with wide angle of incidence	More specific directional sensitivity

Table 1.2: Differences between rods and cones in human

Bipolar ceils

In human retina nine different bipolar cell types have been shown by Golgi staining (Kolb et al., 1992). Eight are cone specific and one is devoted to rods. The rod bipolar is typically a broad cell with its cell body situated central to high in the inner nuclear layer, and produces a tuft of dendrites entering the OPL reaching up to different levels between cone pedicles to reach the stacked rod spherules (Kolb, 1970). In the central retina the rod bipolar dendritic trees are small and contact with up to 20 rods. In contrast in the peripheral retina the dendritic tree is wider and contacts 40-50 rods (Kolb, 1970).

Five of the cone bipolar types are diffuse cone bipolar cells that receive converging information from many cones. The other three cone bipolar types have single cone contacts in a one to one relationship. These are known as midget bipolar cells.

The invaginating midget bipolar type (IMB) connects with the cone pedicle as central invaginating dendrites at ribbon synapses in the cone pedicles (Kolb, 1970). Flat midget bipolar cells (FMB) contact the cone pedicle by means of semi-invaginating, wide-cleft basal junctions. Often FMB dendrites make two contacts with the cone pedicle on either side of the central invaginating dendrite from the other midget bipolar cell (Kolb, 1970).

A cone bipolar cell that is thought to be specific for the short wavelength cones or blue cones (S-cones) has been described in human retina (Kolb et al., 1992). This blue S-cone bipolar typically contacts one cone heavily by several dendrites converging on that particular cone pedicle as central elements at the ribbons. Hence, it is essentially another type of midget bipolar cell, but it differs from regular IMBs and FMBs, in having also two or more wispy dendrites contacting either another cone pedicle or ending blindly in the OPL.

Horizontal cells

All mammalian retinas have two types of horizontal cell. Both types are laterally interconnecting neurons in the outer plexiform layer (Kolb et al., 1980). One is an axonless type (often called A-type). This type solely contacts cones. The other (often called B-type) has dendrites contacting cones, and an axon of several hundred microns length that turned into a terminal structure that contacts only rods (Kolb, 1970).

Horizontal cells are characterised by large surface area gap junctions between dendrites of neighbouring cells. These junctions allow lateral flow of electrical signals within a syncitial network of cells. Receptive field sizes are increased, as is the area over which horizontal cells integrate photic information.

Horizontal cells send visual information back to cones through feedback synapses. This interaction takes place through processes invaginating photoreceptor synaptic endings. Light stimulation is believed to reduce the release of the neurotransmitter gamma amino-butyric acid (GABA) from horizontal cells onto cones, although GABA antagonists typically do not block this feedback. Through the horizontal cell pathway, wide field stimuli exercise an indirect, delayed, depolarising influence on cones, opposing the direct hyperpolarising influence of light stimuli on the cone phototransduction machinery. Horizontal cells impinge synaptically on bipolar cells, feeding visual information forward to these second-order retinal neurons.

Amacrine cells

The amacrine cells are classified into different types according to their morphological characteristics based on the size of their dendritic tree (i.e. small, medium and large), branching characteristics (i.e. tufted, varicose, linear, beaded and radiate), and most importantly on the stratification of their dendrites in the IPL (Mariani, 1990; Kolb et al., 1992). Typically amacrine cells synapse upon other cells including bipolar axons, other amacrine cells or ganglion cell dendrites in the enlarged varicosities at conventional synapses (Dowling and Boycott 1966). Amacrine cells in general are inhibitory neurons.

Ganglion cells

Ganglion cells are the final output neurons of the vertebrate retina. They collect visual information from bipolar cells and amacrine cells, which is integrated within the ganglion cell dendrites and cell body, and transformed into nerve spikes.

Ganglion cells are the most complex information processing systems in the vertebrate retina (Shapley and Perry, 1986). Different cells become selectively tuned to detect subtle trigger features of the visual scene, including colour, size, and direction and speed of motion. Even so signals detected by ganglion cells may not have a unique interpretation. Equivalent signals might result from an object changing brightness, changing shape, or moving. The brain has to determine the most likely interpretation of detected events. Ganglion cell axons terminate in brain visual centres, principally the lateral geniculate nucleus and the superior colliculus. Ganglion cell axons are directed to specific visual centres depending on the visual trigger features they encode.

Muller cells

Muller cells are the principal glial cell of the retina. They are non-neuronal cells and perform diverse functions supporting retinal neurons. They provide architectural support with processed extending across the thickness of the whole retina and forming the limits of the retina at the inner and outer limiting membranes. Muller cell bodies are in the inner nuclear layer. Their processes are intimately related to the cell bodies of neurons in the nuclear layers and surround groups of neural processes in the plexiform layers. The inner limiting membrane is formed by the conical foot plates of the Muller cell and these cells also form foot plates on the large retinal blood vessels at the inner surface of the retina. The surface of the Muller

cell membrane facing the vitreous is covered with a mucopolysaccharide substance, thus forming a basement membrane.

The function of Muller cells includes (modified from Reichenbach and Robinson 1995):

- supplying end products of anaerobic metabolism (breakdown of glycogen) this is to fuel aerobic metabolism in photoreceptors
- scavenging of neural waste products such as carbon dioxide and ammonia
- recycling of amino acid transmitters
- phagocytosis of neuronal debris
- protection of neurons from excessive exposure to neurotransmitters
- release of neurotransmitters
- production of growth factors in response to injury
- synthesis of retinoic acid from retinol
- storage of 11-cis retinal
- storing retinal for cones
- control of homeostasis by controlling the potassium environment
- regulation of potassium distribution across the retinal vitreous interface, the retina and locally in the inner plexiform layer
- completion of the electric field generated by bipolar cells
- induction of "tight" phenotype in retinal vascular endothelium

Astrocytes

Astrocytes are not derived form the retinal neuroepithelium but enter the developing retina from the brain along the optic nerve (Chan Ling, 1994). They have a characteristic morphology with a flattened cell body and fibrous radiating

processes. They express glial fibrillary acidic protein (GFAP) and vimentin, and are almost entirely confined to the nerve fibre layer. They are elongated structures in the central retina, but star-like shaped and smaller in the periphery (Schnitzer 1988). The astrocytes form tubes through which nerve fibre axons travel to the optic nerve head. They are thought also to influence the properties of retinal blood vessels. They may also have a nutritional role as they contain large amounts of glycogen, and may like Muller cells have a role in ionic homeostasis in regulating extracellular potassium levels and metabolism of neurotransmitters like GABA.

Microglia cells

Microglia cells are thought to be of mesodermal origin and thus, strictly speaking are not neuroglial as the astrocytes and Muller cells are. They enter the retina with the mesenchymal precursors of retinal blood vessels in development (Chan-Ling, 1994).

Microglial cells are ubiquitous in the human retina. They may be of two types. One form is thought to enter the retina at earlier stages of development from the optic nerve mesenchyme and lie dormant in the retinal layers for much of the life of the retina. The other form of microglia appear to be blood-borne cells, possible originating from vessel pericytes (Boycott and Hopkins, 1981; Gallego, 1986). Both types can be transformed into a macrophagic like phenotype following injury to the retina, and then they engage in phagocytosis of degenerating retinal neurons.

Comparative retinal anatomy

The basic anatomy of retina in different mammals are very similar. The cell types and functions appear to be also similar. There are, however, specialised features in different animals. Primates and human have a highly differentiated area

called the fovea, in which only cones are present. Fovea is also found in certain species of birds and reptiles, however, no other mammals examined to date has such an arrangement. Nonetheless, in cats and dogs, there is an area with high concentration of cones, called the area centralis. This cone rich area is not found in rodents (Prince 1956).

The percentages of cones are also different in different animals. In human, there is about 6 % of photoreceptors are cones, this proportion reduces to about 4 % in cats and dogs, and to less than 1% in rodents. There are at least three types of cones in human. In cats and dogs, there are at least two types of cones. Some breed of dogs have only one cone population whilst other breeds have three.

The fundal appearance in different species are also markly different. In human, there is no tapetum. The retinal vessels are divided into four main branches to supply the four quadrants of the eye. The central foveal area is avascular.

In cats and dogs, there is a reflective tapetum lucidum in the superior part of the fundus. Its function remains unknown. The tapetal fundus occupies the majority of the superior quadrants of the fundus but does not extend to the periphery. It appears to be granular in appearance. The RPE cells overlying the tapetum is non-pigmented. The non-tapetal fundus is normally deep brown in colour and the granular texture is not as marked as in the tapetal fundus. The pigment in the non-tapetal fundus usually completely obscures the choroidal blood vessels. The retinal vessels are divided into three main branches. In the canine fundus, there are two horizontal retinal vessels supplying the inferior non-tapetal areas and there is a single superior retinal vessels running perpendicular to the horizontal vessels supplying the superior tapetal area (Startup 1969) (Figure 1.2b). Whilst in the feline fundus, there

are also three main branches but the location and pattern differs in different breed (Barnett 1990) (Figure 1.2c).

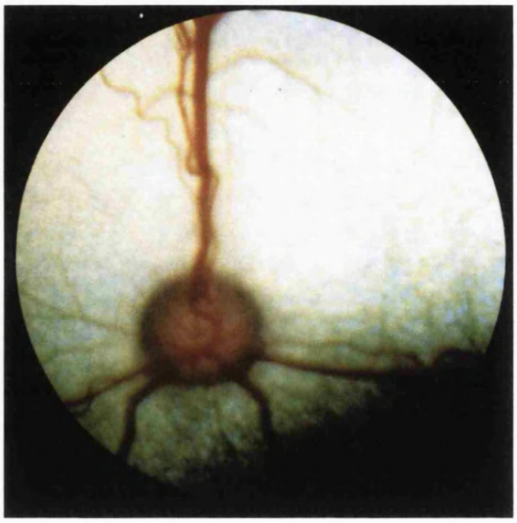

Figure 1.2b: Normal canine fundus

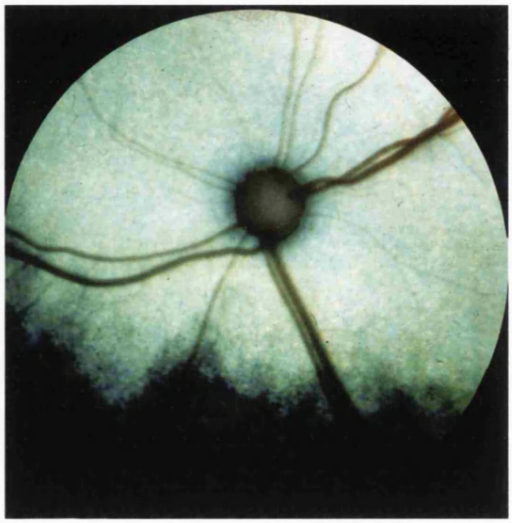

Figure 1.2c: Normal feline fundus

In rodents, there are no tapetum lucidum. As mentioned, no evidence of area centralis can be found either. The vascular pattern resembles a spoke wheel with about 12 radial retinal vessels radiating from the optic disc to the periphery (Price 1956).

1.3 Electrophysiology

The four main electrophysiological tests used clinically in human are electro-oculography (EOG), which reflects activity of RPE / photoreceptor complex; the (full-field) electroretinogram (ERG), which examines the global function of the retina; the focal and pattern electroretinogram (PERG), which assess central (macular) retinal function and additionally ganglion cell function; and the visual evoked cortical potential (VECP or VEP), which enables evaluation of the intracranial visual system including the optic nerves, the optic chiasm and the retro-chiasmal visual pathway.

The International Society for Clinical Electrophysiology of Vision (ISCEV) has introduced Standards for recording these electrophysiological tests (Marmor et al., 1993; Marmor and Zrenner, 1995; Marmor et al., 1996). The Standards procedure attempts to ensure uniformity of technique and thereby enable meaningful inter-laboratory comparisons.

In recent years, the ISCEV standards form the basis of most experimental protocol in animal electrophysiological studies, in order to achieve the same aims as in clinical settings.

In veterinary ophthalmology, ERG only is used clinically to assess global retinal function. In this thesis, only ERG was used and would be discussed here further.

Full-field electroretinography (ERG) in human

Investigation of the retina with electroretinography enables objective functional evaluation of different cell types and layers. The extinction of the ERG in retinal dystrophies, or the presence of marked abnormalities, became apparent with

the development of a clinical ERG recording technique by Riggs (1941) and Karpe (1945) using corneal electrodes mounted in haptic contact lenses, although it had been known since the 19th century that an eye exposed to a bright flash of light generated an electrical potential. Subsequent work refined the stimulus and recording parameters necessary to effect the separation of rod and cone function by manipulation of stimulus parameters and the adaptive state of the eye.

The ERG measures the mass response of the whole retina, reflects photoreceptor and inner nuclear layer retinal function, and allows separate functional assessment of the photopic and scotopic systems. The leading edge of the a-wave of the scotopic ERG arises from hyperpolarisation of the (rod) photoreceptors (Hood and Birch, 1990; Hood and Birch, 1994). The b-wave is probably generated by Muller cells in response to changes in extracellular potassium consequent upon depolarisation of the ON-bipolar cells (Dowling, 1987). There is recent evidence that the hyperpolarising bipolar cells may have a role in "shaping" the photopic cone b-wave (Sieving et al., 1994). The retinal ganglion cells probably do not significantly contribute to the full-field ERG. Furthermore, a lesion confined to the macula will also not significantly affect the full-field ERG.

Recording methods in human

The ERG protocols now commonly adopted in most laboratories include the recommendations by ISCEV, namely the ISCEV Standard ERG (Marmor et al., 1994). This specifies the brightness of a "standard flash", and requires that the response to this flash (the mixed rod-cone maximal response), and to the same flash attenuated by 2.5 log units of neutral density filter (the scotopic rod response) be recorded under full dark adaptation, and that following light adaptation photopic transient and flicker ERGs (cone specific) be recorded. In addition to the presence of

a rod-saturating background in the Ganzfeld, the rods have poor temporal resolution and cannot respond to a 30 Hz flicker.

Most laboratories incorporate the ISCEV Standard responses, which are intended as a minimum data set, into a more comprehensive test protocol. The Standard also specifies recording of the oscillatory potentials, the small wavelets which can be seen superimposed upon the positive going b-wave, and which probably reflect amacrine cell activity. They have limited clinical application.

Corneal electrodes should be used to record the ERG in adults. Typical contact lens electrodes are the Burian-Allen, Jet, or Henkes electrodes, and non-contact lens types are, principally the gold-foil, DTL fibre and H-K loop electrodes. The patient's pupil will initially be dilated and the eye will be fully dark adapted (>20 minutes). The electrodes are then positioned under red light and scotopic rod specific responses recorded using a dim white light (or an equivalent blue stimulus). Stimulus intensity is then increased and the ISCEV maximal response recorded. The patient is then returned to full photopic conditions using the background light of the Ganzfeld, and the photopic transient and flicker responses obtained. ERG quantification concentrates on the amplitude and latency or implicit time of the principal components.

Normal Tracings of ERG in human

The amplitude of a-wave is measured from the isoelectric point-to trough of the negative deflection. The latency of the a-wave is the interval between the beginning of the stimulus to the trough of the negative deflection. The amplitude of b-wave is measured from the trough of a-wave to the peak of the positive deflection. The latency of the b-wave is timing from the beginning of the stimulus to peak of the positive deflection (Figure 1.3 a).

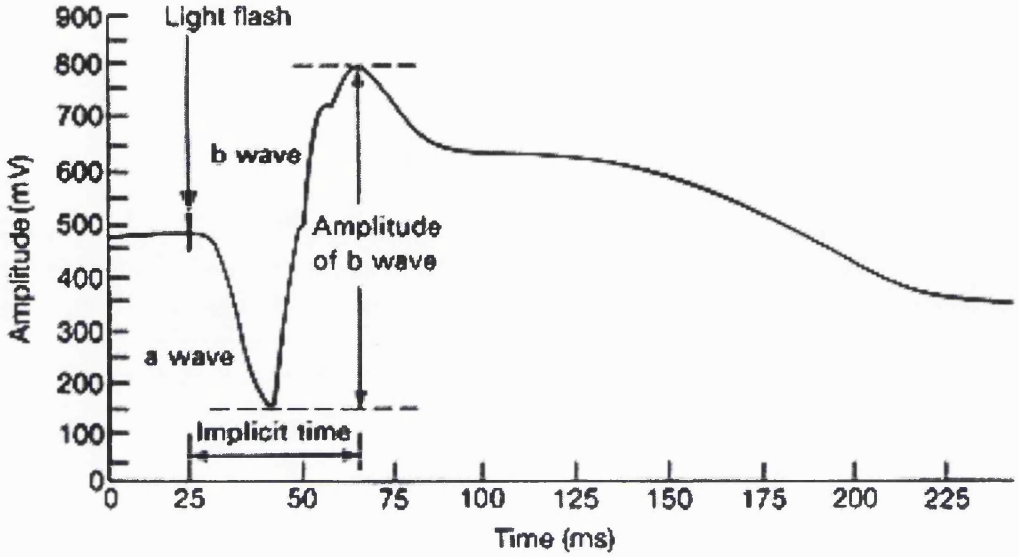

Figure 1.3 a: Diagrammatic representation of the different components of the full field ERG

The amplitude of the 30Hz flicker is measured from the trough to the peak and latency of the 30 Hz flicker is the timing between the two peaks. A normal tracing of ISCEV standard is shown on Figure 1.3 b.

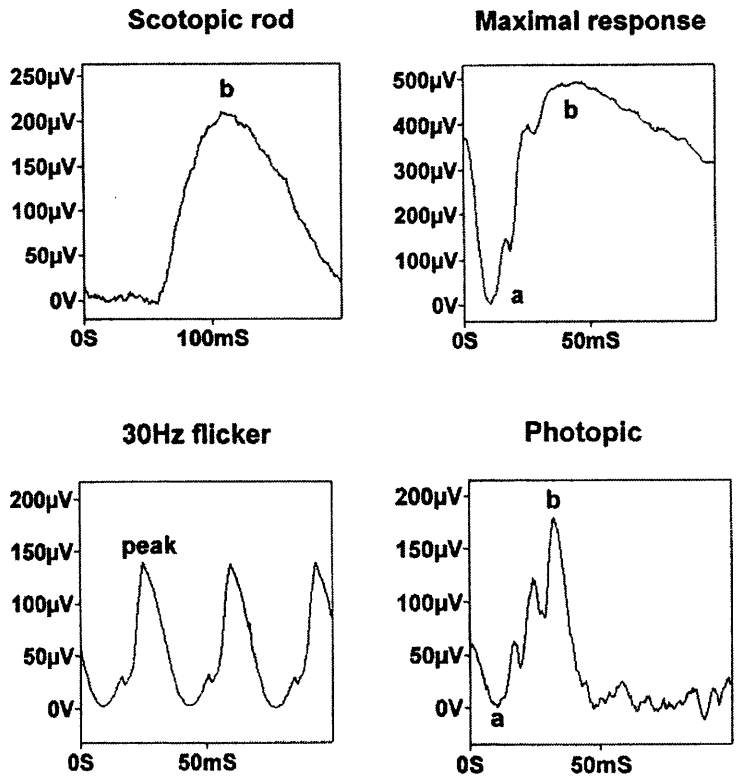

Figure 1.3 b: Normal tracing of ISECV standard full field electroretinogram

Full field ERG in rodents, cats and dogs

ERG can be recorded in animals other than human. The normal amplitudes and latency are different but the waveform are similar. Many laboratories used non-standardised custom made equipment to perform ERG in these animals. It might be adequate to compare different groups of animals within an experiment but the results cannot be compared between laboratories.

Dark adaptation time is an important issue for ERG recording in different animals. It is suggested that full adaptation for rodents might take more than 24 hours and in the cats and dogs, it might take up to 40 minutes. If the dark adaptation time is shorter, the maximum amplitudes would be reduced. Furthermore, the full dark adaptation time might be different in animals with retinal dystrophies.

The light source is also difficult to standardise as the size of the animal differs greatly in different breeds of the same species. Increasingly, most respectable laboratories have moved to use a ganzfeld stimulator so as to provide a diffuse light source with background light control. As the light source is diffuse and come from all directions within the bowl, the exact position of the animal within the bowl is less important and hence the size of the animal's head is no longer a significant issue.

The recording electrodes are of significant concern. Firstly as the animals are normally under general anaesthesia, the eyelids need to be opened either by sutures or by the "side arms" of the recording electrodes. In rodents, a ring electrodes with lid sutures are often used. In cats and dogs, single used Jet electrodes with lid sutures are often used.

General anaesthesia protocol for ERG are well established. In rodents, intraperitoneal pentobarbitone (as recommended by the Dr Berson's laboratory, Harvard University, Cambridge) are often used. In cats and dogs, the anaesthesia is very similar to human protocol with intravenous induction followed by inhaled anaesthesia with halothane. As ERG recording in laboratory settings tend to be prolonged, it is important to ensure that the animal would not become hypothermic. In rodents, warming platform with running warm water can be used. In cats and dogs, warming pad and insulation with cardiac monitoring are often used.

There are recent discussions in ISECV meeting to develop a research protocol and standard for animal ERG recording. However, it failed to agree.

1.4 Retinal degeneration in human

The term "retinal degeneration" are often used to describe a group of inherited diseases in which the photoreceptor degeneration is the main feature. Secondary changes in the RPE, inner retina, vitreous and lens are often seen. Some authors would prefer the term "retinal dystrophies" or "outer retinal dystrophies", however, the term "retinal degeneration" are more commonly used both in clinical and research settings. Hence, in this thesis, the term retinal degeneration and retinal dystrophies are interchangeable.

Nonetheless, it should not be confused with "peripheral retinal degeneration" that is often referred to the peripheral degeneration changes in the retina which might increase the risk of retinal detachment. Some authors would use the term "retinal degeneration" for ganglion cell loss in glaucoma. Although there are similarity in ganglion cell loss and photoreceptor cell death, indeed similar neuroprotection strategies are under investigation in both groups of diseases, the clinical features and the primary disease process are quite different.

Adding to the confusion, some authors used the term retinal dysplasia to describe some animal models of retinal degeneration such as the retinal dysplasia cats, which would be discussed further in this thesis. The authors used this term to describe the conditions in which abnormalities can be found at birth. However, other researchers, including myself, would prefer to restrict this term for conditions which have abnormalities seen at birth but do not deteriorate significantly. As the retinal degeneration in the retinal dysplasia cats deteriorate with age, it should be called the retinal dystrophy cats. However, they were labelled by previous investigators, we have not changed the name in this thesis to maintain consistency with the literature.

Retinal degeneration / dystrophies comprises a variety of disparate genetically determined conditions that differ from one to another in their mode of inheritance, their pattern of visual loss and their ophthalmoscopic appearances. As a group, it is one of the leading causes of blindness during working age in the developed world (Evans et al., 1991). It is possible to subdivide retinal degeneration into groups depending on their clinical features. Some cause loss of function early in disease associated with the scotopic system. Examination reveals defective vision in the mid-zone of the visual field initially and morphological changes in the post-equatorial fundus; most of the diseases in this category are known collectively as retinitis pigmentosa (RP) (Bird, 1995). Other disorders causing loss of photopic function and morphological changes in the central fundus are known as macular dystrophies or cone dystrophies.

This subdivision into "peripheral degenerations" in which the rods may be the primary target of disease and "central degenerations" in which the cones may be the cells initially affected is superficially attractive. However, the function of both rod and cone systems are compromised in most if not all progressive disorders, even in the early stages of their disease. Retinal degeneration, therefore, comprise a spectrum of diseases ranging from predominant rod dystrophies to predominant cone dystrophies with disorders intermediate between the two in which there is varying involvement of the rod and cone systems.

The fundal picture is not a reliable way to distinguish the predominantly affected cell types. In some early cases of cone-rod dystrophies, the central retina appears clinically normal with no peripheral pigmentation but a mildly reduced visual acuity. Furthermore, the number of pathological specimens at the early stages of the diseases are not commonly available. Therefore, the distinction of rod-cone

dystrophies and cone-rod dystrophies are based on electrophysiology alone rather than clinical or pathological features. The distinction between cone-rod dystrophies and cone dystrophies is still a subject of controversy (infra vide for further discussion).

Rod-cone dystrophies / Retinitis Pigmentosa

Donders (1855) described the fundus findings of this condition and first used the term retinitis pigmentosa (RP). Through the years many terms have been used to describe RP, including tapetoretinal degeneration, primary pigmentary retinal degeneration, pigmentary retinopathy, and rod-cone dystrophy. RP is a essentially a heterogeneous group of progressive retinal degenerations sharing a common set of clinical characteristics comprising night blindness, constricted visual fields, pigment deposition in the outer retina, and a diminished or absent of ERG.

It affects about 1 in 4,000 of the general population (Bucker et al., 1984; Merin and Auerbach, 1976) and occurs as autosomal dominant, recessive, X-linked or simplex forms. In addition, digenic inheritance has also been reported (Kajiwara et al., 1994). Mitochondrial disorders (Moraes et al., 1989) have also been described with progressive retinal degeneration; for example in Kearns-Sayre syndrome (Kearns and Sayre, 1958). The term "simplex" is used when there is only one known affected member in a family. If siblings are also affected, then it is termed "multiplex". It is likely that by far the majority of simplex or multiplex RP is autosomal recessive or X-linked. The term "sporadic" implies a condition for which there is no calculable risk of recurrence such as a new mutation.

Clinical features and classification of rod-cone dystrophies

Night blindness is one of the hallmark symptoms of RP. Patients experience night vision difficulties that should not be confused with night-myopia, or difficulty with initial adapting to the dark as seen with diseases of the photopic system.

Progressive loss of visual field is another classical feature. The interval between consciousness of night blindness and visual field loss is enormously variable. In the great majority of cases of RP, the earliest defects of the visual field are relative scotomata that developed in the mid-periphery between 20 and 30 degrees from fixation. These enlarge, deepen, and coalesce to form a ring scotoma. As the ring scotoma enlarges, it extends to the periphery, leaving isolated peripheral islands of vision, usually temporal but occasionally inferior or nasal. The superior visual field is often more abnormal, reflecting the earlier involvement of the inferior retina. The rate of progression of visual field loss is usually slow and relentless. Studies have been performed to quantify the field loss and functional loss over time. Patients do not always notice significant interval loss of peripheral visual field, particularly if their central field is unaffected. Once the visual field is constricted such that central vision is affected, the patient may become aware of any subsequent change with time. This often leads the patient to the conclusion that the rate of degeneration is accelerating. As many patients are unaware of visual field loss, it is important to check their visual fields particularly in the context of driving.

The central vision is not always preserved late into disease in RP, and may in some cases be lost early. The likelihood of retaining good vision to a given age in life is dependent upon the specific type of RP. Patients with sector RP may retain good visual acuity all their life. Patients with adRP are more likely than patients with

autosomal recessive or X-linked RP to retain good acuity beyond 60 years of age. Again this depends on the gene and the mutation.

Furthermore, central visual function can be seriously affected by other associated complications. Cataract, cystoid macular oedema, diffuse retinal vascular leakage, distortion of the inner limiting membrane and macular epiretinal membrane may develop. Retinal pigment epithelial defects may occur at the macula and be associated with loss of acuity.

The classic appearance of advanced RP includes attenuated retinal vessels, pale optic discs, mottling and granularity of the retinal pigment epithelium (RPE), migration of pigmented cells into the inner retina (bone-spicule formation) (Figure 1.4 a), and eventually atrophy of the RPE and choriocapillaris (Figure 1.4 b). In general, there is a high degree of symmetry between the two eyes. In the early stage of RP, the retina might appear either normal or near normal, even when the visual field shows relative scotomata. Patients who have early RP without fundus pigmentary changes are often diagnosed as RP sine pigmento. This is not a specific subtype of RP but just an early stage of the disease. This stage can be short but might last for many years. In most, if not all, cases of RP sine pigmento, pigmentary deposits in the retina will develop eventually.

Many patients with RP will experience, at some time during the course of their disease, light flashes, or photopsias, in their mid-peripheral field of vision adjacent to areas of relative or absolute scotomata.

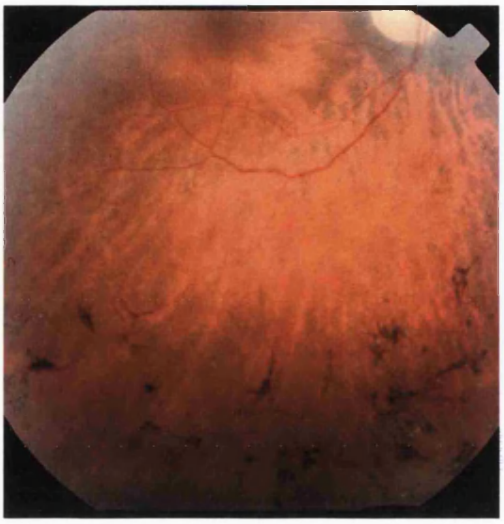

Figure 1.4 a: Fundus picture of patient with RP showing migration of pigmented cells into the inner retina (bone-spicule formation)

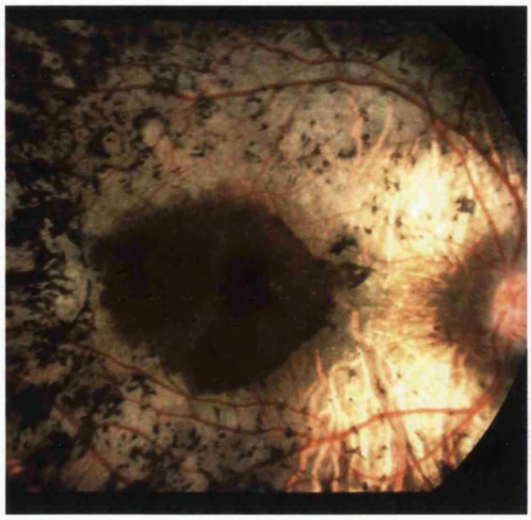

Figure 1.4 b: Fundus picture of patient with RP showing extensive atrophy of the RPE and choriocapillaris

Molecular genetics in Retinitis Pigmentosa

As advances in molecular genetics occur, it is becoming easier to categorise groups, particularly when mutations in genes causing disease are identified.

Autosomal dominant RP (adRP)

The reported relative incidence ranges from 10 to 25% (Pearlman, 1979; Jay, 1982; Boughman and Fishman, 1983). Mutations have been identified in rhodopsin (Dryja et al., 1990), peripherin/*RDS* (Kajiwara et al., 1991) and the neural retina leucine zipper protein (NRL) gene (Bessant et al., 1999) in adRP. The autosomal dominant RP group is reported to be milder and have a later onset. However it is becoming clearer that the severity of disease depends on the gene, the mutation and the position of the mutation in the gene. This has been demonstrated in rhodopsin mutations where some mutations confer a severe phenotype.

Peripherin/RDS is a structural protein in the disc rim of the outer segment discs, and mutations in this gene have been shown to cause adRP. In addition to

causing classical RP, phenotypes include various macular disorders, cone and cone-rod dystrophies and pattern dystrophy. A null mutation in this gene is also presented in the retinal degeneration slow (rds) mouse.

NRL is a retinal transcription factor that interacts with CRX and promotes transcription of rhodopsin and other retinal genes. It is a gene that codes for a basic motif leucine zipper, bZIP, that is highly and specifically expressed in adult retina. Cell culture studies were performed to compare levels of transcription of rhodopsin in the mutant and wild state and it was shown that rhodopsin is over expressed at low levels of NRL in cell systems (Bessant et al., 1999).

Autosomal recessive (arRP)

This is the most common mode of inheritance. The incidence is particularly high in isolated communities and where consanguinity is common. The majority of "simplex" and "multiplex" cases are assumed to be autosomal recessive RP. There is large variation of severity and age of onset, although in autosomal recessive RP vision tends to cause early onset disease progressing to severe visual handicap by the fourth decade. However, mild autosomal recessive disease exists. A number of genes have been found associated with arRP. They include *RPE65* (Gu et al., 1997), rod cGMP phosphodiesterase beta subunit (McLaughlin, 1993 et al), rod cGMP-gated channel alpha subunit (Dryja et al., 1995), cGMP phosphodiesterase alpha subunit (Huang SH et al., 1995), and tubby-like protein 1 (Banerjee et al., 1998). Although satisfactory phenotypic characterisation is not available in human, some of these mutations cause retinal degenerations in rodents (vide infra).

X-linked recessive (xlRP)

The reported relative incidence of x-linked RP of all types of RP ranges from 8 to 20%. As a group, it is possibly the most consistently severe form of the disease with early onset. It is associated with myopia. Mutations in the retinitis pigmentosa GTPase regulator (RPGR) (RP3) (Meindl et al., 1996), and human cofactor C (RP2) (Schwahn et al., 1998) were identified in patients with xlRP. The functions of these genes are not fully understood.

There is also a variation of clinical features in the heterozygotic (carrier) state in different families but intra-family variation is relatively small. In some families, the heterozygotic state can be determined by recognition of a "tapetal" reflex at the macula on fundus examination alone, while others require electrodiagnostic techniques. However, heterozygotes may also be totally normal. Nonetheless, heterozygotes may be symptomatic by the age of 20 years, and severe visual loss has been recorded in middle or late life.

Digenic RP

A combination of mutations at the peripherin/RDS and ROM 1 loci (Kajiwara et al., 1994) has been shown to produce an RP phenotype.

Histopathology of Retinitis Pigmentosa

The process of rod cell death usually begins in the mid-peripheral retina and progresses with time to involve the macula and more peripheral retina, often sparing rods in the far periphery until late in the disease. In all genetic forms of RP, the earliest histopathologic change in the rods is outer segment shortening. Using immunohistochemical techniques, in the normal retina, rhodopsin immunolabeling is restricted to the rod outer segments; in the RP retina, rhodopsin is delocalized to the surface membranes of the rod inner segments and somata (Li, Jacobson and Milam, 1994).

Rods in RP retinas sprout long, axon-like neurite processes that contain rhodopsin and project for considerable distances into the inner retina. The rod neurites have been documented in retinas with RP caused by rhodopsin mutations and other genetic forms of RP, including XL RP where the rhodopsin gene is presumably normal (Li, Kljavin and Milam, 1995). Rod neurite sprouting is common in the peripheral regions of RP retinas where some death of photoreceptors has already occurred, but rod neurites have not been found in the macula of the same retinas, even when this region has undergone significant photoreceptor loss. Cones do not undergo robust neurite sprouting, although some peripheral cone axons are elongated and appear to reach the inner plexiform layer (IPL) (Li, Kljavin and Milam, 1995; Milam et al., 1996). The rod neurites bypass the dendrites of horizontal and rod bipolar cells, the normal targets of rod axons in the outer plexiform layer, and are closely associated with the hypertrophied processes of Muller cells that have undergone gliosis in response to photoreceptor death.

Progressive cone and cone-rod dystrophies

Clinical features and classification of cone and cone-rod dystrophies

Autosomal cone dystrophy has been calculated to occur in 1/10,000 live births with an age dependent penetrance. A large number of cone and cone-rod dystrophies might have been misdiagnosed as retinitis pigmentosa, which is classically a rod dystrophy with secondary cone degeneration.

Most cone dystrophies present in childhood or early adulthood. Pure cone dystrophies are characterised by progressive dysfunction of the photopic system with preservation of scotopic function. Common symptoms and signs in cone dystrophy include photophobia, loss of visual acuity, abnormal to loss of colour vision and loss of central visual field with central scotoma. Fundus findings range from granular abnormalities of the RPE to a bull's eye appearance at the macula. The optic disc may exhibit temporal pallor, and in early onset severe dystrophy, nystagmus may be present. Fluorescein angiography may demonstrate a bull's eye pattern, even when there is little to be seen at the fundus. Psychophysical evaluation in cone dystrophy demonstrates loss of central visual fields, and recovery from bleach is monophasic with a normal rod threshold. Colour vision is usually abnormal early in disease with elevation of protan, deutan or tritan thresholds or any combination of these.

In late disease, rod photoreceptor involvement can be demonstrated in a proportion of cone dystrophies cases (Simunovic and Moore 1998). For this reason cone and cone-rod dystrophies are often considered together. Electrodiagnostic tests are the key to distinguishing cone, cone-rod, and macular dystrophies. Typical electrophysiology features of cone dystrophies in the flicker and photopic electroretinogram include delayed implicit times, reflecting cone dysfunction, and decreased amplitudes reflecting loss of cone cells. These same features are seen in

cone-rod dystrophies, but with rod involvement are also present. In macular dystrophies, the global cone function should be normal but abnormalities in pattern ERG are consistent findings. Nonetheless, some classical examples of macular dystrophies might have cone abnormalities as well (Lois et al., 1999). Some authors suggested that a mild degree of rod involvement that is non-progressive should not alter the diagnosis from cone dystrophies to cone-rod dystrophies. However, the progression of rod involvement in cone-rod dystrophies is often slow, it is therefore difficult to establish unless long-term follow up is available. The degree to which the systems are affected varies between individuals and in different disorders. Hence, it might be best to consider both cone and cone-rod dystrophies together as a continuous spectrum of a group of diseases with different genetic mutations.

Molecular genetics of progressive cone and cone rod dystrophies

Genetic studies to date have shown that autosomal dominant cone and cone-rod dystrophies are genetically heterogeneous. Mutations in peripherin/RDS (Fishman et al., 1997; Nakazawa et al., 1996)(Figure 1.4.2a), GUCA1A (Payne et al., 1998)(Figure 1.4.2b), GUCY2D (Kelsall et al., 1998) and CRX (Freund et al., 1997) are associated with autosomal dominant cone or cone-rod dystrophies. One locus has recently been reported for autosomal recessive cone-rod dystrophy and is on chromosome 17p (Payne et al., 1999), but no responsible gene has been identified. The red cone opsin gene has been associated with some form of X-linked cone dystrophies (Nathans et al., 1992; Neitz and Neitz 1995).

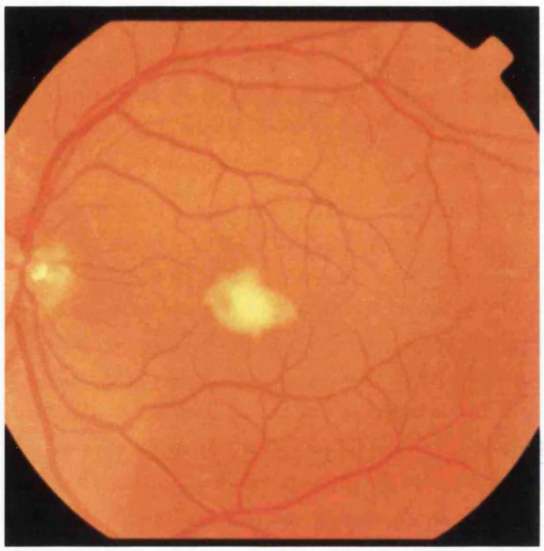

Figure 1.4 c: Fundus picture of a patient, with a clinical diagnosis of pattern dystrophy, who has a peripherin/RDS mutation (Courtesy of Dr Susie Downes)

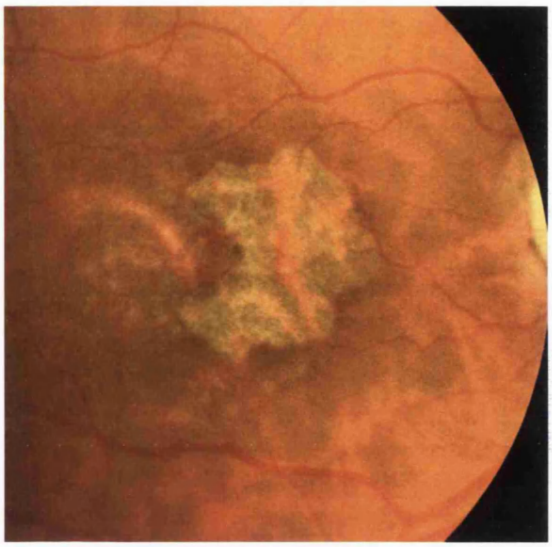

Figure 1.4 d: Fundus picture of a patient with a GUCA1A mutation (Courtesy of Dr Susie Downes)

Histopathology of progressive cone rod dystrophies

Photoreceptor loss and shortened photoreceptor outer segments in the central and far peripheral retinal regions, with relative preservation in mid-periphery, have been described in a patient with cone-rod dystrophy (Rabb et al., 1986). These features were not significantly different from rod-cone dystrophy.

Interestingly, the histopathology of four eyes with different forms of cone-rod dystrophies have been reported recently (Gregory-Evans et al., 1998). In all four cases, there was cone and / or rod loss with outer segment shortening as predicted by the clinical disease. However, the retina of a simplex cone-rod dystrophy of unknown genetic mutation showed enlarged, distorted shape of cone pedicles, along with thickening of some cone axons. This was confirmed by electron microscopy. Furthermore, the cone pedicles also contained spherical inclusions filled with synaptic and coated vesicles of unknown origin or composition. The authors suggested that they might represent invaginating postsynaptic processes. Similar

features were found in a small number of cones in an eye with central areolar choroidal dystrophy but not found in eye with cerebellar ataxia-cone dystrophy, cone-rod dystrophy in Bardet-Biedl syndrome or simplex RP. The significance of these findings is currently unknown, as it is not a uniform feature in all cone-rod dystrophies and the number of cases examined remains small.

1.5 Retinal degeneration models in rodents

In addition to retinal degeneration in human, retinal degeneration is naturally occurring in rodents and companion animals. Furthermore, light damage model in rodents are often used to explore pathogenesis and therapeutic trials. The most commonly studied models are described in the following sections.

Retinal degeneration (rd) mouse

Following its discovery in the 1920s, the retinal degeneration (rd) mouse has been one of the most widely studied retinal degenerations (Farber, Flannery and Bowes-Rickman, 1994). In the rd mouse, there is a rapid loss of rod photoreceptors with a much slower and subsequent loss of cones. Rod degeneration begins on about postnatal day (P) 10, with most of the cells lost by P21 and almost none surviving by P36 (Carter-Dawson, LaVail and Sidman, 1978). The majority of cones are still present at P21, but most of these are lost by 2 to 4 months of age, with a few surviving for the lifetime of the animal (Carter-Dawson and LaVail, 1979). Electrophysiological studies of the superior colliculus of rd mice are consistent with the morphological observations of the rapid rod loss followed by a slower loss of cones (Dragger and Hubel, 1978). The defective gene in the β subunit of the rod cGMP-phosphodiesterase gene has been identified (Bowes et al., 1990; Pittler and Baehr, 1991). Two mutations were found in the rd PDEβ gene. One is a nonsense mutation at codon 347 that introduces a stop codon (Pittler and Baehr, 1991); the second is a retroviral element inserted in intron 1 (Bowes et al., 1993). Both mutations have been found in all strains of rd mice. Further confirmation the mutant PDEβ gene was responsible for rd was provided by introducing a normal copy of the

gene into rd mice and showing that the resulting transgenic mice did not develop retinal degeneration (Lem et al., 1992)

Multiple mutations in the human homologue of the same gene, most resulting in autosomal recessive retinitis pigmentosa (McLaughlin et al., 1993, 1995), and one producing autosomal dominant stationary night blindness (Gal et al., 1994) have been identified.

Retinal degeneration slow (rds) mouse

The retinal degeneration slow (rds) mouse was first described in 1978 (Van Nie et al., 1978) and suffers from a semi-dominant disease characterised by photoreceptor dysplasia followed by degeneration. Mice heterozygous for the mutation (rds/+) develop dysplastic photoreceptor outer segments, which then very slowly degenerate. The homozygous (rds/rds) mice have a more severe phenotype in which they fail to produce any outer segments (Sanyel and Jansen, 1981), and photoreceptor degeneration is apparent by 3 weeks of age (Sanyal et al., 1990). Apoptotic cells can be seen in the outer retinal layer (Figure 1.5). In most studies, albino rds mice were used. The albino elements may accelerate the photoreceptor degeneration. The pigmented rds mouse was characterised as part of this thesis.

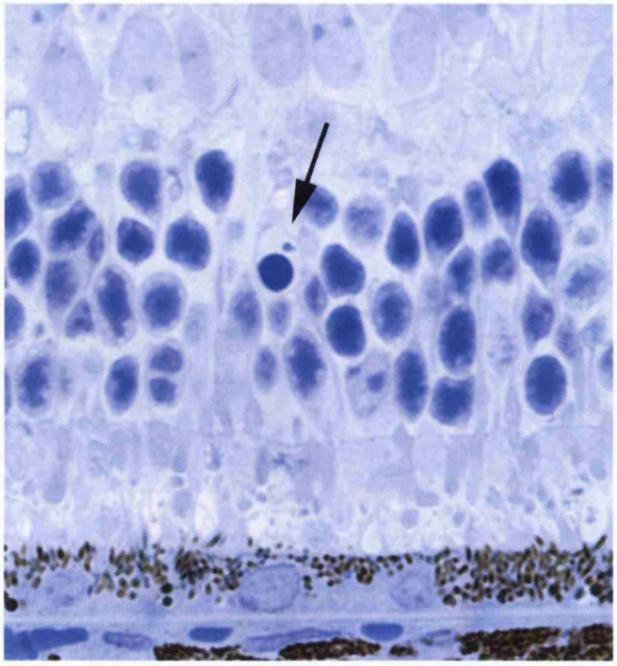

Figure 1.5: Retina of the pigmented rds mouse at postnatal day 45 showing an apoptotic cell (arrow) and the absence of photoreceptors outer segment.

The mutant gene, named peripherin/*rds,* encodes a structural protein present in photoreceptor outer segments (Connell and Molday, 1990; Connell et al., 1991). The rds mutation is due to an insertion of foreign DNA into exon two of the gene creating a null mutation (Travis et al., 1989). Final confirmation that the peripherin/RDS mutation was responsible for the rds phenotype was provided by construction of transgenic rds mice in which a normal peripherin/RDS gene was expressed and resulted in photoreceptor cell rescue (Travis et al., 1992). The semi-dominant nature of the rds mutation is thought to be due to haploinsufficiency.

Following the identification of peripherin/*rds*, screening of the gene in human patients with retinal dystrophies has led to the identification of over 40 different mutations within the gene (Keen and Inglehearn, 1996). Interestingly, different mutations result in a wide range of phenotypes from classical rod-cone dystrophies to cone-rod dystrophies to macular dystrophies (Weleber et al., 1993; Wells et al., 1993).

The Royal College of Surgeons (RCS) rat

The Royal College of Surgeons Rat (RCS) is a well-studied model of heredity retinal degeneration. The dystrophic strain is subject to an autosomal recessive disease resulting in the selective loss of photoreceptors (Bourne et al., 1938). The degeneration occurs after the complete development of the neural retina. It initiates by postnatal day 20 and leads to near complete destruction of photoreceptors by postnatal day 60 (Dowling and Sidman, 1996). The primary defect involves dysfunction of the retinal pigment epithelium (RPE), which fails to phagocytize shed outer rod segments (Heron et al., 1969), resulting in accumulation of debris in the subretinal space and subsequent loss of photoreceptors (Bok and Hall, 1971). Although the molecular defect was only recently been reported (D'Cruz et al, 2000), the primary role of the RPE cells in the RCS rat was identified in 1971 (Mullen and LaVail, 1976), and confirmed by the prevention of photoreceptor degeneration by RPE transplantation (Li and Turner 1988). The molecular defect was found to be in the receptor tyrosine kinase Mertk. The exact function of Mertk is unknown but it appears to play an important in phagocytosis (D'Cruz et al, 2000).

Although earlier studies on the RCS rat suggested that the only neural deficit to be seen was a loss of photoreceptors, inner retinal changes has been detected in recent studies. The inner retinal changes, in particular the loss of retinal ganglion cells appears to be secondary to the inward displacement of retina blood vessels (Villegas-Perez et al., 1996). Some functional studies have investigated the visual capacity of the RCS rat by means of electroretinography (Perlman, 1978) and pupillometry (Trejo and Cicerone, 1982). Such studies have indicated a decreased responsiveness to light by dystrophic RCS rats compared with congenic controls.

1.6 Genetically determined retinal degeneration in companion animals

The term progressive retinal atrophy (PRA) is used in describing retinal degeneration in cats and dogs. The clinical phenotype of PRA in cats and dogs is similar to retinitis pigmentosa in man. As in human outer retinal dystrophies, it represents a group of genetically different retinal disorders having similar disease phenotype. All show the same general ophthalmoscopic abnormalities, and visual deficits characterised initially by rod dysfunction followed by loss of day vision; in the late stages of the disease, the animals are blind, have end-stage retinal degenerative changes, and ,especially in dogs, secondary cataracts .

Progressive retinal atrophy in dogs

Progressive retinal atrophy is much more common in dogs than cats, and is subdivided into developmental and degenerative diseases. The developmental class represents a large aggregate of genetically distinct disorders which are expressed cytologically in the postnatal period, at the time that visual cells are beginning to differentiate. These developmental disorders represent a dysplasia of the rod and/or cone photoreceptors, and each has its own unique disease course and phenotype as assessed by functional and morphologic criteria (Aguirre et al., 1998).

Even though all dysplasias show rather severe structural alterations of the photoreceptor cells, the rate of progression to loss of cones, the benchmark criteria for loss of functional vision, is varied; for example this occurs early in the Norwegian elkhound *(erd),* Irish setter *(rcd1)* and Collie *(rcd2)* retinas, but not until

the equivalent of middle age in Miniature schnauzer (*pd)* and Norwegian elkhound *(rd).*

In contrast, the degenerative group represents defects in photoreceptor maintenance, where the visual cells degenerate after having differentiated normally. This group includes diseases such as the progressive rod-cone degeneration (*prcd*) as found in the Miniature poodle, American and English cocker spaniels, Labrador retriever and the Portuguese water dog (Aguirre et al., 1982; Aguirre and Acland, 1988); and the X-linked PRA in the Siberian husky (Acland et al., 1994). In this group, disease occurs late and the degeneration is slower. Different alleles have been identified at the *prcd* locus (Aguirre and Acland, 1988).

A mutation in codon 807 (Trp807x) of the β-subunit of cyclic GMP-phosphodiesterase (cGMP-PDE) was identified in the Irish setter (*rcd1*). This represents a premature termination of the PDE6B protein by 49 amino acid residues (Suber et al., 1993; Ray et al., 1994). A mutation in the α-subunit of cGMP-PDE was identified in the Cardigan Welsh corgi (Petersen-Jones et al., 1998). Using the candidate gene approach, transducin, PDE6A, PDE6B, PDE6G have been excluded in the Collie and transducin, RDS/peripherin, opsin, PDE6B have been excluded in the Norwegian elkhound (*erd*) and in the *prcd* (Aguirre et al., 1998).

The retinal degeneration seen in the miniature longhaired dachshund dogs (which is characterised in this thesis) traditionally belongs to this group. It is an autosomal recessive early onset retinal degeneration (Curtis and Barnett, 1993). The earliest ophthalmoscopic signs, appearing at approximately 6 months of age and coinciding in some cases with the onset of nyctalopia, included changes in the granular appearance of the tapetal fundus followed by generalized tapetal hyper-

reflectivity and retinal vascular attenuation; later there was irregular loss of pigment in the non-tapetal fundus and optic atrophy.

Using a non-standardized light source, the full field electroretinogram appeared normal in waveform and latency at 10 weeks of age; but by 9 months it was markedly reduced or virtually extinguished. Significant histological changes at 10.5 weeks of age included thinning of the outer nuclear layer, irregularity and attenuation of the rod photoreceptor outer segments and early disorganization of the rod outer segment disc lamellae. By 25 weeks the photoreceptors were grossly degenerate with short rounded inner segments and only residual amounts of outer segment material remaining (Curtis and Barnett, 1993).

This condition in the miniature longhaired dachshund (Curtis and Barnett, 1993) is later in onset than rod-cone dysplasia in Irish setters (mutation in β-subunit of cyclic GMP-phosphodiesterase) (Suber et al., 1993; Ray et al., 1994); but significantly earlier than progressive retinal atrophy in Tibetan terriers and progressive rod-cone degeneration in miniature poodles (Aguirre et al., 1982; Aguirre and Acland, 1988). The exact genetic defect is not known yet, however, linkage has been achieved in a collaboration with Dr Matthew Binns' laboratory. The MLHD locus was mapped to CFA15q23-24.1 by linkage and radiation hybrid mapping, and confirmed by fluorescence in-situ hybridisation (FISH) (Figure 1.6a) (Ryder, Chong, et al., 1999). The CFA15 region shows conserved synteny with human chromosome 12q21-q24, in which no known RP loci have so far been mapped.

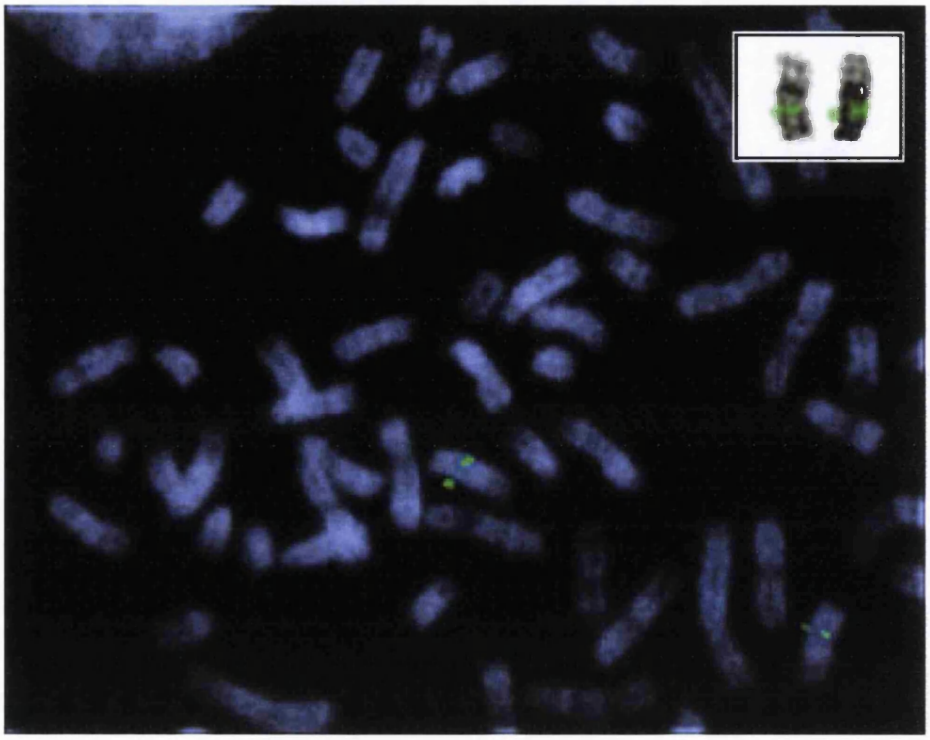

Figure 1.6a: Physical mapping of BAC clone 259-B22 labelled with DIG (main) and DAPI counterstaining (insert) (Courtesy of Dr Matthew Binns)

Progressive retinal atrophy in cats

Two forms of feline PRA have been described and both were initially found in the Abyssinian cats. One is an autosomal dominant form that presents early, and has similar features to the dysplasia group in the dogs (characterisation of this model forms part of this thesis). The gene symbol Rdy has been designated for this model. The other is the autosomal recessive model (Narfström, 1983) that is described in this chapter. The disease in this model has been designated the gene symbol rdAc.

The autosomal dominant early onset feline model of retinal degeneration was described previously (Barnett and Curtis 1985; Curtis et al., 1987; Leon and Curtis 1990, Leon et al., 1991). Clinically, affected kittens show dilated pupils and sluggish pupillary light reflexes from 2 weeks of age. Vision is impaired and an intermittent

rotatory nystagmus develops between 4 to 6 weeks of age. The first signs of fundus abnormality are present at 8 to 12 weeks with increased tapetal reflexes. This is accompanied by progressive attenuation of retinal vessels and optic atrophy (Figure 1.6.b and c). Light microscopy, electron microscopy and electrophysiology were previously performed but most of the work were concentrated on the older cats (from 16 weeks of age onwards).

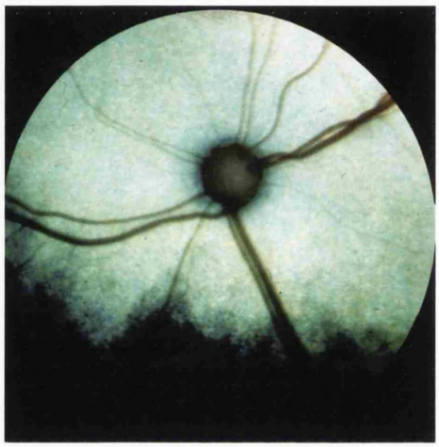

Figure 1.6b: Fundus picture of a normal cat at 6 months of age showing normal optic disc and retinal vessels

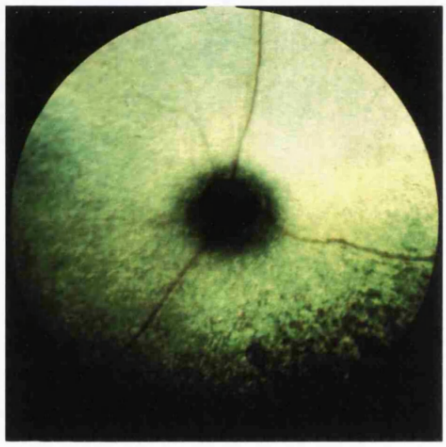

Figure 1.6c: Fundus picture of an affected Rdy cat at 6 months of age showing abnormal tapetal reflex and attenuated retinal vessels

The fundus in the recessive feline PRA appears normal until the animals reach young adulthood, at the age of 1.5 to 2 years. This is followed by a slowly progressive retinal degeneration leading to a generalised retinal atrophy by age of 5 year (Narfström, 1985). Full field ERG revealed a primary effect on the function of rod photoreceptors with a later involvement of the cone system. A significant reduction of maximum dark adapted b-wave amplitudes was found in cats as early as 8-16 weeks when compared to age-matched controls. At this time there was no major difference between affected and controls in scotopic b-wave implicit time (Narfström et al., 1988).

These changes have been verified by ultrastructural studies that showed changes primarily in solitary rod outer segments, while cones and other retinal cells appeared normal (Narfström and Nilsson, 1986). The morphological changes were more severe in mid-peripheral and peripheral areas of the retina compared to the central parts, which seemed to be spared until late in the disease, when there was a generalised loss of both rods and cone photoreceptors.

In affected animals there was an early aberration in the orientation of disc membranes, and a disorganisation of lamellae in some rod outer segments. At the age of 35 days, a difference between affected kittens and normal controls could be observed (Narfström and Nilsson, 1986). However, specific degenerative changes were not observed until after the age of 5 months in the affected kittens.

There was progressive loss of gamma-amino butyric acid (GABA) immunolabeling (Ehinger et al.,1991), and progressive increase of glial fibrillary acidic protein (GFAP) (Ekstrom et al., 1988) immunolabeling in the retina of affected cats. However, the immunoreactivity of interphotoreceptor retinoid binding protein (IRBP) was found to be much reduced in the early stage of the photoreceptor degenerative disease. It progresses to a total loss of immunoreactivity to IRBP in advanced disease (Narfström et al., 1989). Furthermore, levels of both IRBP protein and message were significantly reduced below normal as early as at 4 weeks of age in affected retinas (Wiggert et al., 1994). It was suggested that the early reduction of IRBP could be one of the factors leading to photoreceptor cell death.

Through candidate gene analysis, peripherin / rds, phosducin, rhodopsin, S-antigen, cGMP gated channel beta subunit, transducin subunits alpha, beta and gamma as well as phosphodiesterase, alpha, beta and gamma have been excluded (Narfström, 1998).

1.7 Transgenic animals with retinal degeneration

Genetic manipulation is now possible to alter gene expression of gene in an animal. There are two main approaches. The first method is involved the injection of a foreign DNA construct (gene), that is DNA that contains a regulatory region (promoter) and a coding region for the protein, into fertilised oocytes. The oocytes are then implanted into a foster mother to allow embryo development. The offsprings are tested whether the foreign DNA has been incorporated into the genome. Animals positive for the transgene are bred selectively until a line of transgenic animals are established. Using this technology, a number of transgenic mice (Olsson et al., 1992; Naash et al., 1993), rats (Lewin et al., 1998) and more recently miniature pigs (Petters et al., 1997) carrying a rhodopsin mutation have been produced.

A major disadvantage of this technique is the unpredictable nature of the integration of the transgene into the genome. Multiple copies of the transgene can be incorporated and the location of the integration is uncontrolled. This may result changes in the transcription rate of an endogenous gene at the integration site. In other words, the phenotypes of the transgenic animals might be totally unrelated to the expression of the transgene but due to the altered endogenous gene expression. In fact, there is some worry that the retinal degeneration in the mutant P23H rhodopsin transgenic rats might not be caused by the P23H transgene. Furthermore, the phenotypes of transgenic animals in different species are very different from the human disease. For instance, the P23H rhodopsin mutation is the most common form of adRP in the USA. It is a relatively mild disease. In human, rod-specific ERG can be measured at the early stage of the disease. However, in the transgenic P23H rhodopsin mutant pigs, rod-specific ERG cannot be recorded at any stage (Petters et al., 1997). These factors limit the usefulness of transgenic animals in the

determination of the pathogenesis of human diseases but they can still be useful model for therapeutic trials.

Another approach is to "knock out" or disable the gene. This can be achieved by replacing an endogenous gene with an exogenous DNA fragment that has DNA sequences homologous to the endogenous DNA. The deletion of a gene by this method can be used to determine the function of the gene product by the changes that result from its absence. The main examples in retinal degeneration are those with knock out genes involved in the apoptotic process (vide infra). The main disadvantage is that if the gene product is essential for embryonic development, the animal might never develop normally and the function of the gene product in postnatal life cannot be determined. Furthermore, natural compensation for the missing gene might occur during development. For instance, another gene that codes for a protein in the same family as the targeted gene may upregulate and provide sufficient activity to overcome the lack of the targeted protein. This situation can lead to misinterpretation of the importance of the targeted gene in adult life.

Recently, the target gene can be switched on and off in adult life using promoter that can be controlled by the intake of a drug. This latter method might revolutionise experiments using transgenic animals.

A2: Current and future management of retinal degeneration

2.1 Current management

With few exceptions such as, abetalipoproteinaemia (Illingworth et al, 1980; Gouras et al, 1971), Refsum disease (Hansen et al, 1979) and gyrate atrophy (Kaiser-Kupfer, Caruso and Valle, 1991) there is no treatment by which the primary disorder can be modified. Some symptomatic relief may be derived from cataract extraction (Newsome et al , 1986), and carbonic anhydrase inhibition if there is macular oedema (Cox et al, 1988). Vitamin-A supplementation may slow the progression but this has been the subject of controversy (Berson et al, 1993; Massof and Finkelstein, 1993). Although there is no treatment by which the primary disorder can be manipulated, patients can expect a reasonable account of visual prognosis, accurate genetic counselling, and support for rehabilitation.

Different potential therapeutic approaches to this problem are under investigation. These are based on the possibility of modulation of cell death (the focus of this thesis, see next chapter), transfecting the photoreceptor or retinal pigment epithelial cell with functioning genes, transplanting photoreceptor or retinal pigment epithelial cells into the subretinal space or the use of electronic devices to stimulate the outer retina.

2.2 Gene therapy

In most recessive and some dominant diseases, cell dysfunction is due to lack of functioning genes, and the objective of therapy is the replacement of the defective gene with genes that express normally.

In the retinal degeneration slow (rds) mice, transfection of the fertilised ovum with the appropriate wild-type (normal) gene results in photoreceptor cell rescue (Travis et al, 1992). However, insertion of genes into non-dividing photoreceptor cells in human is a much greater challenge. There are two general approaches by which genes can be introduced into the eye, *ex vivo* or *in vivo*. In the *ex vivo* techniques, the genes are introduced *in vitro* into retinal cells, retinal pigment epithelial (RPE) cells or fibroblasts. The transfected cells are then injected into the eye. *In vivo*, or direct gene transfer, genes are introduced into the cells *in situ* using a vector. The most commonly used gene vectors are replication defective herpes simplex virus, replication defective adenovirus, adeno-associated virus (AAV) and retrovirus. Each has advantages and disadvantages.

Herpes virus can carry a large gene insert (up to 36 kb). However, it rapidly undergoes latency after infection, restricting the long-term expression of the transgenes. This drawback can be overcome by using one of the latency promoters as the helper for transgene expression so it remains active in latency. A cytotoxic response may occur in the target cells that would reduce the longevity of expression (Pepose and Leib, 1994). Furthermore, the wild-type herpes virus carries a significant morbidity in humans. The adenovirus is a relatively safe virus. However, it induces an immune response that prevents re-infection by the same virus, and cytotoxic effect on the target cells reducing longevity of expression (Ali et al, 1997). The current capacity for a gene insert is relatively small (about 8 kb), but with

development of new packaging cell lines, larger insert might be achieved. Further difficulty is that the transfection efficiency in general is low in that only a small proportion of cells is transfected, and expression is brief.

AAV is an attractive alternative, and it is not associated with any apparent pathological response in humans. The recombinant AAV vectors have no virally encoded proteins and hence less immunogenicity. It can infect a variety of cells, and is more efficient in transducing photoreceptors than adenovirus. Wild type AAV integrates into chromosome 19, whilst recombinant AAV may integrate randomly. The latter has the potential risk of disrupting essential genes or causing malignant transformation of the target cells, although there is little evidence of this to date. Chromosomal integration may serve to prolong expression. The maximum size of insert is only 4.7 kb, and it is difficult to prepare high titres without contamination of helper adenovirus (Ali et al, 1997). Retrovirus can only readily infect dividing cells, which restricts its use in the retina.

Delayed photoreceptor cell death in the rd mice (β-PDE defect) by the use of subretinal injection of a recombinant replication defective adenovirus that contains the murine cDNA for the wide-type β-PDE was observed. It is reported that these injections result in β-PDE transcription, and increased PDE activity, although the rescue only lasted for 6 weeks (Bennett et al, 1996). In all these observations, there must always be doubt as to whether the therapeutic effect is due to the expression of the gene or due to injury with the attendant release of growth factors or the immune response to the viral vectors.

On the other hand, in most dominant diseases it is likely that the abnormal gene product produces disease. An anti-sense would be required to negate the influence of the abnormal gene. However, anti-sense molecules are often too big to

discriminate wild-type and mutant mRNA in point mutation, as seen in many autosomal dominant RP (Bird, 1995; Dreunser et al, 1998). Recently, ribozymes present new promises. They can bind and digest targeted mRNA. Specifically designed ribozymes can discriminate between the mutant and wild-type sequences of mRNA associated with autosomal dominant RP (Dreunser et al, 1998). Furthermore, using an AAV vector, ribozymes has been demonstrated to rescue photoreceptor cells in the transgenic rhodopsin P23H mutant rats (Lewin et al, 1998).

Gene therapy could also be used to deliver neurotrophic factor (Cayouette and Gravel, 1997). These approaches can also be combined by re-introduction of genetically modified host cells expressing the neurotrophic factors. The longevity of expression remains the problem, but with *ex vivo* transfection there is a wide scope for manipulation of expression.

2.3 Retinal cell transplantation

The ultimate goal of transplantation is to replace lost photoreceptors with healthy ones, which would acquire the intrinsic properties of photoreceptor cells and have the capacity to re-establish the appropriate cellular connections at the outer plexiform layer. Cell survival appears to be related to donor age. Transplanted foetal tissue appears to survive and differentiate, but fails to show normal orientation in the retina (Aramant et al, 1988). Enzymatic dissociated adult photoreceptor cells can be transplanted by injection into the subretinal space. Such photoreceptors survive and appear to have synaptic terminals but their outer segments degenerate almost completely (Gouras et al, 1991). The functional attributes of these cells have not been tested since the visually directed behaviour of these animals was not assessed. There is also some evidence to suggest cones survived longer following transplantation in rd mouse (Mohand-Said et al, 1997). This might reflect the influence of trophic factors.

However, recent experimental studies have shown more promise in that transplanted cells survive (Gouras et al., 1994; del Cerro et al, 1989; Ivert et al.,1998), integrate synaptically with the remaining retina (Seiler and Aramant, 1998; Kwan, Wang and Lund, 1999) and mediate a simple visual reflex (Kwan, Wang and Lund, 1999)

Despite the paucity of evidence that this technique may be useful, mechanically dissociated foetal retina of 14 to 18 weeks gestation have been grafted in 12 patients with advanced RP. The pre-operative visual acuity was perception of light or worse. No rejection or complications were reported. Five patients had reported subjective improvement of vision. (del Cerro et al, 1997) However, it is impossible to exclude the placebo effect or the influence of injury. Furthermore, no

improvement of vision was found in two patients transplanted with a sheet of adult photoreceptors harvested by vibratome (Kaplan et al, 1997).

Another strategy is the transplantation of RPE cells. In the RCS rat, the RPE failed to phagocytose photoreceptors outer segment material. This accumulation of outer segment material is associated with photoreceptor cell death (Mullen et al, 1976). Transplantation of healthy RPE cells into subretinal space delayed photoreceptor loss, and retinal function, as measured by electroretinogram and pupillary light reflex, is restored following RPE cell grafts in the RCS rats (Sheedlo et al, 1989a; Sheedlo et al, 1989b; Whiteley et al, 1996; Yamamoto et al, 1993). However, this may have limited relevance to most human forms of retinal dystrophy, although it may have a role in slowing the progress of age-related macular degeneration (AMD). The results of human foetal RPE transplantation in 13 AMD patients were recently reported (Algvere et al, 1997). No visual improvement was observed but the graft was reported to survive in most of the patients.

2.4 Artificial vision

It is well known that stimulation of the appropriate area of the visual cortex evokes a visual sensation. Some groups are working on devices that can detect visual signals, and stimulate the visual cortex directly. Thus no viable eye tissue is required, but the signal bypasses the sophisticated coding of the visual system, and the sensations are likely to be complex depending upon the functional attributes of the cell stimulated. This renders the potential success of this approach problematic (Brindley and Lewin, 1968).

There has been extensive media coverage of the artificial retinal implant for blind people. The concept is based on the fact that in RP, the photoreceptors are damaged but the inner retina remains relatively healthy. Electrical stimulation of the inner surface of rabbit retina was the first demonstration the possibilities of retinal implants (Humayun et al, 1994). These experiments were later carried out in humans. Subsequently, intra-ocular electrodes coupled with an optically isolated, constant current generator was used to deliver the pulses. This was performed in 5 patients with bare or no perception of light (3 had RP, 1 had AMD and 1 unspecified retinal dystrophy from birth). Stimulation elicited a visual perception of a spot that was retinotopically correct in 4 subjects who had had previously useful vision. One subject resolved two simultaneous spots giving a visual acuity of about 1/60 (Humayun et al, 1996). However, the methods for permanent implantation of such devices, and their connection with the outside world may pose major technical problems. The latter might be made possible by using an external reception and power unit, the signals are then transmitted into the retinal implant (Figure 2.5). This device assumes the eye is in primary position at all times. Furthermore, the small number of stimulating electrodes that can be used would limit the visual field. Even

these problems are resolved, it is unlikely to simulate foveal function without the presence of photoreceptors.

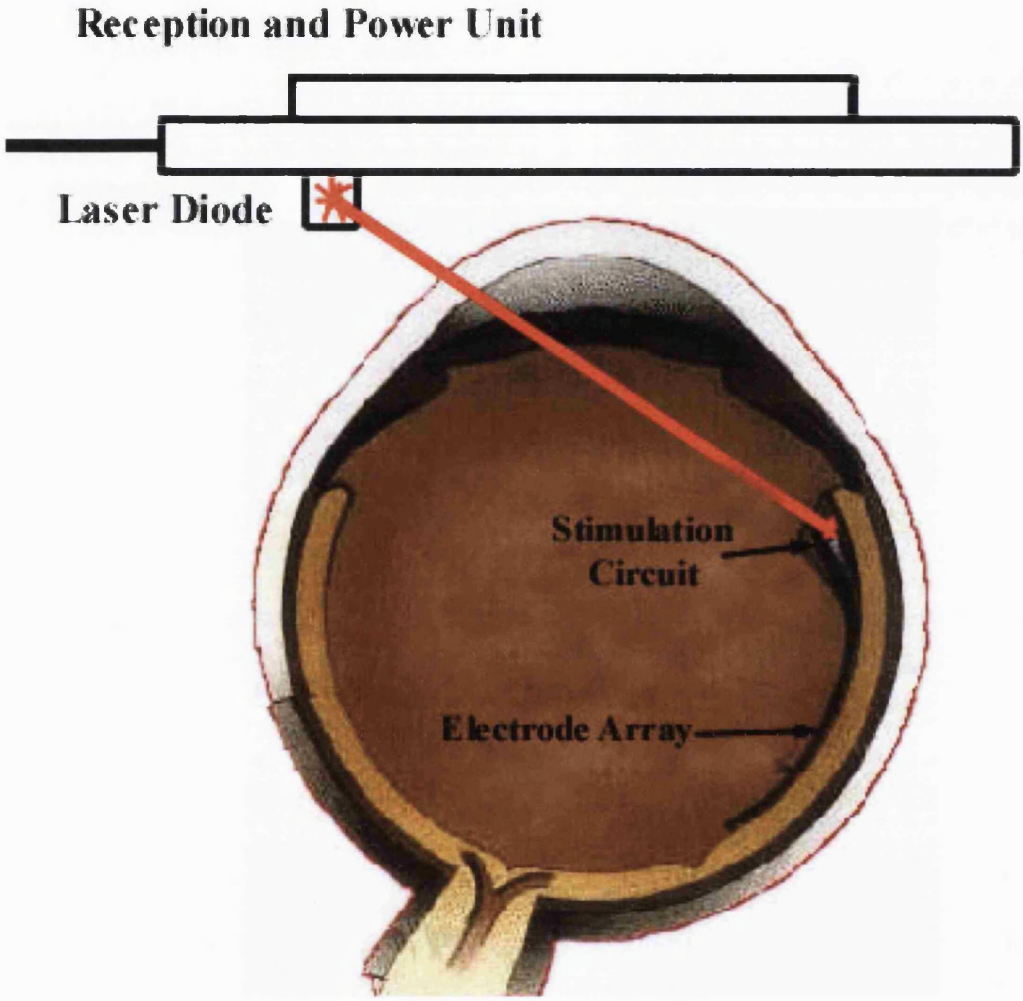

Figure 2.5: A potential artificial retina design (Modification from the Harvard and MIT Collaboration - Retinal Implant Project)

At the moment, none of the techniques mentioned above appear to be very effective for long term modification of the course of the disorder. In the near future (next 5 to 10 years), the scope of gene therapy is limited by the large number of genes and mutations involved in retinal degeneration. Furthermore, the majority of abnormal genes are yet to be found. Eventually, transplantation might be useful to

preserve central vision whilst the artificial retinal implant might be useful to maintain visual field.

A relatively simple approach that can be used in a clinical setting within the next 10 years is certainly required.

A3: Apoptosis and retinal degeneration

3.1 Introduction

Photoreceptor cell death is the final, irreversible event in many blinding diseases including retinal degeneration. The combination of several pieces of indirect evidence make it likely that photoreceptor cells are inherently vulnerable. Firstly, it is remarkable how many different mutations and acquired insults lead to photoreceptor cell death (Bird, 1995). Secondly, there is a wave of cell death in post-mitotic photoreceptor cells when metabolic activity in the retina increases (Maslim and Stone, 1996). This, together with the high focal concentrations of mitochondria in photoreceptors and the very high blood supply to the choriocapillaris suggest that there may be little reserve in the energy supply to photoreceptor cells (Cohen, 1987). The complex inter-relationships between rods, cones, Muller cells and retinal pigment epithelium (RPE) add to the vulnerability in that failure of other cell types then leads to secondary photoreceptor cell loss.

In line with this, apoptosis has been described in a wide variety of hereditary retinal degenerations, including those described in this thesis (Tso et al., 1994; Portera-Cailliau et al., 1994; Chong et al., 1999), in light damage (Hafezi et al., 1997) and following retinal detachment (Chang et al., 1995; Cook et al., 1995) and in other types of retinal degeneration (Xu et al., 1996). The hypothesis that apoptosis can be manipulated as a novel therapeutic approach for retinal degeneration is the basis of the treatment trials in this thesis.

3.2 Apoptosis - an overview

The importance of the genetic basis of cell death was first realised in 1914 by Ludwig Graper. It was rediscovered in 1951 when it became clear that cell death shapes digits and eliminates no longer useful structures. This death was primed by time-dependent intrinsic stimuli, which could be influenced by the environment. John Saunders called this the "death clock" but the term "programmed cell death", introduced by Richard Lockshin, has stuck. Ronald Oppenheim then showed that, during the development of the nervous system, motor neuron production far exceeded that needed to generate the right number of adult neurons (reviewed in Rich et al., 1999).

Structural manifestations of cell death

In 1971, John Kerr, whilst using electron microscopy to study cytotoxicity in the liver, identified at least two modes of cell death. There was good evidence to show that severely injured cells lost control of ion flux across the plasma membrane, swelled and then ruptured. These features were all concordant with descriptions of necrosis. But in the lightly injured or mildly hypoxic livers, cells shrunk to half their original volume, lost contact with their neighbours, rearranged and condensed their nuclear chromatin, and, rarely but significantly, were caught in the act of implosion, emerging as condensed, membrane bounded bodies (Kerr, 1971). Such cells were efficiently phagocytosed and degraded. He then identified the same morphological cell death under a variety of circumstances. To emphasize its commonality and suggest its counterbalancing role to mitosis, the 'shrinkage' process was renamed "apoptosis" (Kerr et al., 1972).

Proteins associated with cell death

Robert Horvitz began dissecting the genetics of developmental cell death in the nematode *Caenorhabditis elegans*. These deaths show some differences from mammalian apoptosis, but also many similarities (Hengartner, 1996). Mutant *C. elegans* strains showing aberrant developmental death were found to result from mutations in a hierarchy of genes. Two of these genes *(ced-3* and *ced-4*, where *ced* stands for 'cell death abnormal') specify irreversible death, whereas another *(ced-9)* is required for survival. All of these, as well as several other nematode death-related genes, have since been shown to have structural and functional mammalian homologues, revealing the commonality of death pathways in animal cells.

APO-1/Fas is cell-surface receptor that can activate apoptosis. It was discovered by two separate groups by screening of panels of antibodies for lymphocytotic effects (Trauth, 1989; Yonehara et al., 1989). APO-1/Fas was subsequently found to be a member of the superfamily of receptors related to the tumour necrosis factor (TNF) receptor (Itoh et al., 1991) which in itself can be activated apoptosis. They all have a cytoplasmic so called "death receptor" domain (Raff, 1992). A route from APO-1/Fas, through a set of rapidly recruited cytosolic proteins, to caspase-8 (also known as FLICE), a regulatory caspase, was identified (Muzio et al., 1996; Boldin et al., 1996). Subsequently, other caspases are found to be involved in this early process of apoptosis and they are collectively called "first level caspases". The activity of this pathway affects sensitivity to lethal stimuli arising elsewhere, including damage to nuclear DNA. Survival factors may also have subtle but essential roles in the maintenance of normal tissue structure, perhaps by ensuring the survival of cells that are appropriately apposed to the basal lamina, but initiating death should cells move away from the lamina.

It was observed that the principal function of the proto-oncogene *Bcl-2* is to prevent death (Vaux et al., 1988). *Bcl-2*, the mammalian homologue of *ced-9*, is the prototype of a family of genes that influence cell survival by their interactions with death effectors.

The mammalian homologue of CED-4 was identified as a mitochondrial protein, apoptosis activating factor-1 (Zou et al., 1997). This, coupled with the knowledge that Bcl-2 family (Kroemer, 1997) are frequently located at the mitochondrial surface, established a fast track to apoptosis from this organelle, which is arguably the strongest candidate for monitoring the energy status of the cell.

The CED-4 pathway arises in stressed mitochondria. The decision to irrevocably commit to apoptosis is played out at the mitochondrial surface in a series of interactions between protective and pro-apoptotic Bcl-2-family proteins (Adams and Cory, 1998). Although the minutiae of these processes are still unknown, changes in membrane permeability are key, leading eventually to release of mitochondrial pro-apoptotic factors such as cytochrome c and apoptosis-inducing factor. Positive-feedback loops seal the fate of the cell. Other organelles such as the endoplasmic reticulum and cell membrane might also play a role.

Cytotoxic T lymphocytes (CTLs), in some instances, kill their targets by inducing apoptosis. Granules that contain apoptotic effectors are deposited at the CTL–target-cell interface. Horvitz revealed that the cysteine protease CED-3 cleaves preferentially at aspartates (Horvitz et al., 1994).

With the identification of sequence identity between CED-3 and interleukin-1b converting enzyme (ICE or caspase-1) (Yuan et al., 1993), it rapidly became clear that the mammalian homologues of CED-3 comprise a family of cysteine proteases, known now as caspases. These cleave next to an aspartate residue and exist as inert

pro-enzymes that are themselves activated by cleavage. However, it appears caspase-3 like, or second level, caspases are more important in apoptosis

Tetrapeptide motifs required for cleavage by caspases were soon identified in many key structural and regulatory proteins. Among these are the caspases themselves, suggesting that the apoptotic effector event is a self-amplifying, autocatalytic cascade. A diagrammatic scheme is shown in Figure 3.2.

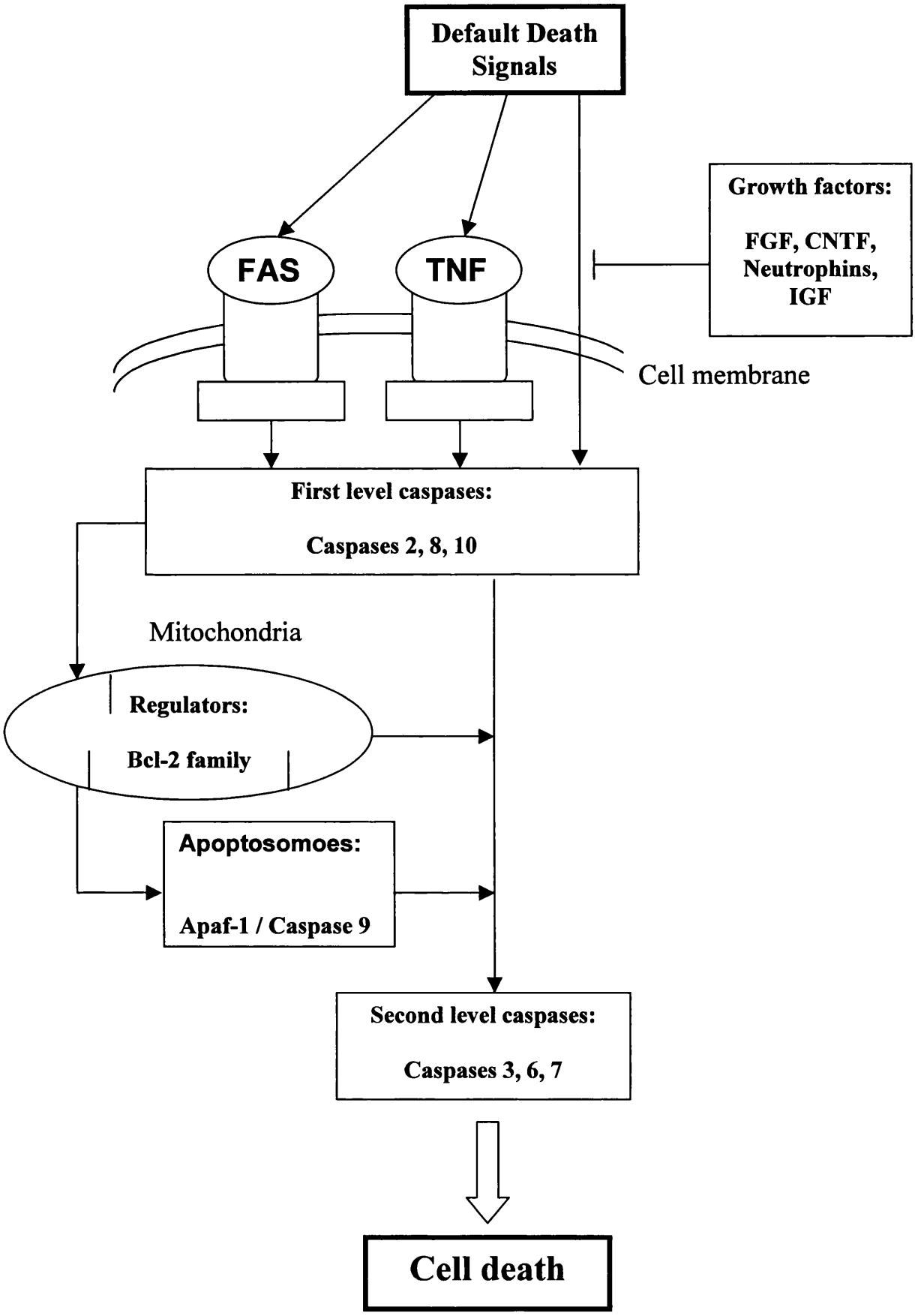

Figure 3.2: A diagrammatic scheme for apoptosis (see text for details)

3.3 Other apoptosis modulators which have been studied in retinal degeneration

Tumour suppressor protein, p53

The p53 tumour suppressor protein is involved in the control of the cell cycle, and has been widely associated with apoptosis in various cell types, especially following DNA damage (Liebermann et al., 1995). However, p53 independent apoptosis has been described (Malcomson et al., 1995).

The retinal degeneration (rd/rd) mice have been interbred with p53 knockout mice to generate p53 -/- rd/rd. The extent and kinetics of photoreceptor cell loss in *rd* mice were indistinguishable in the *p53* +/+ and p53 knockout mice (Hopp et al., 1998). Using similar techniques of crossing the p53 knockout mice with the retinal degeneration slow (rds) mice, the peak in photoreceptor apoptosis, which occurs at 16 days in the p53 +/+ rds mouse, is delayed by 3 days in p53-deficient rds mice. In addition, there is also a delay in the loss of photoreceptor cells at postnatal day 16 and postnatal day 26. However, absence of p53 did not prevent retinal degeneration in the rds mouse. The number of photoreceptor cells in p53 deficient rds mice at 35 days was very similar to that in the p53 +/+ rds mouse (Ali et al., 1998).

Jun, Fos and CREB families

Proteins of both the Jun and Fos families (such as c-Jun, Jun D, Jun B; c-Fos, Fra-1, Fra-2 and Fos B) as well as of the CREB family (such as ATF-2), interact to work collectively as transcription complexes (including AP-1). Homodimers may be formed among members of the Jun family, while proteins of the three families form a variety of heterodimers. The dimers bind to specific elements of DNA in the promoter region of target genes thus regulating their expression (Morgan and Curran,

1991). The transcription factor AP-1 is considered a molecular mediator of cell death in some circumstances (Morgan and Curran, 1995).

The potential role of c-fos in apoptosis may vary from one tissue to another. Continuous expression of c-fos precedes apoptosis in several tissues (Smeyne et al., 1993), and apoptosis is induced by the c-Fos protein in vitro (Preston et al., 1996). On the other hand, c-fos is not essential to induce apoptosis in lymphoid tissues *in vitro* (Gajate et al., 1996).

Similarly, c-fos knockout mice are protected from light damaged retinal degeneration (Hafezi et al., 1997), however, there is no retinal protection of cross breeding the c-fos null mice to the retinal degeneration (rd) mice (Hafezi et al., 1998) despite increased expression of c-fos in the c-fos +/+ rd mice, indicating that c-fos is upregulated but not essential for apoptosis (Rich et al., 1997).

3.4 Growth factors and neurotrophic factors

An unexpected finding was observed during the early attempts of retinal transplantation in which focal injury to retina appeared to protect photoreceptors from degeneration. This was clearly illustrated in the RCS rat when mechanical injury produced by an injection of saline into the subretinal or into the vitreous, or even insertion of a needle without injection led to protection of photoreceptors near the wound (Silverman and Hughes, 1990). This protection was not restricted to genetically determined retinal degeneration. Similar photoreceptor rescue by mechanical injury was observed in the light-induced retinal damage in the rat (Faktorovich et al., 1992), although interestingly this, and related strategies are less effective in the mouse. Furthermore, this is not restricted to photoreceptor cells, a similar self-protective mechanism appears to exist for retinal ganglion cells (Mansour-Robaey et al., 1994). These findings imply that the retina has a self-protective mechanism that can be activated to protect at least partially, photoreceptors and ganglion cells from damage or death. Injury-induced photoreceptor rescue extends beyond the immediate vicinity of the lesion suggesting that soluble factors are involved (Silverman and Hughes, 1990; Faktorovich et al., 1992). As mechanical injury to the eye increases the expression of basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) in the rat retina (Wen et al., 1995) it was logical to assume that these agents might be responsible for the protection.

There are at least four families of survival growth factors might be able to modulate apoptosis:

- Fibroblast growth factors such as bFGF
- Cytokine associated neurotrophic factors such as CNTF

- Neurotrophins such as brain derived neurotrophic factor (BDNF)
- Insulin-like growth factors (IGF) such as IGF-I and IGF-II

Fibroblast growth factors

bFGF is a member of the fibroblast growth factor (FGF) family; a group of heparin-binding, single-chain polypeptides about 150 to 300 amino acids in length. They have a number of important roles in physiological and pathological processes as diverse as cell growth, differentiation, angiogenesis, tissue repair and transformation. At present, nine members in the FGF family are recognised.

The first characterised members of the FGF family were acidic FGF (aFGF, FGF-1) and basic FGF (bFGF, FGF-2). They were purified as mitogens for fibroblasts. The Int-2 (FGF-2), K-FGF (FGF-4), FGF-5, FGF-6, keratinocyte growth factor (KGF, FGF-7), androgen-induced growth factor (AIGF, FGF-8) and glial-activating factor (GAF, FGF-9) have been subsequently identified. These proteins share 30-70% amino acid sequence identity with each other (Brenard and Matthew, 1994).

In the eye, bFGF immunoreactive cells are present in the photoreceptor, inner nuclear and ganglion cell layers. It appears that bFGF is not only expressed by glial cells (Muller cells and astrocytes) but also by rod photoreceptors and subpopulations of cone bipolar cells and amacrine cells. It is however, not expressed in cones (Li, Chang and Milam, 1997). The increase of bFGF expression after mechanical injury is generally rapid but interestingly focal laser photocoagulation does not always increase bFGF expression (Xiao et al., 1996).

Ciliary neurotrophic factor (CNTF)

CNTF was discovered and named for its actions on the parasympathetic neurons of the ciliary ganglia from chick embryos. A variety of actions has

subsequently been identified. In its primary sequence, CNTF does not bear strong homology with any other known protein. The tertiary structure of CNTF and its receptors are, however, similar to that of leukaemia inhibitory factor (LIF) and interleukin-6 (IL-6) (Richardson, 1994); and hence it is often referred as the cytokine associated neurotrophic factor. Human CNTF is a polypeptide with 200 amino acids and its gene has been mapped to chromosome 11q12.2 (Lam et al., 1991). It is synthesised by the Schwann cells and there is a high concentration of CNTF in human optic nerve.

It is however, curious that CNTF is poorly secreted and usually appears to remain within its cell of synthesis. The mechanism controlling CNTF synthesis and secretion are largely unknown, but it appears that the initial response to injury is the release of the CNTF within these cells followed by the upregulation of CNTF mRNA. This may explain the relatively slow rise of CNTF mRNA after mechanical injury (Richardson, 1994). Interestingly, bFGF release can lead to an increase in CNTF mRNA expression (Cao et al., 1997).

CNTF has been shown to promote the survival of a variety of neuronal and non-neuronal cells, (Ernsberger et al., 1989; Oppenheim et al., 1991; Louis et al., 1993) and can slow motoneuron degeneration in the murine mutants wobbler (Mitsumoto et al., 1994) and pmn (Sendtner et al., 1992). CNTF has also been shown to promote the *in vitro* differentiation of O-2A progenitors to type 2 astrocytes (Lillien, Sendtner and Raff, 1990), and sympatho-adrenergic precursors to cholinergic neurons (Saadat, Sendtner, Rohrer, 1989). Disruption of the CNTF gene has been shown to result in a small reduction in motoneuron numbers in adult mice (Masu et al., 1993). CNTF mediates its effects through a tripartite receptor complex composed of a CNTF-binding alpha receptor (CNTFrα), gp130 and LIF receptor

beta (Ip et al., 1992, 1993b; Taga and Kishimoto, 1992; Stahl et al., 1993). LIF receptor beta and gp130 are expressed in a wide variety of tissues (Taga and Kishimoto, 1992) whereas, expression of CNTFrα is more restricted, being confined primarily to the central nervous system (Davis, 1991; Ip et al., 1993b).

Neurotrophins

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family. This family binds to members of a group of transmembrane tyrosine kinase (Trk) receptors and other neurotrophins include nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Jelsma and Aguayo, 1994). They have a wide range of cell functions in the developing, mature and injured nervous system (Jelsma and Aguayo, 1994), and are known to be expressed by glial as well as neuronal cells. Although the function of the neurotrophins expressed by nerve cells has not been defined, the co-expression of some of these factors and their receptors in the same neurons suggests an autocrine or paracrine role (Jelsma and Aguayo, 1994). At least, *in vitro*, cortical neurons secrete BDNF in response to the activation of a voltage-sensitive calcium channel which suggests that neuronal activity could influence neurotrophin expression. The expression of BDNF in the eye is not well characterised. However, the finding that the expression of BDNF mRNA was slightly reduced in the rat retina (Wen et al., 1995) and brain (Ip et al., 1993a) following mechanical injury was unexpected.

Insulin-like growth factor

Insulin-like growth factors (IGF-I and IGF-II) are important for cell proliferation, differentiation and sustained survival of many tissues throughout the body including the eye. IGF-I mRNA is expressed in retinal ganglion cells and

endothelial cells of the choroid and ciliary processes. The IGF-I receptor mRNA has a more extensive distribution, which includes the retinal ganglion cell layer and inner nuclear layer (Burren et al., 1996; 1997). The IGF-I receptor gene is homologous to the *sevenless* gene in *Drosophila*, where it is essential for the differentiation of photoreceptor seven. There is also evidence to suggest that cone photoreceptors can produce IGF-I that strongly influences the production of rod photoreceptors during development (Hoke and Fernald, 1997). The exact role of these factors in the mature retina is less certain, but interestingly scatter laser photocoagulation can increase the intravitreal levels of IGF-I in the miniature pig (Xiao et al., 1996).

A4: Apoptosis modulation in retinai degeneration with growth factors

4.1 Growth factors and neurotrophic factors

The first *in vivo* experimental success of the therapeutic use of growth factors in photoreceptor rescue was the use of bFGF in the Royal College of surgeons (RCS) rat. Although the sham injected eyes displayed localised photoreceptor rescue, there was significantly more survival in the eyes treated with bFGF, either intravitreally or subretinally (Faktorovich et al., 1990). In the light damaged model of retinal degeneration, a high degree of photoreceptor rescue was present with bFGF, BDNF, CNTF, midkine, interleukin-1 beta and aFGF. Lesser protection was seen with NT3, IGF-II and tumour necrosis factor alpha. NGF, epidermal growth factor, platelet-derived growth factor, insulin, IGF-I, heparin and laminin were without significant effect (Faktorovich et al., 1992; LaVail et al., 1991, 1992; Unoki and LaVail, 1994).

CNTF or its derivatives delay photoreceptor cell loss in the retinal degeneration (*rd*), Q344ter mutant rhodopsin transgenic mice (LaVail et al., 1998). BDNF promotes outer segment formation in a feline model of retinal detachment (Lewis et al., 1999). In most studies growth factors have been delivered by intraocular injection of the recombinant protein. CNTF has, however, also been successfully delivered by adenovirus gene therapy (Cayouette and Gravel, 1997).

Growth factor rescue is not specific for photoreceptor cells. There are many examples within the brain and in ischaemic-induced retinal injury, intravitreal injections of bFGF, BDNF or CNTF two days before the ischaemia transiently protects retinal ganglion cells. Post-ischaemia injection of BDNF can prolong the protective effect. Furthermore, BDNF injection up to 3 days after the ischaemia

alone can also protect the inner retina. The retrograde degeneration of the optic nerve after a crush lesion can be also be diminished by BDNF and to a lesser extent by NT-3 or CNTF but not with NGF, aFGF or bFGF (Weibel et al., 1995; Mey and Thanos 1992).

There is increasing evidence to suggest a degree of specificity for different combinations of neurotrophic factors, cell type and mode of injury. For instance, CNTF protects oligodendrocytes from tumour necrosis factor (TNF) mediated injury as well as serum deprivation induced apoptosis, but it does not protect foetal cortical neurons or glioma cell lines from the same insults (D'Souza et al., 1996). Similarly, bFGF is effective in photoreceptor rescue in the RCS and light-damaged rats, but so far no effect has been demonstrated in the rd mouse or light-damaged mice (LaVail et al., 1998). There is also an impression that the retinal ganglion cells are better protected by BDNF, whilst the photoreceptors appear to be better protected by bFGF and CNTF. Furthermore, neurotrophic factors have synergistic effect in neuronal survival *in vitro* (Bianchi and Cohan, 1993; Kato et al., 1994; Mitsumoto et al., 1994; Hartnick et al., 1996; Zurn et al., 1996) but this was not demonstrated in an *in vivo* study of retinal degeneration (LaVail et al., 1998).

It appears that growth factors might be able to delay photoreceptor cell loss. However, due to the small size of the rodent eyes, it is unclear whether the positive effect is due to the growth factor or the trauma effect of the injection. It seems appropriate, before a clinical trial in human, to assess whether growth factors are effective in other animal species, in particular, those have eyes that are similar in size to that of human. Furthermore, the dosage of the growth factors required can be better calculated and the side effects of the growth factors can be assessed. As bFGF has a wide range of actions, in particular, angiogenic and inflammatory responses

which could be potentially harmful in the eye, we chose to use BDNF and CNTF in our initial experiments.

At the early stage of the project, the choice of animal models are rather limited. The transgenic pig is not yet available. We have therefore selected the retinal dysplasia cat (Rdy) cats for the initial examination in view of the fact that it is dominant model and the relatively lower cost in breeding and up-keeping of cats. After refining the dosage and found the more effective neurotrophic factor, we then carried out a functional assessment of the neurotrophic factor therapy in the canine progressive retinal degeneration in the miniature longhaired dachshund (MLHD). As both of the feline and the canine model of retinal degeneration were not fully characterised previously, we have further characterised these two important models of retinal degeneration as part of this thesis.

4.2 Objectives of the project

- To characterise three different models of retinal degeneration in three different animal species
- To carry out treatment trials using neurotrophic factors in the retinal dysplasia (Rdy) cats and miniature longhaired dachshund (MLHD) dogs
- To carry out treatment trials using caspases inhibitors in the pigmented retinal degeneration slow (rds) mice

B. MATERIALS AND METHODS

5.1 Characterisation of the Rdy cats

Initially the main focus of the project is on the characterisation of the retinal dysplasia (Rdy) cats. Affected kittens (n=32) aged between 10 days to 17.5 weeks were compared with normal littermate controls (n=8) aged from 10 days to 13.5 weeks. The animals were bred and studied under the regulation of the United Kingdom Animals (Scientific Procedures) Act 1986 and all animal procedures adhered to the ARVO resolution for the care and use of animals in Vision Research so as all the animal experiments described in this project. All animals were sacrificed with an overdose of systemic phenobarbitone, both eyes were enucleated immediately but only one eye per animal was used in this study. After 24 - 48 hours of immersion fixation in 10% formol saline, the inferior-nasal quadrant was embedded in paraffin wax for immunohistochemistry and TUNEL labeling whilst the superio-nasal quadrant was embedded in Araldite resin using standard protocols. (See Appendix A for details on all of the following methods).

Photoreceptor cell counts of Rdy cats

Photoreceptor cell counts were performed in 1μm thick toluidine blue stained Araldite resin sections. A random location near the centre of the section was selected at low magnification. The section was then moved 550 μm from the random spot. An eye-piece graticule (Graticules Ltd, Tunbridge, England) was employed to demarcate a 25 μm wide retinal strip. The number of photoreceptor cells within this strip was counted. The section was then moved 250 μm toward to the centre, and another retinal strip of 25 μm wide was counted. This was repeated twice to obtain a total of

4 readings. A total of 100 μm of retinal width was counted. The number of photoreceptor cells counted was then multiplied by 10 and expressed as the number of cells per 1 mm of retina. They were counted by two independent observers and the mean values were calculated. The observers were masked to the age of animals.

Demonstration of Apoptosis in Rdy cats

The pyknotic appearance of the nucleus and the presence of double strand breaks in DNA are suggestive of apoptosis. In this study, these two aspects of apoptosis were studied in the Rdy cats. A modified TUNEL (Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labeling) technique was used to detect double strand breaks in DNA. This technique is based on that described by Nakamura and colleagues (1995), but dAdenosine triphosphate replaces dUridine triphosphate. TUNEL-labelled cells were counted in the entire tissue section. The result was expressed as the number of TUNEL-labelled cell per 1 mm of retina.

Pyknotic cells were counted in 1μm thick toluidine blue stained Araldite resin sections. The result was expressed as the number of pyknotic cells per 1 mm of retina. TUNEL-labelled and pyknotic cell were counted by two independent observers and the mean values were calculated. The observers were masked to the age of animals.

Immunohistochemistry of Rdy cats

The distribution of opsin (antiserum courtesy of Dr. D. Bok), synaptophysin, glial fibrillary acidic protein (GFAP) immunoreactivity, and binding of an epithelial marker MNF 118 were investigated using a standard biotin-streptavidin peroxidase method (all antibodies, unless otherwise stated, were obtained from Dako Ltd.,

England). Antigen retrieval pre-treatment with trypsin was performed prior to all primary antibody incubations (Ordonez et al., 1988). Appropriate positive and negative controls were used throughout.

Electron microscopy of Rdy cats

As the electron microscopy of this model has been previously described, only two animals at the age of 5 weeks and 13 weeks were examined. Ultrathin sections were examined in a JEOL 1010 TEM transmission electron microscopy (JEOL (UK) Ltd, Welwyn Garden City, UK) operating at 80kV. Images were recorded onto Kodak 4489 electron image film and printed on Ilford multigrade paper.

Electroretinogram of Rdy cats

Electroretinogram were performed in four animals between the age of 10 days and 9 weeks under general anaesthesia using our purpose built electroretinographic equipment (See Appendix A).

5.2 Neurotrophic factors trials in the Rdy cats

Once the characterisation is completed, we then started our treatment trials using neurotrophic factors on the Rdy cats. All the previous experiments by other investigators in rodents, single injection of neurotrophic factor was used. In our project, it was decided that multiple injections at regular intervals would be carried out in order to maximise the effect of the neurotrophic factor as well as mimic the clinical setting if the work is going to be translated into clinical trials.

Combining the data from Curtis and colleagues (Curtis et al., 1987; Leon and Curtis, 1990; Leon et al., 1991) with our own characterisation data (Chong et al., 1999), we felt it is appropriate to start the first set of experiments at postnatal day 10 when the degeneration is not yet started.

In the early treatment experiment, Axokine (5 μg in 50 μl) was injected intravitreally to the Rdy cats at postnatal day 10 in one eye (n=10). The other eye acted as an untreated control. We could not carry out sham injection on the other eye due to Home Office restriction. The injection was repeated every four weeks. After each intravitreal injection a five day course of topical prednisolone were given to both eyes. Clinical (ophthalmoscopy) and histopathological examinations were carried out at 5.5, 9.5 and 13.5 weeks of age.

After the completion of the initial experiment, we would like to assess whether the neurotrophic factors are effective if the treatment is started at 5.5 weeks of age when the retinal degeneration has already started. In addition, only half the dose of Axokine were used. In addition, we would need to carry out sham injection in some animals as control so as to assess the trauma effect.

In this delayed experiment, we investigated the effects of Axokine (2.5 μg in 25 μl), human BDNF (250 μg in 25 μl) or sham injection of 25 μl of phosphate buffered solution (PBS) at pH 8 (vehicle of Axokine) on one eye of the Rdy cats with the other eye being untreated. Animals (n=17) were randomly assigned to one of the three injection groups. Only one eye was injected, the other eye remained untreated. The injections were started at 5.5 weeks and repeated every four weeks. After each intravitreal injection a five day course of topical prednisolone was given to both eyes. Clinical (ophthalmoscopy) and histopathological examinations were carried out at 9.5, 13.5 and 17.5 weeks of age.

In the third experiment, we injected a small number (n=2) of normal cats with Axokine in order to evaluate complications using an identical protocol as the first experiment.

Injection and histopathology protocol

Animals were anaesthetised by a gas mixture of halothane, nitrous oxide and oxygen. A disposable 0.3 ml insulin syringe with a fixed 29 gauge needle was inserted 2 to 4 mm behind the limbus (depending on the age of the animal) in the superotemporal quadrant aiming towards the optic nerve. When the tip of needle reached the mid-vitreous location, the injection was given in a single swift action. Gentle ocular massage was then carried out for two minutes to reduce the intraocular pressure.

All animals were sacrificed with an overdose of systemic phenobarbitone. The eyes were immersed into 10% formalin in the first experiment and in 4% paraformaldehyde with PBS in the second and third experiments. After 48 hours of fixation, the eyes were hemisectioned circumferentially at the equator. The posterior eye-cup was then divided horizontally and vertically through the optic nerve head.

The superonasal quadrant was embedded in Araldite resin using standard protocol (See Appendix A for further details).

Photoreceptor cell and apoptotic cell counts of the dystrophic animals were performed in 1μm thick toluidine blue stained Araldite sections. The same portions of the eye were counted in all cases. The inferonasal quadrant was embedded in paraffin wax for the modified TUNEL (Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labelling) technique and immunohistochemistry. The same portions of the eye were counted in all cases. The superior and inferior quadrants were orientated in such a way that the sections counted were only separated by the cut edge.

An antibody against glial fibrillary acidic protein (GFAP) (Dako Ltd., England) was used to assess Muller cell activity using a standard biotin-streptavidin peroxidase method. Antigen retrieval pre-treatment with trypsin was performed prior to primary antibody incubation. Appropriate positive and negative controls were used (See Appendix A for further details on all of the following methods).

The counting method used was identical to that used in the characterisation of the Rdy cats (See 5.1 for details). In addition, the two independent observers were masked to the age of the animals and to the injections that they had received.

Retinal fold counting in the Rdy cats

Retinal fold was found in the eyes injected with Axokine. The number of retinal folds was counted in each section of each eye. The results were expressed as the number of folds per 1 mm of retina. The two independent observers were masked to the age of the animals and the injections that they had received.

Statistical analysis

All three parameters (photoreceptor cell count, apoptotic cell count, "TUNEL" positive cell count) in each treatment group in the Rdy cats were compared with the untreated group using paired Student t-tests at individual time-points and then the groups as a whole. All statistical analysis was carried out using Microsoft Excel 97.

5.3 Characterisation of the MLHD dogs

After the results obtained in the Rdy cats, we would like to demonstrate functional changes with Axokine. The MLHD dogs became available to us. The full field ERG was previously reported to be normal at 10 weeks and extinguished at 25 weeks of age (Curtis and Barnett, 1993).

We felt the MLHD dogs would be a good model to test the effect of Axokine injection. Before carrying out the treatment trial, we decided to characterise the model in more details using our custom made electrophysiology set up.

All puppies were bred from either a heterozygous carrier crossed with homozygous affected dogs or from two homozygous affected dogs. Hence, all studied animals were either affected or a carrier. Affected puppies (n=4), aged between 6 weeks to 26 weeks, were examined with carrier littermates (n=6) for comparison. All heterozygous carriers were re-homed after discharged from the Act under Home Office regulations. Single affected animals aged 6 and 24 weeks were sacrificed with an overdose of systemic phenobarbitone and both eyes from each animal were enucleated immediately. The remaining affected animals were kept as breeders for future experiments. One normal dog that was sacrificed for an unrelated medical condition at 8 weeks of age, was examined as a control.

The main focus in this part of the project was electrophysiology as the number of animals available to us was restricted and hence a detailed histopathological characterisation was not possible. However, we have carried out the following examination on the limited number of pathological specimens. One eye of each animal was fixed with 10% formol saline by immersion for 48 hours and then embedded in paraffin wax for immunohistochemistry. The other eye was fixed in a mixture of paraformaldehyde and glutaraldehyde by immersion for 48 hours and

was then embedded in Araldite resin using standard protocols for light and electron microscopy (See Appendix A for details of all of the following methods).

Light microscopy, immunohistochemistry, electron microscopy

The pyknotic appearance and the presence of double strand breaks in DNA suggest apoptosis or programmed cell death (PCD). In this study, we examine these two aspects of apoptosis in the MLHD dogs. A modified TUNEL technique was used to detect double strand breaks in DNA.

The distribution of opsin (antiserum courtesy of Dr. D. Bok), synaptophysin, glial fibrillary acidic protein (GFAP) immunoreactivity was investigated using a standard biotin-streptavidin peroxidase method (all antibodies, unless otherwise stated, were obtained from Dako Ltd., England). Antigen retrieval pre-treatment with trypsin was performed prior to all primary antibody incubations (Ordonez et al., 1988). Appropriate positive and negative controls were used throughout.

Ultrathin sections were examined in a JEOL 1010 TEM transmission electron microscopy (JEOL (UK) Ltd, Welwyn Garden City, UK) operating at 80kV. Images were recorded onto Kodak 4489 electron image film and printed on Ilford multigrade paper.

The cell counting method is identical to that used in the characterisation of the Rdy cats (See 5.1 for details). In addition, the number of cones and rods were counted in the 6 weeks old animal by two independent observers.

Electroretinogram

Both affected and heterozygotes were examined every 2 to 4 weeks between the age of 6 weeks and 24 weeks under general anaesthesia, using our purpose built electroretinographic equipment (See Appendix A for details). In brief, the animal

was anaesthetised and mydriasis achieved with tropicamide 1% eye drops. ERG-Jet corneal electrodes were used. Platinum Iridium sub-dermal ground and reference electrodes were attached at the crown and one centimetre behind lateral canthus respectively. The ganzfeld stimulator was custom built, with a 50 cm bowl, using a Grass PS22C photic stimulator and recorded on PC based data acquisition system (CH Electronics, England).

As mentioned previously, there is no standard recording protocols for research in dogs. After consultation with Dr Gus Aguirre (Cornell University, New York), Dr Keith Barnett (Animal Health Trust, Newmarket) and Dr Graham Holder (Moorfields Eye Hospital, London), it was suggested that the best recording protocol to be used is that based on the human recommendations for electroretinography (ERG) by International Society for Clinical Electrophysiology of Vision standard (Marmor and Zrenner, 1995). It consists of 20 minutes dark adaptation, followed by an intensity series of discrete 'white' stimuli, and 2 and 30 Hz (1.5 $cd.s/m^2$) stimulation, then 8 minutes of light adaptation followed by 2 and 30 Hz (3.0 $cd.s/m^2$) photopic stimuli. The analysis was carried out at a later date when the observer was masked to the identity of the animals.

5.4 Neurotrophic factor trial in the MLHD dogs

Based on the same principle of the treatment trials in the Rdy cats, we used the modified protocol of the delayed experiment in the Rdy cats. The injection dosage of Axokine was the same as that used in the delayed experiment. We decided to start the injection at 10 weeks when the ERG is within the normal limit but the retinal degeneration has already started as demonstrated by histopathology.

Axokine (2.5 μg in 25 μl) was injected intravitreally in the MLHD dogs at postnatal week 10 in one eye (n=3) and sham injection of 25 μl of phosphate buffered solution (PBS) at pH 8 was injected into the other eye. Sham injections were allowed in the contralateral eye to reduce the number of animals used. Specific Home Office permission were granted for this project. The injection was repeated at postnatal week 14. After each intravitreal injection a five day course of topical prednisolone were given to both eyes. Electroretinography was carried out at postnatal weeks 10, 12, 14, 16, 18, and 22. Terminal pathology was obtained at 22 weeks.

The injection and histopathology protocol were identical to that used in the Rdy cats (See 5.1 and 5.2 for details). The electrophysiology protocol was identical to that used in the characterisation of the MLHD dogs (See 5.3 for details). The analysis was carried out in a masked fashion.

5.5 Characterisation of the pigmented rds mice

There is increasing evidence that caspases play an important role in apoptosis. As apoptosis is the main mode of cell death in retinal degeneration, we were interested in the role of caspases in retinal degeneration. Furthermore, activation of caspase-3 was found in axotomized rat retinal ganglion cells in vivo (Kermer 1999) and in the retina of transgenic rats with the rhodopsin mutation s334ter during photoreceptor degeneration (Liu et al., 1999). There is some evidence to suggest that caspases inhibitors might delay cell loss in axotomized rat retinal ganglion cells (Kermer 1998).

A collaboration was established with the Neuroscience Department of SB Pharm Limited. A number of pigmented rds retina at different ages with normal control was sent to the SB laboratory. An upregulation of caspase-3 was also found in the pigmented rds retina. We decided to carry out a pilot study to assess whether caspases inhibitors can slow retinal degeneration in the pigmented rds mouse.

There were a few reason to choose the rds mouse over the rd mouse. The degeneration of the rds mouse is much slower as compared to the rd mouse. There are plans to carry out functional and behaviour studies on them if the treatment trials appear to be successful. These tests would be easier to do in the rds mouse.

In most laboratory, the rds mouse is albino. In our laboratory, both albino and pigmented rds mouse were available. There is some evidence to support that the retinal degeneration in pigmented rds mouse is slower than that of the albino rds mouse (Sanyal, De Ruiter, Hawkins, 1980). In addition, behaviour studies would be easier to be performed in pigmented animals. We had, therefore., decided to use the pigmented rds mouse for our study.

In order to assess the best time point for injection, we have characterised this model as part of the thesis.

Both the pigmented and albino rds mice were provided by Dr Robin Ali, Institute of Ophthalmology, London having been raised from stock kindly donated by Dr Gabriel Travis, University of Texas, Dallas. The albino rds mice were on the BALB/c background. By crossbreeding the albino rds with the wild type pigmented CBA mice, the pigmented rds mice were generated. Tail biopsies were obtained from all the original pigmented breeders and they were tested for the null mutation in the rds gene by using the appropriate primers (carried out in Dr Ali's laboratory).

Albino rds mice (n=25) and pigmented rds mice (n=40) between postnatal day (P) 14 and P90 were raised in 12 hour light - dark cycle and examined histopathologically. Only one eye per animal was used in this study. The eyes were fixed in a mixture of paraformaldehyde and glutaraldehyde by immersion for 48 hours. The anterior segments were removed. The posterior eyecups were processed and embedded in Araldite resin (See Appendix A for details). Semi-thin (1 micron thick) sections were cut and stained with toluidine blue.

A location near the midpoint between the optic nerve and the ora serrata was identified at low magnification. An eye-piece graticule (Graticules Ltd, Tunbridge, England) was employed to demarcate a 25 μm wide retinal strip of at x 400 magnification oriented perpendicular to the plane of the outer nuclear layer. The number of photoreceptor cell nuclei within this strip was counted. The section was then moved 250 μm toward the optic nerve, and another retinal strip of 25 μm width was counted. This was repeated on the other side of the optic nerve to obtain a total of 4 readings. A total of 100 μm of retinal width was therefore counted. The number of photoreceptor cells counted was then multiplied by 10 and expressed as the

number of cells per 1 mm of retina. All counting were performed by two observers masked to the age of the animals. The average was taken.

5.6 Caspases inhibitors trials in pigmented rds mice

We investigated whether inhibitors of caspases delays photoreceptor cell death in the pigmented rds mouse. Three different caspases inhibitors, namely acetyl-Tyr-Val-Ala-Asp-chloromethylkeone (Ac-YVAD.cmk), acetyl-Asp-Glu-Val-Asp-chloromethylkeone (Ac-DEVD.cmk) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethlketone (z-VAD.fmk) were used.

Forty six pigmented rds mice were randomly allocated to three treatment groups at postnatal day 30. One of the inhibitors of caspases (either Ac-DEVD.cmk, Ac-YVAD.cmk or z-VAD.fmk – all kindly provided by SB Pharm Ltd., London) was injected intravitreally into one eye whilst the vehicle, dimethylsulfoxide (DMSO), was injected into the other eye. The animals were raised in 12 hours light-dark cycle and examined histopathologically at P45 and P60. The eyes were fixed in a mixture of paraformaldehyde and glutaraldehyde by immersion for 48 hours. The anterior segments were removed. The posterior eyecups were processed and embedded in Araldite resin (See Appendix A for details). Semi-thin (1 micron) sections were cut and stained with toluidine blue for examination.

The method of photoreceptor cell counting is identical to that used in the characterisation of the pigmented rds mouse (See 5.5 for details). Paired student T-tests were used to evaluate the statistically significance of the results.

C. RESULTS

6.1 Characterisation of the Rdy cats

Apoptosis demonstration and photoreceptor cell loss in Rdy cats

A small number of TUNEL-labelled cells (Figure 6.1a) and pyknotic cells (Figure 6.1b) was found in the outer nuclear layer of both normal and affected animals before 5 weeks of age and no significant difference was observed at these time points. In the affected animals, the number of TUNEL-labelled cells and pyknotic cells increased and peaked around 9 - 10 weeks of age but none were seen in the controls after 5 weeks. The results of pyknotic cell counts and TUNEL positive cell counts of the outer nuclear layer (photoreceptors) are summarised in Figures 6.1c and 6.1d respectively. Inter-observer variability was less than 10% for both the pyknotic cell and TUNEL counting. There were no significant differences between the normal and affected animals in the inner nuclear layer and ganglion cell layer of the retina. Photoreceptor cell counts showed significant cell loss between 5 and 17 weeks (Figure 6.1e).

Between 5 and 17 weeks, about 700 photoreceptor cells were lost per mm of retina. This is equivalent to an average cell loss of 8.3 cells per day per mm. Over the same period of time, the average TUNEL positive cell count was about 2.5 cells per mm. Hence, the estimated time for the cells to be TUNEL positive is about 8 hours. Using a similar calculation, the estimated time for the cells to appear pyknotic is about 12 hours,

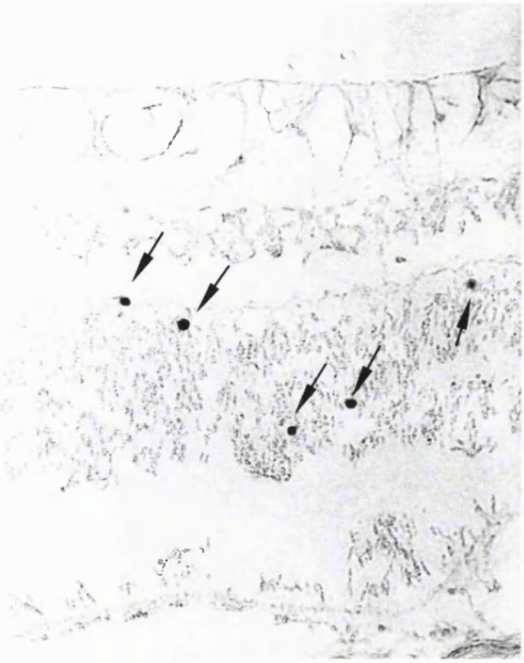

Figure 6.1a: "TUNEL"-positive cells (arrows) in 9 weeks old Rdy cats (x200)

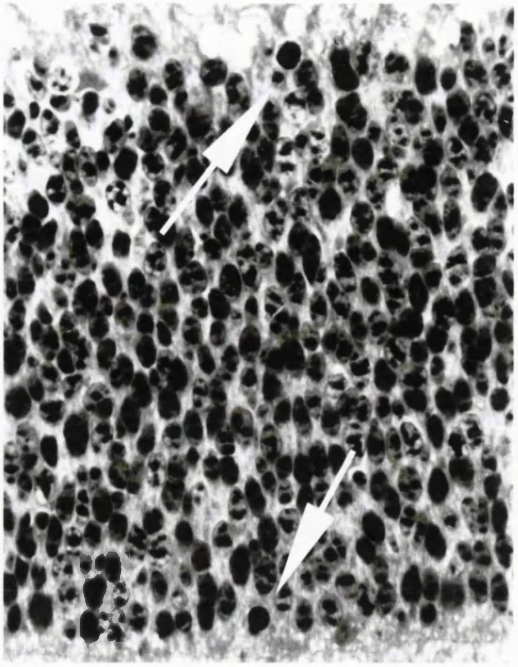

Figure 6.1b: Pyknotic cells (white arrows) in 9 weeks old Rdy cats (x400)

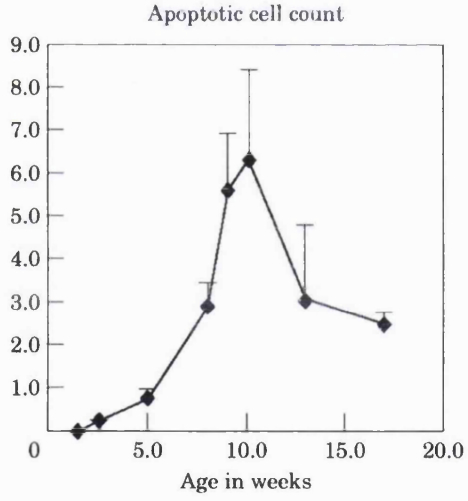

Figure 6.1c: Apoptotic cells per 1 mm of retina (mean + SD) in the Rdy cats

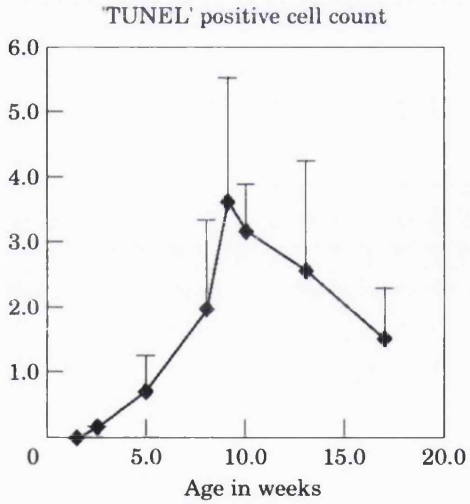

Figure 6.1d: TUNEL positive cell count (Mean + SD) per 1 mm of retina in the Rdy cats

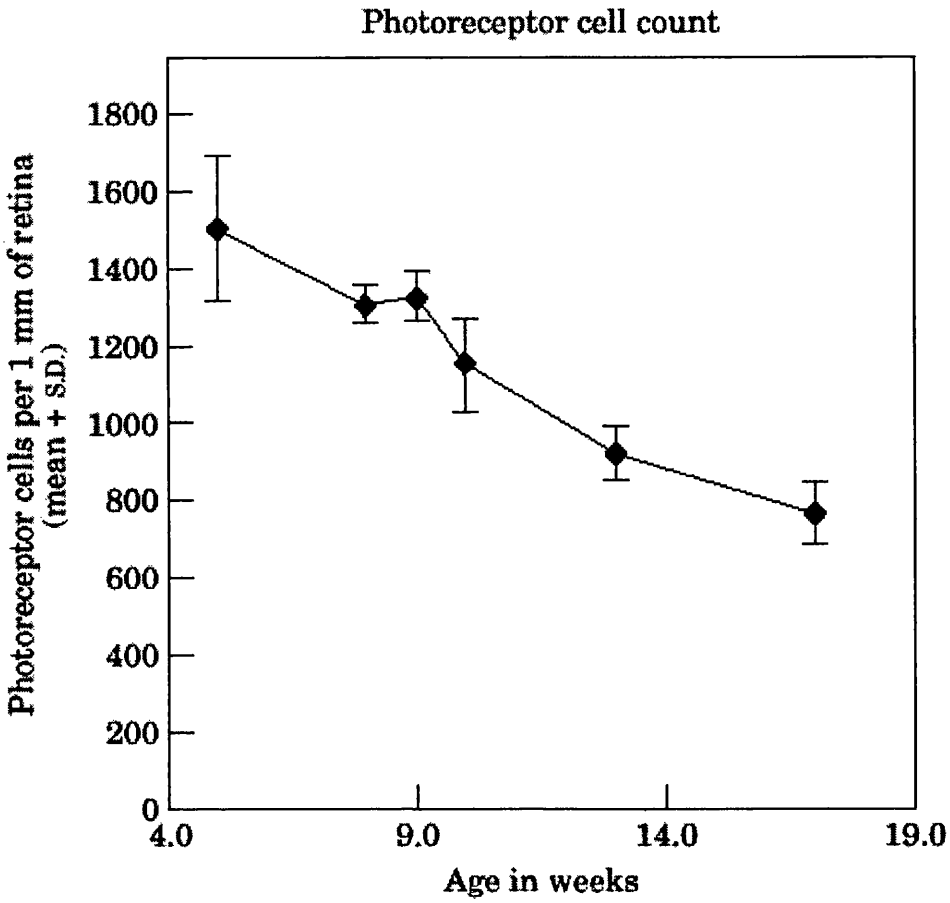

Figure 6.1e: Photoreceptor cell count (Mean +/- SD) per 1 mm of retina in the Rdy cats

Immunohistochemistry of the Rdy cats

Immunohistochemical staining of opsin was almost entirely restricted to the rod outer segments (ROS) in the normal animals (Figure 6.1f). In the Rdy cats, opsin was present in both rod outer and inner segments and there was also accumulation of opsin in the outer nuclear layer and within the presumed photoreceptor cell synaptic terminals in the outer plexiform layer (Figure 6.1g). Some of the rods had sprouted opsin-positive neurites extending towards the ganglion cell layer (Figure 6.1h) and some had branches (Figure 6.1i).

Synaptophysin is a synaptic vesicle protein. In the controls, immunolabeling with anti-synaptophysin was largely restricted to the outer and inner plexiform layers (Figure 6.1j). In the Rdy cats, the immunolabeling of the outer plexiform layer diminished with age and in parallel with the reduction in the number of photoreceptor cells. No significant change in labeling of the inner plexiform layer with anti-synaptophysin was seen (Figure 6.1k).

GFAP was expressed predominantly by the astrocytes in the normal controls (Figure 6.1l). In the Rdy cats, markedly increased immunostaining of GFAP in Muller cells was observed at all time points (Figure 6.1m). Retinal pigment epithelial cell changes were not seen.

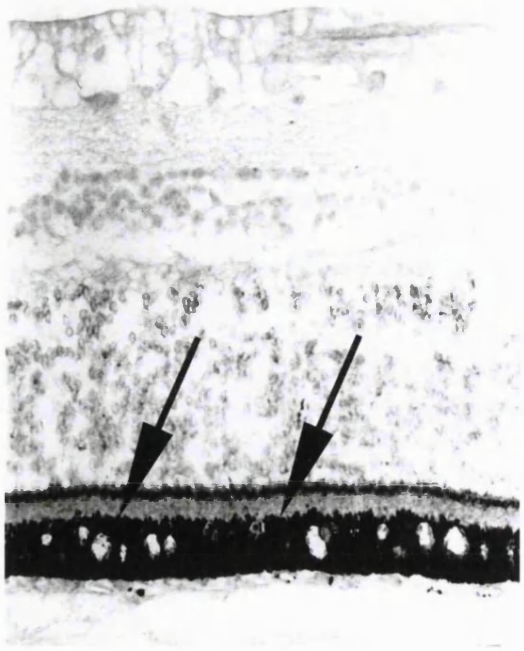

Figure 6.1f: Opsin immunolabeling in normal cats at 13.5 weeks showing localisation in the outer segments (arrow) (x400)

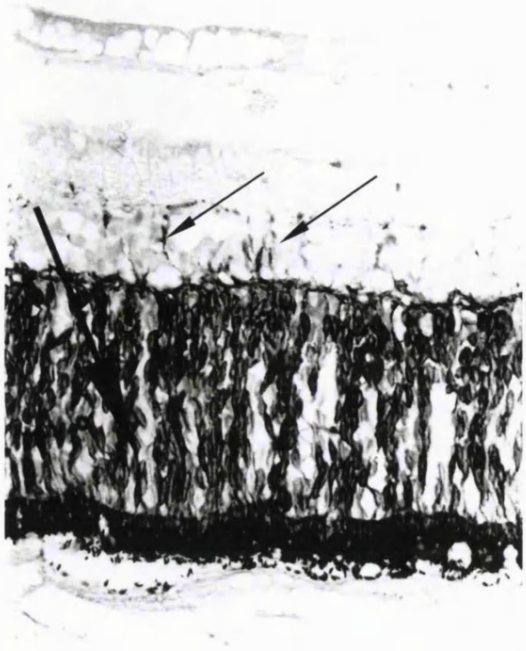

Figure 6.1g: Opsin immunolabeling in the Rdy cats at 13.5 weeks showing widespread distribution of opsin in the outer nuclear layer (large arrow) and neurites (small arrows)(x400)

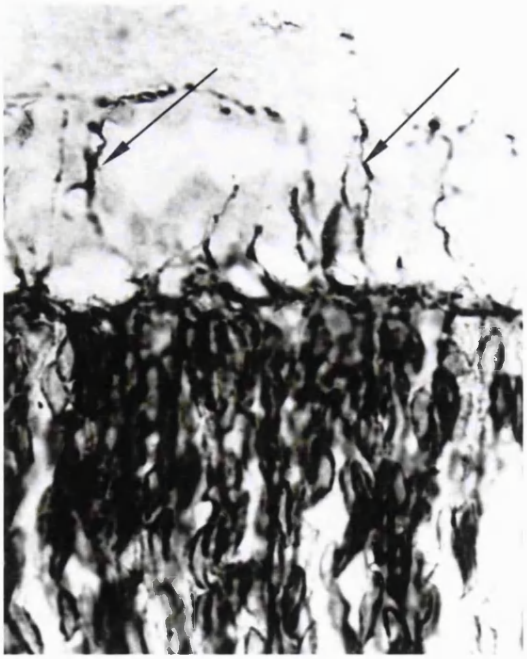

Figure 6.1h: Opsin immunolabeling in the Rdy cats at 13.5 weeks showing neurites (arrows) (x1000)

Figure 6.1i: Opsin immunolabeling in the Rdy cats at 13.5 weeks showing neurites with branching pattern (arrow) (x1000)

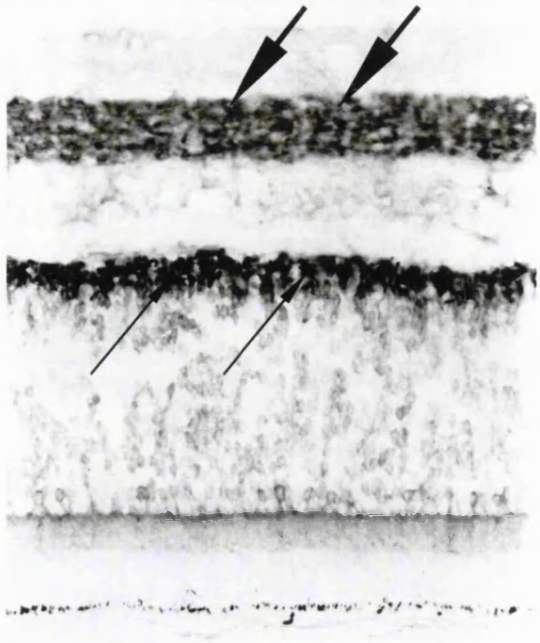

Figure 6.1j: Synaptophysin immunolabeling in normal cats at 13.5 weeks showing normal labelling in inner (large arrows) and outer plexiform layer (small arrows) (x400)

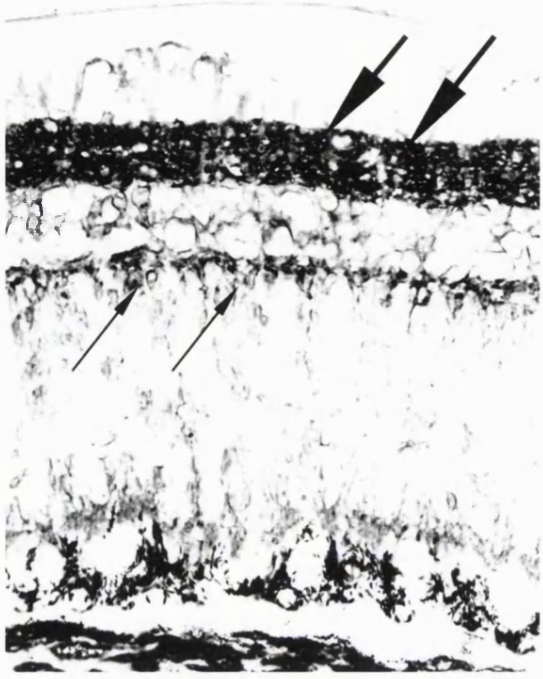

Figure 6.1k: Synaptophysin immunolabeling in the Rdy cats at 13.5 weeks showing normal labelling in inner (large arrows) but reduced labelling in outer plexiform layer (small arrows) (x400)

Figure 6.1l: Glial fibrillary acidic protein immunolabeling in normal cats at 13.5 weeks showing predominantly expressed by astrocytes (arrows) (x400)

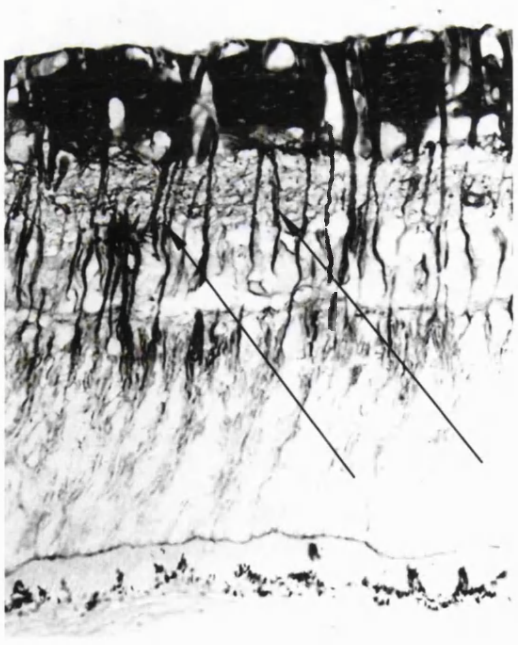

Figure 6.1m: Glial fibrillary acidic protein immunolabeling in Rdy cats at 13.5 weeks showing increase expression by Muller cells (arrows) (x400)

Electron microscopy in the Rdy cats

The inner segment and the connecting cilia were readily identifiable at 5 and 13 weeks of age. The outer segment was present but very disorganised at 5 weeks of age, and by 13 weeks of age, no organised outer segment discs could be found. It was difficult to distinguish rod and cone photoreceptors as both were severely affected.

Figure 6.1n: Electron microscopy of rod outer segments of normal cats at 9 weeks of age. Black bar = 500nm

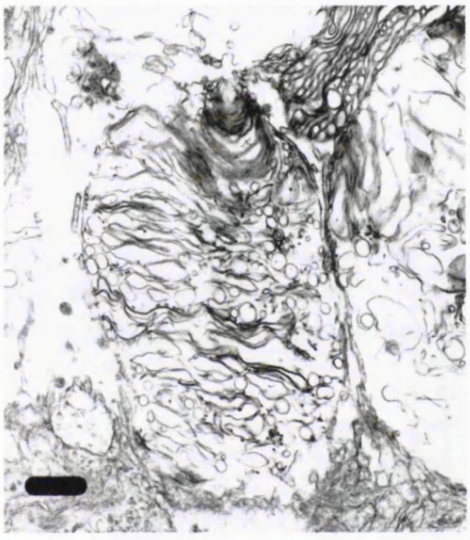

Figure 6.1o: Electron microscopy of rod outer segments of Rdy cats at 9 weeks of age. Black bar = 500nm

Electroretinogram (ERG) in the Rdy cats

In unaffected animals, a significant b-wave is present at the age of 5 weeks. However, a full-field ERG in the affected animals could not be distinguished from noise at all time points between postnatal day 10 to 9 weeks of age.

6.2 Neurotrophic factor trials in the Rdy cats

During the study, the animals tolerated the procedure well and appeared to be pain-free after in the intravitreal injections. The topical prednisolone was given prophylactically, no active inflammation was observed clinically even in the early post-operative period.

Axokine vs Untreated in the Rdy cats

In the first experiment, we assessed whether or not Axokine has any protective effect. Table 6.2a and Figure 6.2a summarize the results of photoreceptor cell counts, apoptotic positive cell counts and "TUNEL" cell counts in the initial experiment. Photoreceptor cell counts were significantly higher in the Axokine treated group at all time-points ($p=0.02$ at 5.5 weeks, $p<0.0001$ at 9.5 weeks and $p=0.003$ at 13.5 weeks). Conversely, apoptotic cell counts ($p=0.01$) and "TUNEL" cell counts ($p=0.04$) were significantly reduced at 9.5 weeks and the "TUNEL" count was significantly lower at 13.5 weeks ($p=0.04$). The first experiment (Axokine vs Untreated) showed significant photoreceptor rescue in Axokine treated eyes.

Age in weeks	5.5 (n=3)	9.5 (n=4)	13.5 (n=3)
APO Count			
Axokine	0.60 ± 0.16	1.08 ± 1.02*	2.16 ± 0.58
Untreated	0.70 ± 0.14	5.92 ± 2.98	3.30 ± 2.74
TUNEL Count			
Axokine	0.16 ± 0.24	0.52 ± 0.28*	0.74 ± 0.38*
Untreated	0.64 ± 0.60	2.16 ± 1.04	1.72 ± 0.64

Table 6.2a: Apoptotic cell count and "TUNEL" positive cell count. First experiment (Axokine vs Untreated) APO = Apoptotic cell count (Mean ± SD); TUNEL = "TUNEL" positive cell count (Mean ± SD). All parameters are expressed as per 1 mm of retina. * p<0.05 for treated vs untreated

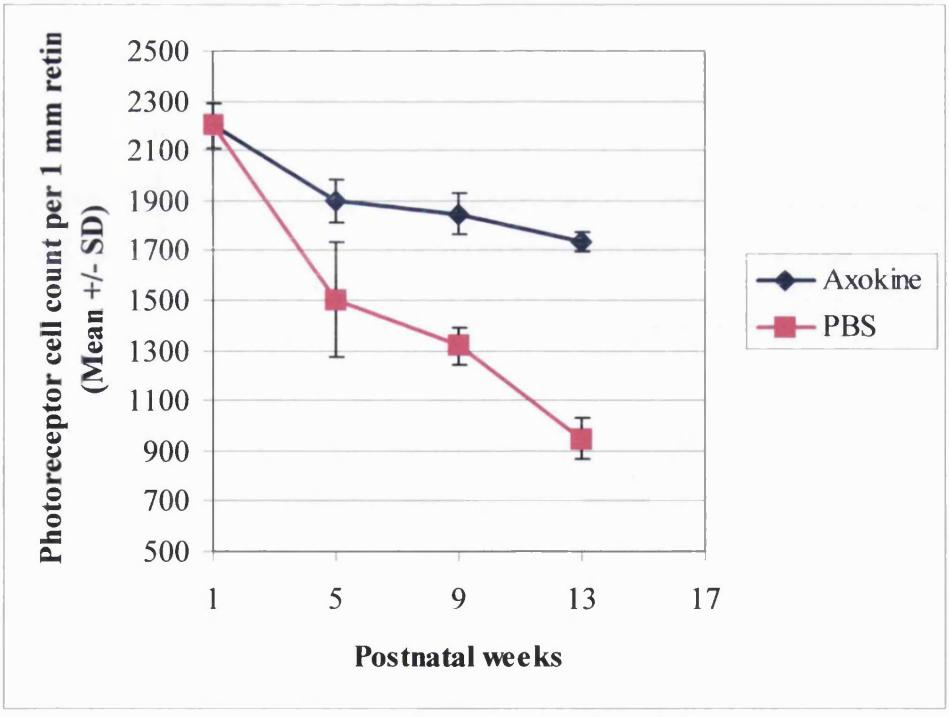

Figure 6.2a: Photoreceptor cell count (Mean +/- SD)

Axokine vs BDNF vs Sham vs Untreated in the Rdy cats

After the initial success with Axokine, in the second experiment, we delayed the beginning of therapy to 5.5 weeks and compared the effects of Axokine (at half the dosage of the initial experiment), human BDNF or vehicle of Axokine. Table 6.2b and Figure 6.2b summarize the results of photoreceptor cell counts, apoptotic cell counts and "TUNEL" positive cell counts in this experiment. The photoreceptor cell counts were statistically significantly higher in Axokine treated group at 13.5 ($p=0.03$) and 17.5 weeks ($p=0.02$). Although the indices of apoptosis were not statistically significant different from the untreated eyes at individual time-points, the entire Axokine treated group had significantly fewer apoptotic cells ($p=0.03$) and "TUNEL" positive cells ($p=0.006$) than the untreated controls. Neither BDNF nor vehicle treatment groups, were statistically significantly different from the untreated eyes in any of the parameters measured.

The second experiment (Axokine vs BDNF vs Vehicle vs Untreated) showed significant photoreceptor rescue in Axokine treated eyes but no effect on BDNF and vehicle treated eyes.

Age in weeks	9.5	13.5	17.5
APO count			
Axokine *	2.76 (n=1)	1.62 ± 0.18 (n=2)	2.32 ± 0.98 (n=3)
BDNF	5.26 (n=1)	1.66 ± 1.26 (n=2)	2.24 ± 0.64 (n=2)
Vehicle	5.5 ± 1.54 (n=2)	2.72± 0.30 (n=2)	3.54 ± 1.16 (n=2)
Untreated	5.3 ± 1.08 (n=4)	2.86 ± 1.16 (n=6)	3.16 ± 0.90 (n=7)
TUNEL count			
Axokine *	3.08 (n=1)	0.48 ± 0.02 (n=2)	0.80 ± 0.54 (n=3)
BDNF	5.10 (n=1)	1.86 ± 0.44 (n=2)	1.70 ± 0.54 (n=2)
Vehicle	6.60 ± 0.84 (n=2)	3.74 ± 2.08 (n=2)	2.16 ± 0.18 (n=2)
Untreated	6.34 ± 0.64 (n=4)	2.52 ± 1.54 (n=6)	1.78 ± 0.60 (n=7)

Table 6.2b: Apoptotic cell count and "TUNEL" positive cell count
The second experiment (Axokine vs BDNF vs Vehicle vs Untreated). APO = Apoptotic cell count (Mean ± SD); TUNEL = "TUNEL" positive cell count (Mean ± SD). All parameters are expressed as per 1 mm of retina. * $p<0.05$ for treated vs untreated.

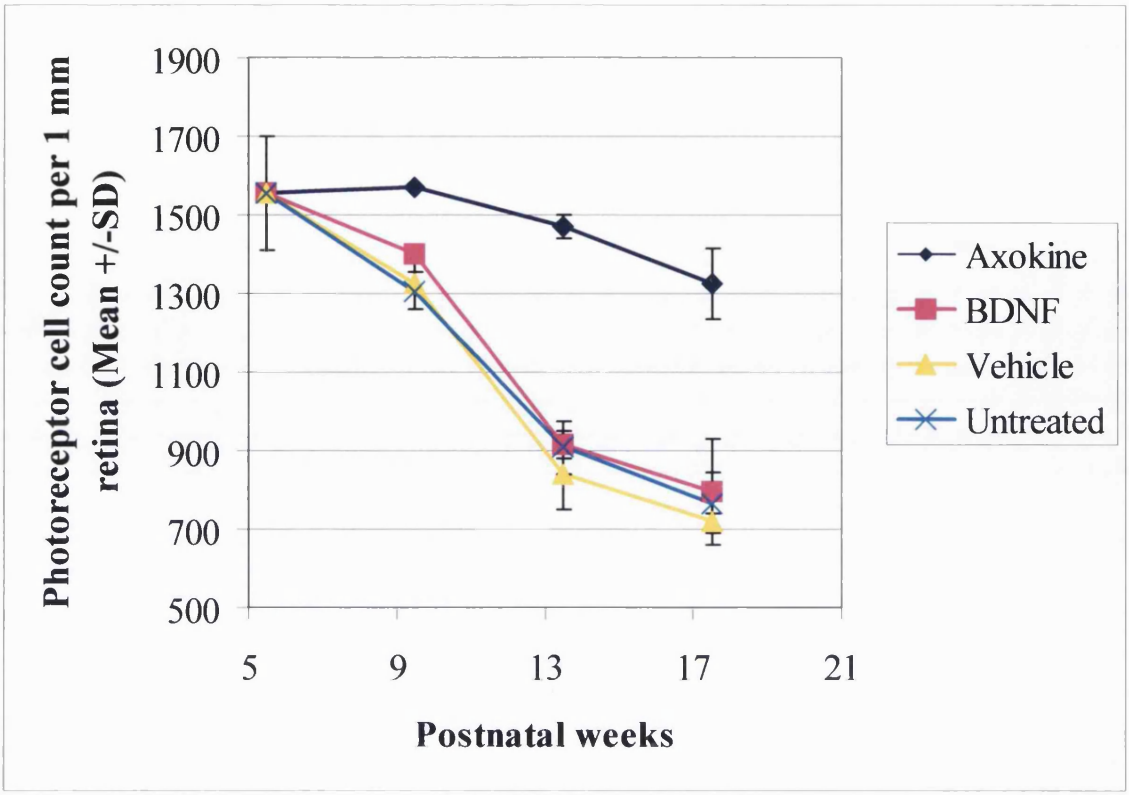

Figure 6.2b: Photoreceptor cell count (Mean +/- SD)

Other clinical and histopathological findings in the Rdy cats

Minimal posterior subcapsular cataracts were observed clinically and histopathologically in all Axokine treated eyes, both dystrophic and normal cats. However, it was not seen in the treated MLHD dogs. The lens opacities were less than 5% of the surface area of the lens in all Axokine treated animals. They affected the posterior subcapsular area only and there was no extension into the nucleus of the lens. None of the cataract obscured the fundal examination. On light microscopy, there was more cells present posterior to the equator of the lens with the associated opacities (Figure 6.2c). Although quantification was not performed, treated animals

in the second experiment (delayed and lower dose) appeared to have less lens opacities.

In all Axokine treated eyes of both dystrophic and normal cats, there were small retinal folds seen in histopathology (Figure 6.2d). These folds were not seen clinically by ophthalmoscopy. No vitritis or any inflammatory response was observed either clinically or histopathologically. In Axokine treated eyes, the mean number of retinal folds per mm of retina in the first and second experiments were 1.802 +/- 0.419 and 0.588 +/- 0.366 respectively. The differences were statistically significant (p=0.0006). Neither cataract nor retinal folds were seen in any of the BDNF injected, vehicle injected or untreated eyes.

Although formal quantification of the ophthalmoscopic findings was not carried out, the overall difference between treated and untreated eyes appeared to be small.

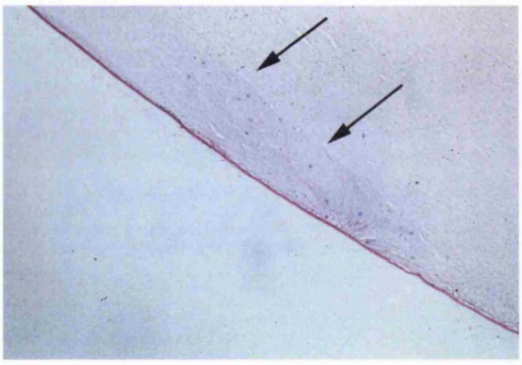

Figure 6.2c: PAS staining of the posterior lens of an Axokine treated eye in the 9.5 weeks old Rdy cats showing cataract formation (x100)

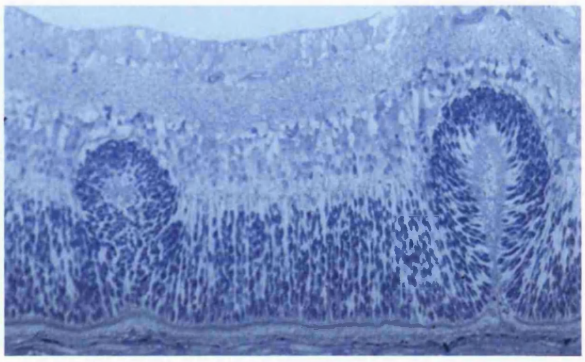

Figure 6.2d: Retina of an Axokine treated eye in the 9.5 weeks old Rdy cats showing retinal folds (x200)

GFAP immunohistochemistry in the Rdy cats

In the untreated normal animals, astrocytes were immuno-positive with GFAP (Figure 6.2e). In the Axokine treated normal animals, there was a marked increase of GFAP immunolabeling in the Muller cells (Figure 6.2f). In the untreated Rdy cats, an increase of GFAP immunolabeling of Muller cells was found (Figure 6.2g), but, there was a suggestion of a further increase of GFAP immunolabeling of Muller cells in the Axokine treated Rdy cats (Figure 6.2h).

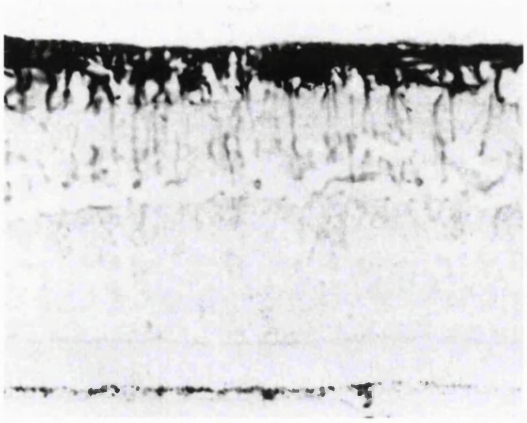

Figure 6.2e: GFAP immunohistochemistry in normal cat at 13.5 weeks of age showing immunolabeling in astrocytes (x200)

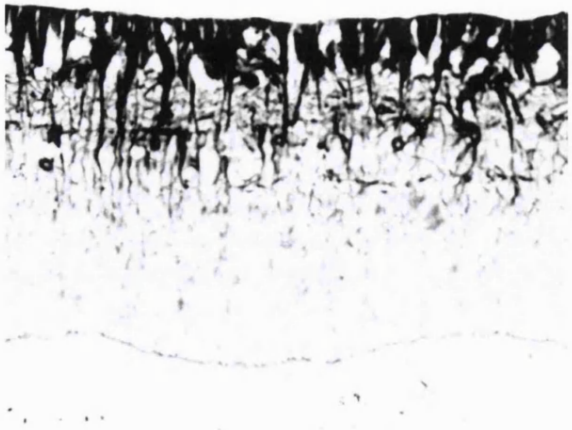

Figure 6.2f: GFAP immunohistochemistry in normal cat at 13.5 weeks of age which was injected with Axokine at 5.5 and 9.5 weeks showing increased immunolabeling in Muller cells (x200)

Figure 6.2g: GFAP immunohistochemistry in Rdy cat at 13.5 weeks of age showing increased immunolabeling in Muller cells (x200)

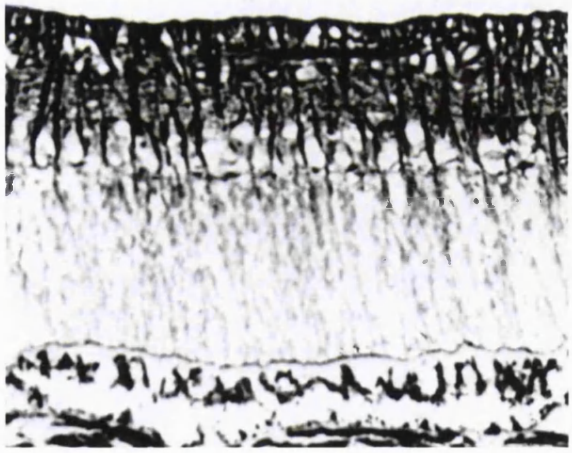

Figure 6.2h: GFAP immunohistochemistry in Rdy cat at 13.5 weeks which was injected with Axokine at 5.5 and 9.5 weeks showing very marked increase of immunolabeling in Muller cells (x200)

6.3 Characterisation of the MLHD dogs

Light microscopy and immunohistochemistry in the MLHD dogs

We found apoptotic cells (as pyknotic nuclei and "TUNEL" positive cells) in the outer nuclear layer in the affected MLHD dogs as early as 6 weeks of age in both cones and rods (Figure 6.3a). However, the thickness of the outer nuclear layer was not significantly difference from that of the control. Furthermore, the nuclear morphology of other rod and cones appeared normal. The ratio of rods with pyknotic nuclei to normal nuclei at 6 weeks of age was 1:420 (+/- 21) whilst the ratio for cones was 1:357 (+/- 34). There were no significantly difference between the ratio of pyknotic nuclei between the two photoreceptor cells population. Counting of normal nuclei in "TUNEL" sections was not possible, but the number of "TUNEL" positive cell per slides are about half of that of pyknotic nuclei, this finding is similar to our findings in the Rdy cats.

The thickness of the outer nuclear layer was substantially reduced by 24 weeks of age (Figure 6.3b). At this point, the morphology of the photoreceptors were grossly abnormal, it is not possible to clearly distinguish rods and cones. It is also not possible to distinguish normal and pyknotic nuclei.

Immunohistochemical staining of opsin was almost entirely restricted to the rod outer segments (ROS) in the normal control (Figure 6.3c). In the affected dogs, opsin was present in both rod outer and inner segments. There was also presented in the cell perikarya within in the outer nuclear layer which could be seen at 6 weeks of age, and became more marked at 24 weeks of age (Figure 6.3d). Synaptophysin, a synaptic vesicle protein, was largely restricted to the outer and inner plexiform layers in the normal control. In the affected dogs, the immunolabeling of the outer

plexiform layer diminished with age and in parallel with the reduction of photoreceptor cells. No significant change was seen in the inner plexiform layer. Glial fibrillary acidic protein (GFAP) was expressed predominantly by the astrocytes in the normal controls. In the affected dogs, markedly increased immunostaining of GFAP in Muller cells was observed.

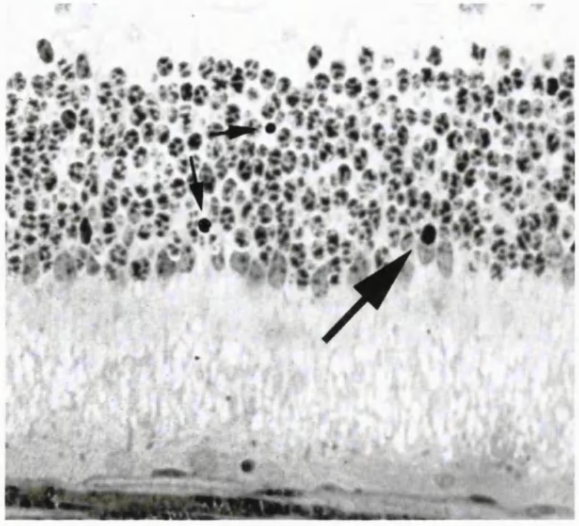

Figure 6.3a: Light microscopy of an affected six week old MLHD dog retina showing pyknotic nuclei of cone (large arrow) and rod (small arrows) photoreceptors (x400)

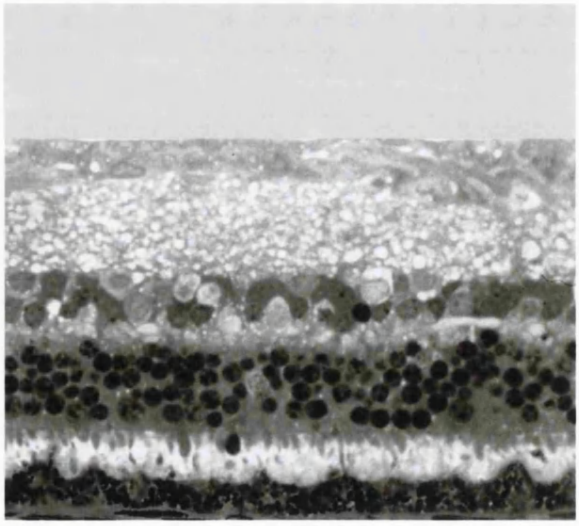

Figure 6.3b: Light microscopy of an affected 24 week old MLHD dog retina showing significantly reduced in the thickness of the outer nuclear layer (x400)

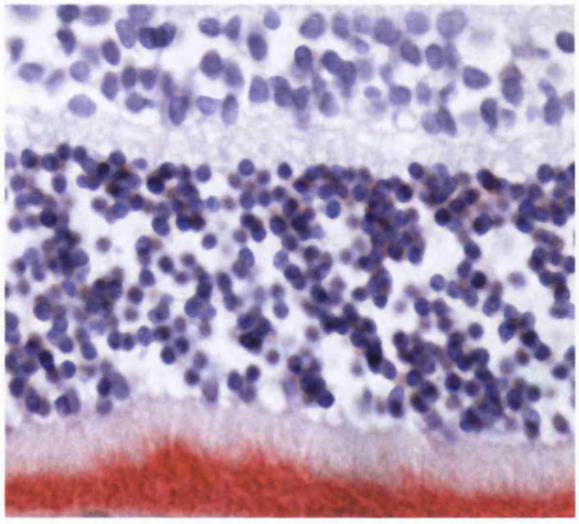

Figure 6.3c: Opsin immunohistochemistry of normal 8 weeks old dog retina showing opsin (red) is localised to the outer segments (x400)

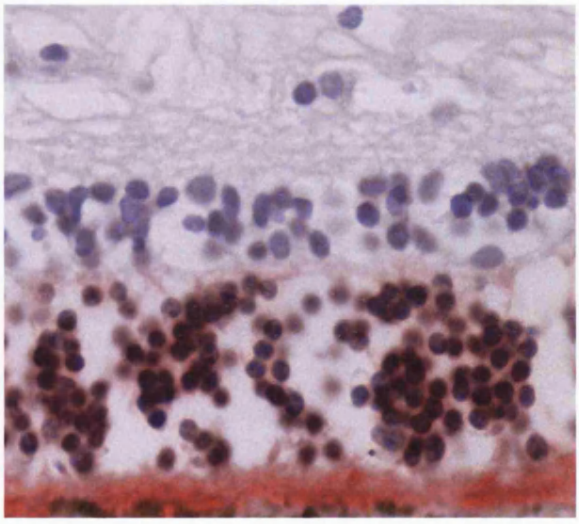

Figure 6.3d: Opsin immunohistochemistry of an affected 24 weeks old MLHD dog retina showing opsin (red) is present to the outer segments but also present in the cell perikarya within the outer nuclear layer (x400)

Electron microscopy in the MLHD dogs

The photoreceptor outer segments (OS) were slightly disorganised and shorter but otherwise appeared normal by electron microscopy (EM) at 6 weeks of age (Figure 6.3eand 6.3f) as compared with control (Figure 6.3g). The OS became totally disorganised by 24 weeks of age (Figure 6.3h). There were apoptotic cells seen (Figure 6.3i) but the cone pedicle appears normal (Figure 6.3j).

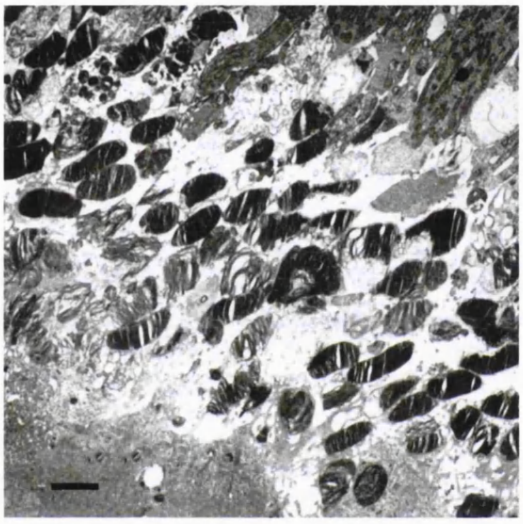

Figure 6.3e: Photoreceptor outer segments in affected dog at 6 weeks of age appears to be slightly shorter and more disorganised (Bar = 2 microns)

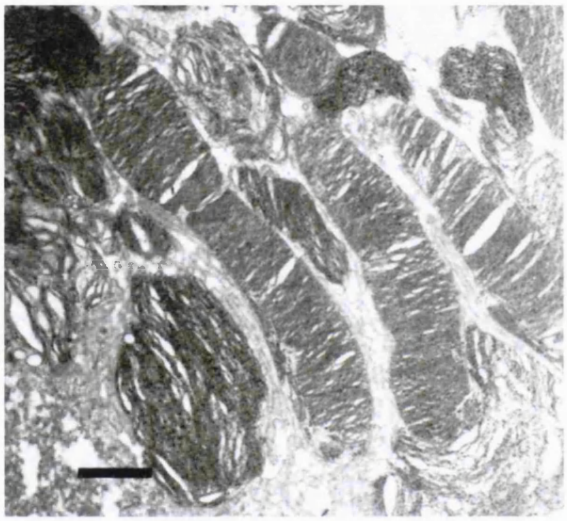

Figure 6.3f: Photoreceptor outer segments in affected dog at 6 weeks of age showing normal looking discs at higher magnification (Bar = 1 microns)

Figure 6.3g: Photoreceptor outer segments in normal dog at 8 weeks of age showing normal outer segments (Bar = 2 microns)

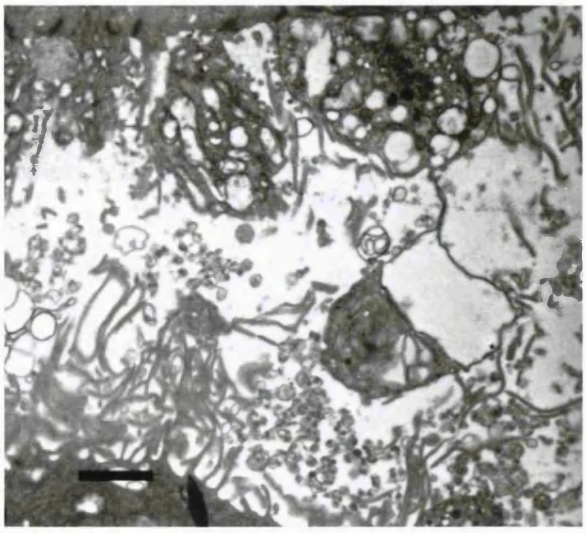

Figure 6.3h: Photoreceptor outer segments in affected dog at 24 weeks of age showing totally disorganised outer segments (Bar = 1 microns)

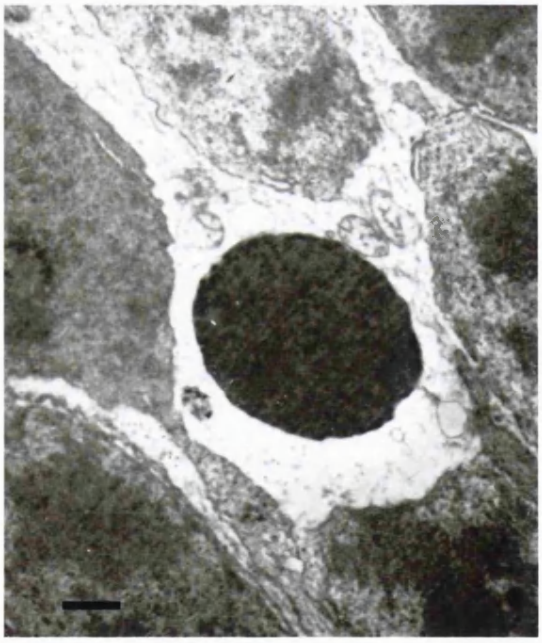

Figure 6.3i: Apoptotic rod photoreceptor in the affected dog at 24 weeks of age (Bar = 1 micron)

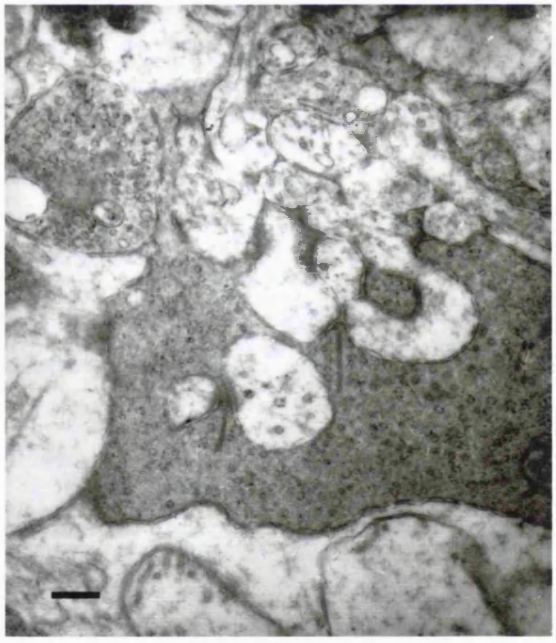

Figure 6.3j: Normal cone pedicle in the affected dog at 24 weeks of age (Bar = 200 nm)

Electroretinogram (ERG) in the MLHD dogs

The ISCEV standard tracing at 10, 14, 17, 20 and 25 were shown in Figure 6.3k - 6.3o). The maximal bright white flash ERG was not significantly different from heterozygote at 10 weeks of age. However, cone specific 30Hz flicker ERG was found to be substantially reduced in the affected dogs as early as 6 weeks of age (data not shown) and became indistinguishable from noise at 14 weeks of age. Furthermore, the maximal bright white flash ERG also diminished progressively and by 25 weeks of age, it was severely reduced. These ERG findings demonstrate the MLHD has an early loss of cone specific ERG, which is followed by a progressive involvement of the rods. This finding is consistent with the definition of cone-rod dystrophy (CORD).

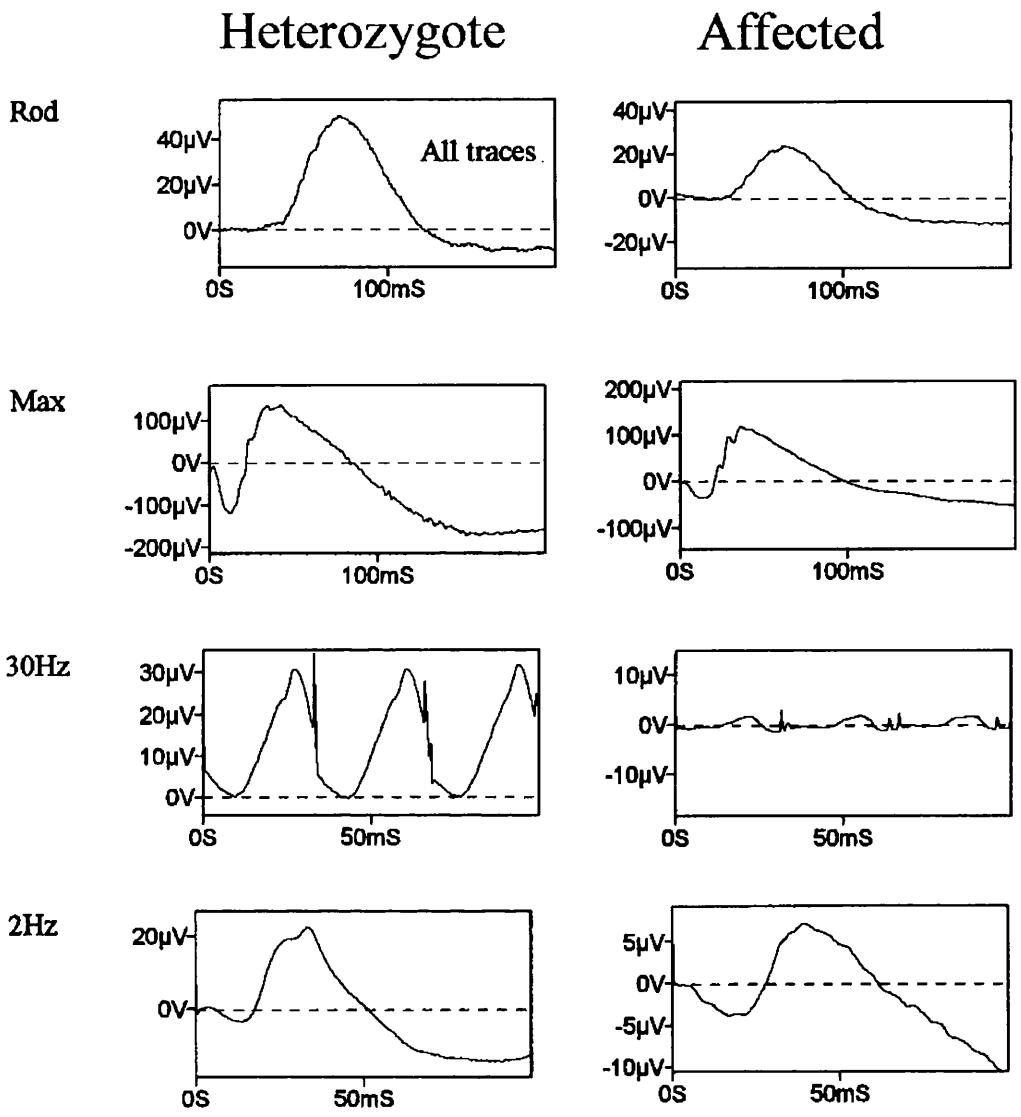

Figure 6.3k: Electroretinogram of heterozygotes and affected MLHD dogs at 10 weeks of age

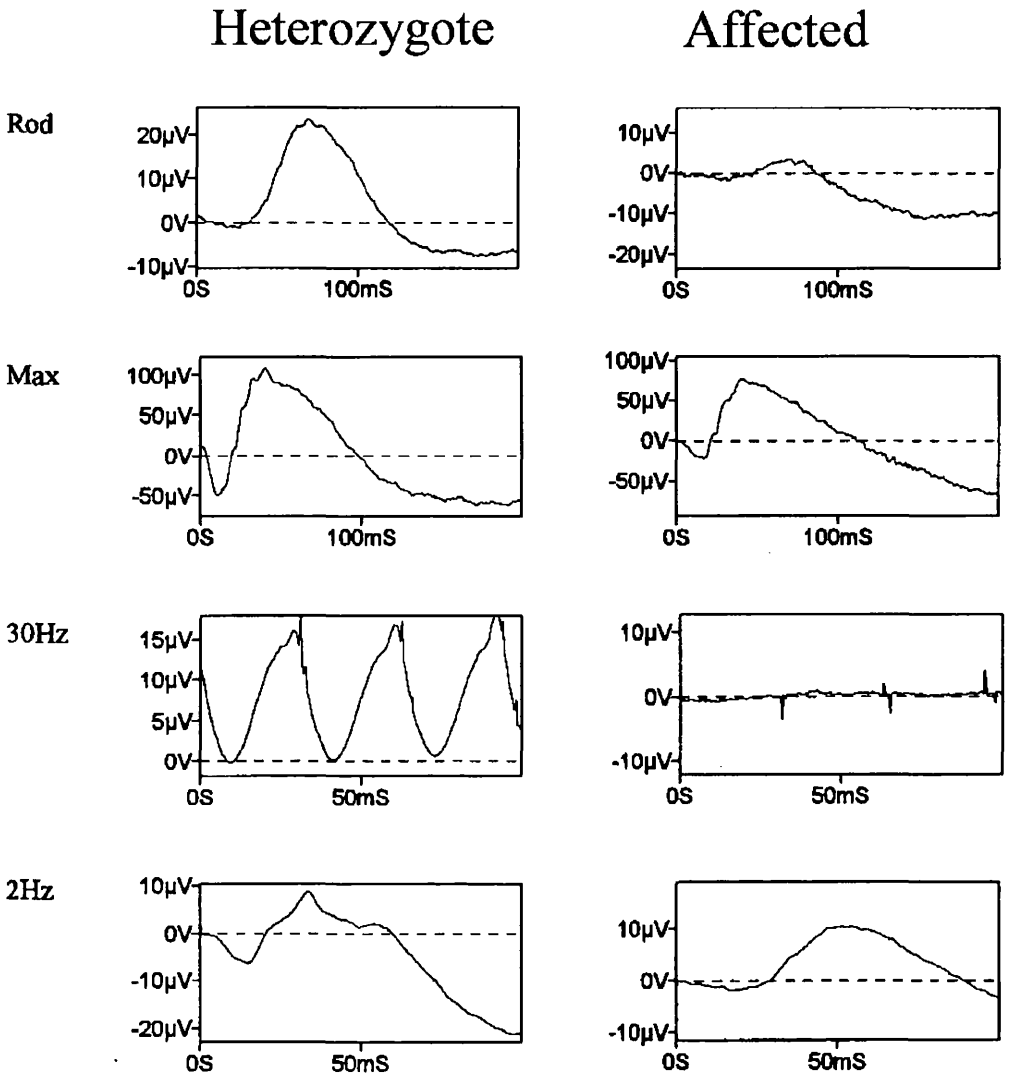

Figure 6.3l: Electroretinogram of heterozygotes and affected MLHD dogs at 14 weeks of age

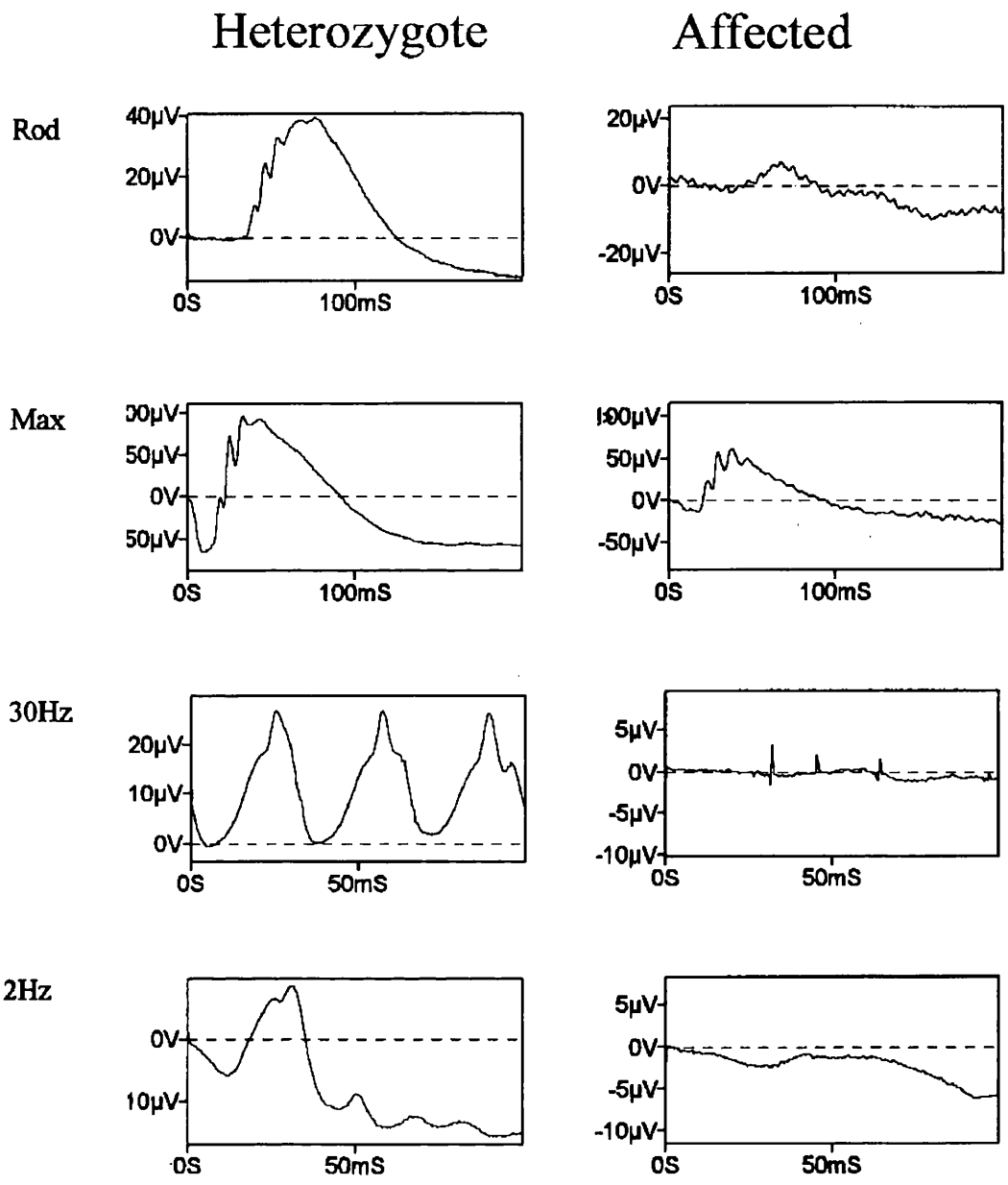

Figure 6.3m: Electroretinogram of heterozygotes and affected MLHD dogs at 17 weeks of age

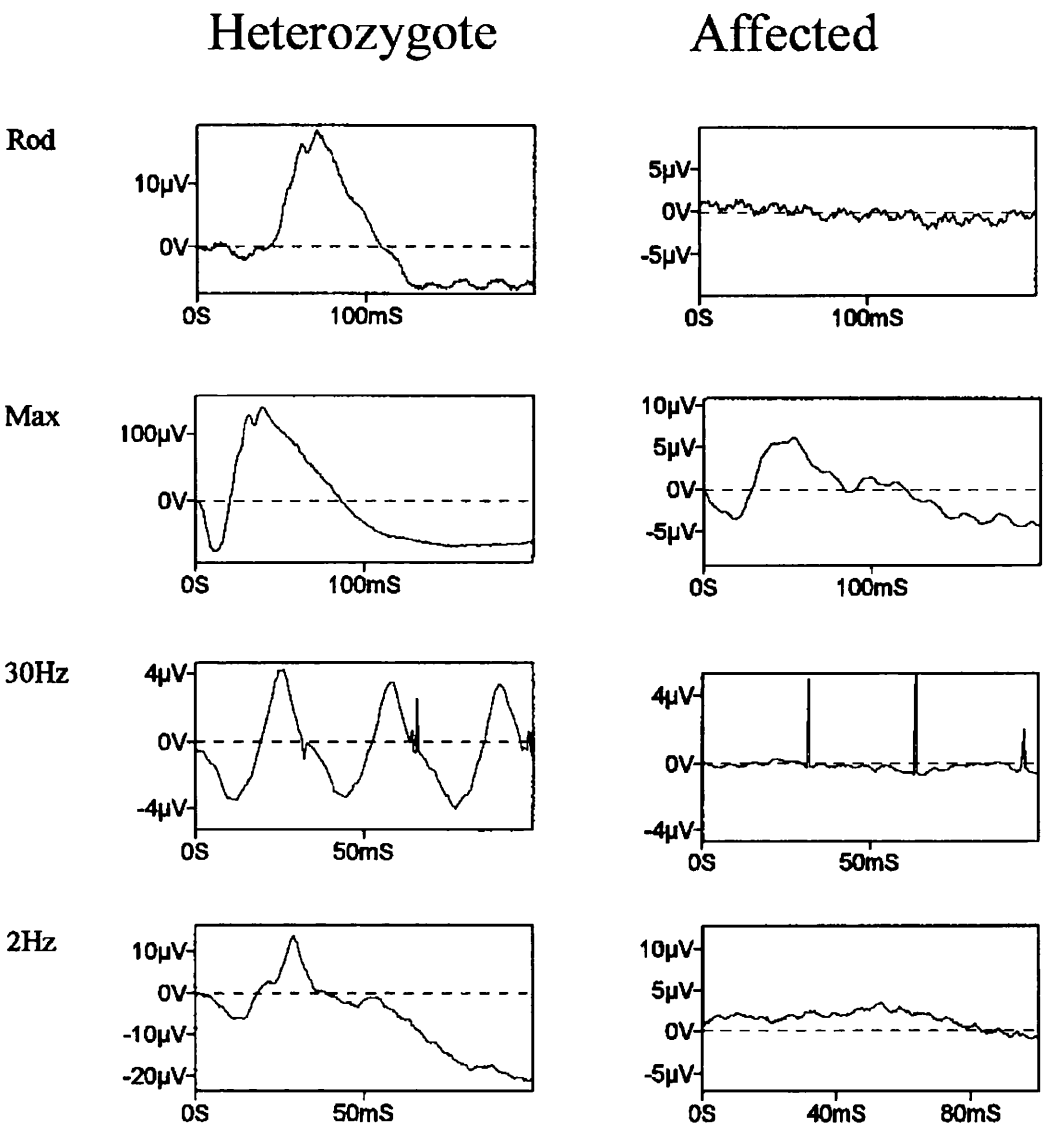

Figure 6.3n: Electroretinogram of heterozygotes and affected MLHD dogs at 20 weeks of age

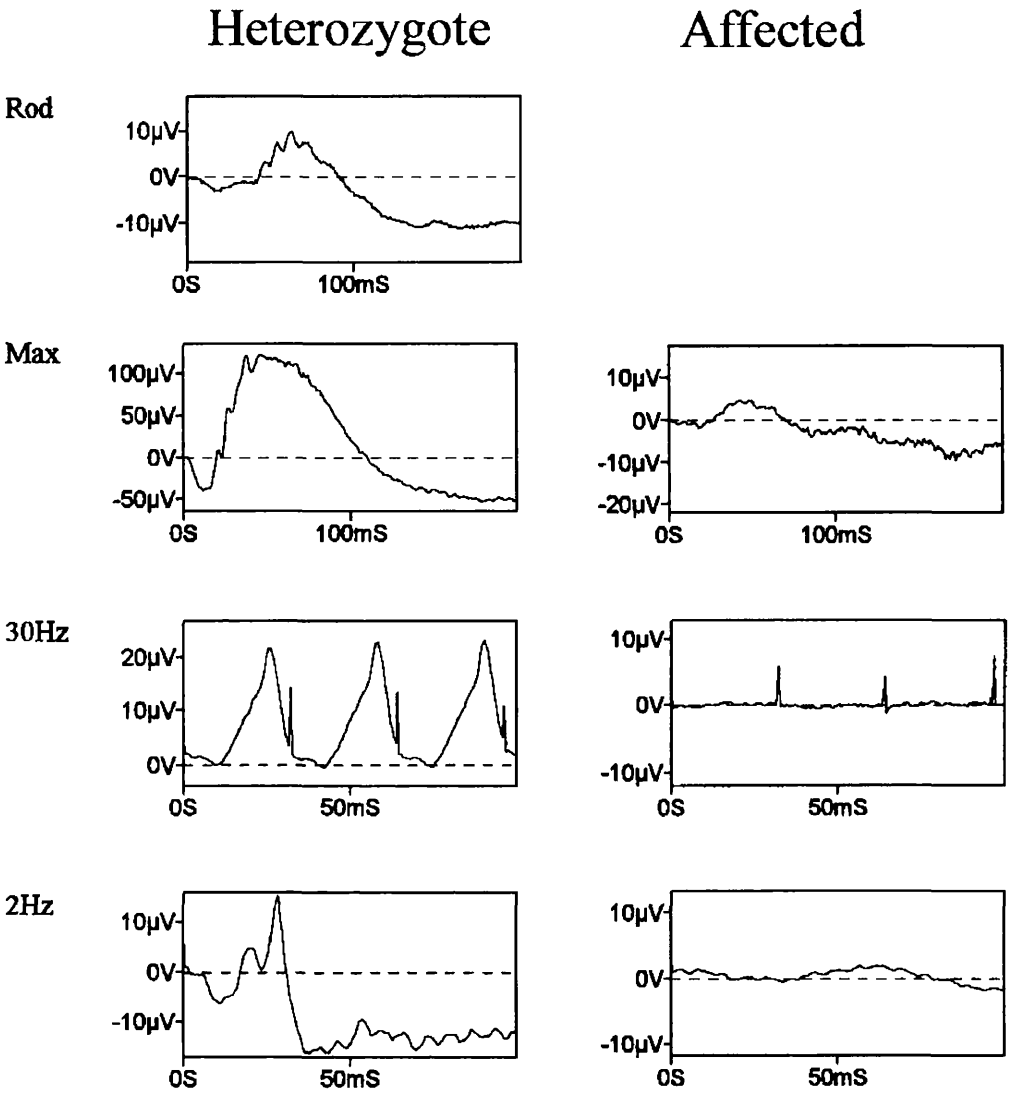

Figure 6.3o: Electroretinogram of heterozygotes and affected MLHD dogs at 25 weeks of age

6.4 Neurotrophic factor trial in the MLHD dogs

Electroretinography in the MLHD dogs

The amplitude of the b-wave at maximal response in the Axokine treated and sham injected eye in one of the three animals is shown in Figure 6.4a. The combined data of Axokine treated eye expressed as the percentage to that of the sham injected eye is shown in Figure 6.4b. The graphs showed an initial reduction of amplitude two week after the first Axokine injection ($p=0.007$). This recovered and showed a larger b-wave than the sham injected eye at 4 weeks after the injection ($p=0.078$). Further injections were given at this point, postnatal week 14. The amplitude was reduced again, two weeks after the second Axokine injection ($p=0.017$). The amplitude of the b-wave recovered at postnatal week 18 (4 week after the second injection), but it was not significantly different from control ($p=0.23$).

Furthermore, after another 4 weeks, at postnatal week 22, the amplitude of the b-wave was still not significantly different from control ($p=0.19$), and in fact, it appeared to be smaller. However, at this point, the amplitudes of the b-wave in either Axokine treated or sham injected eyes were less than 10 microvolts (the normal value would be around 300), this difference is of no clinical or statistical values.

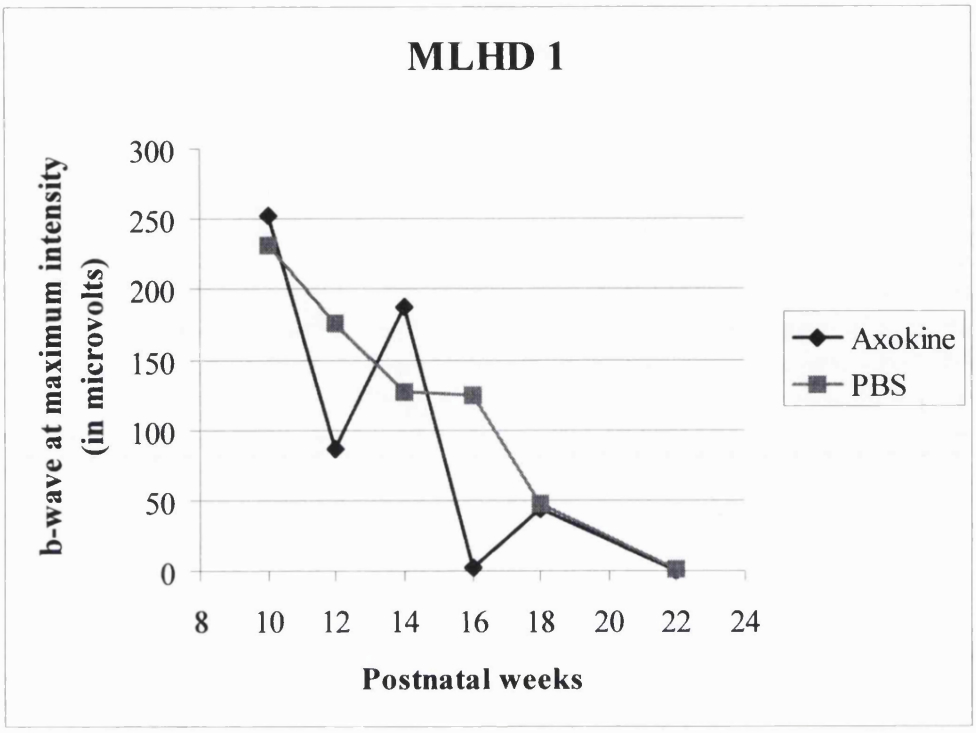

Figure 6.4a: Amplitude of b-wave at maximal intensity of Axokine and sham injected eye in MLHD dog

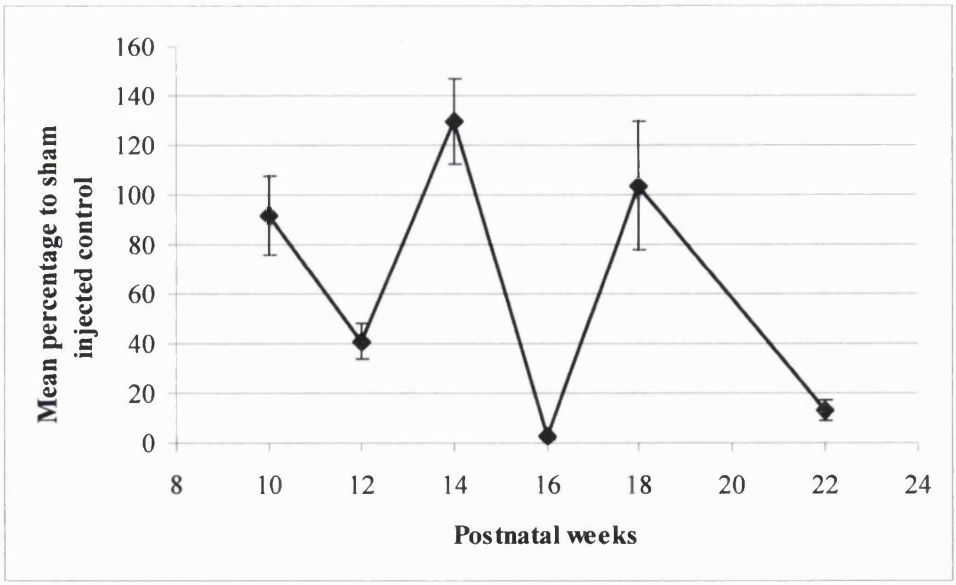

Figure 6.4b: Amplitude of b-wave at maximal intensity of Axokine treated eye expressed as a percentage to the sham injected eye (mean +/- SD) in MLHD dogs. Injections were given at postnatal week 10 and 14

Histopathology in the MLHD dogs

There is no significant difference in photoreceptor cell count between the Axokine treated and sham treated eyes at postnatal week 22.

6.5 Characterisation of the pigmented rds mice

The result of photoreceptor cell counts was plotted in Figure 6.5a. The number of photoreceptors in the pigmented rds mice was significantly higher than that of the albino rds mice at postnatal days 30 ($p<0.001$), 45 ($p<0.001$), 60 ($p<0.001$) and 90 ($p<0.01$).

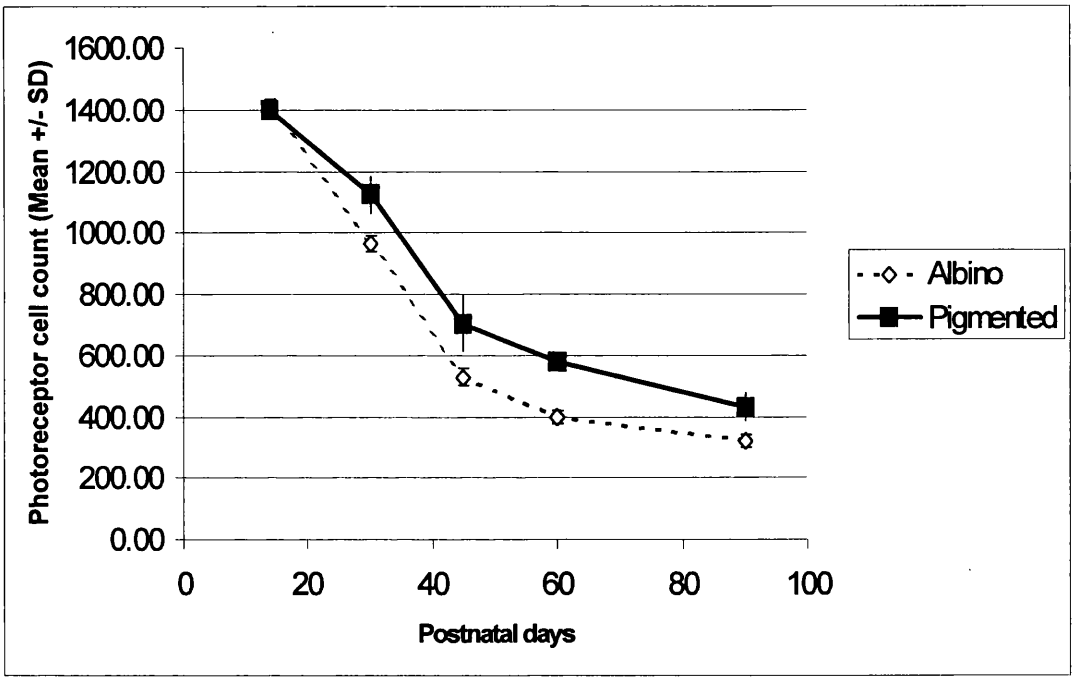

Figure 6.5a: Photoreceptor cell count per 1mm of retina (mean +/- SD) in the pigmented and albino retinal degeneration slow mice

Assuming a steady rate of photoreceptor cell loss between the timepoints, the average cell loss per day during each postnatal period is tabulated in Figure 6.5b.

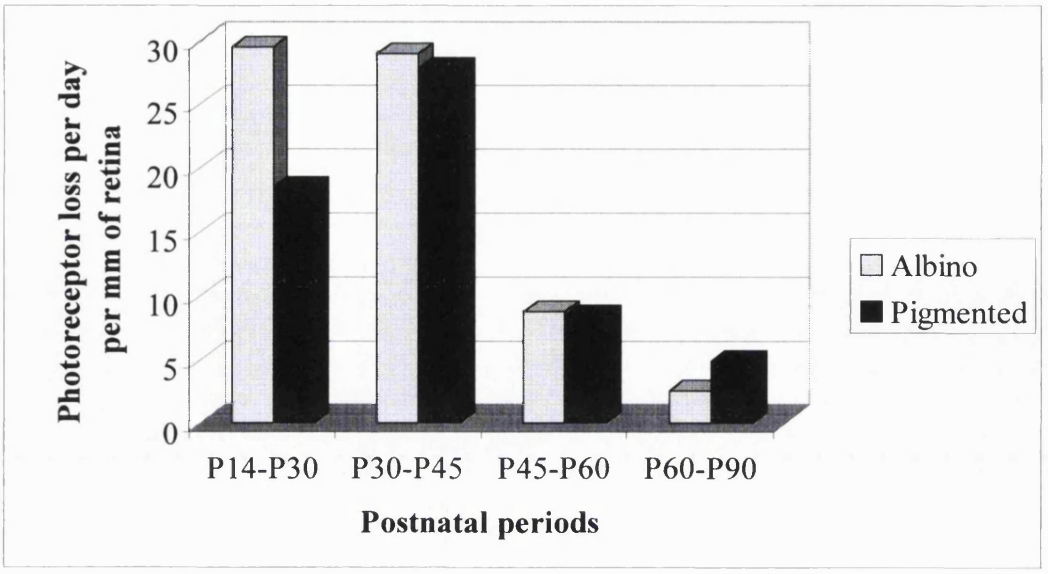

Figure 6.5b: Average number of photoreceptor loss per day per 1 mm of retina in the retinal degeneration slow (rds) mice during the different postnatal (P) periods

6.6 Caspases inhibitors trials in the pigmented rds mice

The results of the photoreceptor cells counts in different treatment groups is summarised in Table 6.6a and plotted in Figure 6.6a. The inter-observer difference was less than 15%. The number of photoreceptors at P60 in the Ac-DEVD.cmk treated eyes was significantly higher than of control. None of the other treatment groups were significantly different from controls. The average cell loss per day between P30 to P45 and P45 to P60 was summarised in Table 6.6b. The histopathology of the retina in an eye injected with DMSO at P30 (Figure 6.6b) and the other eye injected with Ac.DEVD.cmk at P30 (Figure 6.6c) illustrated the photoreceptor rescue in one animal at P60.

	Postnatal day	Control	Treated	P value
Ac.DEVD.cmk	P45 (n=8)	738 +/- 111	755 +/- 122	p=0.42
	P60 (n=9)	597 +/- 109	745 +/- 103	p=0.009 *
Ac-YVAD.cmk	P45 (n=6)	742 +/- 120	789 +/- 127	p=0.45
	P60 (n=8)	583 +/- 105	596 +/- 148	p=0.42
z-VAD.fmk	P45 (n=8)	729 +/- 106	740 +/- 57	p=0.25
	P60 (n=7)	604 +/- 133	600 +/- 105	p=0.96

Table 6.6a: Photoreceptor cell count per 1mm of retina (mean +/- SD) in the pigmented retinal degeneration slow mice injected by either Ac-DEVD.cmk, Ac-YVAD.cmk, z-VAD.fmk or sham injection with DMSO. The p-values of paired Student t-test were also shown. P45=postnatal day 45, P60=postnatal day 60

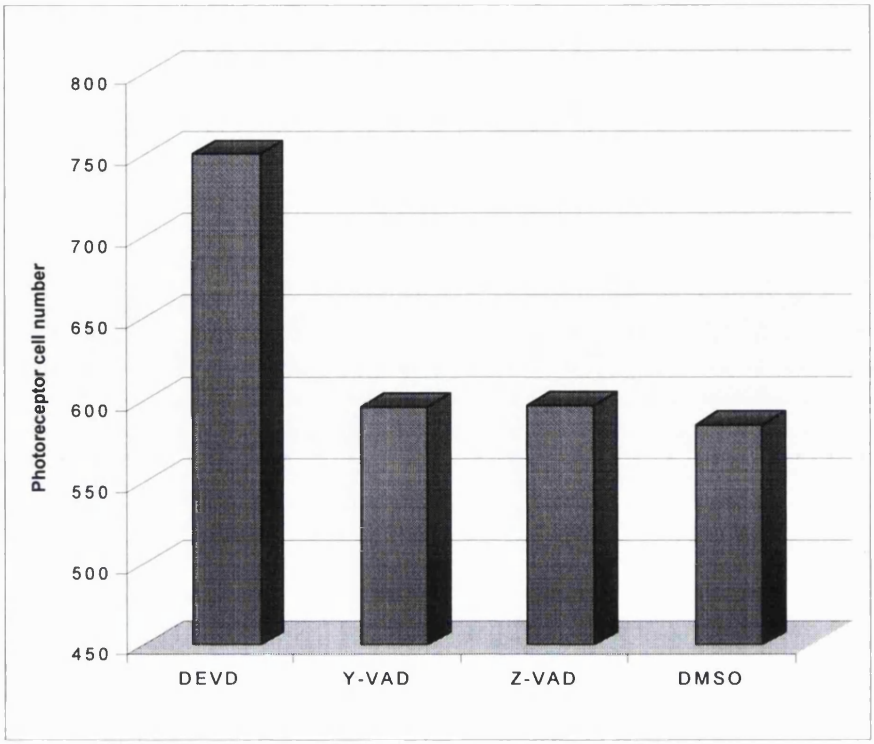

Figure 6.6a: Mean photoreceptor cell count per 1mm of retina in the pigmented retinal degeneration slow mice injected by either Ac-DEVD.cmk, Ac-YVAD.cmk, z-VAD.fmk or sham injection with DMSO at P60

	P30 – P45	P45 – P60
Ac-DEVD.cmk injected	24.5	0.7
Ac-YVAD.cmk injected	22.3	12.9
z-VAD.fmk injected	25.3	9.3
DMSO injected	25.7	9.3
Untreated	27.9	8.4

Table 6.6b: Average cell loss per day between postnatal day (P) 30 and P45, and P45 and P60 in different treatment groups, assuming there were 1123 photoreceptor cell per 1mm of retina at P30.

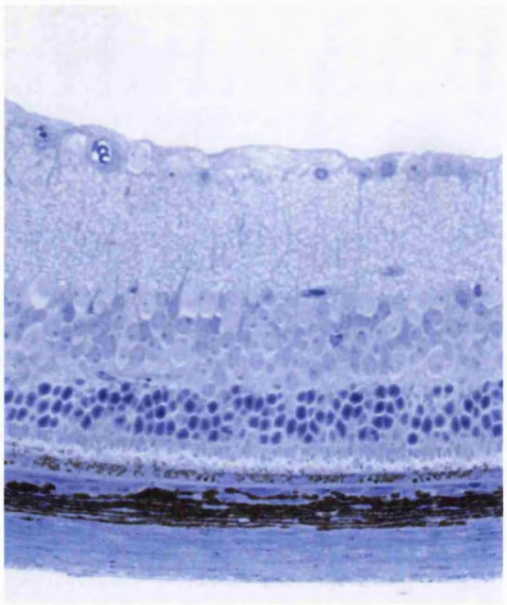

Figure 6.6b: Light microscopy of the pigmented rds mouse at P60 injected with DMSO at P30 showing about 3 to 4 rows of nuclei in the outer retinal layer (x200)

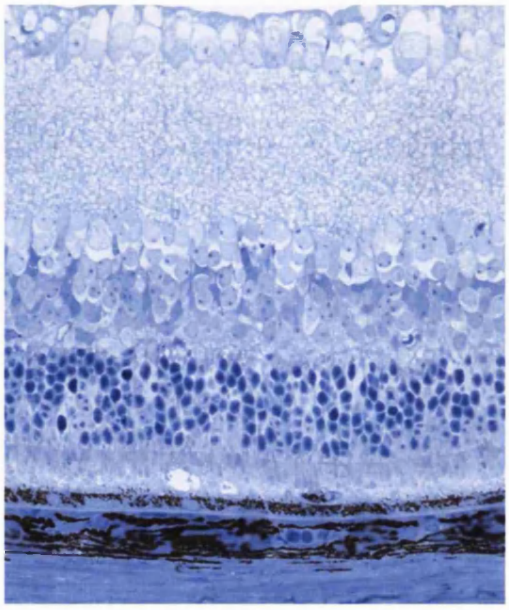

Figure 6.6c: Light microscopy of the pigmented rds mouse at P60 injected with Ac-DEVD.cmk at P30 showing about 5 to 6 rows of nuclei in the outer retinal layer (x200)

6.7 Summary of findings

Retinal dysplasia (Rdy) cat

- Apoptosis is the main mode of photoreceptor cell in this model of retinal degeneration
- The peak of apoptosis is around postnatal week 9
- There is a upregulation of glial fibrillary acidic protein in the Muller cells
- There is a loss of synaptophysin in the outer plexiform layer
- Opsin fails to localise to the outer segment alone and is present all over the cell
- Opsin immunopositive neurites extending into the inner retina are present
- Electron microscopy revealed totally disorganised outer segments
- Electroretinogram is not recordable even at postnatal day 10

Miniature longhaired dachshund (MLHD) dog

- Apoptotic rod and cone photoreceptor cells are present even at postnatal week 6
- There is no significantly difference in the ratio of pyknotic to normal nuclei of rods and cones at postnatal week 6
- There is a upregulation of glial fibrillary acidic protein in the Muller cells
- There is a loss of synaptophysin in the outer plexiform layer at postnatal week 24
- Opsin fails to localise to the outer segment alone and is present all over the cell
- Opsin immunopositive neurites extending into the inner retina are not present
- Well-organised outer segments at postnatal week 6 but become totally disorganised at postnatal week 24
- Cone-specific electroretinogram (ERG) is reduced at postnatal week 6 and becomes indistinguishable from noise at postnatal week 14

- Maximal intensity full field ERG is relatively normal up to postnatal week 10 but progressive reduced and by postnatal week 25, it becomes indistinguishable from noise
- This present the first animal model of cone-rod dystrophy

Neurotrophic factors treatment trials

- Axokine, a human analogue of ciliary neurotrophic factor, appears to delay photoreceptor cell degeneration in the Rdy cats based on photoreceptor cell count
- Axokine suppresses maximal intensity ERG at two weeks after injection but appears to preserve maximal intensity ERG at four weeks after injection in the MLHD dogs
- Repeated injections did not maintain preservation of maximal intensity ERG in the MLHD dogs
- Brain derived neurotrophic factor fails to delay photoreceptor cell degeneration in the Rdy cats based on photoreceptor cell count
- Axokine treated eyes have retinal folds and posterior subcapsular cataract in the Rdy cats but not in the MLHD dogs

Pigmented retinal degeneration slow (rds) mouse

- The retinal degeneration in the pigmented rds mouse is slower that of the albino rds mouse
- The main difference in the rate of degeneration is between postnatal day 15 to 30

Caspases inhibitors treatment trials

- Ac.DEVD.cmk, a caspase 3 inhibitors, appears to delay photoreceptor cell degeneration in the pigmented rds mouse based on photoreceptor cell count

- Ac.YVAD.cmk and z-VAD.fmk fail to delay photoreceptor cell degeneration in the pigmented rds mouse

D. DISCUSSION

7.1 Apoptosis is the main mode of cell death

Apoptosis is a genetically encoded potential of all cells, and is an essential part of embryonic development, cell turnover and of removal of cells infected by virus or harbouring mutations (Raff, 1992). It is characterised morphologically by disintegration of the nucleolus and generalised condensation of the chromatin, associated with cleavage by an endogenous nuclease of nuclear DNA into short chains of nucleosomes in multiples of 180 base pairs (Gavrieli et al., 1992). Condensed nuclei then fragment, giving rise to apoptotic bodies. In contrast to necrosis, the process affects individual cells within a tissue, neighbouring cells remain healthy, and it takes place in the absence of acute inflammation (See A3 for further details).

In all models of hereditary retinal dystrophy examined to date, including human RP, cell loss is through the process of apoptosis (Chang et al., 1993; Li and Milam, 1995; Portera-Cailliau et al., 1994; Tso et al., 1994). The TUNEL technique has been used to demonstrate apoptosis in the retinal degeneration (rd), retinal degeneration slow (rds), transgenic Q344ter rhodopsin mutant mice (Chang et al., 1993; Portera-Cailliau et al., 1994), RCS rats (Tso et al., 1994) and in human RP (Li and Milam, 1995). It labels the 3' ends of DNA fragments in nuclei going through the process of apoptosis in situ (Gavrieli et al., 1992; Nakamura et al., 1995). This technique is more sensitive than the agarose gel electrophoretic analysis of genomic DNA for internucleosomal DNA cleavage (Tso et al., 1994). The nuclei of these cells are, however, TUNEL-labelled for only a short period of time, about 8 hours in this model.

In this study, TUNEL-labelled cell counts generally mirrored the pyknotic cell count. It seems, however, that the TUNEL-labelled cell count peaked slightly earlier at 9 weeks as compared to 10 weeks for pyknotic cell count. There were also fewer TUNEL-labelled cells than pyknotic cells. As TUNEL-labelled the DNA fragmentation, it represents the end of the execution stage of apoptosis. This is followed by the condensation of the cytoplasm and nuclear chromatin as part of the degradation phase of apoptosis. Our findings are in keeping with this hypothesis. However, this is in contrast, to that found in light damage model when TUNEL staining was seen following morphological changes (Hafezi et al., 1997). The authors were unable to explain their observation.

Although the photoreceptor degeneration starts at about 5 weeks, the maximal photoreceptor loss was between 9 to 13 weeks. Apoptosis was maximal at the onset of maximal cell loss, as would be expected. This indicates that apoptosis is the main mode of cell death in the Rdy cats.

Pyknotic nucleus and TUNEL positive cells were also present in the MLHD dogs. Due to the limited material of pathological specimens, it was not possible to identify the peak of apoptosis in this model.

7.2 Immunohistochemistry in Rdy cats and MLHD dogs

Opsin immunolabeling is normally largely restricted to the rod outer segments (ROS). In the Rdy cats, opsin was also present in the rod inner segments and around the cell perikarya. Similar findings have previously been observed in rodent models of retinal degeneration including the rd mice, rds mice, RCS rats and mice with human P23H rhodopsin transgene (Ishiguro et al., 1987; Nir et al., 1989; Nir and Papermaster, 1989; Roof et al., 1994) as well as in human retinitis pigmentosa (Li et al., 1995). In addition to this abnormal perikaryal opsin labeling, the transgenic rhodopsin P23H mutant mouse has accumulation of opsin throughout the outer plexiform layer (Roof et al., 1994). This extensive immunoreactivity was not present in the Rdy cats but there were opsin positive rod neurites sprouting towards the ganglion cell layer. This phenomenon has been described in human retinitis pigmentosa (Li et al., 1995; Milam et al., 1996) but has not been observed in any of the rodent models of retinal degeneration examined to date (Li et al., 1995; Milam et al., 1996). It was also not seen in the MLHD dogs despite significant effort to identify them. It has been suggested that the rapid photoreceptor degeneration in the rodents leaves little time for the rod to attempt making new connections, but with slower retinal degeneration in the human, this is a possibility. This cannot explain why that these neurites were not found in the MLHD dogs.

The precise mechanism of this phenomenon is unknown. It seems likely, however, to reflect changes in the post-synaptic sites on horizontal or bipolar cells. Furthermore, the significance of this phenomenon to the pathogenesis of the disease and its therapeutic implications remain unknown.

The reduction of synaptophysin immunolabeling in the outer plexiform layer of the Rdy cats and the MLHD dogs is also found in other rodent models of retinal

degeneration, including the RCS rat (Sheedlo et al., 1993). No significant changes in the inner plexiform layer were expected as there was no significant cell death in the inner nuclear and ganglion cell layers as compared to normal controls, until end-stage disease (Curtis et al., 1987; Leon and Curtis, 1990). This finding parallels that in moderate severity form of human retinitis pigmentosa, where 88% of the inner nuclear layer remained intact (Santos et al., 1997).

GFAP is the major intermediate filament found in astrocytes. In the retina, GFAP is present only in astrocytes and Muller cells. Muller cells increase GFAP expression in response to focal or generalised retinal injury, such as laser photocoagulation (Humphrey et al., 1993) and light damage (Eisenfeld et al., 1984) as well as in genetically determined retinal degeneration, such as the RCS rats, (Eisenfeld et al., 1984), the recessive feline model of retinal degeneration (Ekstrom et al., 1988) and human retinitis pigmentosa (Milam and Jacobson, 1990).

There is evidence to suggest that the increase of GFAP expression is mediated by growth factors. In focal retinal injury, there is a lag period of about 24 hours before a significant increase of expression is seen (Humphrey et al., 1993) suggesting the injury does not directly increase the transcription of the GFAP gene. The delay suggests one or more intermediate steps. Furthermore, the normal area adjacent to the focal injury also has increased GFAP expression. This would be explained by diffusion of one or more growth factors from the site of focal injury (de Raad et al., 1996). This hypothesis is further supported by the increased expression of basic fibroblast growth factor (bFGF) after retinal injury (Wen et al., 1995). Different growth factors can also have different effects on the Muller cells. For instance, intravitreal injection of bFGF induces GFAP immunoreactivity in Muller cells of normal eyes (Lewis et al., 1992) but intravitreal injection of brain derived

neurotrophic factor reduces GFAP expression in a feline model of retinal detachment (Lewis and Fisher, 1997).

This glial reaction might reflect attempted photoreceptor rescue during retinal injury. However, these activated glial cells can induce scar formation and might even produce neurotoxins. It would be of great interest to investigate further whether this glial reaction is of benefit to the retina or not.

7.3 Electron microscopy of the Rdy cats and MLHD dogs

The electron microscopic findings in the Rdy cats were in general consistent with those previously reported. However, we found some disorganised outer segments in 5 weeks old animal that was not previously seen. Nonetheless, they became so disorganised that they could not be clearly identified at 13 weeks of age. This suggests that the outer segment synthesis is affected at an early stage of the disease but at least it is present unlike the rds mouse.

The morphology of most rods and cones, including their outer segments, were within normal limits at 6 weeks of age in the MLHD dogs. This clearly suggests that the retina is formed normally which is followed by photoreceptor cell death.

We were particularly interested in the cone pedicles in the MLHD dogs as the retina of a human case of simplex cone-rod dystrophy of unknown genetic mutation showed enlarged, distorted shape of cone pedicles, along with thickening of some cone axons (Gregory-Evans et al., 1998). Similar features were found in a small number of cones in an eye with central areolar choroidal dystrophy but not found in eye with cerebellar ataxia-cone dystrophy, cone-rod dystrophy in Bardet-Biedl syndrome or simplex RP (Gregory-Evans et al., 1998). These abnormal cone pedicles were not seen in the MLHD dogs. These observations imply that these abnormalities were not a uniform feature in all cone-rod dystrophies.

7.4 Electroretinogram of the Rdy cats and MLHD dogs

Our findings in the Rdy cats were consistent with that previously described (Curtis et al., 1987). We had hoped that by using a standardised light source and more sophisticated recording equipment, we might obtain electroretinogram from these affected animals but we were unable to do so.

In the MLHD dogs, despite the relatively normal appearance of the cone photoreceptor at the age of 6 weeks, the 30Hz flicker (cone specific) ERG was already significantly affected. The MLHD was previously classified as a rod-cone dystrophy (Curtis and Barnett, 1993) as this aspect of cone function was not previously studied. The findings of the maximal bright white flash ERG are consistent with the previous study. As the cone photoreceptors are more severely affected initially and this is followed by the progressive rod involvement, the features are most consistent with those of a cone-rod dystrophy (CORD). This, therefore, appears to be the first animal model of CORD ever described.

The MLHD dog provides a unique opportunity to study cone-rod dystrophy. Furthermore, the relatively normal maximal bright flash ERG at 10 weeks and almost extinguished maximal bright flash ERG at 25 weeks provides a neat window for therapeutic assessment.

7.5 Neurotrophic factors trials

It appears that multiple intravitreal injections of Axokine (a human CNTF analogue) might reduce apoptotic cell death of photoreceptors in the Rdy cat. However, human BDNF was not effective. These results are similar to those reported in the mouse models of retinal degeneration (LaVail et al., 1998). It is possible that the presence of retinal folds might create bias, but they were not always present in a single slide of Axokine treated animal and the age of the animals were also masked. Furthermore, the second observer did not know the significance of the retinal folds and yet the inter-observer difference was less than 10%. This bias is even more unlikely in the "TUNEL" slides as the slides were not counterstained so the morphology of the retina was not clearly seen.

The number of animal used was relatively small in this project, however, that was largely due to the funding limitation and the Home Office regulation. Despite that, the results were statistically significant.

In the MLHD dog experiment, the initial reduction of the amplitude of the b-wave in the electroretinogram was unexpected. However, this is worrying and has implications if clinical trials are to be taken place. Nonetheless, 4 weeks after the injection, the amplitude of the b-wave was higher than that of the sham injected eye. This apparently beneficial effect, however, did not last despite a repeated injection. Further investigations of these findings are under way, in particular, we are interested in single injection at an earlier age to see whether how long this potential "beneficial effect" lasts.

As the ERG findings of Axokine treated and sham treated were similar at postnatal week 22 when terminal histopathology was taken, it was not surprise that the photoreceptor cell counts were not significantly difference.

In the light damage model of retinal degeneration, both CNTF and BDNF delay photoreceptor degeneration (LaVail et al., 1992, 1998). The ineffectiveness of BDNF in this cat model might be explained by the fact that we were using human BDNF instead of feline BDNF. However, the same preparation at a slightly lower dose (100 μg) of human BDNF promotes outer segment regeneration in an acquired feline retinal degeneration model (Lewis et al., 1999). Although we cannot exclude the possibility that different dosages of BDNF might have an effect in the Rdy cats, it seems unlikely as the difference in dosage was only 2.5 fold. It is possible that BDNF can protect the retina from acute injury as in light damage and retinal detachment, but ineffective in hereditary retinal degeneration.

The difference in response between different insults in various species may be explained by the existence of more than one route leading to apoptosis, the final common pathway of retinal degeneration. The synergistic effect of CNTF and BDNF in enhancing the rate of functional recovery after peripheral nerve transection (Lewin et al., 1997) also suggests that the two neurotrophic factors may act on different pathways. Furthermore, they are known to activate different signal transduction pathways (Ip and Yancopoulos, 1996; Kahn et al., 1997).

Cataract formation and retinal folds were relatively minor, and have not been previously reported following CNTF therapy. These complications are unlikely to be related to surgical trauma as they were present only in Axokine treated eyes in the Rdy cats. Both complications were less severe in the second, lower dose experiment suggesting that the response might be dose dependent. It is interesting that these complications were not seen in the MLHD dogs.

It is possible that the lens and the retina of the cats are still relatively immature at postnatal week 5 when treatment started even in the delayed group. The

retina in the Rdy cats appears immature with oval nuclei at that age. It is possible when the retina is immature, the injected growth factor altered the local concentration of growth factor. This in turn alters the signalling pathway of the photoreceptors so as they lost in orientation and fail to attach to the RPE.

CNTF is known to increase the expression of glial fibrillary acidic protein (GFAP) in Muller cells (Kahn et al., 1997). It is not unexpected that Axokine also caused an increase in GFAP expression in the treated animals and may therefore, directly or indirectly, constitutes other components of a glial injury response, including the release of endogenous growth factors.

In principle, Axokine might cause cataract formation directly or through the secondary release of other growth factors. Although the CNTF alpha receptor has not been demonstrated in the human lens epithelium, a direct effect cannot be excluded. The receptors of other growth factors, including basic fibroblast growth factor (bFGF) are present in lens epithelial cells (Potts et al., 1993). As bFGF can induce proliferation of lens epithelial cells *in vitro* (McAvoy et al., 1991), it is a possible candidate for an intermediate agent.

Similarly, the retinal folds might also be secondary to bFGF production as intravitreal injection of this growth factor causes retinal folds with severe inflammation in the rabbit (Borhani et al., 1993) and also in the cats (Sethi et al., 2000). The morphology of these folds is similar to that of proliferative vitreoretinopathy (PVR) both in experimental animals (Sethi et al., 2000) and human. However, epiretinal membrane is always present in PVR but they were not present in the Axokine treated Rdy cats. As the effect is dose dependent. it is possible that using higher dose of Axokine, epiretinal membrane would be formed. Another explanation is that the epiretinal membrane might take time to form and we

did not find the membrane because we did not wait long enough. The latter would suggest that the retinal folds were present before epiretinal membrane formation. This would be unexpected as it is often believed that the epiretinal membranes contract and pull the retina together forming retinal folds. Furthermore, epiretinal membranes are seen within 21 days after intravitreal injection of bFGF (Sethi et al., 2000).

It is, however, possible that Axokine acts on the Muller cells directly leading to retinal swelling and then folds. Indeed CNTF is known to produce swelling of astrocytes *in vitro* (Levison et al., 1996).

The absence of retinal folds in the Axokine treated MLHD dogs, rodents (personal communication with Dr Matt LaVail, University of California, San Francisco) and the transgenic pigs (personal communication with Dr Fulton Wong, Duke University, North Carolina) might suggest that there are species different in the response to Axokine.

Based on our data, repeated intravitreal injections of Axokine, but not human BDNF, appears to prolong photoreceptor survival in hereditary feline retinal degeneration and it reduced the apoptotic marker counts. Functional rescue could not be demonstrated in the Rdy cats as ERG cannot be recorded. However, a similar dose of Axokine appeared to lead to some degree of short term functional rescue in the MLHD dog after an initial suppression.

7.6 Pigmented rds mice

In the retinal degeneration slow mice, photoreceptor loss begins after the second week of postnatal development that coincides with the time of development of outer segments in wild-type mice (Sanyal, De Ruiter, Hawkins, 1980). Degeneration is more rapid up to the age of postnatal day 45, when the outer nuclear layer is reduced to half of its original thickness; thereafter degeneration progresses more slowly (Sanyal, De Ruiter, Hawkins, 1980). The peak of apoptosis has been found to be around postnatal day 16 to 17 (Chang, Hao and Wong 1993; Portera-Cailliau et al., 1994).

In this study, we confirmed quantitatively the impression of previous investigators that the photoreceptor degeneration of pigmented rds mouse is slower than that in the albino rds mouse (Sanyal and Hawkins, 1986). The difference in the number of photoreceptors can be seen as early as P30 and this is persisted until P90.

The observed difference cannot be explained by the wild type albino mouse has less photoreceptors than that of the wild type pigmented mouse as there were no significant difference of photoreceptor number at P14 before the retinal degeneration begins.

The data indicate that the main difference in the rate of cell loss is between P14 and P30. The light damage effect is probably not marked during the first 14 postnatal days as the eyes of neonatal mouse do not open until P10 to P12. After P14, it is possible that pigmentation delays the peak of apoptosis in the outer nuclear layer similar to that of the rds mouse without the functioning p53 gene (Ali et al., 1998). The mechanism of this delay is unknown. However, the relatively immature retina during this early postnatal period might be more susceptible to light damage. As pigmentation protects rodent retina from intentional exposure of light (Sanyal and

Hawkins, 1986), it is possible that the pigment can also protect the immature retina from normal cyclic lighting.

7.7 Caspases inhibitors trial

In the retinal degeneration slow mice, photoreceptor degeneration begins after the second week of postnatal development that coincides with the development of outer segments (Sanyal, De Ruiter, Hawkins, 1980). Based on our original data, we chose to treat at P30 as there is still significant photoreceptor loss between P30 and P45. Furthermore, the mouse eye at P30 is significantly bigger than that at P15. Treating at P30, therefore, reduces the traumatic effect of the injection. The latter is confirmed as the results were not significantly different between DMSO treated and untreated eyes.

The action of Ac-YVAD.cmk is based on the target sequence in pro-interleukin-1b (YVHD) and it is a cell-permeable and irreversible caspase 1 inhibitor (Thornberry et al., 1992). At extremely high concentration, it can inhibit caspase 3. Ac-DEVD.cmk, is derived from the cleavage site in poly-(ADP-ribose) polymerase (PARP) and is potent cell-permeable and irreversible inhibitor of caspase 3 and members of the caspase 3 subfamily (Lazebuik et al., 1994). Finally, z-VAD.fmk inhibits a wide spectrum of caspases including those of Group II (caspases 3 and 7), and to a lesser extent, those of Groups I and III (Sweeney, Inokuchi and Igarashi, 1998).

Group	Caspases known to be in the group	Tetrapeptide sequences preference	Inhibitors known
I	1, 4, 5	WEHD	Ac-YVAD.cmk, z-VAD.fmk
II	3, 6, 7	DEXD	Ac-DEVD.cmk, z-VAD.fmk
III	2, 8, 10	XEH/TD	z-VAD.fmk

Table 7.7: Classification of caspases based on preference tetrapeptide sequences and their known inhibitors. (Key: A = Alanine, D = Aspartic acid, E = Glutamic Acid, H = Histidine, T = Threonine, V = Valine, W = Tryptophan, X = any amino acid, Y = Tyrosine, Ac-YVAD.cmk = acetyl-Tyr-Val-Ala-Asp-chloromethylkeone, Ac-DEVD.cmk = acetyl-Asp-Glu-Val-Asp-chloromethylkeone, z-VAD.fmk = benzyloxycarbonyl-Val-Ala-Asp-fluoromethlketone)

It was not surprising that YVAD, a caspase 1 inhibitor, had little or no effect on photoreceptor cell count as the role of caspase 1 in neuronal apoptosis is in doubt.

However, it was surprising the zVAD, a broad spectrum caspases inhibitors had little or no effect on photoreceptor cell count whilst DEVD, a caspase 3 inhibitor, delayed photoreceptor cell loss especially between P45 and P60.

Nonetheless, it is in keeping with previous report that DEVD and zVAD can both delay ganglion cell loss after optic nerve axotomy (Chaudhary et al., 1999, Kremer et al., 1998) but that zVAD is less effective (Kermer et al., 1998). It is possible that either zVAD is not effective in photoreceptor degeneration or its effect cannot be assessed in the current experimental model.

This observation can also be explained by their different action against caspase 3. DEVD inhibits caspase 3 directly and reduces PARP protease activity; the latter appears to be necessary for apoptosis. However, zVAD does not inhibit caspase 3 activity but blocks apoptosis either by inhibiting the processing of caspase 3 or at an earlier stage of the apoptotic cascade (Slee et al., 1996).

It is therefore possible that there are slight differences in the nature of the apoptotic cascade in ganglion cells and photoreceptor cells. Similar discrepancy is also seen in neurotrophic factor rescue. Both brain derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) are effective in ganglion cell rescue after optic nerve axotomy (Mey and Thanos, 1992;Weibel, Kreutzberg, and Schwab, 1995) but only CNTF is effective in photoreceptor rescue in retinal degeneration (Chong et al., 1999).

It is also interesting to note that the effect was only seen at P60, 30 days after the injection of caspase-3 inhibitors but not at P45. The delay might suggest the inhibitors might take time to be taken up by the cells. Furthermore, cells which are already committed to cell death beyond the caspases in apoptotic cascade would not be affected by these inhibitors.

E. IMPLICATION OF THE PROJECT AND FUTURE WORK

Our main aims of this project are to study model of retinal degeneration so as to identify their role in testing future therapies and to assess whether neurotrophic factors and caspases inhibitors are potentially useful therapies for retinal degeneration.

We have studied three animal models of genetically determined retinal degeneration in three different animal species. There were many pathological features in common, such as the loss of the outer segments, the thinning of the outer nuclear layer, the presence of apoptotic photoreceptor cells, the upregulation of GFAP, the loss of synaptophysin of the outer plexiform layer and the mislocalisation of the opsin. These similarities cannot be explained by a single gene mutation as the genetic mutation is known in the rds mouse (Travis et al., 1989) and this gene has been excluded in the Rdy cats (personal communication with Dr David Sargan, University of Cambridge) and MHLD dogs (personal communication with Dr Matthew Binns, Animal Health Trust, Newmarket) by candidate gene approach. The Rdy cats and the MHLD dogs are unlikely to be caused by the same mutation as the Rdy cats is an autosomal dominant model whilst MHLD dogs is inherited in autosomal recessive fashion. Hence, our observations suggest that the different genetic mutations can induce similar changes in the retina leading to photoreceptor cell dysfunction and cell death, and the excitation of glial cells.

Nonetheless, there were clear differences in these models. The pigmented rds mice do not ever have rod outer segments (ROS), whilst the ROS are disorganised in the young Rdy cats, but the ROS are within normal limits in the young MLHD dogs. The nuclei of the photoreceptor cells in the Rdy cats are elongated and appear

immature, whilst that of the pigmented rds mice and MLHD dogs appears normal during the early stage of retinal degeneration. Opsin immunopositive neurites extending into the inner retina were only present in the Rdy cats. These latter findings allow us to investigate further the role of these neurites as they are commonly found in human outer retinal dystrophies.

It was a surprise to us to have found the first animal model of cone-rod dystrophy (CORD), the MLHD dogs. The linkage studies, in collaboration with Dr Matthew Binns, might identify a gene that can be used to as a candidate gene in human recessive CORD. Furthermore, this model allows us to study the pathology of CORD in more detail.

Each of these models has their advantages and disadvantages for testing therapies. The pigmented rds mouse is well characterised, the genetic defect is known and the cost of breeding and upkeeping of mice is relatively small. They are likely to be used for testing novel therapy as in this project. However, it is likely that transgenic rodents would be more commonly studied in the future as they become generally available. Characterisation of these transgenic animals would take time and effort but they might be more closely resemble to human diseases.

The Rdy cat is inherited in an autosomal dominant fashion and the breeding of cats is much easier than that of other companion animals. The main disadvantages are that they do not have normal or near normal retinal anatomy before the retinal degeneration begins. Furthermore, there is no recordable ERG to assess function objectively. Nonetheless, they will be useful as an intermediate testing ground to refine dosage and to find the most effective agents after positive results have been shown in the rodents.

The MLHD dog is a useful model for testing therapy before clinical trials. The retinal anatomy is relatively normal at postnatal week 6. Retinal function can be measured at postnatal week 6 and progressively deteriorated to ERG extinguished by postnatal week 25. This allows a good treatment window for investigation. However, they are much more difficult to breed that cats. In addition, as a recessive model, the breeding program leads to the production of a large number of carriers. Under the current Home Office regulation, these carriers have to be castrated at 6 months of age before re-homing. All this increases the upkeep costs. It is estimated that each affected MLHD dogs used in this project costs well over £2000 as compared to about £300 for the Rdy cats. More complex surgical procedures can also be performed in the MLHD dogs.

In terms of transgenic animal, so far only the transgenic P23H rhodopsin mutant pig have eyes similar in size to human eyes. It was very costly to produce and even then the rod specific ERG is not recordable at any stage in these pigs limit the use of them. As it stands, it is not a better model of retinal degeneration than the MLHD except the genetic defect in the MLHD is so far unknown. However, it is likely that would become available to us in the near future. Furthermore, the exact genetic mutation of the model is not particularly important if the therapy to be tested is targeting at delaying cell loss.

Axokine appears to have some degree of anatomical and functional rescue in the Rdy cats and MLHD dogs respectively. It is true that the number of animals used were small and there is possible bias in cell counting in the Rdy cat experiments. Further studies using small dosage of Axokine might be required to see whether it is possible to have anatomical rescue without causing cataract or retinal folds. The Axokine trial in the MLHD dogs was really a pilot study. Further studies are

required to confirm the "functional rescue" at 4 weeks after the injection. It would be worthwhile using different dosages to reduce the initial ERG suppression. Furthermore, we did not know how long a single injection of Axokine works if it does. Single injection experiments would be required. These experiment framework can be used for future testings of other neurotrophic factors or indeed other reagents.

Nonetheless, there is clear limitation to animal research. For instance, the retinal degeneration in both the Rdy cats and MHLD dogs is very rapid in comparison to that commonly seen in human patients with retinitis pigmentosa and it is difficult to extrapolate to the clinic with confidence. However, the effect observed might be enough to slow the progression in humans to an extent that it would confer some visual benefit. It is a judgement call when would animal research stop and clinical trials begin. One would argue that when a reagent can produce significant rescue in a number of animal species and there are no significant side effects in the animal, clinical trial would be justified to be considered. At the moment, Axokine has not reached this stage yet. Indeed, it might never reach that stage.

Furthermore, repeated intravitreal injections will not be practical in clinical settings. An improved delivery system is required before this neurotrophic factor will play a significant role in the future management of outer retinal dystrophies. One of the options is to transplant cells, such as fibroblasts or Schwann cells, that have modified *in vitro* to produce steady concentration of neurotrophic factor. Similarly gene therapy approach for transfecting RPE / Muller cells to produce neurotrophic factors might be another alternative. The MLHD dogs would be useful for these experimental settings in the future.

As Axokine appears to delay cells from going into apoptosis, it is likely that it might be useful in the neuroprotection of ganglion cells in glaucoma and ischaemic

diseases, and in photoreceptor degeneration in retinal detachment before surgery can be performed. Its role in these optic nerve and retinal diseases will need further investigation.

The rationale of using growth factors is to prevent apoptosis. For the treatment of human retinal degenerations, it has the potential advantage over alternative biological approaches to therapy in that the cause of the disorder need not be known. If it could be shown that a single growth factor was effective in all animal available models of genetically determined retinal dystrophy, the initiation of a trial on the human disease could be justified. It is clearly an advantage that there is a wide variety of animal models available with different fundamental genetic defects. It is disappointing that the effect in MLHD dog was not as striking as that seen in cat and mouse.

The results of the inhibitors of caspases treatment trials indicated that caspase 3 and its subfamily might play an important role in photoreceptor degeneration. By blocking them with DEVD delays photoreceptor loss in the pigmented rds mouse. A positive result suggests that further investigation in this area is appropriate. Nonetheless, a negative result in this experimental setting does not necessary mean that z-VAD and YVAD are ineffective in all retinal degenerations. The dosage used might not be appropriate and the preparation used might be ineffective for cellular penetration. Other dosages and preparation should be investigated. Furthermore, anatomical rescue can only indicate that the cells are present but they might not be functioning at all. Electrophysiological and behavioural studies are required to evaluate this further.

There is more to know. In particular, do the animals see better after a period of treatment? This is especially relevant in those cases with mutations affecting the

phototransduction cascade. How and where do the growth factors work? Is it on the photoreceptors themselves or on their environment? How do the infrequent doses delivered by injection into the vitreous compare with physiological levels of release? Can untoward side effects, such as cataracts, be avoided? None of these questions are unanswerable, but they need to be addressed before a therapeutic regimen can be applied in humans.

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Appendix A: Techniques employed

A1 Light microscopy, immunohistochemistry and TUNEL techniques

All specimens were fixed in either formalin or 4% paraformaldehyde for 24 to 48 hours before processing.

Processing into paraffin wax

Specimens were placed into labelled cassettes. They were loaded onto tissue processor for overnight dehydration, ante-medium and wax infiltration using the following standard program:

- 70% methylated spirits at room temperature for 50 minutes
- 85% methylated spirits at room temperature for 60 minutes
- 99% methylated spirits at room temperature for 60 minutes
- 100% ethanol 1 at room temperature for 90 minutes
- 100% ethanol 2 at room temperature for 120 minutes
- Equal parts ethanol and xylene at room temperature for 90 minutes
- Xylene 1 at room temperature for 90 minutes
- Xylene 2 at room temperature for 90 minutes
- Paraffin wax 1 at 60°C for 120 minutes
- Paraffin wax 2 at 60°C for 120 minutes
- Paraffin wax 3 at 60°C for 60 minutes

Specimens were transferred to molten wax in the embedding station. They were placed in a suitable sized embedding mould, correctly orientated, and along with their reference label transferred to the cold stage to allow the wax to solidify.

APES coating of microscope slides for immunohistochemistry (Maddox and Jenkins, 1987)

- Immerse rack of clean microscope slides in 2% aminopropyltriethoxysilane (Sigma A3648) in acetone for 10 seconds.
- Wash slides in 3 different pots of acetone for a total of 10-15 minutes.
- Air dry in incubator overnight

Dewaxing and rehydration of sections

- Place slide staining rack in dewaxing xylene 1 for 10 minutes
- Transfer to dewaxing xylenes 2 and 3 for a further 10 minutes each.
- Transfer to 1:1 xylene and absolute alcohol for 2 - 5 minutes
- Transfer through descending concentrations of methylated spirit to water spending over 2 minutes in each.
- Wash in tap water for over 5 minutes
- Rinse in distilled water before performing staining technique.

Mayer's haematoxylin and eosin (Mayer, 1903)

Solutions required:

Haematoxylin Solution:

Haematoxylin	1 g
Sodium iodate	0.2 g
Aluminium potassium sulphate	50 g
Citric acid	1 g
Chloral hydrate	50 g
Distilled water	1000 ml

Dissolve haematoxylin, iodate and alum in water overnight. Add citric acid and chloral hydrate and boil for 5 minutes. Cool and filter before use.

Eosin solution:

Eosin yellowish	5 g
Sodium acetate	8.2 g
Distilled water	990 ml
Acetic acid	6 ml

Adjusted to pH 4.6 - 5

Method:

- Sections for dewaxing and rehydration (see above)
- Stain in haematoxylin for 5 - 30 minutes depending upon ambient conditions and age of solution.
- Wash in tap water for over 10 minutes.

- Differentiate between cytoplasmic and nuclear staining by exposure to acid alcohol for a few seconds.
- Wash in water until sections are blue.
- Check, microscopically, that only nuclei are stained.
- Repeat haematoxylin staining and/or acid alcohol differentiation until result is achieved.
- Wash in tap water.
- Stain in eosin for 2 - 5 minutes.
- Rinse in water and differentiate in 70% alcohol until connective tissue and cytoplasmic staining exhibits more than one shade of red.
- Dehydrate, "clear" and mount.

Periodic acid schiff (PAS) staining (McManus, 1946)

Schiff's reagent:

- 400 ml of distilled water
- Boil, remove from heat then add basic fuchsin 2 g
- When solution reaches 50'C add 40 ml of 1 N hydrochloric acid
- When solution reaches 24'C add 4 g of sodium metabisulphite
- Leave solution overnight.
- Clear solution using 4g.of activated charcoal
- Filtered
- Store at 4'C.
- Bring to room temperature for use.

PAS Staining Method:

- Sections for dewaxing and rehydration (see above)
- Oxidise in periodic acid for 10 minutes
- Wash in water.
- Wash in distilled water.
- React with Schiff's reagent for 5 minutes at room temperature
- Wash in running water for 15 minutes
- Stain nuclei in Mayer's haematoxylin and differentiate as required.
- Wash in water.
- Dehydrate, "clear" and mount.

Trypsin Pretreatment (Curran and Gregory, 1977; Mepham et al., 1979; Ordonez et al., 1988)

Trypsin solution

Trypsin	0.05 g
Calcium chloride	0.05 g
0.2M tris	14 ml
0.1N HCl	16 ml
Distilled water	20 ml

Adjust to pH 7.8

Method:

- Sections for dewaxing and rehydration (see above)
- If performing peroxidase method block endogenous peroxidase activity with 0.5% hydrogen peroxide for 25 minutes
- Wash in water for 10 minutes
- Equilibrate sections in tris buffer pH 7.8 at 37°C for 10 minutes
- Expose to trypsin solution at 37°C for 10 minutes
- Wash in cold running water for 10 minutes
- Perform immunohistochemical technique.

Streptavidin biotin complex peroxidase method (Guesdon et al., 1979, Bains and Miliar, 1988)

Streptavidin biotin peroxidase complex (Dako K0377)

PBS buffer pH 7.3	5ml
Reagent A	45µl
Reagent B	45µl

Method:

- Sections for dewaxing and rehydration (see above)
- Block endogenous peroxidase activity with 0.5% hydrogen peroxide for 25 minutes
- Wash in water for 10 minutes
- Pretreatment if required
- Wash in PBS pH 7.3 for 10 minutes
- Marvel 5% in PBS block for 20 minutes
- Primary antibody at appropriate concentration and time
- Wash in PBS pH 7.3 for 10 minutes
- Secondary biotinylated antibody depending on primary antibody for 45 minutes
- Wash in PBS pH 7.3 for 10 minutes
- Tertiary Streptavidin biotin peroxidase complex (see above) in PBS pH 7.3 for 45 minutes
- Wash in PBS pH 7.3 for 10 minutes
- Peroxidase demonstration with either diaminobenzidine (DAB) in brown, or AEC in red

Diaminobenzidine (DAB) demonstration (Graham and Karnovsky, 1966)

DAB Solution:

0.2 M tris	12.5 ml
0.1 N hydrochloric acid	20 ml
distilled water	17.5 ml
adjust pH to 7.6	
DAB	0.3 gm

Method:

- Filter DAB solution
- Add 0.5 ml of 3% hydrogen peroxide before use
- Incubate sections in substrate solution at room temperature
- Timing of incubation depends on primary antibody and section
- Wash sections in water for 10 minutes
- Counterstain sections if required
- Wash in water
- Dehydrate, "clear" and mount

Aminoethyl carbazole (AEC) demonstration (Graham et al, 1965)

AEC Solution:

0.1 N acetic acid	10.5 ml
0.1 M sodium acetate	39.5 ml
adjust pH to 5.2	
Aminoethyl carbazole	15 mg
Dimethyl formamide	3 ml

Method:

- Filter AEC solution
- Add 0.5 ml of 3% hydrogen peroxide before use
- Incubate sections in substrate solution at room temperature
- Timing of incubation depends on primary antibody and section
- Wash sections in water for 10 minutes
- Counterstain sections if required
- Wash in water
- Mount in aqueous mountant
- When dry mount directly in DPX from xylene

TUNEL (Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labeling) technique

A modified TUNEL technique was used to detect double strand breaks in DNA. This technique is based on that described by Nakamura and colleagues (Nakamura et al., 1995) but dAdenosine triphosphate replaces dUridine triphosphate.

Method:

- Dewaxed with xylene and rehydrated through a graded series of alcohol (see above)
- Endogenous peroxidase activity was blocked by 0.5% hydrogen peroxide for 25 minutes in the dark at room temperature
- Treated with 20 μg/ml of proteinase K in phosphate buffered saline (PBS) at room temperature for 15 minutes
- Wash in PBS for 10 minutes
- Buffered with terminal deoxynucleotidyl transferase (TdT) buffer for 10 minutes
- Labeling reaction with 10 mM of dAdenosine triphosphate (dATP), 0.4 mM of biotin 14 dATP and TdT for 60 minutes at 37 °C
- The positively labelled cells were demonstrated by a standard biotin-streptavidin peroxidase method visualised by diaminobenzidine (DAB)

A2 Transmission electron microscopy

Tissue samples destined for examination by transmission electron microscopy were fixed in a mixture of 1% paraformaldehyde and 3% glutaraldehyde buffered to pH 7.4 with 0.1M sodium cacodylate-HCl for periods of 1 to 3 days.

Processing and embedding in Araldite resin

- Thorough rinse in 0.1M sodium cacodylate buffer
- Osmicated for 2 hours with an 1% aqueous solution of osmium tetroxide
- Dehydrate in 50% alcohol for 10 minutes
- Dehydrate in 70% alcohol for 10 minutes
- Dehydrate in 90% alcohol for 10 minutes
- Dehydrate in 95% alcohol for 10 minutes
- Dehydrate in 100% alcohol for 10 minutes
- Dehydrate in 100% alcohol for 10 minutes
- Dehydrate in 100% alcohol for 10 minutes
- Propylene oxide for 20 minutes
- Propylene oxide for 20 minutes
- Left in a 50:50 mixture of propylene oxide and araldite for 12 hours
- Transfer to 100% araldite for 4 to 6 hours
- Transfer to embedding moulds at 60°C for 12 hours

Section cutting, staining and photography

Semithin and ultrathin sections were cut using a Leica ultracut S microtome (Leica, Milton Keynes, UK) fitted with the appropriate type of diamond knife (diatome, TAAB laboratories, Aldermaston, UK).

Semithin sections were stained with a mixture of 1% borax and 0.5% toluidine blue dissolved in 50% ethanol at 60°C for 20 seconds. After rinsing with 50% ethanol, sections were dried on a hot plate and mounted in DPX medium.

Ultrathin sections for TEM were contrasted by sequential immersion in saturated uranyl acetate in 50% ethanol followed by 5 minutes in Reynold's lead citrate prior to examination in a JEOL 1010 TEM (JEOL (UK) Ltd, Welwyn Garden City, UK) operating at 80kV. Images were recorded onto Kodak 4489 electron image film and printed on Ilford multigrade paper.

Unless otherwise stated, all fixatives, solvents, resins, grids and stains EM film and Ilford photographic paper were obtained from Agar Scientific Limited, Stansted, UK.

A3 Electroretinogram and clinical examination

The subject was anaesthetised and mydriasis achieved by tropicamide 1% eye drops. Topical anaesthesia was also instilled. A superior limbal suture was placed in each eye to maintain the ocular position. The subject was then placed on a sliding table attached to the ganzfeld stimulator (Figure A3a, A3b and A3c). ERG-Jet or custom made corneal electrodes were used for electroretinography (ERG). Platinum Iridium sub-dermal ground and reference electrodes were attached at crown and at one centimetre behind lateral canthus respectively. The ganzfeld was custom built, with a 50 cm bowl, using a Grass PS22C photic stimulator. Light intensity was controlled by PS22C electronics and neutral density filters, giving a maximum 'white' intensity of 12.5 cd.s/m^2. Photopic background intensity was 21 cd/m^2. Signals were amplified by a factor of 2000 to 50000 times, bandpass 0.5Hz to 3.0KHz, and recorded on PC based data acquisition system (CH Electronics, England). (Figure A3d)

The recording protocol, based on the human recommendations for ERG by International Society for Clinical Electrophysiology of Vision (ISCEV) standard (Marmor and Zrenner, 1995), consisted of 20 minutes dark adaptation, followed by an intensity series of discrete 'white' stimuli, ranging from 0.003 to 12.5 cd.s/m^2, (inter-flash interval 1 to 15 seconds), 2 and 30 Hz (1.5 cd.s/m^2) stimulation, then 8 minutes of light adaptation followed by 2 and 30 Hz (3.0 cd.s/m^2) photopic stimuli.

Both the cats and dogs were examined by direct ophthalmoscopy and slit-lamp biomicroscopy when required. In order to prevent bleaching of the retina, clinical examination is usually carried out with the animal under anaesthesia after the intravitreal injection or the electroretinography had been performed

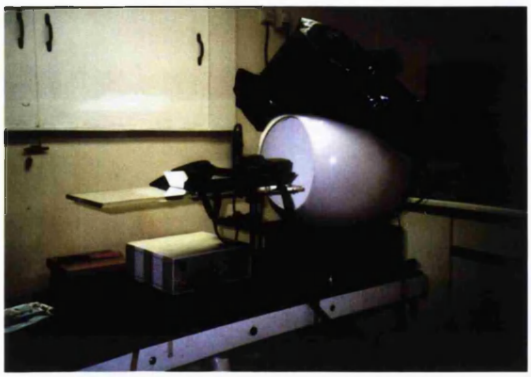

Figure A3a: Custom built ganzfeld with sliding table

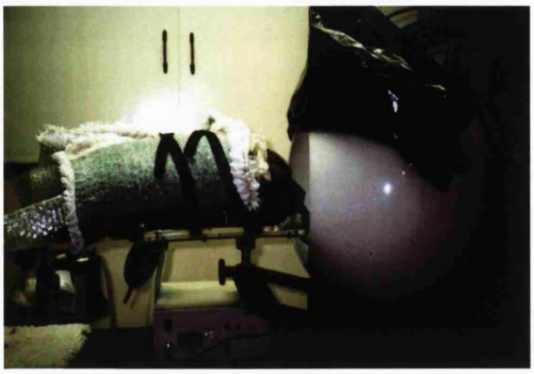

Figure A3b: Subject secured in sliding table before placement of electrodes

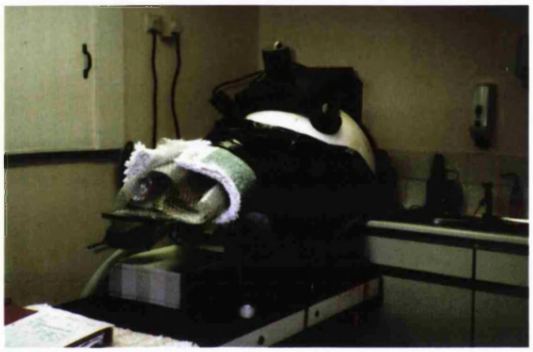

Figure A3c: Subject in position within the ganzfeld ready for recording

Figure A3d: The ERG recording machine. From left, monitor connected to the infra-red camera within the ganzfeld to monitor the position and condition of the subject, the Grass stimulator and amplifier, and the PC based data acquisition system

Appendix B: Publications and Presentations

B1 Published articles

- NHV Chong, RA Alexander, L Waters, KC Barnett, AC Bird, PJ Luthert. **Repeated intravitreal injections of ciliary neurotrophic factor lead to long-term photoreceptor cells survival in hereditary retinal degeneration.** ***Investigative Ophthalmology & Visual Science 1999; 40: 1298-1305***
- NHV Chong, RA Alexander, KC Barnett, AC Bird, PJ Luthert. **An immunohistochemical study of an autosomal dominant feline rod / cone dysplasia (Rdy cats).** ***Experimental Eye Research 1999; 68(1); 51-7***
- NHV Chong, AC Bird. **Current and future management of inherited outer retinal dystrophies.** ***British Journal of Ophthalmology, 1999, 83; 120-22.***
- PJ Luthert, NHV Chong. **Photoreceptor rescue.** ***Eye 1998; 12: 591-596.***

B2 Oral and poster presentations with printed abstracts

- NHV Chong, L Waters, CR Hogg, KC Barnett, AC Bird, GE Holder, PJ Luthert. **Axokine suppresses electroretinogram temporarily in retinal degeneration.** ***Investigative Ophthalmology & Visual Science (Supple) 2000 Vol 41, 4726 S889***
- NHV Chong, RA Alexander, AS Kwan, PJ Luthert. **Caspases inhibitors do not consistently slow photoreceptor cell loss in retinal degeneration.** ***Investigative Ophthalmology & Visual Science (Supple) 1999 Vol 40, 3789 S717.***
- NHV Chong, AC Bird, PJ Luthert. **Apoptosis manipulation as a therapeutic approach to retinal degeneration**. Oral Presentation in the RCOphth Annual Congress 1999

- NHV Chong, RA Alexander, L Waters, KC Barnett, AC Bird, PJ Luthert. **CNTF analogue, but not BDNF, delays photoreceptor cell death in retinal degeneration.** ***Investigative Ophthalmology & Visual Science (Supple) 1998 Vol 39, 2689***
- NHV Chong, L Waters, KC Barnett, AC Bird, PJ Luthert. **Axokine delays photoreceptor cell death in Rdy cats.** ***Investigative Ophthalmology & Visual Science (Supple) 1997 Vol 38, 1445***
- NHV Chong, AC Bird, KC Barnett, PJ Luthert. **Apoptosis in a feline model of retinal dystrophy (Rdy).** Poster Presentation in the RCOphth Annual Congress 1997
- PJ Luthert, NHV Chong, L Waters, CR Hogg, KC Barnett, AC Bird, GE Holder. **Pathological and electrophysiological features of a recessive canine cone-rod dystrophy** ***Investigative Ophthalmology & Visual Science (Supple) 2000 Vol 41, 46 S9***
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PERSPECTIVE

Management of inherited outer retinal dystrophies: present and future

N H Victor Chong, Alan C Bird

The inherited outer retinal dystrophies comprise a large number of disorders characterised by a slow and progressive retinal degeneration. They have been arbitrarily divided into macular dystrophies, retinitis pigmentosa (RP), and cone/rod dystrophies on the basis of their phenotype. They are the result of mutations in genes that are presumed to express in either the photoreceptor cells or the retinal pigment epithelium.[1] Retinitis pigmentosa is believed to affect about one in 3500 of the population,[2] and the prevalence of remaining dystrophies is probably similar. The mode of inheritance can be autosomal dominant, autosomal recessive, X linked recessive,[3] or digenic.[4 5]

With few exceptions such as α,β lipoproteinaemia[6 7] and Refsum disease,[8] there is no treatment by which the primary disorder can be modified. Some symptomatic relief may be derived from cataract extraction,[9] and carbonic anhydrase inhibition if there is macular oedema.[10] Vitamin A supplementation may slow the progression although this has been the subject of controversy.[11 12] Although there is no treatment by which the primary disorder can be manipulated, the patients can expect a reasonable account of visual prognosis, accurate genetic counselling, and support for rehabilitation. In addition, those affected are also keen to know the progress in research for a cure particularly as they have become progressively more aware of the potential for treatment through the various self help associations such as the British Retinitis Pigmentosa Society, the internet, and the press.

Researchers around the world are working on different potential therapeutic approaches to the problem. These are based on the possibility of modulation of cell death by growth factors, transfecting the photoreceptor or retinal pigment epithelial cell with functioning genes, transplanting photoreceptor or retinal pigment epithelial cells into the subretinal space, or the use of electronic devices to stimulate the retina.

Apoptosis and its manipulation

In some disorders in both humans and animals there is increasing evidence that the metabolic defect consequent upon the mutation causes cell death by apoptosis. This accounts for cone loss in patients with RP due to rhodopsin mutation.[13] This is also illustrated by loss of photoreceptors in the RCS (Royal College of Surgeons) rat in which the primary defect is in the retinal pigment epithelium.[14]

Apoptosis, or programmed cell death, is a genetically encoded potential of all cells, and is an essential part of embryonic development, cell turnover, and of removal of cells infected by virus or harbouring mutations.[15 16] Morphologically, it is characterised by disintegration of the nucleolus and generalised condensation of the chromatin. This is due to incision of nuclear DNA by an endogenous nuclease into short chains in multiples of 180 base pairs. In contrast with necrosis the process affects isolated cells with healthy neighbouring cells and in the absence of acute inflammation.

The observation that apoptosis may be manipulated by growth factors suggested an alternative therapeutic approach. The first indication that this may be viable was the demonstration that injection of basic fibroblast growth factor (bFGF) into the eye resulted in long term survival of photoreceptors in the RCS rat.[17] A potential disadvantage of bFGF is its lack of specificity since it influences many cell types. For instance, in the rabbit it can cause cataract and proliferative vitreoretinopathy.[18] Neurotrophins, including brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin 4/5 (NT4/5) appear to be more attractive since they have a relatively specific influence on neural tissue.[19] They are present in the CNS, and play important roles in neural development, differentiation, and survival.[19] Another important neurotrophic factor is the ciliary neurotrophic factor (CNTF) which is closely related to interleukin 6.[20] There is an increasing body of experimental work that illustrates the potential value of this approach. Intravitreal injections of BDNF, CNTF, or bFGF transiently protect the retina from pressure induced ischaemic injury.[21] Both BDNF and CNTF protect the retina from light damage in albino rats and mice.[22 23] BDNF also promotes the regrowth of outer segments of photoreceptors in a feline retinal detachment model.[24] CNTF delays photoreceptor degeneration in rd (retinal degeneration) mice,[23] transgenic rhodopsin Q344ter mutant mice,[23] and Rdy (retinal dystrophy) cats.[25]

This form of treatment of retinal degeneration has the advantage that it may be effective in disease with unknown genetic mutation. It is unfortunate that the effect of the neurotrophic factors is relatively short lived, lasting only about a month in animals[22 23] although the effect may be much longer in humans. Repeated monthly intravitreal injections would present ergonomic difficulties, and inevitably be accompanied by attendant risks such as retinal detachment and endophthalmitis. Biological and biochemical slow delivery systems are being developed that may overcome these problems.[26]

Gene therapy

In most recessive and some dominant diseases, cell dysfunction is due to lack of functioning genes, and the objective of therapy is the replacement of the defective gene with genes that express normally.

In the retinal degeneration slow (rds) mice, transfection of the fertilised ovum with the appropriate wild type (normal) gene results in photoreceptor cell rescue.[27] However, insertion of genes into non-dividing photoreceptor cells in human is a much greater challenge. There are two general approaches by which genes can be introduced into the eye,

ex vivo or in vivo. In the ex vivo techniques, the genes are introduced in vitro into retinal cells, retinal pigment epithelial (RPE) cells, or fibroblasts. The transfected cells are then injected into the eye. In vivo, or direct gene transfer, genes are introduced into the cells in situ using a vector. The most commonly used gene vectors are the replication defective herpes simplex virus, the replication defective adenovirus, adeno-associated virus (AAV), and retrovirus. Each has advantages and disadvantages.

Herpes virus can carry a large gene insert (up to 36 kb). However, it rapidly undergoes latency after infection restricting the long term expression of the transgenes. This drawback can be overcome by using one of the latency promoters as the helper for transgene expression so it remains active in latency. A cytotoxic response may occur in the target cells that would reduce the longevity of expression.[28] Furthermore, the wild type herpes virus carries a significant morbidity in humans. The adenovirus is a relatively safe virus. However, it induces an immune response that prevents reinfection by the same virus, and cytotoxic effect on the target cells reducing expression longevity.[29] The current capacity for a gene insert is relatively small (about 8 kb), but with development of new packaging cell lines, a larger insert might be achieved. However, transfection efficiency in general is low in that only a small proportion of cells are transfected and expression is brief. The AAV is an attractive alternative as it is not associated with any pathological response in humans. The recombinant AAV vectors have no virally encoded proteins and hence less immunogenicity. They can infect a variety of cells, and are more efficient in transducing photoreceptors than adenovirus. Wild type AAV integrates into chromosome 19, while recombinant AAV may integrate randomly. The latter has the potential risk of disrupting essential genes or causing malignant transformation of the target cells, although there is little evidence of this to date. Chromosomal integration may serve to prolong expression. The maximum size of insert is only 4.7 kb, and it is difficult to prepare high titres without contamination of helper adenovirus.[29] Retrovirus can only infect dividing cells, which restricts its use in the retina.

The most promising observation to date is the reported delay of photoreceptor cell death in the rd mice (b-PDE defect) by the use of subretinal injection of a recombinant replication defective adenovirus that contains the murine cDNA for the wide type b-PDE. It is reported that these injections result in b-PDE transcription, and increased PDE activity, although the rescue only lasted for 6 weeks.[30] In all these observations, there must always be doubt as to whether the therapeutic effect is due to the expression of the gene or to injury with the attendant release of growth factors or the immune response to the viral vectors.

On the other hand, in most dominant diseases it is likely that the abnormal gene product produces disease. An antisense protein would be required to negate the influence of the abnormal gene. However, antisense molecules are often too big to discriminate wild type and mutant mRNA in point mutation, as seen in much autosomal dominant RP.[1 31] Recently, ribozymes present new promises. They can bind and digest targeted mRNA. Specifically designed ribozymes can discriminate between the mutant and wild type sequences of mRNA associated with autosomal dominant RP.[31] Furthermore, using an AAV vector, ribozymes rescue photoreceptor cells in the transgenic rhodopsin P23H mutant rats.[32]

Gene therapy could also be used to deliver neurotrophic factor.[33] These approaches can also be combined by reintroduction of genetic modified host cells expressing the neurotrophic factors. The longevity of expression remains the problem, but with ex vivo transfection there is a wide scope for manipulation of expression.

Retinal cell transplantation

The ultimate goal of transplantation is to replace lost photoreceptors with healthy ones, which would have the capacity to re-establish the appropriate cellular connections at the outer plexiform layer. Cell survival appears to be related to donor age. Transplanted fetal tissue appears to survive and differentiate, but fails to show normal orientation in the retina.[34] Enzymatic dissociated adult photoreceptor cells can be transplanted by injection into the subretinal space. Such photoreceptors survive and appear to have synaptic terminals but their outer segments degenerate almost completely.[35 36] However, the functional attributes of these cells have not been tested since the visually directed behaviour of these animals was not assessed. There is also some evidence to suggest that cones survived longer following transplantation in the rd mouse.[37] This might reflect the influence of trophic factors.

Despite the paucity of evidence that this technique may be useful, mechanically dissociated fetal retina of 14–18 weeks' gestation have been grafted in 12 patients with advanced RP. The preoperative visual acuity was perception of light or worse. No rejection or complications were reported. Five patients had reported subjective improvement of vision.[38] However, it is impossible to exclude the placebo effect or the influence of injury. Furthermore, no improvement of vision was found in two patients transplanted with a sheet of adult photoreceptors harvested by vibratome.[39]

Another strategy is the transplantation of RPE cells. In the RCS rat, the RPE failed to phagocytose photoreceptor outer segment material. This accumulation of outer segment material results in photoreceptor cell death.[14] Transplantation of healthy RPE cells into subretinal space delayed photoreceptor loss and retinal function, as measured by electroretinogram and pupillary light reflex, is restored following RPE cell grafts in the RCS rats.[40–43] However, this may have limited relevance to most human forms of retinal dystrophy, although it may have a role in slowing the progress of age related macular degeneration. The results of human fetal RPE transplantation in 13 patients with age related macular degeneration were recently reported.[44] No visual improvement was observed but the graft was reported to survive in most of the patients.

Artificial vision

It is well known that stimulation of the appropriate area of the visual cortex causes a visual sensation. Some scientists are working on devices that can detect visual signals, and stimulate the visual cortex directly. Thus, no viable eye tissue are required, but the signal bypasses the sophisticated coding of the visual system, and the sensations are likely to be complex depending upon the functional attributes of the cell stimulated. This renders the potential success of this approach problematic.

There has been extensive media coverage of the artificial retinal implant for blind people. The concept is based on the fact that in RP, the photoreceptors are damaged but the inner retina remains relatively healthy. Electrical stimulation of the inner surface of rabbit retina was the first demonstration of the possibilities of retinal implants.[45] These experiments were later carried out in humans. Subsequently, intraocular electrodes coupled with an optically isolated, constant current generator were used to deliver the pulses. This was performed in five patients with bare or no perception of light (three had RP, one had age related macular degeneration, and one unspecified retinal

dystrophy from birth). Stimulation elicited a visual perception of a spot that was retinotopically correct in four subjects who had had previously useful vision. One subject resolved two simultaneous spots giving a visual acuity of about 1/60.[46] However, the methods for permanent implantation of such devices, and their connection with the outside world may pose major technical problems. Furthermore, the number of stimulating electrodes and the visual field covered are likely to be very limited. Even if these problems are resolved, it is unlikely to simulate foveal function without the presence of photoreceptors.

Conclusion

We are still a long way from the cure of retinitis pigmentosa, although it is encouraging to the patients and their carers that there is a worldwide effort to devise new methods of management. Although none of the techniques appear to be very effective for long term modification of the course of the disorder, the vast majority of retinal dystrophies are slowly progressive, and many patients retain good visual function for a large part of their lives. A small change in the metabolic environment of the retina may have a great influence on the dynamics of the degeneration.

In the short term (5–10 years), the use of growth factor is possibly the most promising approach, although in the long term it may not be the best solution. Transplantation might be useful to preserve central vision while the artificial retinal implant might be useful to maintain visual field. Gene therapy might ultimately be the best treatment, but currently it is an immature genie yet to deliver its promises.

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An Immunohistochemical Study of an Autosomal Dominant Feline Rod/Cone Dysplasia (Rdy Cats)

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An autosomal dominant, early onset feline model of rod/cone dysplasia has been described. The clinical features, light and electron microscopy, and the electrophysiology were documented. We have now examined in more detail the histopathological and immunohistochemical changes during the early phase of the disease using antibodies against opsin, synaptophysin, glial fibrillary acidic protein (GFAP) and an epithelial marker (MNF118). We have also demonstrated programmed cell death by a modified TUNEL (Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labelling) technique.

In the Rdy cats, there was significant photoreceptor degeneration between 5 and 17 weeks of age. The TUNEL-labeled cell and pyknotic cell counts in the outer nuclear layer peaked at around 9 weeks of age. Accumulation of opsin in the entire outer nuclear layer of the retina was noted with opsin-immunolabeled rod neurite sprouting. There was a reduction in synaptophysin immunoreactivity in the outer plexiform layer. The Muller cells were activated and expressed GFAP. No significant change of immunolabeling of MNF118 was found. These findings closely parallel those seen in human RP.

Key words: animal model; retinal degeneration; immunohistochemistry; apoptosis.

1. Introduction

Retinitis pigmentosa (RP) is a group of hereditary retinal degeneration characterised by poor night vision and restricted visual fields (Bird, 1995). It has a prevalence of about 1 in 3500 (Berson, 1993) and is one of the leading causes of working age blindness in the developed world (Evans et al., 1996). There is currently no effective treatment.

An autosomal dominant early onset feline model of retinal degeneration has been described previously (Barnett and Curtis, 1985; Curtis, Barnett and Leon, 1987; Leon and Curtis, 1990; Leon, Hussain and Curtis, 1991). Clinically, affected kittens slow dilated pupils and sluggish pupillary light reflexes from 2 weeks of age. Vision is impaired and an intermittent rotatory nystagmus develops between 4 and 6 weeks of age. The first signs of fundal abnormalities are present at 8 to 12 weeks with increased tapetal reflexes. This is accompanied by progressive attenuation of retinal vessels and optic atrophy.

In order to further characterize this model of retinal degeneration, in the current investigation, the immunohistochemical changes in the retina and photoreceptor cell death are studied using markers of apoptosis.

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2. Materials and Methods

Affected kittens ($n = 32$) aged between 10 days to 17·5 weeks were examined with normal littermates ($n = 8$) aged at 10 days to 13·5 weeks for comparison. The animals were bred and studied under the regulation of the United Kingdom Animals (Scientific Procedures) Act 1986 and all animal procedures adhered to the ARVO resolution for the Care and Use of Animals in Vision Research. All animals were killed with an overdose of systemic phenobarbitone, both eyes were enucleated immediately but only one eye per animal was used in this study. After 24–48 h of immersion fixation in 10% formol saline, the inferior-nasal quadrant was embedded in paraffin wax for immunohistochemistry and TUNEL labeling whilst the superio-nasal quadrant was embedded in Araldite resin using standard protocols.

Photoreceptor Cell Counts

Photoreceptor cell counts were performed in 1 μm thick toluidine blue stained Araldite resin sections. A random location near the centre of the section was picked at low magnification. The section was then moved 550 μm from the random spot. An eye-piece graticule (Graticules Ltd, Tonbridge, England) was employed to obtain a retinal strip of 25 μm wide. The number of photoreceptor cells within this strip was counted. The section was then moved 250 μm toward to the centre, and another retinal strip of 25 μm wide was counted. This was repeated twice to obtain a total

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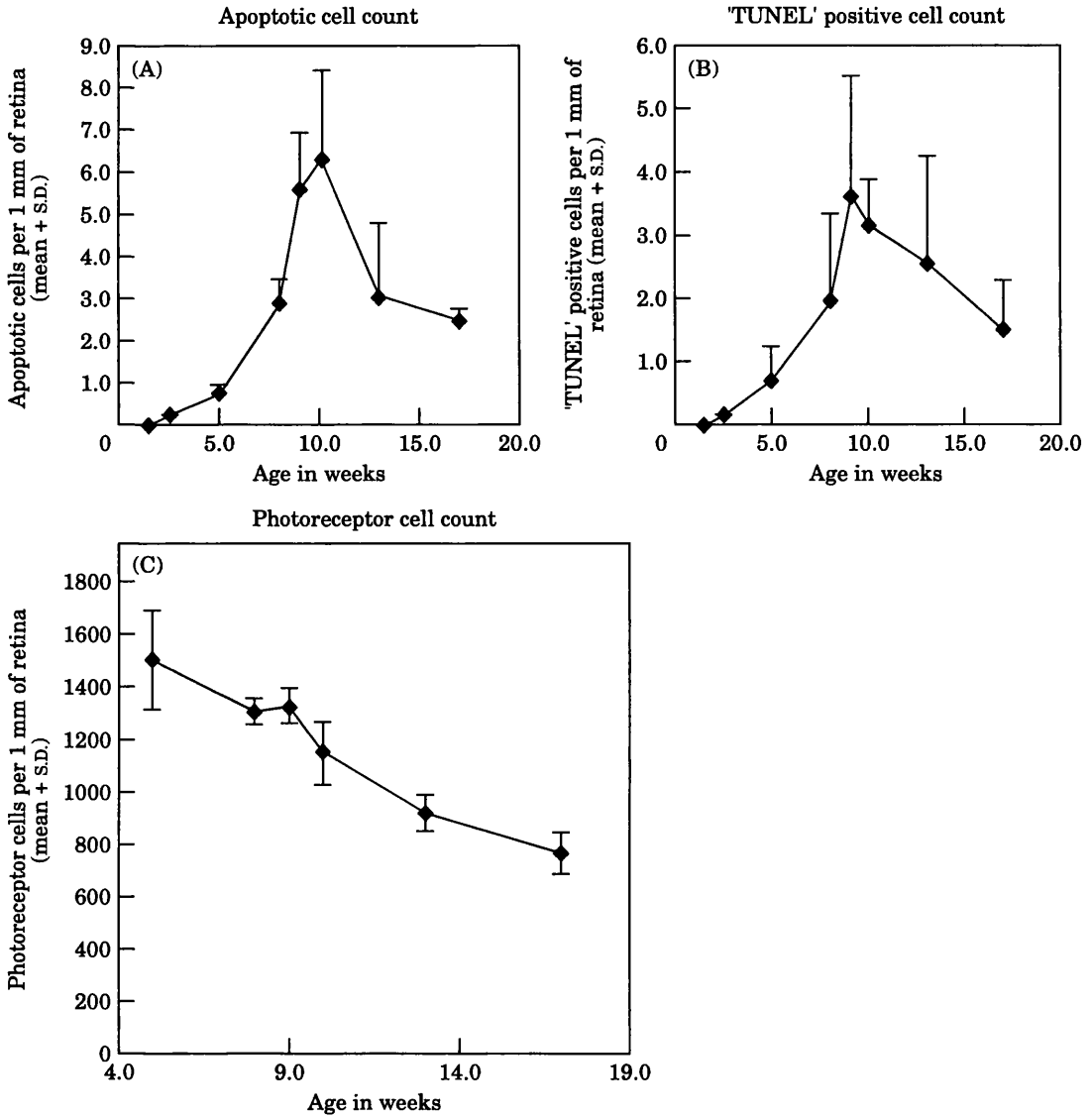

FIG. 1. (A) Pyknotic cell count in the nuclear layer (Mean+S.D.); (B) TUNEL-labeled cell count in the outer nuclear layer (Mean+SD); (C) Photoreceptor cell count (Mean±S.D.) per 1 mm of retina in the Rdy cats.

of 4 readings. A total of 100 μm of retinal width was counted. The number of photoreceptor cells counted was then multiplied by 10 and expressed as the number of cells per 1 mm of retina.

Demonstration of Programmed Cell Death

The pyknotic appearance and the presence of double strand breaks in DNA suggest programmed cell death (PCD). In this study, we examine these two aspects of PCD in the Rdy cats. A modified TUNEL (Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labeling) technique was used to detect double strand breaks in DNA. This technique is based on that described by Nakamura and colleagues (Nakamura, Sakai and Hotchi, 1995), but dAdenosine triphosphate replaces dUridine triphosphate. Briefly, the tissue sections were dewaxed with xylene and rehydrated through a graded series of alcohol. Endogenous peroxidase activity was blocked by 0·5% hydrogen peroxide for 25 minutes in the dark at room temperature. Tissue sections were treated with 20 μg ml^{-1} of proteinase K in phosphate buffered saline (PBS) at room temperature for 15 minutes followed by PBS wash. They were buffered with terminal deoxynucleotidyl transferase (TdT) buffer for 10 minutes followed by labeling reaction with 10 mM of dAdenosine triphosphate (dATP), 0·4 mM of biotin 14 dATP and TdT for 60 minutes at 37°C. The positively labeled cells were demonstrated by a standard biotin-streptavidin peroxidase method visualised by diaminobenzidine (DAB). TUNEL-labeled cells were counted in the entire tissue section. The result was expressed as the number of TUNEL-labeled cell per 1 mm of retina.

Pyknotic cells were counted in 1 μm thick toluidine blue stained Araldite resin sections. The result was expressed as the number of pyknotic cells per 1 mm of

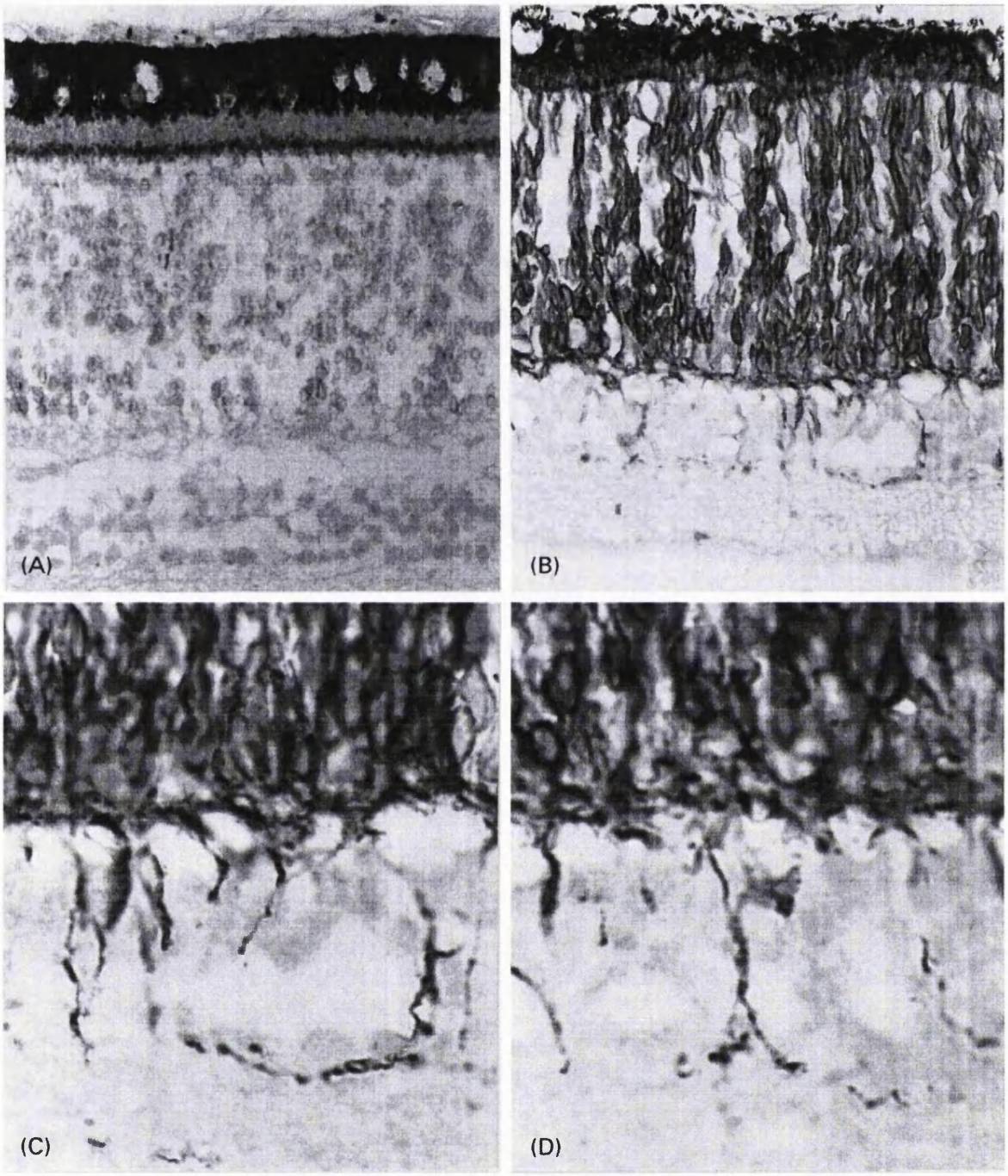

FIG. 2. Opsin immunolabeling. (A) Normal cat at 13·5 weeks × 400; (B) Rdy cat at 13·5 weeks × 400; (C) Rdy cat at 13·5 weeks × 1000; (D) Rdy cat at 13·5 weeks × 1000.

retina. TUNEL-labeled and pyknotic cell were counted by two independent observers and the mean values were calculated.

Immunohistochemistry

The distribution of opsin (antiserum courtesy of Dr D. Bok), synaptophysin, glial fibrillary acidic protein (GFAP) immunoreactivity, and binding of an epithelial marker MNF 118 was investigated using a standard biotin-streptavidin peroxidase method (all antibodies, unless otherwise stated, were obtained from Dako Ltd, England). Antigen retrieval pre-treatment with trypsin was performed prior to all primary antibody incubations (Ordonez, Manning and Brookes, 1988). Appropriate positive and negative controls were used throughout.

3. Results

A small number of TUNEL-labeled cells and pyknotic cells was found in the outer nuclear layer of both normal and affected animals before 5 weeks of age and no significant difference was observed at these time points. In the affected animals, the number of TUNEL-

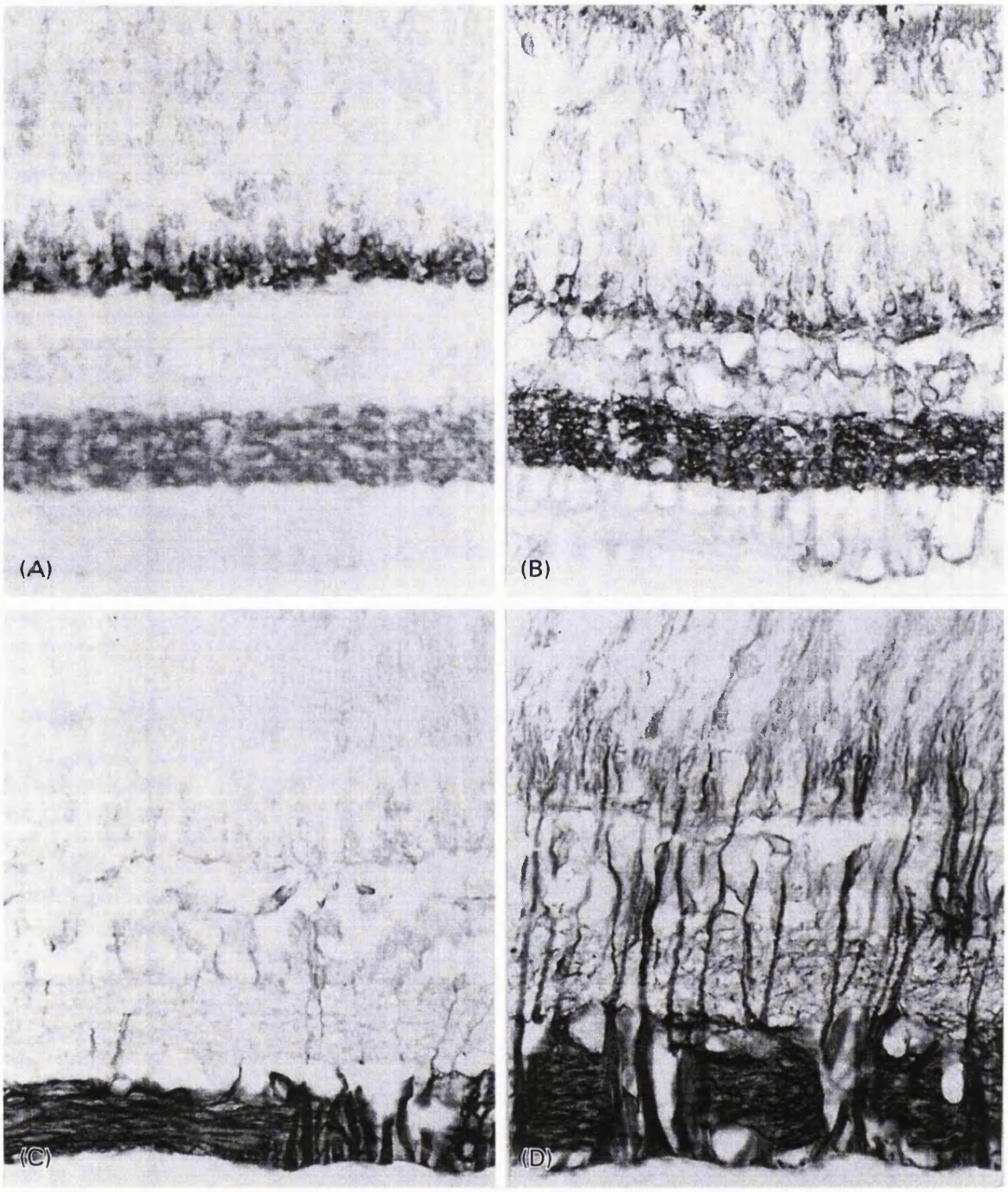

FIG. 3. Synaptophysin and glial fibrillary acidic protein (GFAP) immunolabeling × 400. (A) Normal cat at 13·5 weeks with synaptophysin immunolabeling; (B) Rdy cat at 13·5 weeks with synaptophysin immunolabeling; (C) Normal cat at 13·5 weeks with GFAP immunolabeling; (D) Rdy cat at 13·5 weeks with GFAP immunolabeling.

labeled cells and pyknotic cells increased and peaked around 9–10 weeks of age but none were seen in the controls after 5 weeks. The results of pyknotic cell count and TUNEL positive cell count of the outer nuclear layer (photoreceptors) are summarised in Figs 1(A) and 1(B) respectively.There were no significant differences between the normal and affected animals in the inner nuclear layer and ganglion cell layer of the retina (data not shown). Inter-observer variability was less than 10% for both the pyknotic cell and TUNEL counting. Photoreceptor cell counts showed significant cell loss between 5 and 17 weeks [Fig. 1(C)].

Between 5 and 17 weeks, there was about 700 photoreceptor cells were lost per mm of retina, which was equivalent to an average cell loss of 8·3 cells per day per mm. Over the same period of time, the average TUNEL positive cell count was about 2·5 cells per mm. Hence, the estimated time for the cells to be TUNEL positive was about 8 hr. Using similar calculation, the estimated time for the cells to appear pyknotic morphologically was about 12 hr.

Immunohistochemical staining of opsin was almost entirely restricted to the rod outer segments (ROS) in the normal controls [Fig. 2(A)]. In the Rdy cats, opsin was present in both rod outer and inner segments and there was also accumulation of opsin in the outer nuclear layer and within the presumed photoreceptor cell synaptic terminals in the outer plexiform layer [Fig. 2(B). Some of the rods had sprouted opsin-positive neurites extending towards the ganglion cell layer [Fig. 2(C)] and some had branches [Fig. 2(D)].

Synaptophysin is a synaptic vesicle protein. In the controls, immunolabeling with anti-synaptophysin was largely restricted to the outer and inner plexiform layers [Fig. 3(A)]. In the Rdy cats, the immunolabeling of the outer plexiform layer reduced with age and in parallel with the reduction of photoreceptor cells. No significant change in labeling of the inner plexiform layer with anti-synaptophysin was seen [Fig. 3(B)].

GFAP was expressed predominantly by the astrocytes in the normal controls [Fig. 3(C)]. In the Rdy cats, markedly increased immunostaining of GFAP in Muller cells was observed at all time points [Fig. 3(D)]. Retinal pigment epithelial cell changes were not seen (data not shown).

4. Discussion

PCD is a genetically encoded potential of all cells, and is an essential part of embryonic development, cell turnover and of removal of cells infected by virus or harbouring mutations (Raff, 1992). It is characterised morphologically by disintegration of the nucleolus and generalised condensation of the chromatin, associated with cleavage by an endogenous nuclease of nuclear DNA into short chains of nucleosomes in multiples of 180 base pairs (Gavrieli, Sherman and Ben-Sasson, 1992). Condensed nuclei then fragment, giving rise to apoptotic bodies. In contrast to necrosis, the process affects individual cells within a tissue, neighbouring cells remain healthy, and it takes place in the absence of acute inflammation.

In all models of hereditary retinal dystrophy examined to date, including human RP, cell loss is through the process of PCD (Chang et al., 1993; Portera-Cailliau et al., 1994; Tso et al., 1994). The TUNEL technique has been used to demonstrate PCD in the retinal degeneration (rd), retinal degeneration slow (rds), transgenic Q344ter rhodopsin mutant mice (Chang, Hao and Wong 1993; Portera-Cailliau et al., 1994), RCS rats (Tso et al., 1994) and in human RP (Li and Milam, 1995). It labels the 3′ ends of DNA fragments in nuclei going through the process of PCD in situ (Gavrieli, Sherman and Ben-Sasson, 1992; Nakamura, Sakai and Hotchi, 1995). This technique is more sensitive than the agarose gel electrophoretic analysis of genomic DNA for internucleosomal DNA cleavage (Tso et al., 1994). The nuclei of these cells are, however, TUNEL-labeled for only a short period of time, about 8 h in this model.

It is believed that TUNEL-labeled cells represent an early stage of PCD. This is followed by the condensation of the cytoplasm and nuclear chromatin. In this study, TUNEL-labeled cell counts generally mirrored the pyknotic cell count. It seems, however, that the TUNEL-labeled cell count peaked slightly earlier at 9 weeks as compared to 10 weeks for pyknotic cell count. There were also fewer TUNEL-labeled cells than pyknotic cells.

Although the photoreceptor degeneration starts at about 5 weeks, the maximal photoreceptor loss was between 9 to 13 weeks. PCD was maximal at the onset of maximal cell loss, consistent with our findings. This indicates that PCD is the main mode of cell death in this model of retinal degeneration. As the photoreceptor degeneration presents early, this would be a useful model for the study of PCD manipulation and therapeutic intervention.

Opsin immunolabeling is normally largely restricted to the rod outer segments (ROS). In the Rdy cats, opsin was also present in the rod inner segments and around the cell perikarya. Similar findings have previously been observed in rodent models of retinal degeneration including the rd mice, rds mice, RCS rats and mice with human P23H rhodopsin transgene (Ishiguro, 1987; Nir et al., 1989; Nir and Papermaster, 1989; Roof, Adamian and Hayes, 1994) as well as in human retinitis pigmentosa (Li, Kljavin and Milam, 1995).

In addition to this abnormal perikaryal opsin labeling, the transgenic rhodopsin P23H mutant mouse has accumulation of opsin throughout the outer plexiform layer (Roof, Adamian and Hayes, 1994). This extensive immunoreactivity was not present in the Rdy cats but there were opsin positive rod neurites sprouting towards the ganglion cell layer. This phenomenon has been described in human retinitis pigmentosa (Li, Kljavin and Milam, 1995; Milam, Cideciyan and Jacobson, 1996) but has not been observed in any of the rodent models of retinal degeneration examined to date (Li, Kljavin and Milam, 1995). It has been suggested that the rapid photoreceptor degeneration in the rodents leaves little time for the rod to attempt making new connections, but with slower retinal degeneration in the human, this is a possibility. The precise mechanism of this phenomenon is unknown. It seems likely, however, to reflect changes in the post-synaptic sites on horizontal or bipolar cells. Furthermore, the significance of this phenomenon to the pathogenesis of the disease and its therapeutic implications remain unknown.

The reduction of synaptophysin immunolabeling in the outer plexiform layer of the Rdy cats is also found in other rodent models of retinal degeneration, including the RCS rat (Sheedlo et al., 1993). No significant changes in the inner plexiform layer were expected as there was no significant cell death in the inner nuclear and ganglion cell layers as compared to normal controls, until end-stage disease (Curtis, Barnett and Leon, 1987; Leon and Curtis, 1990). This

finding parallels that observed in moderate human retinitis pigmentosa, where 88% of the inner nuclear layer remained intact (Santos et al., 1997).

GFAP is the major component of glial intermediate filaments found in astrocytes. In the retina, GFAP is present only in astrocytes and Muller cells. However, Muller cells increase GFAP expression in response to focal or generalised retinal injury, such as laser photocoagulation (Humphrey et al., 1993) and light damage (Eisenfeld, Bunt Milam and Sarthy, 1984) as well as in genetically determined retinal degeneration, such as the RCS rats (Eisenfeld, Bunt Milam and Sarthy, 1984), the recessive feline model of retinal degeneration (Ekstrom et al., 1988) and human retinitis pigmentosa (Milam and Jacobson, 1990).

There is evidence to suggest that the increase of GFAP expression is mediated by growth factors. In focal retinal injury, there is a lag period of about 24 hr before a significant increase of expression is seen (Humphrey et al., 1993) suggesting the injury does not directly increase the transcription of the GFAP gene. The delay suggests one or more intermediate steps. Furthermore, the normal area adjacent to the focal injury also has increased GFAP expression. This would be explained by diffusion of one or more growth factors from the site of focal injury (de Raad et al., 1996). This hypothesis is further supported by the increased expression of basic fibroblast growth factor (bFGF) after retinal injury (Wen et al., 1995). Different growth factors can also have different effects on the Muller cells. For instance, intravitreal injection of bFGF induces GFAP immunoreactivity in Muller cells of normal eyes (Lewis et al., 1992) but intravitreal injection of brain derived neurotrophic factor reduces GFAP expression in a feline model of retinal detachment (Lewis and Fisher, 1997).

This glial reaction might reflect attempted photoreceptor rescue during retinal injury. However, these activated glial cells also induce scar formation and might even produce neurotoxins. It would be of great interest to investigate further whether this glial reaction is of benefit to the retina or not.

In comparison to rodent eyes, not only are cat eyes larger in size, the well characterised visual neurophysiology of cats will be of value in the assessment of pathophysiology and efficacy of novel therapeutic agents. The findings in the Rdy cats closely parallel those seen in human RP.

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Repeated Injections of a Ciliary Neurotrophic Factor Analogue Leading to Long-term Photoreceptor Survival in Hereditary Retinal Degeneration

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Purpose. To determine whether ciliary neurotrophic factor (CNTF) or brain-derived neurotrophic factor (BDNF) treatment leads to long-term photoreceptor survival in hereditary retinal degeneration.

Methods. An autosomal dominant feline model of rod-cone dystrophy was used throughout the study with two normal animals. In the first experiment, intravitreal injections of a human CNTF analogue (Axokine; Regeneron Pharmaceuticals, Tarrytown, NY) were administered to one eye of each animal (n = 10) beginning on postnatal day 10 and were repeated every 4 weeks. Clinical and histopathologic examinations were performed at 5.5, 9.5, and 13.5 weeks. In the second experiment, animals (n = 17) were randomly assigned to receive intravitreal injections of either Axokine (at half the initial dose), human BDNF, or the vehicle for Axokine to one eye at 5.5 weeks. The same therapy was repeated every 4 weeks in each group. Clinical and histopathologic examinations were performed at 9.5, 13.5, and 17.5 weeks. Photoreceptor survival was assessed by cell counting. Apoptotic cells were identified by morphology and a modified TdT-dUTP terminal nick-end labeling (TUNEL) technique. In the third experiment, two normal animals were treated with Axokine as in the first experiment. Glial fibrillary acidic protein (GFAP) immunohistochemistry was performed to assess glial cell reaction.

Results. In the first two experiments, Axokine significantly prolonged photoreceptor survival ($P < 0.01$) and reduced the presence of apoptotic cells ($P < 0.05$) and TUNEL-positive cells ($P < 0.05$). In the second experiment, results in the the BDNF- and sham-injected eyes were not significantly different from those in the untreated eyes. Minimal posterior subcapsular cataract and mild retinal folds were found in all Axokine-treated eyes in both dystrophic and normal animals. These complications were milder in the second experiment when injections were started later and at a reduced dose. GFAP immunolabeling was also increased in all Axokine-treated eyes.

Conclusions. Axokine, but not BDNF, delays photoreceptor loss in this hereditary retinal degeneration. Repeated injections maintain the protective effect. (*Invest Ophthalmol Vis Sci.* 1999;40:1298-1305)

Retinitis pigmentosa (RP) is a group of hereditary disorders involving retinal degeneration characterized by poor night vision and restricted visual field.[1] It affects approximately 1 in 3500 people.[2] There is currently no effective treatment, and it is one of the leading causes of working-age blindness in the developed world.[3] Despite the increasing number of disease-causing mutations that have been identified, the pathophysiology of photoreceptor death in this group of conditions is largely unknown.[4]

There is convincing evidence that the genetic defects can lead indirectly to cell death. This is evident, for example, in cone loss in patients with retinitis pigmentosa due to mutations in the *rhodopsin* gene, which is expressed only in rods.[5] It is of interest that in these and other retinal degenerations photoreceptor cell death occurs by apoptosis.[4-9] These observations imply that therapeutic modification of the process leading to apoptosis or the apoptotic pathway itself may modify the course of these disorders.

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TABLE 1. Apoptotic Cell Count and TUNEL-Positive Cell Count

	5.5* (n = 3)	9.5 (n = 4)	13.5 (n = 3)
First experiment: Axokine-treated versus untreated eyes			
Apoptotic cell count			
Axokine	0.60 ± 0.16	1.08 ± 1.02†	2.16 ± 0.58
Untreated	0.70 ± 0.14	5.92 ± 2.98	3.30 ± 2.74
TUNEL Count			
Axokine	0.16 ± 0.24	0.52 ± 0.28†	0.74 ± 0.38†
Untreated	0.64 ± 0.60	2.16 ± 1.04	1.72 ± 0.64
	9.5*	**13.5**	**17.5**
Second experiment: Axokine versus BDNF versus vehicle versus no treatment			
Apoptotic cell count			
Axokine†	2.76 (1)	1.62 ± 0.18 (2)	2.32 ± 0.98 (3)
BDNF	5.26 (1)	1.66 ± 1.26 (2)	2.24 ± 0.64 (2)
Vehicle	5.5 ± 1.54 (2)	2.72 ± 0.30 (2)	3.54 ± 1.16 (2)
Untreated	5.3 ± 1.08 (4)	2.86 ± 1.16 (6)	3.16 ± 0.90 (7)
TUNEL count			
Axokine†	3.08 (1)	0.48 ± 0.02 (2)	0.80 ± 0.54 (3)
BDNF	5.10 (1)	1.86 ± 0.44 (2)	1.70 ± 0.54 (2)
Vehicle	6.60 ± 0.84 (2)	3.74 ± 2.08 (2)	2.16 ± 0.18 (2)
Untreated	6.34 ± 0.64 (4)	2.52 ± 1.54 (6)	1.78 ± 0.60 (7)

All data are means ± SD cell counts in 1 mm of retina with sample number in parentheses.
* Age in weeks.
† $P < 0.05$, treated versus untreated eyes.

An unexpected finding was observed during the early attempts at retinal transplantation for photoreceptor rescue. Focal injury to the retina appears to protect nearby photoreceptors from degeneration. This was clearly illustrated in the Royal College of Surgeons (RCS) rat in which mechanical injury produced by an injection of saline into the subretinal space or into the vitreous or even insertion of a needle without injection led to protection of photoreceptors near the wound.[10] This protection is not restricted to genetically determined retinal degeneration. Similar photoreceptor rescue by mechanical injury was observed in light-induced retinal damage in the rat.[11] Injury-induced photoreceptor rescue extends beyond the immediate vicinity of the lesion, suggesting that diffusible factors may be involved.[10,11] Because mechanical injury to the eye increases the expression of basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) in the rat retina,[12] it is logical to assume that these agents may be responsible, at least in part, for this protection.

A single intravitreal injection of bFGF delayed photoreceptor loss in the RCS rat.[13] Subsequently, bFGF, CNTF, and brain-derived neurotrophic factor (BDNF) were found to protect photoreceptors from light-induced retinal damage in the rat[14] and the mouse.[15] In addition, CNTF appears to be protective in the retinal degeneration (*rd*) mouse and the transgenic mouse with the Q344ter rhodopsin mutation.[15]

To date, neurotrophic factors have been shown to be effective only in rodents in which a single injection is used for short-term rescue. To determine whether this phenomenon is specific to rodents and whether these factors can achieve long-term rescue, we studied the capacity of multiple intravitreal injections of a human CNTF analogue (Axokine) and human BDNF to provide long-term protection of photoreceptors in an autosomal dominant feline model of rod-cone dystrophy (*Rdy* cats). Axokine (Regeneron Pharmaceuticals, Tarrytown, NY) is a modified form of human CNTF to enhance its specific activity.[16]

METHODS

The *Rdy* cat[17–20] was used throughout the study with two normal animals. In brief, the features are those of an autosomal dominant rod-cone dystrophy with photoreceptor degeneration beginning at 5 weeks of age. The peak of apoptosis occurs at approximately 9 weeks[21]. The inner retina is relatively well preserved until end-stage disease.

The animals were bred and studied under the regulation of the UK Animals (Scientific Procedures) Act 1986 and all animal procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

All the intravitreal injections were performed by one of the authors (NHVC). Animals were anesthetized by a gas mixture of halothane, nitrous oxide, and oxygen. A disposable 0.3-ml insulin syringe with a fixed 29-gauge needle was inserted 2 mm to 4 mm behind the limbus (depending on the age of the animal) at the superotemporal quadrant and directed toward the optic nerve. When the tip of needle reached the midvitreous, the injection was administered in a single, swift action. A gentle ocular massage was performed for 2 minutes to reduce the intraocular pressure.

In the first experiment (early treatment), Axokine (5 μg in 50 μl vehicle) was injected intravitreally in one eye at postnatal day 10 (n = 10). The other eye acted as an untreated control. The injection was repeated every 4 weeks. After each intravitreal injection a 5-day course of topical prednisolone were given to both eyes. Clinical (ophthalmoscopy) and histopathologic

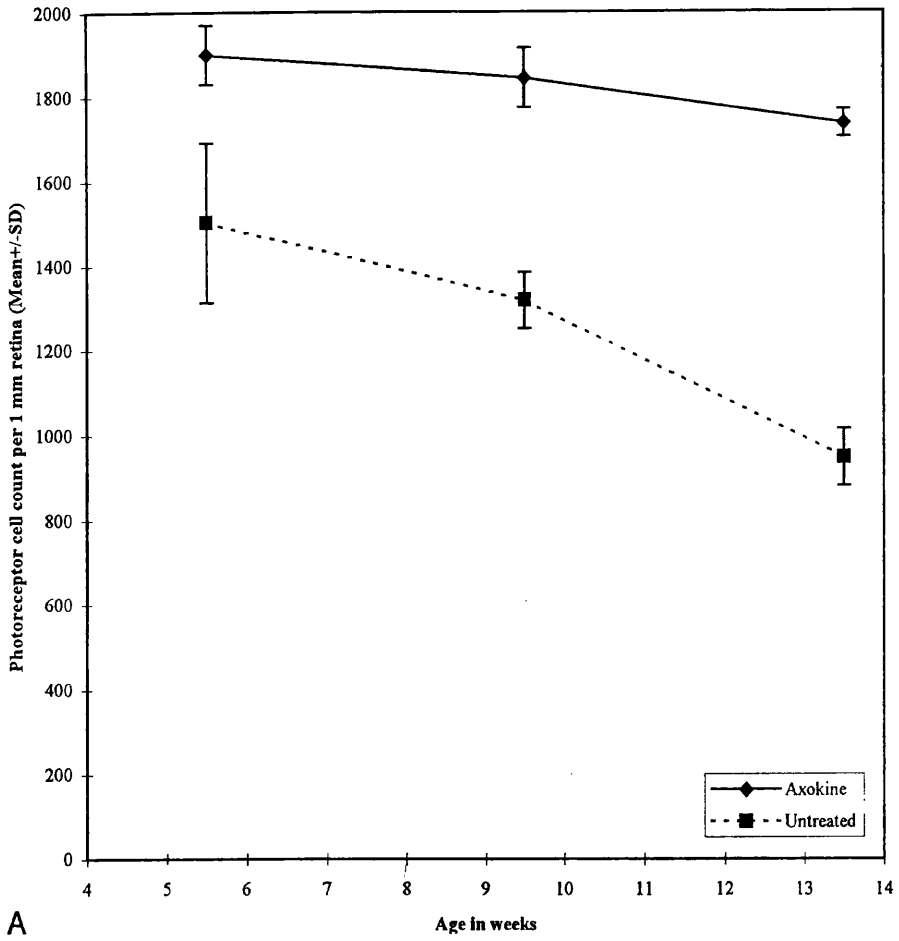

FIGURE 1. Photoreceptor cell count (mean ± SD). (A) The first experiment (Axokine versus no treatment) showed significant photoreceptor rescue in Axokine-treated eyes. (B) The second experiment (Axokine versus BDNF versus vehicle versus no treatment) showed significant photoreceptor rescue in Axokine-treated eyes but no effect in BDNF- and vehicle-treated eyes.

examinations were performed in cats at 5.5, 9.5, and 13.5 weeks of age.

In the second experiment (delayed treatment), we investigated the effects of the Axokine (2.5 μg in 25 μl), human BDNF (250 μg in 25 μl) or sham injection with 25 μl phosphate-buffered solution (PBS) at pH 8 (vehicle of Axokine) in one eye, with the other eye untreated. Animals (n = 17) were randomly assigned to one of the three injection groups. Only one eye was injected, the other eye remained untreated. The injections were started at 5.5 weeks and repeated every 4 weeks. For 5 days after each intravitreal injection, topical prednisolone was applied to both eyes. Clinical (ophthalmoscopy) and histopathologic examinations were performed in cats at 9.5, 13.5, and 17.5 weeks of age.

In the third experiment, we injected a small number (n = 2) of normal animals with Axokine to evaluate complications using identical protocol as the first experiment.

All animals were killed with an overdose of systemic phenobarbitone. The eyes were immersed in 10% formalin in the first experiment and in 4% paraformaldehyde with PBS in the second and third experiments. After 24 to 48 hours of fixation, the eyes were hemisectioned circumferentially at the equator. The posterior eyecup was then divided horizontally and vertically through the optic nerve head. The superonasal quadrant was embedded in Araldite resin using a standard protocol. Photoreceptor cell and apoptotic cell counts of the dystrophic animals were performed in 1-μm-thick toluidine blue-stained Araldite sections. The same portions of the eye were counted in all cases. The inferonasal quadrant was embedded in paraffin wax for the modified Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labeling technique and immunohistochemistry. The same portions of the eye were counted in all cases. The superior and inferior quadrants were oriented in such a way that the sections counted were only separated by the cut edge.

An antibody against glial fibrillary acidic protein (GFAP) (Dako, UK) was used to assess Müller cell activity using a standard biotin-streptavidin peroxidase method. Antigen retrieval pretreatment with trypsin was performed before primary antibody incubation. Appropriate positive and negative controls were used.

Photoreceptor Cell Count

A random location near the center of the section was chosen at low magnification. The section was then moved 550 μm from the random spot. An eyepiece graticule (Graticules, Tonbridge, UK) was used to obtain a 25-μm-wide retinal strip. The number of cells within this strip was counted. The section was then

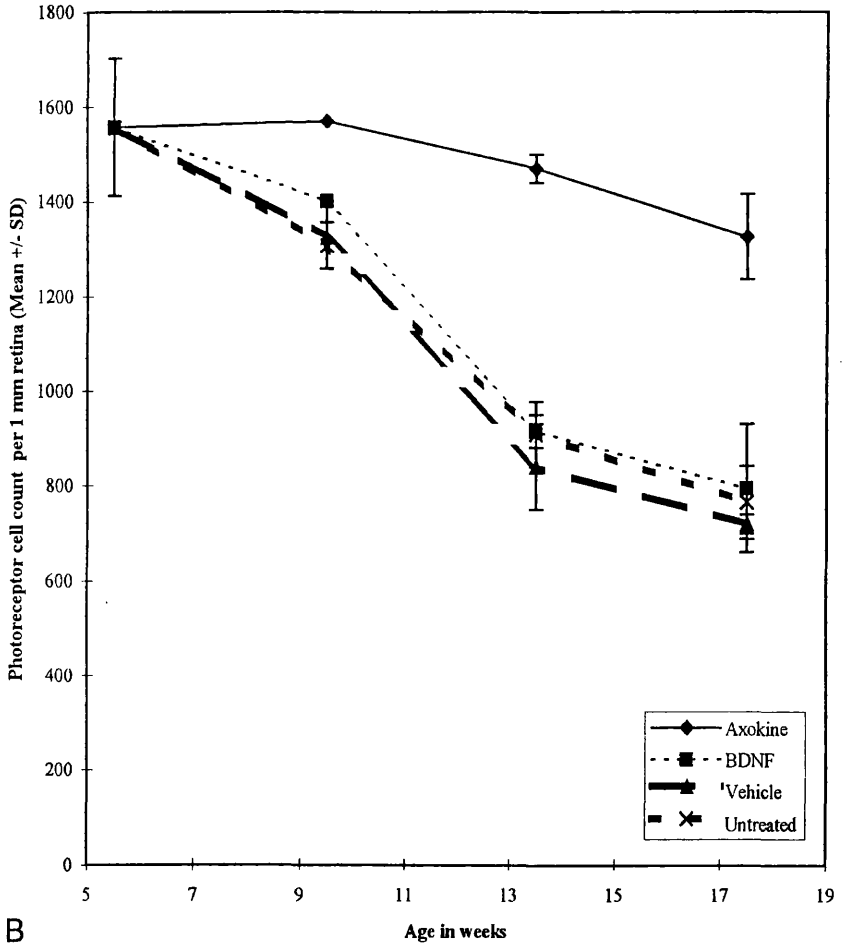

FIGURE 1. (*Continued*)

moved 250 μm toward the center, and another 25-μm-wide retinal strip was counted. This was repeated twice to obtain a total of four readings. One hundred micrometers of retinal width was counted. The number of photoreceptor cells counted was then multiplied by 10 and expressed as the number of cells per 1 mm of retina.

Identification of Apoptosis

Apoptotic cells were identified on morphologic grounds as cells with condensed pyknotic nuclei. The whole section was counted. The result was expressed as the number of apoptotic cells per 1 mm of retina.

An in situ 3′-tailing reaction was used to detect double-strand breaks in DNA.[22] In this modification of the TdT-dUTP terminal nick-end labeling (TUNEL) technique, dUTP was replaced with deoxyadenosine triphosphate (dATP). Sections of rat duodenum were used as positive control specimens, and omission of the transferase enzyme from the reaction mixture served as a negative control. Cells in the entire section were counted. The result was expressed as the number of TUNEL-positive cells per 1 mm of retina.

Counts of photoreceptor cells, apoptotic cells, and TUNEL-positive cells were performed using the microscope setting of ×40 objective with a ×10 eyepiece, providing a 0.5-mm diameter field. All apoptotic cell counts and TUNEL-positive cell counts were performed independently by two observers. The difference in results between the two observers was less than 10%, and the mean was taken as the final result.

Retinal Fold Counting

The number of retinal folds was counted in each section of each animal. The results were expressed as the number of folds per 1 mm of retina.

Statistical Analysis

All three parameters (photoreceptor cell count, apoptotic cell count, TUNEL-positive cell count) in each treatment group were compared with those in the untreated group using paired Student's *t*-test at individual time points and then the groups as a whole. All statistical analysis was performed using commercial software (Excel 95; Microsoft, Redmond, WA).

RESULTS

During the study, the animals tolerated the procedure well and appeared to be pain free. Topical prednisolone was applied prophylactically and no active inflammation was observed clinically, even in the early postoperative period.

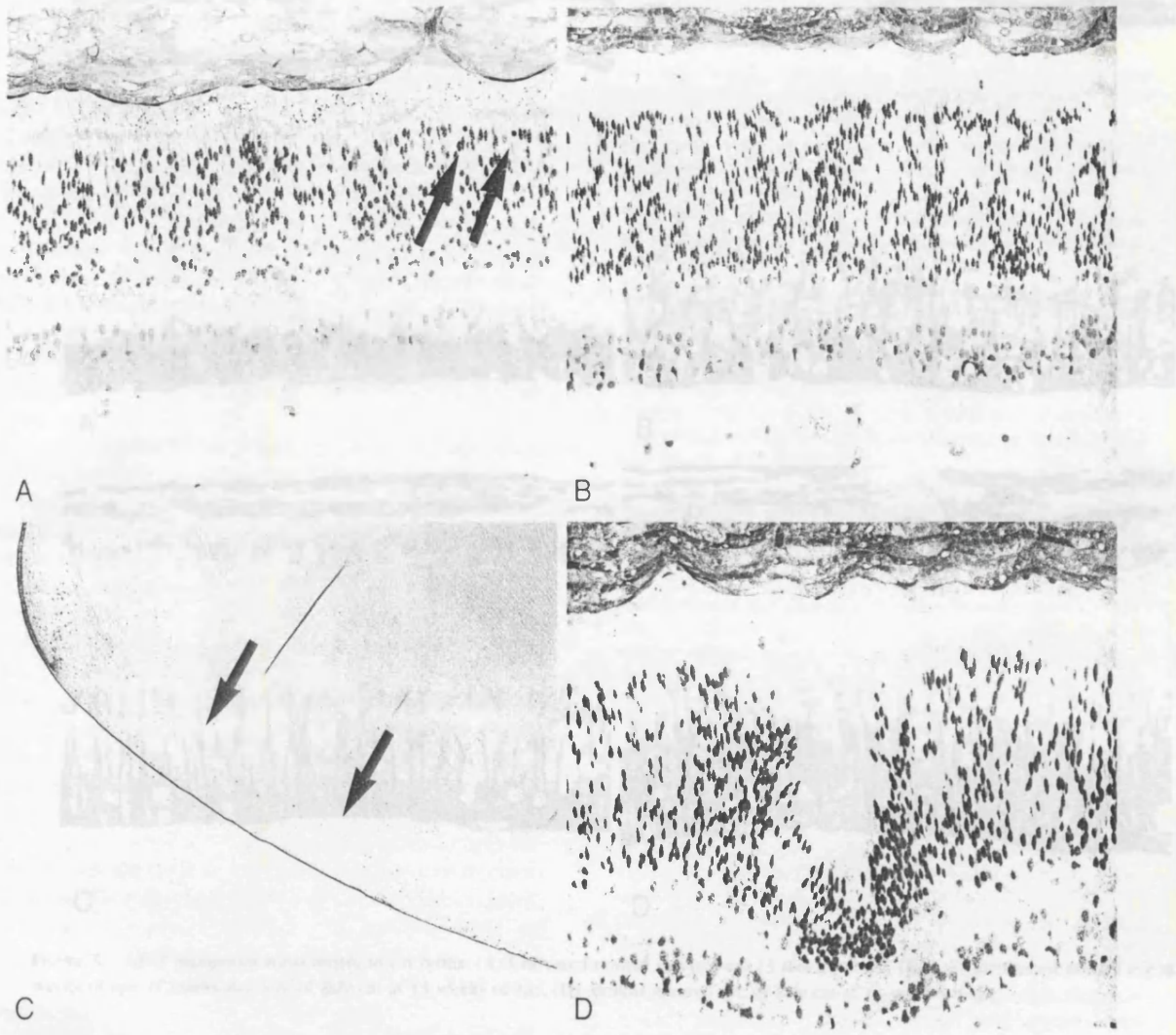

FIGURE 2. Histopathology of *Rdy* cats. (**A**) Retina of the untreated eye in a cat 17 weeks of age showing apoptotic cells (*arrows*). (**B**) retina the of Axokine-treated eye at 17 weeks of age; (**C**) lens of the Axokine-treated eye at 17 weeks of age showing posterior subcapsular cataract (*arrows*); (**D**) retina of the Axokine-treated eye at 17 weeks of age showing retinal fold.

Cell Counts in Axokine-Treated Versus Untreated Eyes

In the first experiment, we assessed whether Axokine had a protective effect. The results of photoreceptor cell counts, apoptotic positive cell counts, and TUNEL cell counts in the initial experiment are summarized in Table 1A and Figure 1. Photoreceptor cell counts were significantly higher in the Axokine-treated group at all time points ($P = 0.02$ at 5.5 weeks; $P < 0.0001$ at 9.5 weeks; $P = 0.003$ at 13.5 weeks). Conversely, apoptotic cell counts ($P = 0.01$) and TUNEL cell counts ($P = 0.04$) were significantly reduced at 9.5 weeks, and the TUNEL count was significantly lower at 13.5 weeks ($P = 0.04$).

Cell Counts in Axokine-Treated Versus BDNF-Treated Versus Sham-Treated Versus Untreated Eyes

After the initial success with Axokine, in the second experiment, we delayed the beginning of therapy to 5.5 weeks and compared the effects of Axokine (at half the dosage of the initial experiment), human BDNF, and vehicle of Axokine. The results of photoreceptor cell counts, apoptotic cell counts, and TUNEL positive cell counts are summarized in Table 1B and Figure 1B. The difference in photoreceptor cell counts was statistically significant in the Axokine-treated group at 13.5 ($P = 0.03$) and 17.5 weeks ($P = 0.02$; Figs. 2A, 2B). Although the indices of apoptosis were not statistically significant in the untreated eyes at individual time points, the entire Axokine-treated group had significantly fewer apoptotic cells ($P = 0.03$) and TUNEL-positive cells ($P = 0.006$) than did the untreated controls. The difference between results in the BDNF- and vehicle-treated groups and those in the untreated eyes were not statistically significant in any of the parameters measured.

Other Clinical and Histopathologic Findings

Minimal posterior subcapsular cataracts were observed clinically and histopathologically in all Axokine-treated eyes in dystrophic and normal animals. The lens opacities were less than 5% of the surface area of the lens in all Axokine-treated

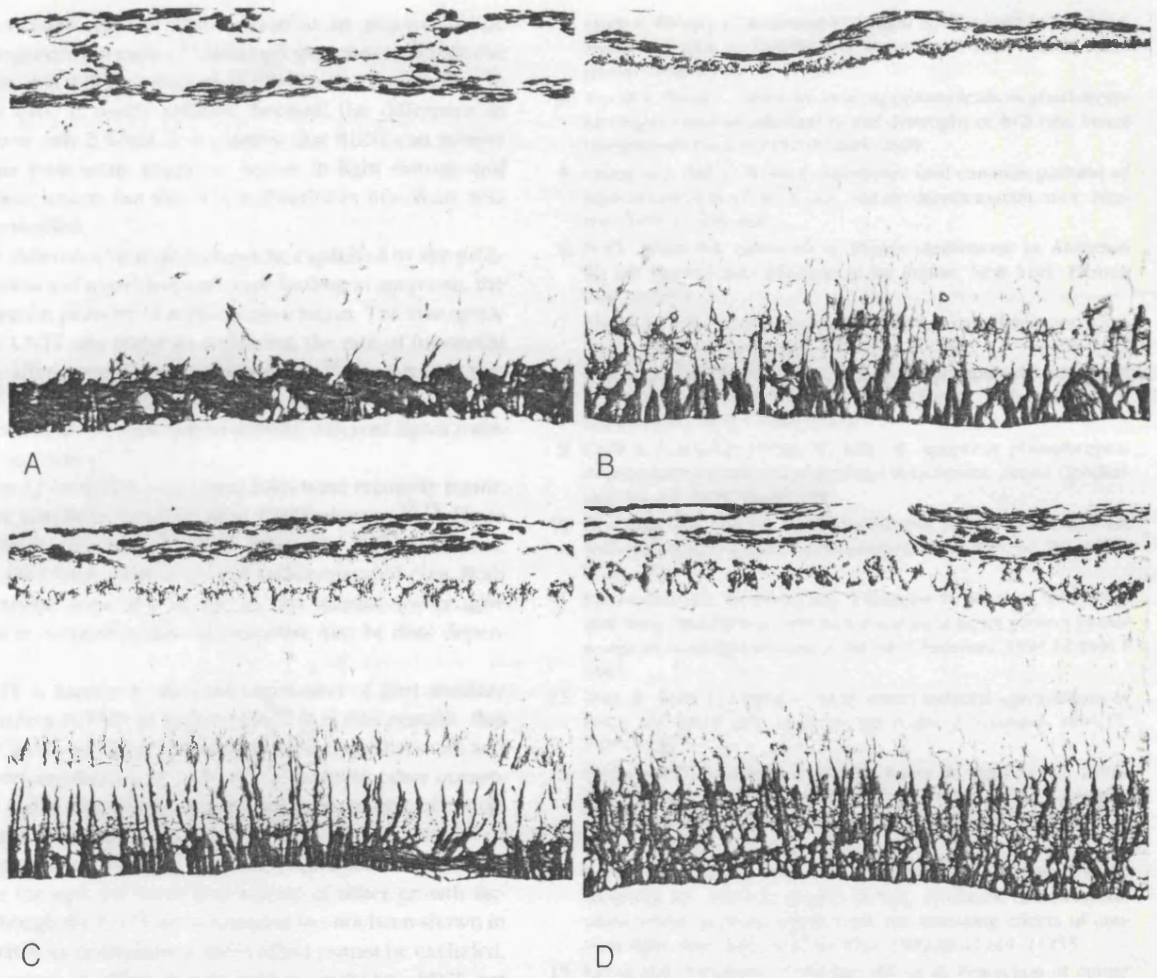

FIGURE 3. GFAP immunohistochemistry in cat retina. (A) Untreated normal eye in a cat 13 weeks of age; (B) Axokine-treated normal eye at 13 weeks of age; (C) untreated eye of *Rdy* cat at 13 weeks of age; (D) Axokine-treated eye of *Rdy* cat at 13 weeks of age.

animals. They affected the posterior subcapsular area only, and there was no extension into the nucleus of the lens. None of the cataracts obscured the fundus examination. In light microscopy, there were more cells present posterior to the equator of the lens with the associated opacities (Fig. 2C). Although quantification was not performed, treated animals in the second experiment (delayed and lower dose) appeared to have fewer lens opacities.

In all Axokine-treated eyes in dystrophic and normal animals, there were small retinal folds seen in histopathologic analyses (Fig. 2D). These folds were not detected in clinical examination by ophthalmoscopy. No vitritis or inflammatory response was observed either clinically or histopathologically. In Axokine-treated eyes, the mean number of retinal folds per millimeter of retina in the first and second experiments was 1.802 ± 0.419 and 0.588 ± 0.366, respectively. The difference was statistically significant ($P = 0.0006$). Neither cataract nor retinal folds were seen in any of the BDNF-injected, vehicle-injected, or untreated eyes.

Although no formal quantification of the ophthalmoscopic findings was obtained, the overall difference between treated and untreated eyes appeared to be small.

GFAP Immunohistochemistry

In the untreated normal animals, astrocytes were immunopositive for GFAP (Fig. 3A). In the Axokine-treated normal animals, there was a marked increase of GFAP immunolabeling in the Müller cells (Fig. 3B). In the untreated *Rdy* cats, an increase of GFAP immunolabeling of Müller cells was found (Fig. 3C), and there was a suggestion of a further increase of GFAP immunolabeling of Müller cells in the Axokine-treated *Rdy* cats (Fig. 3D).

DISCUSSION

In the present study, Axokine (a human CNTF analogue) reduced apoptotic cell death of photoreceptors in the *Rdy* cat, and multiple injections provided long-term protection. However, human BDNF was not effective. These results are similar to those reported in the mouse models.[15] In the light damage model of retinal degeneration, both CNTF and BDNF delay photoreceptor degeneration.[14,15] The ineffectiveness of BDNF in this cat model may be explained by the fact that we were using human BDNF instead of feline BDNF. However, the same preparation at a slightly lower dose (100 μg) of human BDNF

promotes outer segment regeneration in an acquired feline retinal degeneration model.[23] Although we cannot exclude the possibility that different dosages of BDNF may have an effect in the *Rdy* cats, it seems unlikely, because the difference in dosage was only 2.5-fold. It is possible that BDNF can protect the retina from acute injury, as occurs in light damage and retinal detachment, but that it is ineffective in hereditary retinal degeneration.

The difference in response may be explained by the probable existence of more than one route leading to apoptosis, the final common pathway of retinal degeneration. The synergistic effect of CNTF and BDNF in enhancing the rate of functional recovery after peripheral nerve transection[24] also suggests that the two neurotrophic factors may act on different pathways. Furthermore, they are known to activate different signal transduction pathways.[25,26]

Cataract formation and retinal folds were relatively minor, and have not been reported after CNTF therapy.[14,15] These complications are unlikely to be related to surgical trauma, because they were present only in Axokine-treated eyes. Both complications were less severe in the second, lower dose experiment, suggesting that the response may be dose dependent.

CNTF is known to increase expression of glial fibrillary acidic protein (GFAP) in Müller cells.[25] It is also possible that Axokine increases GFAP expression in the treated animals and may therefore, directly or indirectly, constitute other components of a glial injury response, including the release of endogenous growth factors.

In principle, Axokine may cause cataract formation directly or through the secondary release of other growth factors. Although the CNTF alpha receptor has not been shown in the human lens epithelium, a direct effect cannot be excluded. The receptors of other growth factors, including bFGF are present in lens epithelial cells.[27] Because bFGF can induce proliferation of lens epithelial cells in vitro,[28] it is therefore a possible candidate for an intermediate agent.

Similarly, the retinal folds may also be secondary to bFGF, because intravitreal injection of this growth factor causes retinal folds with severe inflammation in the rabbit.[29] This effect is also dose dependent. The absence of inflammation in the *Rdy* cats may reflect a species difference. It is, however, possible that Axokine acts on the Müller cells, leading directly to retinal swelling and then folds. CNTF is known to produce swelling of astrocytes in vitro.[30]

Based on our data, repeated intravitreal injections of Axokine, but not human BDNF, provide long-term photoreceptor survival in hereditary feline retinal degeneration and may be a therapy for human retinitis pigmentosa.

Acknowledgment

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Retinopathy Associated with Enterococcus Enteropathy in the Neonatal Rat

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PURPOSE. Preretinal neovascularization has been previously observed in neonatal rats with spontaneously occurring diarrhea. This neovascularization appears analogous to retinopathy of prematurity (ROP), which occurs in human neonates. A new enterococcus species, designated *Enterococcus rattus*, has been isolated from the duodenum of these rats. In the present controlled study, the effect of the enteropathy induced by this organism on the retinal vasculature in the neonatal rat was further investigated.

METHODS. One hundred fifty newborn Sprague-Dawley rats were randomly assigned to 6 expanded litters (n = 25). On the second day of life, animals were gavaged with either 100 μl of *E. rattus* suspension (1.0×10^7 colony forming units, inoculated group, n = 100 rats) or 100 μl saline (control group, n = 50 rats). All rats were raised in room air and were killed on day 13 of life. Duodenal and blood samples were cultured. The retinal vasculature was assessed using fluorescent microscopy and ADPase staining in a masked manner. Two additional inoculated litters and one control litter were studied for evaluation of arterial blood gases and validation of the grading method for preretinal neovascularization.

RESULTS. One hundred percent of rats in the inoculated group developed severe diarrhea and had duodenal cultures positive for *E. rattus* compared with 0% in the control group. Preretinal neovascularization similar to ROP occurred in 55% of rats in the inoculated group compared with 2% in the control group (P = 0.001). Retinal vascular areas were reduced in the inoculated group (mean ± SD, 89% ± 5% versus 96% ± 2%; $P <$ 0.001). Rats in the inoculated group demonstrated severe growth retardation (final weight, 9.7 ± 2.2 versus 16.7 ± 2.7 g, $P <$ 0.001). Inoculated animals also experienced acidosis (pH 7.31 ± 0.06 versus 7.39 ± 0.06 control, P = 0.04).

CONCLUSIONS. A previously undescribed enterococcal enteropathy was associated with preretinal neovascularization similar to ROP in the neonatal rat. This supports an independent role for factors other than inspired oxygen in the development of ROP. (*Invest Ophthalmol Vis Sci.* 1999;40:1305-1309)

Although hyperoxia is considered to be a major risk factor in the development of retinopathy of prematurity (ROP) in human neonates, the incidence of ROP is increasing despite the judicious use of oxygen.[1] ROP occurs more frequently in the sickest and smallest of premature infants.[1] Clinical and experimental studies have suggested that other factors, such as sepsis, carbon dioxide, acidosis, and postnatal growth retardation, may play a role in the pathogenesis of ROP.[2-6]

The analysis of factors by retrospective clinical studies is limited by potentially confounding variables. The use of animal models of ROP may help evaluate the contributions of a single factor in pathogenesis. Severe preretinal neovascularization analogous to ROP is observed in the rat model of oxygen-induced retinopathy (OIR) after exposure to cyclic hyperoxia and hypoxia. Room air controls in this model do not develop neovascularization.[4]

We have previously reported the observation of preretinal neovascularization in neonatal rats with spontaneously occurring diarrhea.[7] These rats were never exposed to hyperoxia or hypoxia. We isolated the causative bacterium from the duodenal content of these rats, and it was determined to be a previously undescribed *Enterococcus* by analysis of the DNA sequence of the 16S ribosomal RNA gene.[8] This bacterium has been designated *Enterococcus rattus*. In this report, we describe a controlled study of *E. rattus* gavage on the retinal vasculature of the neonatal rat.

METHODS

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision

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