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Studies on mechanisms of cell death in retinal degeneration

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Abstract

Retinitis pigmentosa (RP) is a heterogeneous group of inherited retinopathies affecting approximately 1.5 million people worldwide. RP is associated with genetic mutations leading to progressive deterioration of the photoreceptors followed by degeneration of the retinal pigment epithelium (RPE). The molecular mechanisms by which these mutations drive photoreceptors to death are not well understood. Accordingly, effective therapeutic strategies to stop the progression of RP and/or restore vision have yet to be established. The current study examines mechanisms of cell death in two models of RP that affect genes in visual cycle: Pro23His (P23H) rat and *rd1* mice.

The P23H transgenic albino rat strains are widely used models for the most common mutation of the rhodopsin (*RHO*) gene associated with autosomal dominant form of RP. However, detailed phenotypic characterization of the most commonly used strain (line 3, P23H-3) is lacking. Before investigating what underlying cell death signaling pathways contribute to photoreceptors death in this animal model, P23H-related functional and structural changes were evaluated using electroretinography (ERG) and *in vivo* imaging. In the control Sprague-Dawley (SD, control) rats, photoreceptor and post-photoreceptor ERG responses enhanced from postnatal day (P)18 to 1 month, when they reached adult levels. By contrast, the function of the rod and cone pathways in the P23H-3 rat showed progressive decline from 1 month of age, which correlated with loss of photoreceptors in the outer nuclear layer (ONL) from 2 months onwards. Quantitative analysis of dying P23H-3 photoreceptors in the ONL, using TUNEL assay, revealed a biphasic pattern of the cell death, with a rapid initial phase, peaking at P18, followed by a slower progressive phase. However, activation of caspase-3 and nuclear translocation of apoptosis inducing factor (AIF) were detected rarely in the nuclei of the ONL at P18, suggesting that the classical intrinsic apoptotic pathway may not be the major

contributor. Consistent with this, analysis of mitochondrial membrane potential ($\Delta\psi_m$) using flow cytometric analysis of JC-1 fluorescent assay showed similar staining patterns for both P23H-3 and age-matched SD control cells, with no significant differences between the ratio of red/green fluorescence, that is no difference in $\Delta\psi_m$. To further investigate other potential cell death pathways involved in the degeneration of photoreceptors in P23H-3 rats, gene expression profiling was performed using a rat cell death qPCR array on RNA isolated from P14 and P18 P23H-3 and age-matched SD rat retinæ. The results revealed significant dysregulation in the expression levels of several genes associated with autophagy and necrosis.

The *rd1* (*Pde6b*^{rd1/rd1}) mouse is a well-characterised animal model of human autosomal recessive RP bearing a gene mutation in the β -subunit of phosphodiesterase 6 (*Pde6b*). As previous studies have debated the importance of apoptotic pathways in this model, the roles of two central molecules (BAX and BAK) was investigated in mitochondrial-mediated apoptosis during normal maturation of the retina and during *rd1*-associated photoreceptor degeneration. C567Bl6 mice lacking *Bak* (*Bak*^{-/-} and *Bak*^{-/-} *Bax*^{+/-}) exhibited normal architecture and lamination of retina, with no significant differences in thickness of retinal layers compared to wild-type (WT) at P14. Analysis of *Bak* and *Bax* expression by droplet digital PCR (ddPCR) in *rd1* mice at P14 and P18 revealed elevated expression of *Bak*, but not *Bax*, compared to the WT controls. Genetic removal of *Bak* in *rd1* mice, with or without loss of one *Bax* allele, resulted in a significant and transient increase in ONL thickness compared to mice *rd1* at P14, that was not maintained at P18. However, in double-knockout mice (*rd1/Bak*^{-/-} *Bax*^{-/-}) the ONL thickness was similar to the WT, despite the presence of the *rd1* mutation. Moreover, a substantial thickening was detected in inner retinal layers including inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL) when compared to age-matched *rd1* and WT controls. The data indicate that in the *rd1* mouse model, BAK and BAX function

redundantly in intrinsic cell death pathways, which are a major contributor to photoreceptor death over and above normal developmental cell death in the maturing retina.

To investigate the potential to pharmacologically target intrinsic cell death mechanisms, the efficacy of a BAK antagonist was examined *in vivo*. Intravitreal injection of a specific BAK inhibitor (WEHI-1250993) into *rd1/Bax^{-/-}* mice at P16 significantly reduced photoreceptor death at P18 when compared to vehicle controls. While these results provide proof of principle that intravitreal administration of antiapoptotic agents may be effective for ameliorating photoreceptor death, further studies are needed to develop BAX antagonists and determine the long-term effectiveness of such therapeutic approaches.

In conclusion, P23H mutation in RHO induced a progressive deterioration of retinal function and structure in transgenic rats. Multiple mechanisms of cell death possibly form a complicated network of molecular events to mediate degeneration of photoreceptors in P23H-3 animal model. However, the mitochondrial-mediated apoptosis is central to both naturally occurring developmental as well as *rd1*-associated cell death in mouse retina. Targeting these cell death pathways may slow the progression of photoreceptor death in individuals with RP.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature:

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Date: ...

Preface

According to the rules and regulations that govern the Doctor of Philosophy degree at the University of Melbourne, the assessment of my contribution to this thesis was made as follows:

Chapter 2: 100%

Chapter 3: 80%, genotyping of mutant mice was performed by Dr. Stephane Chappaz and his colleagues.

Conference presentations

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List of abbreviation

ARVO	Association for Research in Vision and Ophthalmology
AC	amacrine cells
ADRP	autosomal-dominant retinitis pigmentosa
AIF	apoptosis-inducing factor
AMD	age-related macular degeneration
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APAF-1	apoptotic protease activating factor-1
ARC	animal resources center
ARRP	autosomal-recessive retinitis pigmentosa
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2-associated X
BCL-2	B-cell lymphoma 2
BH	BCL-2 Homology
CARD	caspase recruitment domain
CARD	caspase recruitment domain
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
cDNA	complementary DNA
CED	cell death abnormal
CNS	central nervous system
DD	death domain
ddPCR	droplet digital PCR
DED	death effector domain
DISC	death-inducing signaling complex
ER	endoplasmic reticulum
ERG	electroretinogram
FADD	FAS-associated death domain
FSC	forward scatter
FW	forward
GPCR	G-protein coupled receptor
H&E	Haemotoxylin and Eosin
HC	horizontal cells
Hprt	hypoxanthine guanine phosphoribosyltransferase
IAP	inhibitor of apoptosis
ICE	interleukin-1 β -converting enzyme
INL	inner nuclear layer
IP	intraperitoneal
IPL	inner plexiform layer
IS	inner segment
OCT	ocular coherence tomography
OMM	outer mitochondrial membrane
ONL	outer nuclear layer
OPL	outer plexiform layer
OPs	oscillatory potentials
OS	outer segment
P23H	Pro23His
P23H-3	Pro23His-line 3

PCR	polymerase chain reaction
PDE	phosphodiesterase
PFA	paraformaldehyde
REV	reverse
RGC	retinal ganglion cells
RHO	rhodopsin
RIN	RNA integrity number
RIP	receptor-interacting protein
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
SD	Sprague-Dawley
SEM	standard error of the mean
SSC	side scatter
TRADD	TNF receptor-associated death domain
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UPR	unfolded protein response
WT	wild-type
XLRP	X-linked retinitis pigmentosa

Chapter 1.

Literature review and scope of work

1.1. Introduction

Photoreceptor death is thought to cause approximately 50% of all cases of irreversible vision loss in the western world. It contributes to vision loss in inherited retinal degeneration, as well as age-related macular degeneration. Retinitis pigmentosa (RP) is the most common form of inherited retinopathy, characterized by progressive loss of photoreceptors (rods and cones), which leads to severe visual impairment (Bravo-Gil et al., 2017; Guadagni et al., 2015). RP has been found to be caused by more than 3000 genetic mutations, affecting around 70 genes (Daiger et al., 2013a). Presently, there is no treatment for RP (Sahni et al., 2011) and what is needed is an understanding of the basic underlying biology that causes the disease to explore and develop novel therapeutic strategies.

Several studies have demonstrated that apoptosis, a type of programmed cell death, is one of the main death pathways involved in pathogenesis and progression of photoreceptors demise in RP (Chang et al., 1993b; Portera-Cailliau et al., 1994; Travis, 1998a). However, pharmacological approaches targeting apoptosis have only shown minor protective effects against photoreceptor degeneration. Moreover, recent investigations have demonstrated that other types of cell death, including autophagy and necrosis, are also implicated in RP-related photoreceptor loss. Together, photoreceptor death in RP seems to be mediated by different pathways of cell death, which may have cross-talk and/or functional redundancy. Accordingly, further studies are required to understand the complex mechanisms by which photoreceptors die in the retina.

Through the years, experimental animal models have contributed into developing knowledge about pathophysiology of RP and developing novel therapies. The overarching aim of this thesis is to understand the molecular mechanisms that contribute to photoreceptor loss in inherited retinal degeneration using rodent models of RP. General structure of retina and

visual pathway will be discussed below, followed by an overview of retinal degeneration and cell death pathways involved.

1.2. Structure of the vertebrate retina

The vertebrate retina is a layered light-sensitive neural tissue covering the posterior surface of the eye that collects light from the surrounding environment and transmits it to the brain allowing visual perception. At a cellular level, the retina contains five classes of neurons; photoreceptors, which absorb light; horizontal, bipolar and amacrine cells, which are interneurons collecting signals from photoreceptors; and ganglion cells, which are the output neurons of the tissue carrying visual information to the brain. These neurons are organized in five alternating layers of cell bodies (nuclei) and synapses/axons. Photoreceptor cell bodies are localized in the outer nuclear layer (ONL), while interneurons reside in the inner nuclear layer (INL), and retinal ganglion cells (and some of displaced amacrine cells) occupy the innermost ganglion cell layer (GCL) (Sanes et al., 2015a). Photoreceptor-interneuron synapses are found in the outer plexiform layer (OPL), while interneuron-ganglion cell synapses constitute the inner plexiform layer (IPL) (**Figure 1.1**).

In the retina, two pathways mediate synaptic transmission: the vertical and lateral pathways. Signal transmission from photoreceptors to bipolar cells forms the vertical pathway, which is modulated by the inhibitory functions of horizontal (in the OPL) and amacrine (in the IPL) cells, which make up the lateral pathway (Wässle et al., 1991).

1.2.1. The vertical pathway

1.2.1.1 Photoreceptor cells

Photoreceptors are the photosensitive neurons in the retina, specialized for converting light stimuli into neuronal signals. Rods and cones are two main classes of photoreceptors (Ahnelt et al., 2000; Wassle, 2004). Rods express the photopigment rhodopsin and are exquisitely sensitive to light, thus facilitate dim-light vision. Cones express one of three cone-opsins, each of which respond maximally to a specific wavelength of light. Hence, cones mediate perception of bright-light, high acuity colour vision (Schnapf et al., 1988; Wassle et al., 1991).

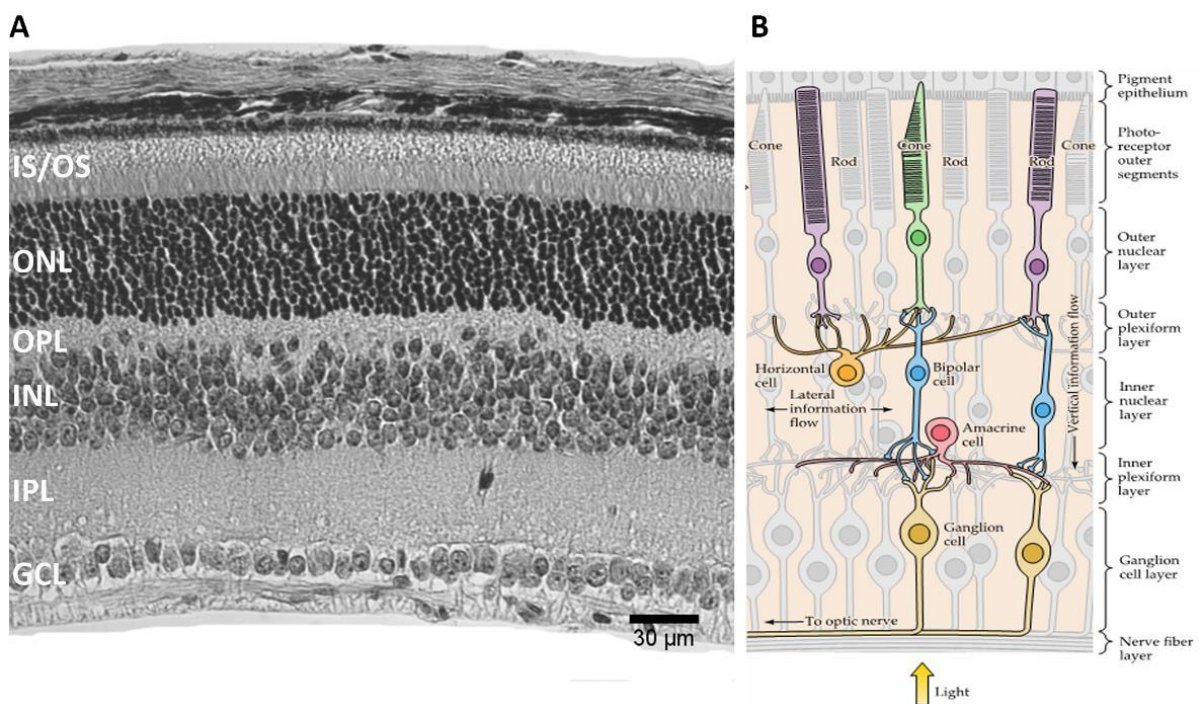


Figure 1.1: The structure and the neurons of the mammalian retina

(A) Structure of the adult mouse retina stained by Haematoxylin and Eosin (B): Schematic diagram showing the basic neuronal organization of the vertebrate retina. Abbreviations: IS: photoreceptor inner segments; OS: photoreceptor outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 30 µm (B: adapted from Neuroscience Purves 3rd edition).

Morphologically, photoreceptors in all vertebrates share similar cellular compartments, namely the outer segment (OS), inner segment (IS), soma, axon, and synaptic terminal. The

OS is filled with stacks of membranous disks containing the photopigments rhodopsin or cone-opsins which capture photons of light and convert them into electrical impulses. The IS contains the cellular organelles, the Golgi, endoplasmic reticulum (ER), and mitochondria, and is where cellular protein and lipids are synthesized (Baker et al., 2013).

The biochemical process of transducing a light signal into an electrical output is known as the phototransduction cascade and occurs in the cell membranes and pigment-loaded disk membranes of the photoreceptors OS (Arshavsky et al., 2002a). The phototransduction cascade is a G-protein-signalling pathway mediated by photopigment (rhodopsin or cone opsins), a chromophore called 11-cis retinal, transducin and cGMP phosphodiesterase (PDE6) (Grossniklaus et al., 2015).

Under dark conditions, photoreceptors remain continuously in a depolarized state because of influx of Na^+ and Ca^{2+} ions into the cell through cGMP-gated channels and maintain a constant synaptic release of glutamate. In rods, photon absorption induces a conformational change in the 11-cis retinal to all-trans retinal, leading to activation of rhodopsin which in turn catalyses the activation of transducin. Transducin, stimulates the activity of PDE to hydrolyse cGMP to GMP and thus decrease the level of cytoplasmic cGMP. This results in the closure of the cGMP-gated Na^+ and Ca^{2+} channels on the plasma membrane, photoreceptors become hyperpolarized, and inhibiting glutamate release from their terminals. This visual signal is subsequently processed by the before mentioned interneurons and ganglion cells before being transmitted to the brain via the optic nerve (Arshavsky et al., 2002b).

1.2.1.2 Bipolar cells

Retinal bipolar cells function as the second-order neurons in the visual pathway, relaying inputs from photoreceptors and horizontal cells to the inner retina (Strettoi et al., 2010). In the mammalian species, there are approximately 9-12 subtypes of cone bipolar cells (Boycott et al., 1991; Ghosh et al., 2004; Grunert et al., 1994; Hartveit, 1997; MacNeil et al.,

2004), whereas only one type of rod bipolar cell has been identified (Kolb, 1970). Reflecting this difference, each rod interacts with the dendrite of one individual rod bipolar cell at its synaptic terminal (spherule), while cones can make several connections with dendrites of cone bipolar cells at their synapses (pedicle) (Ghosh et al., 2004).

Functionally, bipolar cells fall into two fundamental groups: ON and OFF type cells, which differ in their response to light (Slaughter et al., 1981). ON bipolar cells respond to light stimulation with a graded depolarization as they express the metabotropic glutamate receptor, mGluR6 (Nakajima et al., 1993; Nomura et al., 1994), which negatively couples to a cationic-selective channel via the G-protein α_0 ($G\alpha_0$) (Vardi, 1998; Vardi et al., 1993). By contrast, OFF bipolar cells express the ionotropic AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) or kainate glutamate receptors and become hyperpolarized in response to light (DeVries, 2000; Hack et al., 1999).

1.2.1.3 Ganglion cells

Retinal ganglion cells are the final output neurons of the retinal vertical pathway, being responsible for the transmission of visual information to the brain. In the IPL, their dendrites make a dense synaptic network with bipolar and amacrine cells to collect sensory input (Sanes et al., 2015a).

Based on morphology, gene expression profile, regular spacing and physiological properties ganglion cells can be classified into distinct types (Sanes et al., 2015b) and it has been shown that different physiological RGC types have specific morphologies (Cleland et al., 1975). In the mouse retina, at least 25 distinct subtypes of ganglion cells have been identified (Roska et al., 2014). Physiologically, there are two main subtypes of GCs in the mammalian retina; ON- and OFF-center which are tuned to respond to increases and decreases in light intensity, respectively (Kuffler, 1953).

1.2.2. *The lateral pathway*

1.2.2.1 *Horizontal cells*

Horizontal cells are horizontally elongated neurons in the retina, located at the border between OPL and INL, and mediate lateral interconnection of photoreceptors (Perlman et al., 2009). In most vertebrate retinæ, at least two types of HCs have been described: A-type, which are axonless, and B-type, which have an elongated axon (Boycott, 1988). Generally, the somata and dendritic ends of both types interact with cone pedicles, whereas the axonal terminals (if present) synapse with rod spherules (Muller et al., 1993). Horizontal cells receive synaptic signals from cone photoreceptors via ionotropic kainate-AMPA receptors on their dendrites and provide inhibitory feedback (Hack et al., 2001; Vardi et al., 1992). While it has been suggested that horizontal cells release the inhibitory neurotransmitter, GABA, to negatively modulate the excitability of photoreceptors (Wu, 1992), the exact mechanism(s) is still unknown.

1.2.2.2 *Amacrine cells*

Amacrine cells (AC) are the intrinsic interneurons, which show the most functional and morphological variety in the retina (Wässle et al., 1991). The cell bodies of ACs occupy either the innermost part of the INL or GCL and their processes extend to the IPL (Wässle et al., 1991), where they process synaptic input from bipolar cells and other amacrine cells and in turn send signals to ganglion cells as well as providing feedback to bipolar cells (Dowling et al., 1966; MacNeil et al., 1998; Zhang et al., 2002). In the vertebrate retina, the majority of amacrine cells express GABA or glycine and function as inhibitory neurons (Brecha et al., 1988; Crooks et al., 1992); however, some also co-express excitatory neurotransmitters such as dopamine and acetylcholine (Chun et al., 1988; Kolb, 1997; O'Malley et al., 1989; Vaney et al., 1988). This expression of multiple neurotransmitters suggests that amacrine cells release

different sets of neurotransmitters to achieve their primary function of bipolar and ganglion cell modulation.

1.2.2.3 Retinal glial cells

The vertebrate retina comprises two main types of support cells: macroglia including astrocytes and Müller cells; and microglia, the yolk sac-derived resident immune cells in the retina. All retinal glial cells are involved in regulation of the micro-environment of the retina (Bringmann et al., 2006).

1.2.2.3.1 Macroglia

Müller cells are the specialized radial glial cells of the vertebrate retina. Their cell bodies are localized in the INL, with their processes distributing through all retinal layers (Bringmann et al., 2006). This anatomical arrangement provides close interactions between Müller cells and retinal neurons, synapses, other glia, and blood vessels, important for maintaining retinal structure and function (Bringmann et al., 2006). Müller cells have a variety of functions in the retina including: providing retinal neurons with nutritional and metabolic support (Poitry-Yamate et al., 1995; Tsacopoulos et al., 1996), maintaining ion and water homeostasis (Kofuji et al., 2002; Newman et al., 1996; Newman, 1996; Newman et al., 1984), regulation of the retinal blood flow (Paulson et al., 1987), recycling of photopigments (Das et al., 1992), secreting neuroactive (Newman, 2003; Newman et al., 1998; Stevens et al., 2003) and vasoactive factors (Eichler et al., 2000; Eichler et al., 2004), protecting neuronal cells against oxidative damage (Pow et al., 1995; Schütte et al., 1998) and processing of neuronal signals (Biedermann et al., 2002; Brew et al., 1987; Qian et al., 1993; Rauen et al., 1998).

Astrocytes are the second type of macroglia in the retina. Unlike Müller cells, astrocytes do not derive from the retinal embryonic epithelium, rather originate from optic stalk lineages, and migrate into the retina from the optic nerve head (Stone et al., 1987; Turner et al., 1987; Watanabe et al., 1988). Astrocytes are primarily positioned in the nerve fiber layer;

where their processes make interact extensively with blood vessels and form the retinal blood barrier (Vecino et al., 2016). Astrocytes have essential roles in maintaining ion homeostasis, regulation of blood vessel growth during retinal development and transmitting neuronal signals in the retina (Ridet et al., 1997).

1.2.2.3.2 Microglia

Microglial cells are the resident immune cells of the CNS and retina (Kettenmann et al., 2011). In the healthy retina, they are limited to the inner retinal layers, below the sub-retinal space (Chen et al., 2002) and exhibit a “resting” phenotype characterised by a branched morphology with many processes (Wake et al., 2009). They are capable of secreting neuroprotective and anti-inflammatory substances, thus participate in host defense against invasion of microorganisms and regulation of immune response (Streit, 2002). Under pathological conditions, the microglia can be activated rapidly and change their morphology. Activated microglial cells are very motile, release inflammatory cytokines and phagocytose debris of degenerated/damaged cells (Fu et al., 2014; Sternberg, 2006; Streit, 2002).

1.2.3. Retinal pigment epithelium

The retinal pigment epithelium (RPE) is a specialized epithelial monolayer, densely filled with pigment granules. The RPE is located between the outer segments of the photoreceptors and the choroid (Weiter et al., 1986) and has several crucial survival functions, including transporting of nutrients and metabolites (Wimmers et al., 2007), absorbing of light energy and protecting retina from photooxidative damage, converting all-trans-retinal into 11-cis-retinal (Sim et al., 2010), phagocytosis of shed photoreceptor outer segments (Bok, 1993), and secreting neurotrophic, inflammatory and vasculotropic growth factors (Adamis et al., 1993; Bost et al., 1994; Dawson et al., 1999) .

Based on the data presented above, it is evident that the structure of the mammalian retina is critical to the proper detection and processing of light. Alterations in the retinal structure that occur due to injury or degeneration leads to significant visual impairment.

1.3. Retinal degenerations

Retinal degenerations are a leading cause of irreversible vision impairment characterized by neuronal dysfunction and/or irreversible loss of retinal cells and exemplified in diseases such as retinitis pigmentosa, age-related macular degeneration (AMD), diabetic retinopathy and retinal detachment (Luthert et al., 1998; Seiler, 2014). Although retinal degeneration can be attributed to a range of environmental or genetic causes, the discussion below is restricted to the most common inherited forms of retinitis pigmentosa affecting millions of people worldwide.

1.3.1. *Retinitis pigmentosa*

Retinitis pigmentosa (RP) refers to a heterogeneous family of hereditary retinal dystrophies, with a prevalence of 1 in 3000 to 8000 individuals worldwide (Ali et al., 2017). Nyctalopia (night blindness), which often is first noticed in adolescence, is a classic early clinical symptom of RP, followed by visual field constriction due to loss of peripheral vision and eventually complete loss of vision because of deterioration in central, high acuity vision (Jacobson et al., 2010). These clinical symptoms reflect the progressive degeneration of retinal cells with dysfunction and loss of rod photoreceptors and RPE during the early stages of RP, followed by loss of cone photoreceptors at a later stage (Ali et al., 2017; Daiger et al., 2013b; Ferrari et al., 2011; Fletcher et al., 2011). RP is generally considered as a retinal disease with a powerful Mendelian hereditary component. Currently, mutations in more than 150 genes are

known to cause RP, which can be inherited in an autosomal-dominant (ADRP), autosomal-recessive (ARRP) or X-linked (XLRP) manner (Fletcher et al., 2011; Roger et al., 2014). Proteins encoded by these genes have diverse functions; while some are specifically involved in visual pathway (e.g., phototransduction cascade, structural proteins, visual cycle, outer segment renewal, transcription factors), others are general proteins with broad expression profiles (e.g., splicing factors, nucleotide metabolism) (Marigo, 2007). Although most of the genetic mutations affect proteins that are expressed exclusively in rod photoreceptors and thus lead to their degeneration, cone photoreceptors also die, even though they may not express the mutated protein (Narayan et al., 2016). The mechanisms that lead to cone death are not well understood, but prevailing theories include: (1) dependency of cone survival factors secreted by rods (Keegan et al., 2003; Mohand-Said et al., 1998; Mohand-Said et al., 2000; Ohnaka et al., 2012; Traverso et al., 2003), (2) cone starvation (Ait-Ali et al., 2015; Punzo et al., 2008; Venkatesh et al., 2015), (3) oxidative damage (Komeima et al., 2007; Komeima et al., 2006; Lee et al., 2011; Shen et al., 2005), (4) activation of retinal microglia (Peng et al., 2014) and (5) rod-produced toxins (Narayan et al., 2016; Punzo et al., 2008; Venkatesh et al., 2016).

Owing to the genetic heterogeneity and functional diversity of proteins involved in RP, the biochemical pathways underlying the different types of RP remain elusive. While photoreceptor cell death is the major hallmark of RP, the processes that lead to cell death are complicated and can vary depending on the specific causative genes. Various pathways, including oxidative stress, inflammatory response, endoplasmic reticulum (ER) stress, caspase-dependent and/or -independent pathways of apoptosis, necroptosis and autophagy, have been identified to mediate photoreceptor death in RP, but their cellular and molecular events still remain poorly characterized (Nakazawa, 2014).

Currently, there is no appropriate treatment that can halt the progression of retinal degeneration or restore vision in RP patients (Arshavsky et al., 2002b). However, several

alternative approaches are being explored, which are dependent upon the stage of the disease. In the early phases of RP, when photoreceptors are still functional, the first strategy aims to modify the causative biochemical abnormality in the visual cycle by pharmacological treatment, including vitamin A (Berson et al., 1993), a diet high in omega-3 fatty acids (Berson et al., 2004), or gene therapy (Musarella et al., 2010; Sahni et al., 2011). Ocular gene therapy, using viral vectors, has shown promising therapeutic effects in mice, dogs (Acland et al., 2005; Acland et al., 2001b; Bennicelli et al., 2008; Roddy et al., 2017), and humans (Hauswirth et al., 2008; Maguire et al., 2008; Russell et al., 2017).

Another approach attempts to prevent or slow down photoreceptor cell death using specific inhibitors of programmed cell death, applying or inducing neurotrophic growth factors, as well as reducing retino-toxic products and oxidative damage (Bok et al., 2002; LaVail et al., 2000; Liang et al., 2001a; Liang et al., 2001b; Masuda et al., 2017). In advanced stages, when most of the photoreceptors have died or become non-functional, other therapeutic options including retinal transplantation and the use of electronic retinal implants (Caspi et al., 2009; Yanai et al., 2007) have been explored (Musarella et al., 2010; Sahni et al., 2011).

While some of these approaches have been/are being trialed in humans (Acland et al., 2001a; Cideciyan et al., 2009; Hauswirth et al., 2008), the ongoing development of such therapies is still highly reliant on testing ‘proof of concept’ in appropriate animal models prior to trials in human patients. Indeed, much of our understanding about ocular diseases such as RP, comes from many years of primary investigations on animals presenting with similar signs of those in human.

1.3.2. *Animal models of Retinitis Pigmentosa*

Animal models provide a valuable means for studying the genetic basis and pathogenesis of retinal diseases on the one hand and evaluating novel, potentially

protective/therapeutic approaches on the other. As animals like mice, rats, cats, and dogs naturally suffer from a wide range of retinal diseases with similar traits to the human diseases, understanding the detailed cellular events in such animal models can improve our knowledge about underlying mechanisms in their human counterparts (Dalke et al., 2005; Fauser et al., 2002; Fletcher et al., 2011). The use of animal models is also considered a powerful tool in investigating the effects of environmental factors such as diet (Li et al., 1998b) or light (Hafezi et al., 1997; LaVail et al., 1999) on the retinal degenerative process. Additionally, over the last few years many transgenic and knockout animal models have been generated to identify the role of particular genes within the retina (Dalke et al., 2005; Fauser et al., 2002; Fletcher et al., 2011).

Given the lower cost of animal housing and the relatively rapid progression of retinal degeneration, rodents, and particularly mice, have been the most prominent animal models for investigating retinal degeneration in many laboratories worldwide. However, most rodent models are less suitable for studying cone-related diseases because they have a rod-dominant retina, in which cone-rich areas like the human fovea do not exist (Dalke et al., 2005; Fauser et al., 2002; Pennesi et al., 2012). Such structural differences between human and rodent eye should always be kept in mind when extrapolating findings from animal studies to humans.

In most animal models of RP, retinal degeneration is caused by inactivation or dysfunction of a single gene encoding proteins involved in rod structural integrity (e.g. *Prph*), phototransduction (e.g. *Pde6B*), maintaining RPE structure (e.g. *Rpe65*) or glial function (e.g. *Crb1*). Animal models for RP are generally classified based on the function of the affected protein (Fletcher et al., 2011). In the current study, the Pro23His and *rd1* animal models of RP were used and will be discussed in more detail below.

1.3.2.1 *Pro23His rat, a model for genetic rhodopsin mutations*

Rhodopsin (RHO), the photopigment expressed in rod photoreceptors, belongs to the class A of G-protein coupled receptors (GPCRs), a large family of cell surface signaling receptors (Darren et al., 2013; Zhou et al., 2012). It is a serpentine protein consisting of 348 amino acid residues and has seven transmembrane domains, which traverse the membrane of the disks within the photoreceptor outer segment. The protein also has a covalently bound 11-cis retinal molecule, which is a derivative from vitamin A (Wang et al., 1980).

Mutations in human *RHO* were first reported in 1990 (Dryja et al., 1990) and are currently considered one of the most common causes of RP, accounting for approximately 15% of all inherited human retinal degenerations (Chapple et al., 2001b). Among all different *RHO* mutations, a single-base C-to-A transversion, resulting in substitution of proline for histidine in codon 23 is the most frequent, causing autosomal dominant RP (**Figure 1.2**) (Dryja et al., 1990). It has been suggested that the RHO (P23H) protein is misfolded, retained in different subcellular compartments such as ER and is unable to bind 11-cis-retinal (Kaushal et al., 1994b; Sung et al., 1991a; Sung et al., 1993a). The misfolded RHO can be degraded through the ubiquitin-proteasome system or accumulates in the cytosol (Illing et al., 2002; Saliba et al., 2002). Several transgenic rodents have been generated that express human or mouse P23H RHO to better understand the molecular mechanisms by which the P23H mutation leads to photoreceptor death (Fletcher et al., 2011).

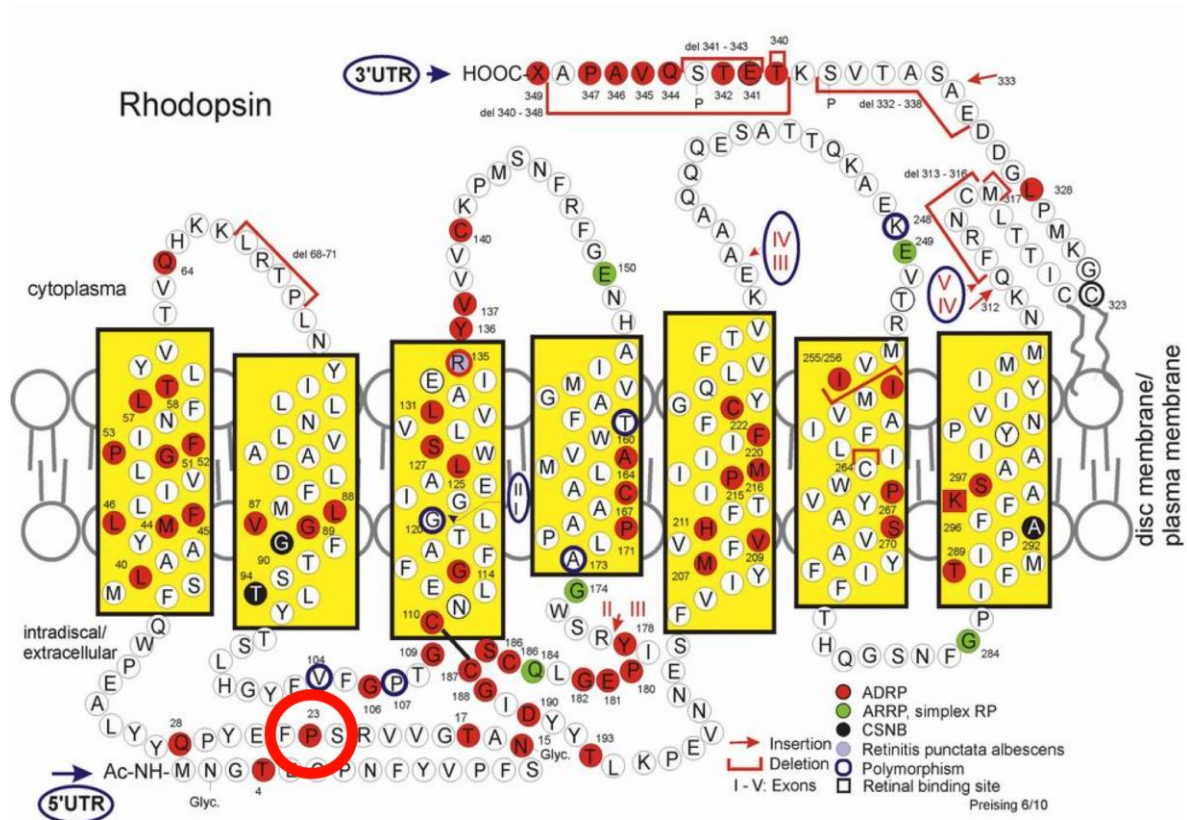


Figure 1.2: Mutations in rhodopsin

A single-base C-to-A transversion, resulting in substitution of proline for histidine in codon 23 (red circle) is the most frequent mutation in rhodopsin, causing autosomal dominant RP (Adapted from <http://www.retina-international.org/files/sci-news/rhomut.htm>).

The P23H albino rat model is a transgenic Sprague-Dawley (SD) rat generated by incorporation of a C57BL/6J mouse rhodopsin transgene carrying the P23H mutation. Three lines of albino P23H transgenic rats were generated, each developing retinal degeneration at different rates. Line 1 is a model of fast retinal degeneration, line 2 has a slow degeneration, while line 3 model has an intermediate disease time course. P23H rat models are widely used as animal models for translational studies and investigation of therapeutic approaches to rescue photoreceptors in autosomal dominant RP. In all three lines of P23H rat, rod photoreceptors degenerate progressively over several months, and thus mimic the clinical progression in RP patients harboring the RHO (P23H) mutation. Visual function, which correlates with photoreceptor loss, is also abnormal in these animals (Lewin et al., 1998b; Orhan et al., 2015a; Steinberg et al., 1996).

In line 1 heterozygous albino rats, rod outer segments are reduced by 40% by 4 weeks of age compared with wild-type SD rats, whilst the ONL has thinned by approximately 40% indicative of photoreceptor death. By 29 weeks of age, only two rows of ONL cell bodies remained with these being identified predominantly as cones (Machida et al., 2000). Photoreceptor loss correlates with impaired retinal function with dysfunction detectable as early as 4 weeks of age by the electroretinogram (ERG) (Machida et al., 2000; Orhan et al., 2015a). Under dark-adapted conditions (rod photoreceptors), maximum rod a- and b-waves amplitudes are significantly reduced and progressively decline to 29 weeks, by which age there is an 80% reduction in b-wave amplitude. In addition to the outer retina, the retinal ganglion cell layer undergoes a progressive deterioration during and following photoreceptor loss (Kolomiets et al., 2010).

In photopic conditions (cone photoreceptors), ERG response is normal at 4 weeks of age, but begins to reduce by 8 weeks of age when the ONL thickness is already reduced by 50%. This suggests that a substantial number of rods are lost prior to significant cone dysfunction in this rat model (Machida et al., 2000). However, a recent study suggested that cone function may be altered earlier in the disease process, with b-wave amplitude reduced and implicit time increased as early as 1 month of age, with this dysfunction increasing with age (Orhan et al., 2015a).

Not surprisingly, structural, and functional loss occur more rapidly in the P23H line 1 homozygote strain than observed in the heterozygote. Homozygous P23H-1 rats undergo a quick and progressive loss of photoreceptors. ONL thickness halved by 21 days, with a subsequent reduction in rod bipolar dendrites along with a decrease in photoreceptors-ON-bipolar cell synapses (Cuenca et al., 2004a). By P30, only 40% of photoreceptor nuclei remain, while by 150 days, only a single layer of rods and cones remains in the ONL with higher numbers in the peripheral retina (Pennesi et al., 2008). Some rod bipolar cells persist, and they

form aberrant synaptic complexes with cones and horizontal cells. With the progress of degeneration, further alterations occur in the inner retina, including a diminution and disruption in AII amacrine cell population and thinning of the IPL (Cuenca et al., 2004b). Furthermore, it was shown that at P30, the RGC layer has already a lower number of cells compared to the SD rats, possibly due to restricted blood supply during retinal development. Loss of RGCs starts from P180, suggestion their degeneration happens following almost complete loss of photoreceptors and the subsequent disorganization in the inner retina (García-Ayuso et al., 2010).

Similarly, homozygous P23H-1 rats show an earlier and more severe visual dysfunction compared to heterozygous rats, with both rod a- and b-wave amplitudes about half of that recorded in heterozygotes animals at P21. Reduction of rod a-wave, rod and cone b-waves and oscillatory potentials (OPs) amplitude are evident at 23 days. Rod scotopic a-wave and OPs responses are absent by 38 days, while b-wave amplitudes continue to decrease until the response is purely cone driven (by 150 days) and remain steady up to 250 days (Cuenca et al., 2004b). In homozygotes rats, the cone pathway never reaches functional maturity with cone-related responses already severely diminished at P21, even though cones and downstream connectivity are unaffected at the structural level. Thus, rod degeneration severely impairs cone responses, prior to any obvious morphological changes in cone pathways (Pinilla et al., 2005). Although more severe reduction occurs in rod-related responses compared with cone-related responses at all ages, surprisingly both display a similar rate of decline with age. There is, however, a biphasic temporal pattern of functional reduction, with a very rapid (P21–P60) period of loss followed by a slower (P60–P200) change in ERG parameters, reflecting the rod-cone pathology evident in RP.

All the studies discussed above used P23H-1 rats with an albino background; however, this line was also generated on a Long Evans background in order to determine the effect of

pigmentation on the pathology (Cuenca et al., 2014b). Anatomical and functional assessment of the retina in this strain suggested albinism exacerbates the rate of P23H-related degeneration. Mixed scotopic a- and b-waves amplitudes are initially higher in pigmented rats compared to albino rats, however both undergo a similar decline with age. By contrast, at early and late time points photopic b-wave maximum amplitudes had higher proportions as compared to the age-matched wild types. These findings indicate that the lack of pigmentation possibly affects cone function more than rod function over time (Cuenca et al., 2014b).

Line 3 albino rats have a lower level of transgene expression than that found in line 1, leading to a slower degeneration of retina. By P30, scotopic rod a-wave maximum amplitude is already significantly lower than SD controls and continues to drop progressively with age (Chrysostomou et al., 2009; Machida et al., 2000). From P16 to P120 the amplitude of the rod a- and b-wave is reduced by 45% and 20% respectively, followed by a further 60% and 50% reduction between P180 and P540. The total reduction in amplitude over 540 days of life in the P23H-3 rat is 80% for the rod a-wave and 55% for the post-receptor b-wave (Chrysostomou et al., 2009). No significant differences were found in photopic cone-related b-wave and phototransduction sensitivity of P23H-3 rats compared to the wild type between P28 and P203 (Machida et al., 2000). However, between P380 and P540, the photopic cone b-wave amplitude declines by 45% in the ERG of P23H-3 retina (Chrysostomou et al., 2009).

With respect to retinal structure, P23H-3 exhibits only a 15% reduction in the number of photoreceptor cells and slightly shortened ROS at P30. The ONL thins faster between P30 and P56 and then slows with ongoing aging. By P203, the ONL thickness and ROS length are half of the control values (Machida et al., 2000), while photoreceptor loss proceeds steadily with ONL being decreased by 68% compared to SD rats by 230 days (Chrysostomou et al., 2009).

P23H line 2 shows a very slow rate of degeneration such that their retinae have almost normal appearance up to P60. By P180, there is 25% loss of photoreceptors nuclei in the ONL and by P380, 1-2 layers of nuclei remain in the ONL of superior posterior regions. Consistent with gradual structural changes in P23H-2 retinae, functional loss also occurs slowly. At P90, the scotopic and photopic ERG response amplitudes found to be almost unaffected (Anderson et al., 2002; LaVail et al., 2018; McGill et al., 2012).

1.3.2.2 *rd1* mouse, a model for phototransduction cascade mutations

Autosomal recessive RP is associated with mutations in genes mostly encoding proteins essential for the rod phototransduction cascade. A major component of this cascade is the enzyme cGMP phosphodiesterase-6 (PDE6), which catalyzes the hydrolysis of cGMP to GMP. PDE6 is a tetramer comprising two separate catalytic subunits (PDE6A, PDE6B) and two gamma inhibitory subunits; all are required for normal photoreceptor function (Cote, 2004). Mutations in *PDE6A*, *PDE6B* and *PDE6G* have been identified in autosomal recessive forms of human RP (Dryja et al., 1999; McLaughlin et al., 1995). Homozygous mutations in *PDE6* subunits result in a non-functional PDE6 protein and accumulation of cGMP (Bowes et al., 1990; Farber et al., 1974; 1976), subsequently leading to constant depolarization of photoreceptor membranes, which may induce apoptotic cell death as a result of metabolic stress or uncontrolled influx of cations into photoreceptor OS (Acosta et al., 2005).

Similarly, mutations in *Pde6* subunits lead to retinal degeneration in mice. The first mouse model of retinal degeneration was the *rodless* (gene symbol *r*) mouse identified in 1924 (Keeler, 1924). Later, the same mutation was identified in the *retinal degeneration* (*Pde6b*^{*rd1*}, or more commonly known as *rd1* or *rd*) mouse carrying a nonsense mutation in exon 7 of the *Pde6b* gene encoding the beta subunit of cGMP-PDE (Pittler et al., 1991). Since the discovery of the initial *Pde6b* mutation, various other gene mutations have been shown to lead to

congenital retinal disorders in mice, with the animal models designated *rd1*–*rd16* (Dalke et al., 2005).

The *rd1* mouse is one of the most commonly used animal models for autosomal recessive RP. In this model, deterioration of rods initiates at P10 and progresses very rapidly such that by P25 all rod photoreceptors are lost (Carter-Dawson et al., 1978b ; Farber et al., 1994). Cone death is detected around P35, with most of cone photoreceptors lost by P100 (Farber et al., 1994). In accord with anatomical changes, the ERG response of the *rd1* mouse retina is significantly impaired compared to control mice as early as P16 (eye opening occurs around P14). At P18, while the rod response is basically absent in *rd1* mice, the cone response is still relatively normal. However, by P21 neither rod or cone responses are detectable by ERG (Gibson et al., 2013). During and following photoreceptor degeneration in the *rd1* mouse, substantial anatomical remodeling occurs in the inner retina, including neurite sprouting of horizontal and amacrine cells, abnormal development of dendrites of rod bipolar cells and dendrite retraction of horizontal and bipolar cells (Fariss et al., 2000; Greferath et al., 2015; Kalloniatis et al., 2016; Marc et al., 2003; Strettoi et al., 2000; Strettoi et al., 2002a).

While it is known that the loss-of-function mutation of PDE6 protein leads to an accumulation of cGMP, with resultant photoreceptor death, the molecular mechanisms that lead to cell death in the *rd1* retina are still unclear. Several expression profiling experiments show dysregulated gene expression (Azadi et al., 2006; Hackam et al., 2004; Rohrer et al., 2004) in the *rd1* retina suggesting a wide range of genes become dysregulated including ion channels, transporters, transcription factors as well as genes involved in growth differentiation, cellular stress neuroinflammation and cell death. More recently poly-ADPribose-polymerase (PARP) and histone deacetylases (HDACs) (Sancho-Pelluz et al., 2010) have also shown to be dysregulated raising the possibility of wide-ranging gene expression alterations due to epigenetic changes. The general consensus from these studies is that loss of photoreceptors in

the *rd/rd* mouse arises from Ca^{2+} toxicity followed by subsequent activation of multiple destructive pathways including apoptosis and neuroinflammation. How these insults and processes activate various cell death pathways still remains to be determined.

1.4. Programmed cell death

Photoreceptor cell death is the common outcome of mutations that cause RP and found to be mediated by programmed cell death (PCD) (Clarke et al., 2000).

Programmed cell death plays an essential role during development and subsequently in maintaining tissue homeostasis in multicellular organisms (Jin et al., 2005). During development, it modulates cell numbers through eliminating extra cells to produce organs and tissues, particularly during organogenesis. Programmed cell death is also a protective process, removing injured or infected cells that can be potentially dangerous in adult tissues (Fuchs et al., 2011). Failure of this regulation results in a wide range of human diseases such as immunological and developmental disorders, neurodegeneration and cancer (Jin et al., 2005). There are three main types of cell death defined by morphological features including autophagy, necrosis and apoptosis (Kroemer et al., 2009). There is a growing body of evidence suggesting that there are complex cross-talk mechanisms that link autophagy, necrosis and apoptosis such that they can collaborate, antagonize or support each other to regulate programmed cell death (Nikoletopoulou et al., 2013).

1.4.1. Overview of autophagy

Autophagy (Greek; meaning ‘self-eating’) is an evolutionarily conserved catabolic pathway by which cytosolic organelles and long-lived or misfolded proteins are engulfed within double membrane vacuoles, called autophagosomes and then transported to the lysosome for degradation.

Autophagy occurs at low basal levels in almost all cells, playing a vital role in development as well as adaptation in response to diverse forms of stress such as starvation (Duprez et al., 2009; Russo et al., 2013). Thus far, at least 32 different autophagy-related genes (Atg) have been identified in yeast, many of which are conserved in mammals (Russo et al., 2013). Although autophagy undoubtedly has a vital pro-survival role in cells, it has also been considered as a type of programmed cell death based on the finding that numerous autophagic vacuoles are commonly present in dying cells (Debnath et al., 2005). According to a wide range of genetic and biochemical investigations, excess autophagy seems to mediate cell death in certain conditions, especially when cells are not able to die through apoptosis (Murakami et al., 2013b). For a detailed review of autophagy see (Mizushima et al., 2010).

1.4.2. Overview of necrosis

Necrotic cell death or necrosis (Greek; meaning “dead”) has traditionally been considered as a passive form of cell death, occurring in an accidental, unregulated, energy-independent manner (Duprez et al., 2009; Nikolettou et al., 2013). Morphologically, necrosis is characterized by swelling of the cytoplasm and organelles, an increase in cell volume, followed by cell membrane disruption and release of the cytosolic constituents into the surrounding extracellular space. Thus, necrosis is usually accompanied by inflammation in tissues with dying cells (Kroemer et al., 2009). This type of cell death occurs in various

normal conditions including tissue regeneration, immune response, and embryogenesis as well as pathological processes (Pequignot et al., 2003).

Cytokines, TNF receptor family members (TNF, FAS, TRAIL), stimulation of purinergic and glutamate receptors and some survival factors (insulin or NGF) can induce necrotic cell death (Proskuryakov et al., 2003). In certain conditions, necrotic cell death can be triggered in response to a regulated interplay of signaling transduction pathways. It has been demonstrated that TNF- α can initiate either apoptosis or necrosis depending on cell type (Laster et al., 1988). Blocking death receptor-induced apoptosis by caspase inhibitors can induce an alternative necrotic cell death in murine L929 fibrosarcoma cells (Vercammen et al., 1998). Later, it was discovered that this type of receptor-dependent necrotic cell death is mediated by the stimulation of receptor-interacting protein 1 (RIP1) (Holler et al., 2000), which in turn is regulated by RIP3 (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). This RIP kinase-mediated necrosis is termed as ‘necroptosis’ or ‘programmed necrosis’ (Chan et al., 2003; Vercammen et al., 1998). It has been suggested that necrosis is associated with some physiological and pathological conditions like retinal degeneration (Murakami et al., 2013a; Vandenabeele et al., 2010). For a more detailed review the reader is directed to (Edinger et al., 2004).

1.4.3. Overview of apoptosis

‘Apoptosis’ is a term first coined by Kerr et al. in 1972 to describe specific morphological features of cell death associated with fragmentation of DNA. Coming from the Greek words “apo” meaning ‘off’ or ‘separation’ and “ptosis” meaning ‘falling’, the term translates to ‘falling off’ similar to ‘leaves falling from a tree’ (Duque-Parra, 2005; Kerr et al., 1972).

Morphologically, apoptosis is characterized by rounding of the cell, reduction of cellular volume, chromatin condensation, nuclear fragmentation, maintenance of ultrastructural integrity of cytoplasmic organelles and blebbing of cell membrane to partition cellular fragments of cytoplasm (apoptotic bodies). Apoptotic bodies are rapidly recognized and digested by neighboring cells and phagocytes. Through this process the cell essentially remains intact and does not release its constituents into the surrounding tissue and thus there is no inflammation, nor immune response (Duprez et al., 2009; Kroemer et al., 2009; Li et al., 2008).

1.4.3.1 The apoptotic pathways

A huge number of studies have been conducted to explore the molecular mechanisms involved in induction, mediation, and regulation of the apoptotic signaling pathways. The nematode (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*), and the mouse (*Mus musculus*) have been used widely as animal models for studying apoptotic pathways (Fuchs et al., 2011).

The primary understanding of the molecular mechanisms of apoptosis in mammalian cells has been derived from genetic studies of programmed cell death during *C. elegans* development (Jin et al., 2005). In 1986, it was demonstrated that programmed death of somatic cells in *C. elegans* is an invariant fate precisely regulated by cell lineage (Ellis et al., 1986).

Programmed cell death in *C. elegans* is controlled by three Cell Death abnormal (CED) proteins, named CED-3 (caspase-9 homologue in mammals), CED-4 (Apaf-1 homologue in mammals) and CED-9 (antiapoptotic Bcl-2 homologue in mammals). In pre-apoptotic cells CED-9 is associated with CED-4, thereby inhibiting the activation of CED-3. In response to particular developmental signals to trigger cell death, EGL-1 (BH3-only member homologue) binds CED-9, thereby releasing CED-4 and resulting in the activation of the protease CED-3 (**Figure 1.3**) (Fuchs et al., 2011; Jin et al., 2005; Li et al., 2008). While *C. elegans* has the

basic core components of the programmed cell death apparatus, this basic process has been overlaid by increasingly complicated mechanisms in the mammalian system to trigger and control the pathway (Jin et al., 2005). Two main evolutionarily conserved families lie at the heart of this pathway in mammals, namely the caspase and BCL-2 families of proteins.

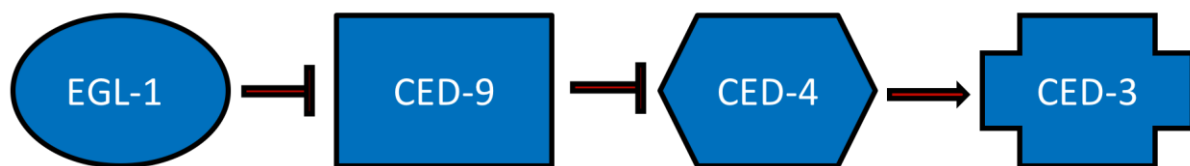


Figure 1.3: Schematic of the core components of apoptosis in *C. elegans*

In an apoptotic cell, binding of EGL-1 to the CED-9–CED-4 complex results in the release of CED-4, which in turn activates CED-3, leading to cell death (Jin et al., 2005).

1.4.3.2 Caspase family of proteins

Caspases (cysteine-aspartate-specific proteases) are a family of proteins that have a critical role in apoptotic pathways. In 1993, it was demonstrated that *C. elegans ced-3* gene encodes a cysteine protease (Yuan et al., 1993), a homologue of interleukin-1 β -converting enzyme (ICE) which was discovered independently in a human cell line (Cerretti et al., 1992; Thornberry et al., 1992). Overexpression of ICE (later renamed caspase-1) was found to be sufficient to initiate apoptosis in rat embryonic fibroblast cells (Rat-1 cell line) (Miura et al., 1993).

Such structural and functional homology between mammalian caspase-1 and CED-3 of *C. elegans* led to identification of multiple homologous proteins in mammals named the “caspase family”. To date, 10 murine caspases (caspase-1, -2, -3, -6, -7, -8, -9, -11, -12 and -14) and 11 human caspases (caspase-1, -2, -3, -4, -5, -6, -7, -8, -9, -10 and -14) have been identified. Caspase-4 and -5 in human are functional orthologs of caspase-11 and -12 in mouse, while the mouse genome lacks an orthologue of human caspase-10. Other caspases with similar numbers are functional orthologs of each other in human and mouse (Duprez et al., 2009; Jin et al., 2005; Li et al., 2008; Murakami et al., 2013b).

The caspase family proteins are expressed as catalytically inactive zymogens containing an N-terminal prodomain of variable length, followed by a large subunit (p20) and a small C-terminal subunit (p10). They are activated by a proteolytic cleavage at specific Asp residues and/or allosteric conformational changes. The active enzymes are heterotetramers containing two large and two small subunits with two active sites per molecule (Li et al., 2008).

There are at least two alternative methods for classification of caspases. In the first one, caspases are divided into two subfamilies based on their known main functions (Li et al., 2008):

1. Pro-apoptotic caspases (caspase-2, -3, -6, -7, -8, -9, -10) are recognized as having primary roles in promoting cell death signaling transduction.
2. Pro-inflammatory caspases (caspase-1, -4, -5, -11, -12) control maturation of cytokine during inflammation.

As caspases are involved in numerous other cellular activities separate to the pro-apoptotic or pro-inflammatory functions and there is considerable crosstalk among caspases from both classes, an alternative grouping system has been suggested, in which caspases are simply classified into two groups based on the length of the prodomains which also correlates with their role in the apoptotic signaling cascade (Li et al., 2008):

1. Initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, and -12) have long prodomains, comprising one of the two protein-protein interaction motifs: the death effector domain (DED) in caspase-8 and -10 or the caspase recruitment domain (CARD) in caspase-1, -2, -4, -5, -9, -11, and -12 (**Figure 1.4**).
2. Effector or executioner caspases (caspase-3, -6, and -7) possess short prodomains (**Figure 1.4**).

Generally, the initiator caspases are activated by interaction with their regulatory adaptor proteins through the DED or CARD domain. Once activated, initiator caspases cleave specific Asp residues of downstream effector caspases, which in turn can cleave a wide range

of protein substrates and induce apoptosis (Duprez et al., 2009; Jin et al., 2005; Li et al., 2008; Murakami et al., 2013b; Reed, 2000).

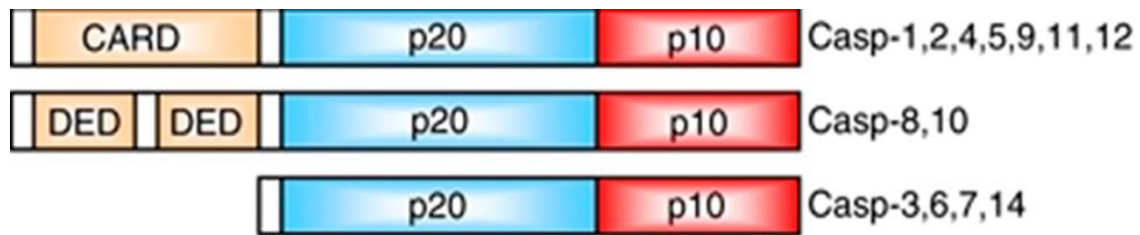


Figure 1.4: Structural domains of caspase family in mammals

Initiator caspases possess long prodomains containing death effector domains (DEDs) or caspase recruitment domains (CARDs), while executioner caspases have shorter prodomains (Li et al., 2008).

1.4.3.3 BCL-2 family of proteins

The BCL-2 (B-cell lymphoma 2) family consists of more than 30 proteins, all of which share at least one of the four conserved regions, termed Bcl-2 homology domains (BH1–BH4). Many members of this family also have a hydrophobic carboxyl terminal domain to deliver them to intracellular membranes such as the outer mitochondrial membrane (OMM), endoplasmic reticulum (ER), lysosomal membrane and nuclear envelope. Based on structural and functional aspects, the BCL-2 family members are divided into three subfamilies (**Figure 1.5**) (Czabotar et al., 2014):

1. **Initiators (pro-apoptotic BH3-only proteins):** Members of this subfamily only contain a conserved BH3 domain and are essential initiators of the apoptotic pathway. BAD (BCL-2-associated death promoter protein), BIK (also known as BLK or NBK), BID, tBID, HRK (also known as death protein-5 (DP5), BIM (BCL-2 interacting mediator of cell death, also known as BOD), BMF, NOXA and PUMA (p53 up-regulated modulator of apoptosis protein, also known as BBC3) are some of the well-known proteins in this group.
2. **Guardians (multi-domain pro-survival proteins):** The anti-apoptotic or pro-survival members of this subfamily share all four BH domains and are responsible for

preserving outer mitochondrial membrane integrity. To date, at least six anti-apoptotic proteins have been identified in human including, BCL-2, BCL-XL (BCL-extra-long), BCL-W (also known as BCL2L2), MCL1 (myeloid cell leukaemia sequence 1), A1 (BCL-2 related protein A1, also known as BFL1 in humans) and BCL-B (also known as BCL2L10) (Czabotar et al., 2014; Donovan et al., 2004).

3. Effectors (multi-domain pro-apoptotic proteins): BAX (BCL-2-associated X protein), BAK (BCL-2 homologous antagonist/killer protein) and BOK are the members of this subfamily containing all four BH domains (Czabotar et al., 2014; Youle et al., 2008).

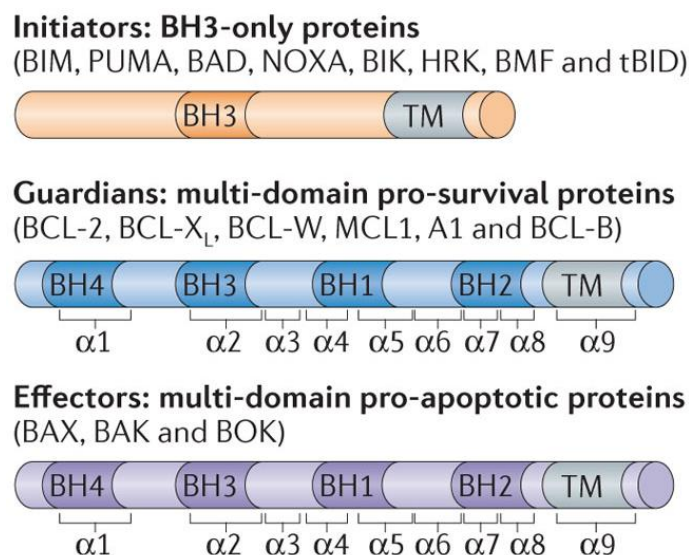


Figure 1.5: Members of BCL-2 family

Based on structural and functional aspects they are divided to three subfamilies. While the multi-domain pro-survival and pro-apoptotic members of the family contain all four BCL-2 homology regions (BH1–BH4), the BH3-only proteins only possess the BH3 helix, by which they interact with the multi-domain BCL-2 family members (Czabotar et al., 2014).

1.4.3.4 Caspase-dependent apoptosis pathways

Although, there are likely various pathways for caspase activation in mammalian cells, only two have been clarified in depth, namely the extrinsic pathway (death receptor pathway) and the intrinsic pathway (the mitochondrial pathway) (Elmore, 2007).

1.4.3.4.1 *The extrinsic apoptotic pathway*

The extrinsic or receptor-mediated pathway is triggered by the binding of extracellular ligands to death receptors located on the surface of the cell's plasma membrane (**Figure 1.6**). Death receptors are a family of type I transmembrane proteins that share a similar cysteine-rich extracellular domain and have a 60–80 amino acid death domain (DD) within the cytoplasmic region of the protein that is crucial for transmitting the death signal from the cell outer membrane to the intracellular signaling pathways.

Thus far, eight members of the death receptor family have been identified including, tumor necrosis factor receptor 1 (TNFR1; aka DR1, CD120a, p55 and p60), Fas (aka DR2, APO-1 and CD95), DR3 (aka APO-3, LARD, TRAMP and WSL1), TNF-related apoptosis inducing ligand receptor 1 (TRAILR1; aka DR4 and APO-2), TRAILR2 (aka DR5, KILLER and TRICK2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor.

The Fas and TNFR1 receptors are most commonly used in models that describe the molecular mechanism of the extrinsic pathway. For example, the binding of Fas ligand to Fas leads to the binding of the adapter protein FADD (Fas-associated death domain protein), while the binding of TNF to TNFR1 leads to the binding of the adapter protein TRADD (TNF receptor-associated death domain). Association with FADD or TRADD induces dimerization of the Fas/TNFR1 receptors through their death effector domains (DEDs), association with procaspase-8 (procaspase-10 in human) to form the death-inducing signaling complex (DISC), resulting in the autocatalytic activation of procaspase-8. Active caspase-8 in turn can directly cleave caspase-3 or other effector caspases that ultimately leads to cell death (Elmore, 2007; Lavrik et al., 2005; Lawen, 2003; Li et al., 2008; Telegina et al., 2017; Wajant, 2006) (**Figure 1.6**).

1.4.3.4.2 *The intrinsic apoptotic pathway*

In addition to the extrinsic pathway, apoptosis can be initiated through the intrinsic or mitochondrial-mediated pathway, which is independent of death receptor signaling. This apoptotic pathway can be directly induced in response to intracellular signaling stimuli such as cellular stress, DNA damage, viral infections, growth-factor deficiency, activation of oncogenes, hypoxia, oxidative stress and specific cytokines or hormones. This pathway is tightly controlled and regulated by the BCL-2 family (Duprez et al., 2009; Kiraz et al., 2016; Saraste et al., 2000).

Three models have been suggested to explain how members of the BCL-2 family interact with each other to control this apoptotic pathway:

1. Direct activation model: In this model, pro-apoptotic effectors BAX and BAK are directly activated by specific members of BH3 only proteins, mainly the truncated form of BID (tBID), BIM and PUMA. BH3-only proteins are primarily sequestered by pro-survival proteins, such as BCL-2 to inhibit activation of BAX and BAK (Certo et al., 2006; Gavathiotis et al., 2008; Kim et al., 2006; Kim et al., 2009; Kuwana et al., 2005; Letai et al., 2002). It is believed that some BH3-only proteins, including BAD and NOXA, interact with their pro-survival relatives selectively, while other BH3-only proteins such as BIM, tBID and PUMA can neutralize all pro-survival subfamily members (Certo et al., 2006; Chen et al., 2005; Kuwana et al., 2005).
2. Indirect activation model: According to this model, pro-survival members also need to sequester any activated form of BAX or BAK containing exposed BH-3 domain (Fletcher et al., 2008; Wang et al., 1998). Therefore, all the pro-survival members are required to be neutralized by BH3-only proteins before mitochondrial outer membrane permeabilization is induced by free BAX and BAK. This model

suggests regulation of apoptosis is mediated by a simple competition, whereby higher levels of BH3-only proteins leads to increased interaction with pro-survival proteins and blocking their inhibition of activated BAX or BAK. BAX is thought to be inhibited by all pro-survival proteins, whereas BAK can be only suppressed by MCL1, A1 and BCL-XL (Chen et al., 2005; Simmons et al., 2008; Willis et al., 2005; Willis et al., 2007).

3. Unified model: It is believed that both direct and indirect activations models function through a tripartite network of interactions among the three BCL-2 subfamilies. Thus, based on this model pro-survival members sequester both BH3-only proteins and activated BAX and BAK (Czabotar et al., 2014; Llambi et al., 2011; Shamas-Din et al., 2013). In healthy cells, BAK is situated in the outer mitochondrial membrane (Griffiths et al., 1999a), whereas BAX exists as a cytoplasmic monomer (Suzuki et al., 2000). When cells receive enough death signals, initiator BH3-only proteins (pro-apoptotic), particularly BIM or tBID, migrate to and sit on the outer mitochondrial membrane where they recruit cytoplasmic BAX.

Regardless of the exact regulatory mechanisms, once apoptosis is signalled, BAX and BAK undergo conformational changes and oligomerization, ultimately leading to mitochondrial outer membrane permeabilization by forming proteolipid pores within the membrane, which is a crucial initiation point in this pathway. In addition to recruiting BAX, both initiator and effector BH3-only members also sequester pro-survival proteins such as BCL-XL at the outer mitochondrial membrane, preventing them from inhibiting BAX and BAK oligomerization. Permeabilization of the outer mitochondrial membrane results in soluble mitochondrial proteins such as cytochrome C, OMI/HTRA2, GSPT1/eRF3, SMAC/DIABLO (second mitochondria-derived activator of caspases) and endonuclease G,

being released from the intermembrane space into the cytosol, where they initiate the caspase activation cascade.

The most well-defined example of this activation process involves cytochrome C. Once in the cytosol, cytochrome-c binds to apoptotic protease activating factor-1 (APAF-1), a cytosolic protein containing a caspase recruitment domain (CARD), leading to ATP association. This in turn, triggers APAF-1 oligomerization to form a wheel shaped structure called the “apoptosome”. Consequently, multiple procaspase-9 molecules are recruited into the apoptosome through the CARD domains of APAF-1, which promotes their autoactivation. Active caspase-9 subsequently activates downstream executioner caspases such as caspase-3 and -7, which later cleave other major intracellular substrates (**Figure 1.6**).

In addition to the above pathway, mitochondrially released SMAC/DIABLO proteins also bind to a group of cytosolic proteins known as inhibitor of apoptosis proteins (IAPs) to block their inhibitory action on active caspases. IAPs are normally responsible for suppressing inadvertent activation of caspase-9 and caspase-3 by binding them through specialist BIR (Baculovirus IAP repeat, BIR) domains (Jin et al., 2005; Shamas-Din et al., 2013; Wang, 2001).

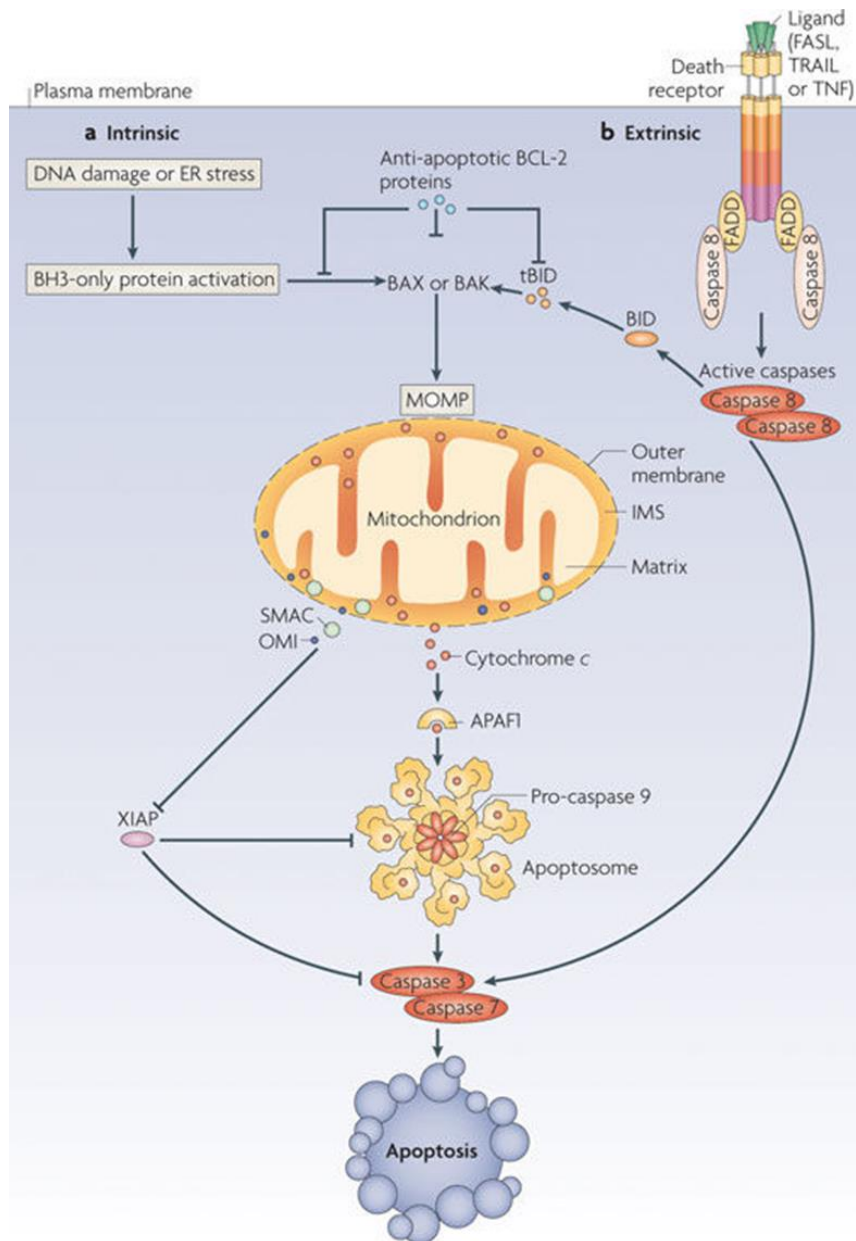


Figure 1.6: The extrinsic and intrinsic pathways of apoptosis

(A): The extrinsic apoptotic pathway is triggered by the interaction of death receptors with their related ligands, leading to recruitment of FADD and then caspase-8. Caspase 8 is dimerized and activated which in turn can cleave caspase-3 and caspase-7 which results in apoptosis. Cleavage of caspase-8 can also mediate crosstalk between the extrinsic and intrinsic pathways by activation of the BH3-only protein BID; (B): Intrinsic pathway. Upon intrinsic death signals BH3-only members of BCL-2 family are activated, leading to suppression of the pro-survival proteins, activation of the pro-apoptotic effectors BAX and BAK and eventually mitochondrial outer membrane permeabilization. Following this, cytochrome c is released from intermembrane space, binds APAF-1 and apoptosome is formed which in turn recruits and activates caspase-9. Subsequently, caspase-9 cleaves executioner caspases, caspase-3 and caspase-7, leading to their activation and apoptosis (Tait et al., 2010).

1.4.3.4.3 Cross-talk between intrinsic and extrinsic pathways

Although the intrinsic and extrinsic pathways initiate apoptosis in different ways, both lead to activation of executioner caspases, such as caspase-3. Prior to this convergence, several

studies have indicated there is crosstalk between these two pathways at both initiation (early) and execution (late) stages.

In 1998, two different cell types were identified based on the requirement of the intrinsic pathway activation for apoptosis induced by extrinsic pathway. In type I cells (SKW6.4 and H9) mitochondria are not involved in Fas-mediated apoptosis (extrinsic), whereas type II cells (Jurkat and CEM) require the release of cytochrome C from mitochondria to eventually induce cell death via Fas. At the molecular level, activation of Fas in type I cells leads to sufficient levels of FADD and caspase-8 recruited to the DISC to trigger apoptosis, while in type II cells there are insufficient levels and stimulation of the intrinsic pathway is required to induce cell death (Scaffidi et al., 1998). This extrinsic-intrinsic cross-talk relies on DISC-mediated initiation of the mitochondrial-mediated pathway via the BCL-2 pro-apoptotic protein, BID. Specifically, while type II cells have insufficient levels of FADD/caspase-8 within the DISC, to trigger apoptosis directly, the level of recruited caspase 8 is sufficient to cleave cytosolic BID, which then translocates to mitochondria where it induces mitochondrial dysfunction and ultimately activates the intrinsic pathway (Li et al., 1998a; Luo et al., 1998). This idea of extrinsic/intrinsic cross talk has been strengthened by the discovery that molecules such as SMAC/DIABLO, which can neutralize caspase-3 and caspase-9 inhibitors, are also released from mitochondria in response to death receptor (extrinsic) activation (Deng et al., 2002; Du et al., 2000). Furthermore, activation of caspase-8 has been identified in response to some intrinsic cell death signals to accelerate further effector caspase activation (Cowling et al., 2002; Tang et al., 2000). Moreover, this crosstalk seems to also occur between the upstream molecules of the extrinsic and intrinsic pathways. For instance, p53, a tumor suppressor activated in response to DNA damage, not only induces the intrinsic pathway via activation of pro apoptotic molecules such as BAX and PUMA, but also up-regulates mediators of extrinsic pathway such as FasL (**Figure 1.6**) (Jin et al., 2005; Kasibhatla et al., 1998; Roy et al., 2000).

1.4.3.5 *Caspase-independent apoptotic pathway*

Although activation of caspases undoubtedly plays a critical role in apoptotic pathways, programmed cell death has been identified in the absence of caspase activity (Chipuk et al., 2005). In this process, other proteases and some endonucleases have been shown to be involved.

Calpains: Calpains are a group of non-lysosomal calcium dependent cysteine proteases consisting of 14 different families, of which μ -calpain (calpain I) and m-calpain (calpain II) are ubiquitously expressed (Suzuki et al., 2004). They exist in the cytosol as inactive zymogens and are activated by a conformational change upon interaction with Ca^{2+} (Guroff, 1964; Moldoveanu et al., 2002).

Several studies have revealed that in certain cell types, apoptosis can be mediated by calpains in response to specific death signals. Caspases and other non-caspase proteins including APAF-1, BCL-2 family members such as BAX and BID, p53 and p73, can be cleaved by calpains, leading to either their activation or inactivation (Broker et al., 2005).

Cathepsins: Cathepsins are a group of endolysosomal peptidases that includes cysteine, aspartate and serine proteases. They are involved in lysosomal membrane permeabilization, which leads to lysosomal-mediated cell death. A number of cathepsins including cysteine cathepsins B and L, as well as aspartate cathepsin D, have been identified to engage in apoptosis probably via activation of pro-apoptotic protein BID or blocking cleavage of anti-apoptotic BCL-2 members (BCL-2, BCL-XL and MCL-1) (Aits et al., 2013; Appelqvist et al., 2012; Cirman et al., 2004; Conus et al., 2008). It has also been reported that release of lysosomal cathepsins can be induced by activated calpains through a “calpain-cathepsin cascade” (Broker et al., 2005; Yamashima, 2004; Yamashima et al., 1998).

Apoptosis-inducing factor (AIF): AIF is a flavin adenine dinucleotide-dependent oxidoreductase synthesized from a nuclear gene and imported to the mitochondrial

intermembrane space, where it plays a vital role in cell survival via the structural and functional regulation of complex I and III in the respiratory chain (Modjtahedi et al., 2006; Vahsen et al., 2004). In 1999, AIF was identified as the first mitochondrial protein that induces cell death independent of caspases (Susin et al., 1999). Upon various death signals, AIF is cleaved by calpains (cytosolic and/or mitochondrial) and cathepsins forming tAIF (Polster et al., 2005; Yuste et al., 2005). Following mitochondrial outer membrane permeabilization, tAIF translocates into the nucleus where it mediates chromatinolysis and large-scale DNA fragmentation (Cregan et al., 2004; Joza et al., 2009) (Delavallee et al., 2011; Murakami et al., 2013b).

Endonuclease G: Similar to AIF, endonuclease G (Endo G) is a protein encoded by a nuclear gene and localizes to mitochondria in living cells. It is involved in mitochondrial DNA replication and repair. Upon a cell receiving death signals, it is released from mitochondria and translocates to the nucleus (Lemarié et al., 2004). Several investigations have shown that Endo G can initiate apoptosis and DNA cleavage as a caspase-independent factor (Cregan et al., 2004; Li et al., 2001; Parrish et al., 2003; Wenzel et al., 2005).

1.4.4. Apoptosis and the normal development of the retina

Development of the vertebrate retina is mediated by naturally occurring apoptosis, which eliminates excessive and non-functional neural cells (Perry et al., 1983; Rakic et al., 1983; Young, 1984). The involvement of caspase-3 in normal development of retina was evaluated in caspase-3-deficient (*Casp 3^{-/-}*) mice. Developmental-related apoptotic cell death of the INL was delayed by nearly 2 weeks; however still occurs so that the retinal organization remained normal. Optic nerve, GCL and nerve fiber layers exhibited normal morphology, suggesting that genetic ablation of caspase-3 activates alternative mechanisms of cell death to resume normal development (Zeiss et al., 2004b). During normal maturation of BALB/c and

C57Bl6 retina, a remarkable decrease was evident in the expression level of Apaf-1, caspase-3 and -9 which may be a mechanism by which apoptosis is firmly controlled in mature photoreceptors (Donovan et al., 2002).

Contribution of the BCL-2 family proteins in mediating apoptotic cell death during normal development of retina was first identified when increased cell numbers were found in the GCL and INL of transgenic mice overexpressing BCL-2 in the retina (Strettoi et al., 2002b). Donovan et al. (2006) demonstrated that isoforms of BIM (particularly BimEL) are expressed during the peak period of postnatal cell death in the normal retina (P2-P7, BALB/c mice) but undergo a rapid reduction in expression of both mRNA and protein for BIML and BIMEL between P8 and P10. This study reported retinal development is delayed in mice that lack the apoptotic activator protein BIM (*Bim*^{-/-}), confirming the importance of BIM proteins in removing cells during developmental cell death. In these mutant mice, the retina is significantly thicker because of less developmental apoptotic cell death during early postnatal days.

Loss of the proapoptotic molecule, *Bax*, was found to prevent developmental cell death in the inner retina, GCL and INL (Hahn et al., 2003; Mosinger Ogilvie et al., 1998). However, similar analyses of another proapoptotic family member, *Bak*, suggest it may not be as important since *Bak*^{-/-} mice showed similar retinal structure to wild-type (Hahn et al., 2003). Reflecting the importance of *Bak* and *Bax* in controlling apoptosis, studies have shown them to have over-lapping roles in many tissues during development (Lindsten et al., 2000). *Bak*^{-/-}/*Bax*^{-/-} mice tend to die perinatally with multiple developmental defects (persistent interdigital webbing, imperforate vagina and excess neuronal and hematopoietic cells). Surprisingly ~10% of *Bak*^{-/-}/*Bax*^{-/-} mice survive to adulthood (Lindsten et al., 2000). Examination of retinae from mice with *Bak*^{-/-}, *Bax*^{-/-} and *Bak*^{-/-}/*Bax*^{-/-} mutations revealed that the double mutants had even greater increased thickness of the total retina and IPL when compared to *Bax*^{-/-} mice (Hahn et

al., 2003). This appears to be due to almost total abrogation of TUNEL staining during the period of developmental cell death (Hahn et al., 2003).

1.4.5. *Apoptosis and photoreceptor cells loss in retinitis pigmentosa*

As discussed above, programmed cell death is an essential process for retinal development; however, it is also the underlying cause of vision loss in many ocular disorders such as RP, age-related macular degeneration (ARMD), diabetic retinopathy and retinal detachment. Intensive efforts have been made to understand the retinal cell death mechanisms in order to develop new therapeutic strategies to inhibit or delay neuronal degeneration. The results of these studies have demonstrated that photoreceptor demise in different animal models of RP is associated with caspase-dependent and/or caspase-independent of apoptosis.

1.4.5.1 *Caspase-dependent apoptosis pathways in retinitis pigmentosa*

Using different animal models of RP, several research groups have investigated the role of the caspase family in photoreceptor death in retinal degeneration. However, the findings have been inconsistent, with activation of different caspases being identified in different models. For example, activation of caspase-3 was identified in transgenic rats carrying the murine S334ter rhodopsin mutation, (Liu et al., 1999), caspase-1 and -2 in Royal College of Surgeons (RCS) rats (Katai et al., 1999) , caspases -3,-6 and -8 in rats treated with NMU (Yoshizawa et al., 1999; Yoshizawa et al., 2000), and caspase-3, -8 and -12 in *rd1* mice (Jomary et al., 2001a; Kim et al., 2002; Lohr et al., 2006; Sanges et al., 2006a). Caspase ablation/inhibition experiments also consistent with diverse cell death mechanisms in RP. In the T17M RHO mouse model of RP, ablation of caspase-7 rescued both retinal function and structure, possibly through reprogramming the unfolded protein response and a reduction of JNK-induced apoptosis (Choudhury et al., 2013). Also, genetic inactivation of caspase-12 in

these animals maintained the integrity of retinal structure and delayed photoreceptor cell degeneration via reduction of caspase-3/7 and calpain activity (Bhootada et al., 2015). Additionally, in *rd5* mice (*Tubby* gene mutant) inhibition of caspase-3 activity by Z-DEVD-FMK significantly decreased photoreceptor loss, suggesting that caspases are key mediators of retinal degeneration in these animals (Bode et al., 2003).

However, the literature is far from clear on the role played by caspase-dependent apoptosis in RP. For example, Doonan et al. (2003) indicated that caspases are not activated during photoreceptor degeneration in *rd1* mice. Moreover, intraperitoneal injection of a caspase-3 inhibitor or genetic elimination of caspase-3 provided only moderate and transient photoreceptor protection in *rd1* mice (Yoshizawa et al., 2002; Zeiss et al., 2004a). Similarly, caspase-3 activity was found to play a minimal, if any, role in P23H rat retinal dystrophy (Kaur et al., 2011a). Further *in vitro* work using primary C57BL6 retinal cells cultured under serum free conditions, reported the failure of several caspase inhibitors, zVAD-fmk, DEVD-CHO and BD-fmk, to block or alter photoreceptor death, suggesting that caspase dependent apoptosis was not involved in retinal cell death (Carmody et al., 2000). Rather this study suggested the degeneration was mediated by oxidative stress.

Together, these findings suggest that while caspase-dependent pathways may play a role in photoreceptor cell death, they are not the only cell death pathway activated in retinal degenerations (Lohr et al., 2006).

1.4.5.2 Caspase-independent apoptosis pathways in retinitis pigmentosa

Caspase-dependent apoptosis, as discussed above, was classically introduced as a common mechanism of photoreceptors death in retinal degeneration of human, as well as related animal models. However, photoreceptor cell death has been shown to be caspase-independent in several animal models of retinal degeneration. The activation of calpain and cathepsin D has been identified in *rd1* mice (Doonan et al., 2005; Paquet-Durand et al., 2006;

Sharma et al., 2004). Higher calpain activity coincided with a significant drop in the expression of calpastatin, an endogenous calpain inhibitor, has been reported during retinal degeneration (Kaur et al., 2011a; Paquet-Durand et al., 2006). Moreover, co-activation of caspases and calpain have been described in *rd1* mice, S334ter and retina-derived 661W cells following growth factor starvation, suggesting cross-talk between caspase and calpain pathways possibly to magnify the apoptotic cascade (Gomez-Vicente et al., 2005; Kaur et al., 2011a; Sharma et al., 2004).

As mentioned previously, AIF is a mitochondrial intermembrane protein, which in response to certain lethal signals translocates into the nucleus to induce caspase-independent apoptotic cell death (Cande et al., 2002). Nuclear translocation of both AIF and caspase-12 has been reported in dying photoreceptors of *rd1* in response to higher intracellular levels of Ca^{2+} and calpain activation. Consistent with this, calpain inhibitors suppressed AIF and caspase-12 activity and, apoptosis in vitro and in vivo (Sanges et al., 2006b). Nuclear translocation of AIF also occurs in apoptotic photoreceptors of RCS rats (Murakami et al., 2008) and, both mitochondrial and cytosolic fractions of RCS rat retina were found to contain altered activity of calpains during retinal degeneration (Mizukoshi et al., 2010).

Interestingly, the mitochondrial calpain became activated prior to the cytosolic calpain, presumably to cleave AIF and thus permit induction of apoptosis. Furthermore, inhibition of calpain suppressed nuclear AIF localization and reduced photoreceptor cell loss in RCS rats (Mizukoshi et al., 2010; Ozaki et al., 2012b). Taken together, these findings indicate that photoreceptor degeneration in RP can be mediated by caspase-independent pathways.

1.4.5.3 Roles of the BCL-2 family of proteins in retinitis pigmentosa

As discussed above, members of the BCL-2 family, pro- and anti-apoptotic proteins, are key regulators of apoptosis by controlling the mitochondrial membrane integrity. Their

involvement in the propensity of photoreceptor cells to undergo a caspase-dependent or -independent death have been demonstrated in several studies (Danial et al., 2004).

To investigate the involvement of BCL-2 protein in photoreceptor death, two separate groups generated transgenic mice overexpressing BCL-2 on a retinal degeneration background. While overexpression of BCL-2 slowed down photoreceptor loss in *rd1* and rhodopsin mutant mice in one study (Chen et al., 1996b), it did not rescue photoreceptor cells from *rd1*-induced apoptotic death in another study (Joseph et al., 1996a). Later, it was shown that a combination of BAG-1 and BCL-2 transgenes significantly delayed the progression of retinal degeneration in the S334Ter mice, suggesting that BCL-2 needs ancillary members of BCL-2 family for complete activity (Eversole-Cire et al., 2000). Further investigation of the Bcl2 family involvement showed the expression of *Bim* was not altered in the *rd1* retina, during the period of photoreceptor cell death (P10-P21), suggesting that BIM may not take part in retinal degeneration caused by *Pde6 β* mutation (Doonan et al., 2007).

Although expression of BAX persists in the normal retina until P30-P60 (Donovan et al., 2006), experiments using *Bax*-deficient mice, suggest that it may not be involved in photoreceptor degeneration in the *rd1* model as there was no rescue in the photoreceptor degeneration by P21 (Mosinger Ogilvie et al., 1998). By contrast, a recent study indicated BAX is activated in three murine genetic models of RP (*rd1*, P23H^{Tg}, and *Rho*^{-/-}), possibly through a protease cascade containing calpain I and cathepsin D, but not calpain II (Comitato et al., 2014). Such discrepancy in the literature suggests that retinal degeneration is not dependent on a single apoptotic mediator. Similar to BAX, BAK is also expressed in the postnatal retina until P30-60 (Donovan et al., 2006), but the individual function of BAK in the retinal degeneration is yet to be explored. BAK and BAX have been found to function redundantly in many tissues, including retina (Hahn et al., 2004; Hahn et al., 2003). However, the influence of *Bax* and *Bak* double null mutations on retinal degeneration in genetic RP

models has not been examined. Thus, there is a need to investigate the individual and combined functions of these two pro-apoptotic factors in retinal degeneration.

1.5. Aims of study

Photoreceptor degeneration is the final stage in retinal degenerative diseases leading to visual impairment. This cell death has been considered as a process mediated by the classical caspase-dependent apoptotic pathway. However, as discussed above, recent studies have revealed the involvement of caspase-independent apoptosis and also non-apoptotic pathways, including necrosis or autophagy in photoreceptor cell death. Considering the redundancy and interplay between all these pathways, it is essential to explore what pathway is key to photoreceptor cell death so that possible therapies can be designed to inhibit or slow this neuronal loss.

The overall aim of the current study was phenotypic characterisation of albino rats heterozygous for the P23H mutation (line 3, P23H-3) and to examine the molecular mechanisms of photoreceptor death in RP using P23H-3 rat and *rd1* mouse models with an emphasis on apoptosis. Therefore, three aims were defined as below:

Aim 1: Characterization of retinal function and structure of P23H-3 rat, an animal model of RP. The experiments addressing this aim are described in Chapter 2.

Aim 2: Identification of cell death pathways in retinal degeneration of P23H-3 rat. The experiments addressing this aim are described in Chapter 2.

Aim 3: Investigating the role of pro-apoptotic BAK and BAX in retinal degeneration of *rd1* mouse. The information addressing this aim are described in Chapter 3.

Chapter 2.

Characterization of retinal function, structure, and cell death pathways in the P23H-3 rat, an animal model of retinitis pigmentosa

2.1. Introduction

Inherited retinal dystrophies are a family of neurodegenerative diseases in which genetic mutations affecting the RPE or photoreceptors lead to irreversible dysfunction or loss of neuronal cells within the retina. The most common form of these retinal dystrophies is retinitis pigmentosa (RP), a hereditary and genetically heterogeneous group, featuring degeneration of rod and cone photoreceptors (Hamel, 2014; Kalloniatis et al., 2016). It is estimated that over 2 million patients are suffering from RP in the world (Sorrentino et al., 2016). While the progression of RP varies among different individuals, continuous loss of photoreceptors eventually causes severe visual impairment (Hamel, 2014; Maugeri et al., 2000). At present, there is no proven treatment for most of RP patients; however, a variety of therapeutic approaches are under investigation.

In RP, numerous mutations have been described in genes, encoding proteins involved in different cellular mechanisms, such as the phototransduction cascade (e.g. *PDE6A*, *PDE6B*, and *RHO*) (Jones et al., 2017). Of particular importance is rhodopsin (*RHO*), a transmembrane protein synthesized in the ER, modified post-translationally and transported to the outer segment of rod photoreceptors (Fukuda et al., 1979; Kaushal et al., 1994c; Krebs et al., 2004; Liang et al., 1979). The proper expression of *RHO* is crucial for the detection of light photons and their conversion into electrical signals (phototransduction). Mutations in the *RHO* gene, are the most prevalent cause of RP and account for almost 30-40% of patients with the autosomal dominant form of the disease (Dryja et al., 2000b; Noorwez et al., 2008). Substitution of proline to histidine in amino acid 23 (P23H-*RHO*) is the most common mutation of *RHO*. P23H-*RHO* folds improperly at the N-terminal, the binding site of 11-cis-retinal chromophore and is unable to form functional visual pigment (opsin + chromophore). The misfolded P23H protein, unlike wild type, fails to be transported to the plasma membrane; instead it forms oligomeric aggregates within the ER and Golgi (Kaushal et al., 1994b; Roof et

al., 1994; Sung et al., 1991a). This aberrant protein transport results in recruitment of ER-resident chaperones to suppress protein accumulation and the ubiquitin/proteasome system is activated to eliminate the aggregate-prone proteins from the cell (Anukanth et al., 1994; Chapple et al., 2001a). Retention of P23H RHO in the cytosol can induce ER stress and activate the unfolded protein response (UPR), which in turn leads to photoreceptor death (Frederick et al., 2001; Lin et al., 2007; Tam et al., 2006).

Reflecting the autosomal dominant phenotype, several studies have demonstrated a dominant-negative impact of P23H-RHO on WT protein, reporting that co-expression of WT and P23H RHO led to formation of aggregates including the WT RHO and abnormal transport of WT protein. In addition to these alterations, it was shown that there was elevated proteosomal degradation and steady-state ubiquitination of the WT protein in the presence of P23H-RHO (Illing et al., 2002; Saliba et al., 2002).

Rat transgenic models carrying a transgene expressing a mouse rhodopsin gene with a C-A substitution at codon 23 (P23H amino acid conversion) were first described in 1996 (Steinberg et al., 1996). Three different lines were obtained and while line 2 appeared to have a normal phenotype, lines 1 and 3 showed slightly different rates of degeneration, with line 1 being more rapid (1-3 cell ONL layers at 2 months) than line 3 (5-7 cell layers at 2 months) (Steinberg et al., 1996). Further characterization of line 3 indicated that transgene expression begins at P5, but morphological changes are not detectable until after P30 (Lewin et al., 1998a; Machida et al., 2000). Scotopic ERG data indicated a loss of rod a-wave amplitude of 74% over 4-29 weeks (Machida et al., 2000). While this animal model has been widely used, a detailed analysis of the time course of the structural and physiological phenotype progression has not yet been conducted.

In the current study, the retinal function of P23H-line 3 animals was investigated from P18 to 3 months of age. To correlate observed alterations in retinal function to structure, *in*

vivo and histological structural analysis was performed. Finally, to explore the potential mechanisms involved in neuronal death in P23H-3, the involvement of key cell death pathways was examined.

2.2. Materials and Methods

2.2.1. *Animals*

All experimental procedures were conducted in accordance with the association of Vision Research and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and The University of Melbourne Animal Ethics Committee (Ethics: 1312958). Sprague-Dawley (SD) rats were obtained from the Animal Resources Center (ARC, Canning Vale WA Australia). Heterozygous Pro23His-line 3 transgenic rats (P23H-3) were generated by crossing homozygous P23H-3 with SD rats. The original P23H-3 transgenic rat strains were produced by Prof. Matthew LaVail, Beckman Vision Centre, University of California San Francisco School of Medicine in collaboration with Xerogen Biosciences (formerly Chrysalis DNX Transgenic Sciences, Princeton, NJ), National Eye Institute. Breeding stock for the P23H-3 used in this thesis, were kindly donated by Dr. Krisztina Valter-Kocsi and Prof. Jonathan Stone at The Australian National University, Canberra. Both the transgenic and wild-type SD rats were housed and bred in the Biomedical Sciences Animal Facility at the University of Melbourne on a 12-hour light-dark cycle with a controlled temperature ($22 \pm 1^\circ\text{C}$). Food and water were available ad libitum.

2.2.2. *Electroretinographic recording (ERG)*

To evaluate the function of retinal neuronal cells, full field electroretinograms (ERGs) were recorded from P23H-3 and age-matched SD rats at P18, 1, 2 and 3 months of age ($n \geq 8$ at each age group). ERG is used to measure the electrical response of retinal neurons to light stimulus initiated by photoreceptor cells and followed by inner retina (amacrine and bipolar cells) and ganglion cells (Weymouth et al., 2008). Animals were dark-adapted overnight (minimum of 12 hours) and deeply anesthetized under dim red light by an intraperitoneal (IP)

injection of a 12:1 mixture of ketamine (60 mg/kg; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia,) and xylazine (5 mg/kg; Troy Laboratories, NSW, Australia). For P18 animals, the mixture was further diluted 1:10 in sterile saline (0.9%, B. Braun Australia Pty Ltd, NSW, Australia). The corneal reflex was anesthetized by Alcaine (0.5%, Alcon Laboratories, Victoria, Australia) and the pupil was dilated via administration of atropine (1%, Aspen Pharmacare, NSW, Australia) and phenylephrine hydrochloride (10%, Bausch & Lomb, NSW, Australia). Rats were placed in a custom-made restraining device including a heating pad to maintain body temperature. To avoid dehydration of cornea, GenTeal® lubricating eye gel (0.3%, Alcon Laboratories, Victoria, Australia) was applied after the ERG was recorded.

ERG waveforms were recorded by placing a silver/silver-28 chloride (Ag/AgCl) electrode on the cornea (active) and a reference electrode in the mouth (inactive). Full-field ERG responses were obtained by a 2.1-log cds/m² flash from a Nikon camera (Nikon SB900; Nikon, Lidcombe, NSW, Australia) delivered through a custom made Ganzfeld. Responses were amplified (gain $\times$ 5,000; -3 dB at 1 Hz and 1 kHz, AD Instruments, Castle Hill, NSW, Australia) and digitized at 10 kHz. A twin consecutive flash protocol with 0.8-second inter-stimulus interval was used to evaluate the contribution of rod and cone photoreceptor in the ERG response independently (Lyubarsky et al., 1996; Phipps et al., 2004). The first flash elicited mixed responses from both rod and cone pathways, while the second flash elicited only the cone pathway responses. To isolate rod responses, the cone response was subtracted from the initial mixed response (Jobling et al., 2013; Vessey et al., 2015). For each animal, three measurements were taken, and the final value was represented as an average response. ERG recordings were monitored using Scope software (version 3.6.10, AD Instruments Pty, Ltd) on an Apple Macintosh computer (Apple, Inc., Cupertino, California, USA).

2.2.2.1 ERG component analysis

The ERG waveform reflects the summed electrical response of all neuronal cells in the retina following a light stimulus. To assess individual components of the recorded ERG responses, published equations were used for modelling the rod and cone waveforms.

The rod photoreceptor response was analyzed according to a model, PIII, by which a modified computational description explains phototransduction cascade (Hood et al., 1990).

The equation was:

$$\text{Equation 1: } PIII(i,t) = \{1 - \exp[-I \cdot S \cdot (t - td)^2]\} \cdot Rmax$$

PIII gives the photocurrent generated by the photoreceptors as a function of intensity, i ($\text{cd} \times \text{s} \times \text{m}^2$) and time, t (in seconds). $Rmax$ (μV) is the saturated amplitude of the PIII, S (sensitivity) represents the gain of the phototransduction process ($\text{m}^2 \times \text{cd}^{-1} \times \text{s}^{-3}$), and td (seconds) is a short delay that accounts for the latency between the stimulus and the response initiation.

The b-wave (PII model) and oscillatory potentials (OPs) reflect the activity of ON-bipolar cells and amacrine cells, respectively, in the inner retina (Hood et al., 1990). PII model was isolated by digital subtraction of PIII from the raw waveform and subsequently fitted using an inverted gamma function. OPs were derived from leading edge of the rod b-wave from data collected in response to $2.1 \log \text{cd} \times \text{s}/\text{m}^2$. Three main OPs (OP2, OP3, OP4) were isolated by fitting an inverted gamma function (Weymouth et al., 2008) to the rod PII, and then digital subtraction of this curve from the raw waveform data. The isolated OPs were analyzed to calculate both amplitude (μV) and implicit time (ms), the time until the maximum response was reached. Results were expressed as the summed OPs amplitude and summed OPs implicit time.

In rodents, the retina mainly consists of rod photoreceptors, with only a small population of cone photoreceptor cells. Additionally, the magnitude of the a-wave and

phototransduction amplification is significantly larger for rod photoreceptors compared to that of cones. As a result, the cone a-wave (PIII) was too small to be isolated reliably in rats. However, the cone inner retinal response pathway has a greater amplification enabling a cone post photoreceptor response to be detected. The cone b-wave (PII), could be analyzed by measuring the maximum amplitude (μV), from the pre-stimulus baseline to the peak of the waveform and the implicit time (ms), the time until the maximum response was reached.

2.2.3. In vivo fundus imaging of retina

After completion of ERG recording, P23H-3 and SD rat eyes at P18, 1, 2 and 3 months of age ($n \geq 8$ at each age group) were imaged using a fundus camera attached to an ocular coherence tomography (OCT) imaging module, providing a high-resolution view of the retina in addition to cross sectional thickness data. OCT is a non-invasive method allowing quantitative assessment of retinal morphology to identify changes that may occur in ocular diseases. Anesthetized rats were positioned in the restraining device and GenTeal® lubricating eye gel (0.3%, Alcon Laboratories, Victoria, Australia) was applied. OCT and fundus images were captured on a Micron III rodent fundus camera (Phoenix Research Labs, California, USA) using specialty Micron III (Phoenix Research Labs, California, USA) and StreamPix™ software (Norpix Inc., Quebec, Canada), respectively. OCT images were analyzed using a custom ImageJ script to measure the thickness (μm) of the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), inner and outer photoreceptor segments (IS\OS) including the retinal pigment epithelium (RPE) and total retina.

2.2.4. Tissue collection and preparation

Prior to tissue collection, animals were deeply anesthetized by IP injection of ketamine and xylazine as described previously (12:1 mixture of ketamine, 60 mg/kg and xylazine, 5 mg/kg) and sacrificed with an intracardial injection of Lethobarb (pentobarbitone phosphate, 120 mg/kg Virbac Pty.Ltd., NSW, Australia). The eyes were enucleated, and the anterior segment was dissected and gently removed along with the lens and vitreous. The posterior eyecups containing the neural retina were placed in the specific fixative dependent on the purpose of experiment (see below for details).

2.2.5. Immunofluorescence

2.2.5.1 Cryostat sectioning

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay and caspase-3 staining were performed on fixed, frozen sections. For cryosectioning, the posterior eyecups were fixed in fresh 4% paraformaldehyde in phosphate buffer (PB, 0.1 M; pH 7.4) for 30 min for TUNEL and 4 hours for caspase-3 staining at room temperature. Fixed eyes were washed with PB and then immersed in increasing gradients of sucrose in PB for cryoprotection, 10% and 20% for an hour at room temperature followed by overnight incubation in 30% at 4°C. Eyecups in 30% sucrose were subsequently snap frozen in liquid nitrogen and stored at -80°C until use.

For frozen retinal sections, eyecups were cut in half through the optic nerve using a scalpel blade under dissecting microscope, one half was embedded in Tissue-Tek O.C.T compound (Sakura Seiki, Tokyo, Japan) within a plastic embedding mold and frozen in liquid isopentane (Fisher Scientific, Loughborough, UK) chilled in liquid nitrogen. Frozen blocks were sectioned at 12 µm thickness on a cryostat (Hyrax C60, Zeiss, Germany), collected on

poly-L-lysine coated glass slides (Polysine® Adhesion slides, Thermo Scientific, VIC, Australia) and kept at -20°C.

2.2.5.2 Terminal deoxynucleotidyl transferase dUTP nick-end (TUNEL) assay

To visualize dying cells, TUNEL assay (DeadEnd™ Fluorometric TUNEL System; Promega Corp., NSW, Australia) was performed as per manufacture's instructions. Briefly, frozen cryostat sections (n > 5 animals per group) were defrosted at room temperature for an hour and a hydrophobic border was drawn around the tissue using a PAP-Pen (Invitrogen, California, USA). The sections were washed twice in phosphate buffer saline (PBS, 1X, pH 7.4) and immersed in 0.2% Triton-X in PBS for 20 min. The slides were rinsed with PBS for 2 x 5 min. and incubated in equilibration buffer (Promega) for 10 min. The rTdT incubation buffer was prepared according to the manufacturer's instructions, applied to the sections, and incubated at 37°C for one hour in the dark. Subsequently, sections were immersed in 2x SSC (Promega) for 15 min. and then washed in PBS for 3 x 5 min. For labeling cell nuclei, sections were further incubated with DAPI (Life Sciences, Victoria, Australia) for 20 min. Finally, sections were rinsed with PBS for 2 x 5 min., mounted with fluorescence mounting medium (Dako, Agilent Technologies, Santa Clara, CA, USA) and covered with a 24 mm x 50 mm glass coverslip (Menzel-Gläser, Braunschweig, Germany). Sections were imaged immediately to minimise any fading of the label.

2.2.5.3 Caspase-3 staining

For caspase-3 immunostaining, frozen sections were defrosted, heated in a 0.01% M citrate buffer (65°C, pH 6) for 10 min and cooled to room temperature for 30 min. A PAP-Pen (Invitrogen, California, USA) was used for drawing a hydrophobic barrier around the tissue. The sections were rinsed 3 times (5 min. each) with PB and incubated with primary antibody (**Table 2.1**) overnight in a humid chamber at room temperature. Prior to incubation, primary antibodies were diluted in antibody buffer containing 3% v/v normal goat serum (NGS), 1%

w/v bovine serum albumin (BSA), 0.05% w/v sodium azide and 0.5% v/v Triton-X in 0.1M PB to obtain intended concentration (**Table 2.1**). The following day, sections were washed 3 x 5 min. in PB and incubated for 90 min, at room temperature in the dark, in a mixture of the nuclear dye 4',6-diamidino-2-phenylindole (DAPI, Life Sciences, Victoria, Australia) and secondary antibody (**Table 2.1**) diluted in antibody buffer 1:300 and 1:500 respectively. Subsequently, tissues were washed 3 x 5 min. in PB and cover-slipped with a 24 mm x 50 mm glass coverslip (Menzel-Gläser, Braunschweig, Germany) using fluorescent mounting media medium (Dako, North, Sydney, NSW, Australia).

Table 2.1: List of primary and secondary antibodies used for immunofluorescence

Primary Antibody	Dilution	Secondary Antibody	Dilution
Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb (#9664, Cell Signaling Technology)	1:100	Alexa Fluor® 488 Goat anti-rabbit (Invitrogen, Molecular Probes®, Oregon, USA)	1:500
Mouse monoclonal antiAIFM1(#SAB5300151, sigma-aldrich)	1:100	Alexa Fluor® 488 Goat anti-mouse (Invitrogen, Molecular Probes®, Oregon, USA)	1:500

2.2.5.4 Apoptosis inducing factor staining

For apoptosis inducing factor (AIF) staining, paraffin sections were used. The whole eye cups were fixed overnight in Davidson's fixative containing 8% formaldehyde, 31.5% ethanol and 2 M acetic acid. They were then dehydrated by immersion in graded alcohols (70%, 95% and 100%), cleared in xylene and embedded in paraffin wax. Serial sections of 5 µm thickness were cut, collected on poly-L-lysine coated glass slides, and incubated overnight at 37°C. Sections were deparaffinized in xylene, rehydrated by washing in graded ethanol (100%, 90% and 70%) and finally distilled water (dH₂O). Sections were heated in a 10 mM Tris-HCl buffer (60°C, pH 9) for 10 min. and cooled to room temperature for 30 min

before conducting immunofluorescence for AIF as described above for caspase-3. Details of antibodies are provided in **Table 2.1**.

2.2.6. Confocal microscopy and imaging

Following TUNEL labelling, tile scans of the entire retinae were captured immediately by a laser scanning confocal microscope (Zeiss LSM510, Meta, Carl Zeiss, Oberkochen, Germany) using a x 20 air objective at a resolution of 512 x 512 pixels. For viewing antibody staining, retinal sections were viewed and imaged on the Zeiss LSM800 (Carl Zeiss AG, Jena, Germany), using a 40x oil immersion objective at a resolution of 1024 x 1024 pixels.

2.2.7. Quantification of apoptotic cells

To quantify the number of TUNEL positive cells, three mid-sagittal sections through the eye-cup (preferably with identifiable optic nerve head within the section) from each animal were used for analysis. To assess the distribution of dying cells with respect to the retinal eccentricity, the length of half section (from the optic nerve to the tip of the periphery) was measured and divided into three segments of identical length as central, mid-peripheral and peripheral regions using ImageJ software. The number of TUNEL-labeled cells in the ONL was counted in each segment using the cell counter plugin of ImageJ. The quantifications from the same segment of three different sections were averaged to obtain a single value and the results were expressed per millimeter of retinal length. To calculate the total number of TUNEL positive cells of ONL in each retinal section, obtained values for each region was summed up, averaged for three relative sections of each animal ($n > 5$ per group), and then the final value was presented as per millimeter of retinal length. Similarly, to evaluate the progression of photoreceptor loss, relative to retinal eccentricity, the thickness of the ONL was

also measured, in each division at P18, 1 and 2 months. The measurements from the same segment of three different sections were averaged to obtain a single value.

2.2.8. JC-1 - mitochondrial membrane potential assay

To analyze mitochondrial membrane potential ($\Delta\Psi_m$) changes occurring in the early stages of apoptosis, the JC-1 assay (MitoProbe™ JC-1 Assay Kit, ThermoFisher, Australia) was performed according to the manufacture's instruction with minor modifications.

At P14 and P18 of age, P23H and SD rats ($n = 6$ for each experimental group) were anesthetized and killed as previously described. The eye balls were enucleated, placed in cooled cell culture medium (Medium 199, Sigma, Australia) and the anterior segment was removed. Retinae were isolated from the posterior eye and cut into small pieces using a scalpel blade and a single cell suspension produced using the Papain Dissociation System (Worthington Biochemical Corporation, Lakewood, NJ, USA). Briefly, fragments from each retina were transferred to the digestion tube containing 800 μ l papain and DNase-I and incubated in a rotator (700 rpm) at 37°C for 60 min. Afterwards, the solution with the partially digested retina was mechanically dissociated using a 1 ml micropipette and a long glass fire polished Pasteur pipette. The solution was centrifuged using a microcentrifuge at 300xg (2000 rpm) for 5 min. and the pellet was resuspended in 1 ml of inhibitor mixture to stop the reaction. Cells obtained from each sample were counted on a Countess™ automated cell counter (Invitrogen, Australia) according to the provided protocol. To perform the JC-1 assay, approximately 1×10^6 cells per sample were suspended in 1 mL warm PBS. 10 μ l of 50 μ M JC-1 (0.5 μ M final concentration) was added to each tube and cells were incubated 30 min. at 37°C with 5% CO₂. For the positive control tube, 2 μ l of 50 mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP, supplied with the kit) was added simultaneously with JC-1. Cells then were washed once by adding 2 ml of warm PBS, centrifuged at 300xg (2000 rpm)

for 5 min. and resuspended in 200 µl PBS. DAPI was added to each sample as a nuclear counterstain. Cells were analyzed on a fluorescence assisted cell sorting (BD FACS Aria III Cell Sorter) with 488 nm excitation using emission filters appropriate for Invitrogen Alexa Fluor 488 dye (530 nm for green and 695 nm for red). Collected data were analyzed using FlowJo (V.10.4.2) software.

2.2.9. Quantitative real-time PCR

Animals at P14 and P18 (n = 9 per group) were anaesthetized and killed as previously described. To isolate retinal total RNA, posterior eyecups of P23H-3 and SD rats were dissected in sterile, ice-cold PBS, to separate the neural retina from the RPE. Retinae were separated from the posterior eyecup of P23H and SD rats, snap frozen in liquid nitrogen, and total RNA was extracted using the RNeasy® Mini Kit (Qiagen, VIC, Australia) as per manufacturer's instructions. All samples underwent on column DNase-I treatment at room temperature for 15 min. to remove genomic DNA contamination (Qiagen, VIC, Australia).

To quantify RNA concentration and assess purity, the Agilent 2200 Tape Station (Agilent Technologies 5067-5583 D1000, Germany) was used according to manufacturer's instructions. The Tape Station analysis software gives an RNA integrity number (RIN), an algorithm designed to estimate the integrity of the total RNA as a value between 1 to 10. Only samples with RIN number above 8 were considered of sufficient quality to be used for this study. After RNA analysis, samples were stored at -80°C until use.

The rat cell death pathway finder RT² profiler PCR array (Cat#: PARN-212ZE, Qiagen, VIC, Australia) was used to profile the expression of 84 key genes involved in programmed cell death pathways incorporating apoptosis, autophagy, and necrosis. RNA samples from P23H and SD rats (P14 and P18, n = 9 for each group) were pooled within their respective experimental groups (n = 3 pooled samples per group) to maximize the amount of RNA and

minimize bias due to biological variation. Total RNAs (400 ng) was reverse transcribed into complementary DNA (cDNA) using the RT2 First Strand cDNA Kit (Qiagen) according to the manufacturer's instructions (RT2 Profiler PCR Array Handbook; Qiagen). The cDNA samples were mixed with RT2 SYBR Green Mastermix (Qiagen) and then added across the 384-well plate. Each 384-well plate contained 4 replicates of 96-well assays including 84 different genes of interest, 5 housekeeper control genes, 1 genomic DNA control, 3 reverse transcription controls and 3 positive PCR control. The cDNA was amplified using the Applied Biosystems ViiA 7 Real-Time PCR System (Thermofisher) at 95°C for 15 min., followed by 95°C for 15 seconds and 60°C for 1 min. (40 cycles). Three independent arrays were run for each experimental group in order to collect statistically valid data. The data were analyzed using RT² Profiler PCR Array Data Analysis version 3.5 from QIAGEN website (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). Gene expression obtained for experimental groups were normalized to the average threshold cycle (Ct) value of the housekeeping genes and expressed as fold-change with respect to the control group.

2.2.10. Intravitreal injection of BAK inhibitors in P23H-3 rats

Two different inhibitors of BAK (WEHI-1249625 and WEHI-1250993) were supplied by Drs. Ben Kile and Guillaume Lessene from Monash University and WEHI, respectively. Prior to injection, a stock of each compound was prepared by dissolving the compound powder in 5% DMSO (99.9 % for molecular biology, Sigma, Australia) in sterile saline (0.9%, B. Braun Australia Pty Ltd, NSW, Australia) to achieve the intended concentration. To calculate the effective concentration at the retina, the vitreal volume of P23H-3 rats at P16 was estimated as ~25 µl.

At P16 of age, P23H-3 rats were anesthetized as previously described. The corneal reflex was anesthetized with topical administration of Alcaine (0.5%; Alcon Laboratories,

Victoria, Australia) and the dilation of pupil was achieved via administration of atropine (1%, Aspen Pharmacare, NSW, Australia) and phenylephrine hydrochloride (10%, Bausch & Lomb, NSW, Australia). The vehicle-diluted compound (1 µl) was injected intravitreally into the left eye using a 30-G needle attached to a 5 µl Hamilton micro syringe (Reno, NV, USA) via a polyethylene tube. The right eye received 1 µl of vehicle (5% DMSO in sterile saline) as the vehicle. The animals were then kept on the heat pad at 37°C to maintain the body temperature and monitored during recovery. Injected animals and their eyes were monitored daily for 2 days post-injection. At P18 of age, animals were killed under deep anesthetic and eyes were collected and prepared for TUNEL analysis as previously described.

Table 2.2: Experimental design for intravitreal injection of BAK antagonist into P23H-3

BAK inhibitor	Molecular mass	Solvent	Estimated concentration in the retina	Number of injected animals
WEHI-1249625	593.7	5% DMSO in saline	6 mM	10
WEHI-1250993	541.64	5% DMSO in saline	1 µM	8

2.2.11. Statistical analysis

Statistical analysis and graphing were performed using GraphPad Prism 7 (San Diego, CA, USA). All data were expressed as the mean ± standard error of the mean (SEM). Analysis and curve fitting of the ERG data was carried out in a spreadsheet (Excel 2007; Microsoft, Redmond, WA). A two-way analysis of variance (ANOVA) was used to determine statistical significance among experimental groups, where the two independent variables were genotype and age, followed by a Tukey's post-hoc test for further multiple comparisons. P-values less than 0.05 were considered statistically significant and represented * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ in all graphs.

2.3. Results:

2.3.1. Rod pathway response is attenuated from 1 month in P23H-3 rats

In order to determine P23H-associated functional changes, scotopic ERGs were recorded from P23H-3 and SD rats at P18, 1, 2 and 3 months of age. Animals were dark-adapted overnight, and the mixed responses of rod and cone were recorded. To analyze rod- and cone-related response separately, a paired-flash ERG paradigm was used. In both P23H-3 and SD retinæ, rod ERG responses were apparent and well developed at P18, only a few days after eye opening. Representative rod pathway waveforms for the SD and P23H-3 rat are represented in **Figure 2.1** and showed that overall amplitude of the rod response in P23H-3 is less than WT rats at 1, 2 and 3 months of age.

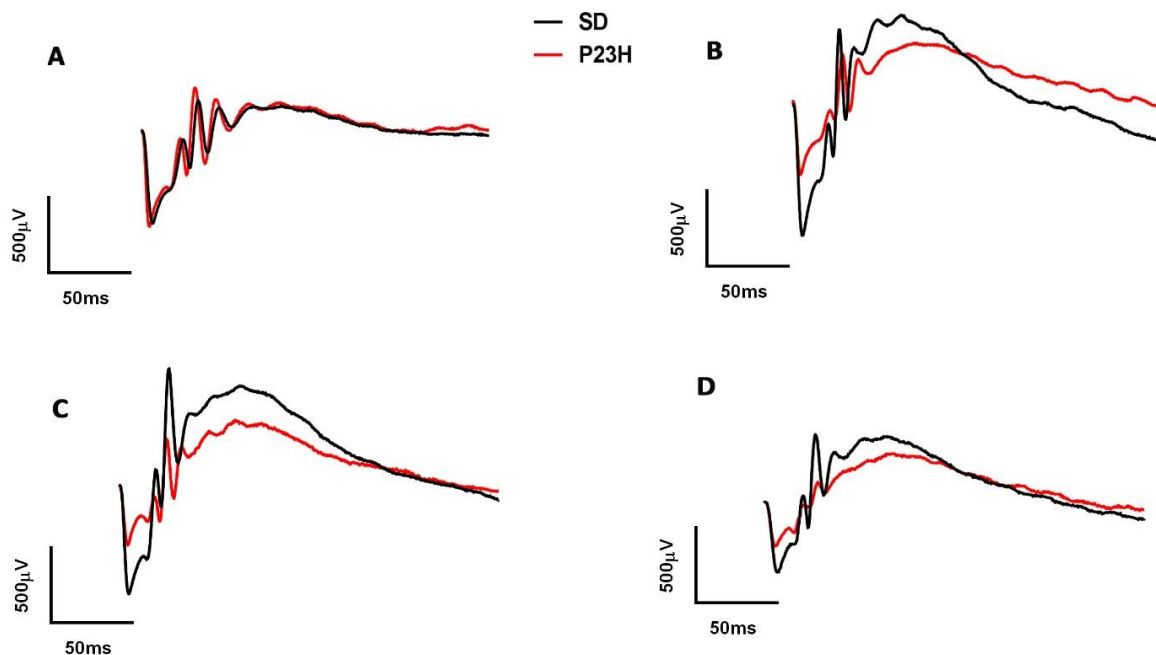


Figure 2.1: Alterations in rod responses of P23H-3 rats with age

Representative rod responses in SD (black) and P23H-3 (red) rats at P18 (A), 1 month (B), 2 months (C) and 3 months (D). Overall response of the rod pathway in P23H-3 is less than SD rats from 1 month of age.

When the retinal response was modelled and quantified, the rod a-wave (PIII) amplitudes showed a progressive age-related decrease from 1 to 3 months in both strains, while

there was a specific effect of genotype with the response in the P23H-3 significantly reduced compared to SD from 1 to 3 months of age ($p < 0.0001$ for all significant differences; **Figure 2.2A**). At 1 month, the rod PIII amplitude was reduced by ~ 30%, while the response decreased by ~40% at 2 months and ~ 60% at 3 months of age (**Figure 2.3**). Additionally, from P18 to 1 month the rod a-wave amplitude significantly elevated in SD rats ($p < 0.01$; **Figure 2.2A**).

Although the rod amplitude decreased in the P23H-3 retinae, intriguingly, the rod PIII (a-wave) sensitivity was significantly greater at P18 in the P23H-3 ($p < 0.0001$) but was similar to the SD thereafter. In 18-day-old SD rats, rod sensitivity was significantly lower than animals at 1 month ($p < 0.001$; **Figure 2.2B**).

To examine rod post-photoreceptor alterations in P23H-3 rats, the rod b-wave (PII) was analyzed which reflects the function of the rod depolarizing bipolar cells in the inner retina (Hood et al., 1996; Robson et al., 1995). To isolate rod-related b-wave, the cone response was subtracted from the mixed response. The rod b-wave (PII) also showed progressive age-related decrease in amplitude in both SD and P23H-3 rats from 1 to 3 months. Compared to the SD responses, the P23H-3 responses were significantly reduced from 1 to 3 months of age ($p < 0.0001$; **Figure 2.2C**). At 1 month, the rod PII amplitude was reduced by 22%, which worsened (-38%) at 2 and 3 months of age (**Figure 2.3**). From P18 to 1 month, the rod b-wave showed a significant increase in both P23H-3 ($p < 0.01$) and SD ($p < 0.0001$) animals (**Figure 2.2C**). Although the rod PII implicit times were slightly greater in P23H-3 retinae compared to SD at all ages examined, the data were only significant at 1 month of age ($p < 0.05$). Moreover, both strains had longer b-wave implicit time at P18 compared to 1 month ($p < 0.01$ for P23H-3 and $p < 0.0001$ for SD; **Figure 2.2D**).

In addition, the function of amacrine cells in P23H-3 rats was assessed by isolation of oscillatory potentials (OPs) from the leading edge of the PII and then obtained values were summed as shown in **Figure 2.2E**. Analysis of summed OPs showed, they had significantly

lower amplitudes in P23H-3 retinæ compared to the SD controls at all ages studied, except P18 (42%, 51% and 62%, $p < 0.0001$). In SD control rats, the summed OPs amplitude showed a significant increase from P18 to 1 month ($p < 0.0001$). While both P23H-3 and SD animals had significantly longer OPs implicit time at P18 compared to 1 month ($p < 0.001$ and $p < 0.0001$ respectively), it remained unaffected in P23H-3 rats when compared to the age-matched normal SD rats (**Figure 2.2F**). Together the data show significant age-related decline in activity of the rod pathway in P23H-3 rats from 1 month of age.

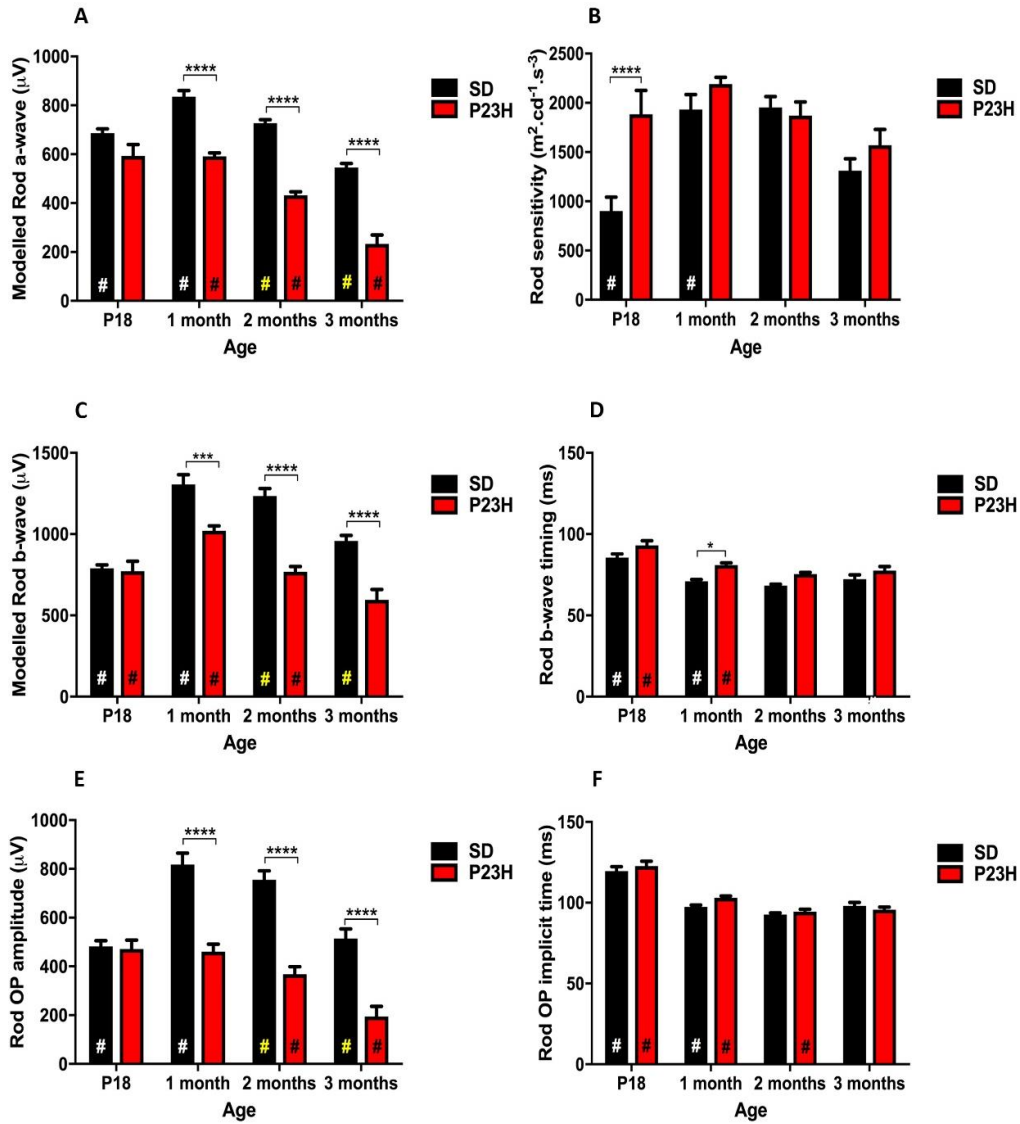


Figure 2.2: Quantitative analysis of functional rod responses in P23H-3

Quantification of rod ERG responses in SD (black) and P23H-3 (red) rats showing change in rod a-wave amplitudes (A), rod sensitivity (B), rod b-wave amplitudes (C), rod b-wave time-to peak (D), rod oscillatory potential amplitudes (E) and OP implicit times (F). Rod a-wave amplitudes were significantly reduced in the P23H-3 compared to the SD from 1 month (A). There were no significant differences in the rod a-wave sensitivity between the P23H-3 and SD rats, except at P18, which was significantly greater in P23H-3 (B). The rod b-wave amplitudes were significantly attenuated in the P23H-3 rat in comparison with the SD from 1 month onwards (C). Rod b-wave implicit time was only significantly increased in the P23H-3 compared to the SD at 1 month (D). Summed rod OPs amplitude of P23H-3 was significantly lower than those of SD rats at 1, 2 and 3 months (E). Rod OPs implicit time of P23H-3 was not significantly altered at any age (F) compared to SD controls. The rod a-wave (PIII) (A), rod b-wave (PII) (C) and summed rod OPs (E) amplitudes as well as rod OPs implicit time (F) showed a progressive age-related reduction from 1 to 3 months in both strains. The histograms indicate the mean $\pm$ SEM, Two-way ANOVA, Tukey's post hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, $n \geq 10$ in each group. *, significant difference for genotype; #, significant difference for age.

To determine whether the neurons in the inner retina undergo a selective functional deterioration over and above that predictable due to the loss of the rod photoreceptor function, the P23H-3 rod post-photoreceptor responses (b-wave and summed OPs) were expressed as a

percentage of the mean SD control animal response and compared statistically. While no significant differences were found in the percentage loss of rod PIII and PII amplitudes at 1 month and 2 months, the PII deficit at 3 months showed a lower reduction than what was expected due to PIII loss ($p < 0.05$; **Figure 2.3**). Although no significant differences were found in the percentage loss of rod PIII and OPs amplitudes, the reduction in OPs amplitudes were significantly greater than PII amplitude loss at all ages examined ($p < 0.01$, $p < 0.05$ and $p < 0.01$ respectively; **Figure 2.3**). This suggests that neuronal cells in the inner retinae of P23H-3 transgenic rats remain intact.

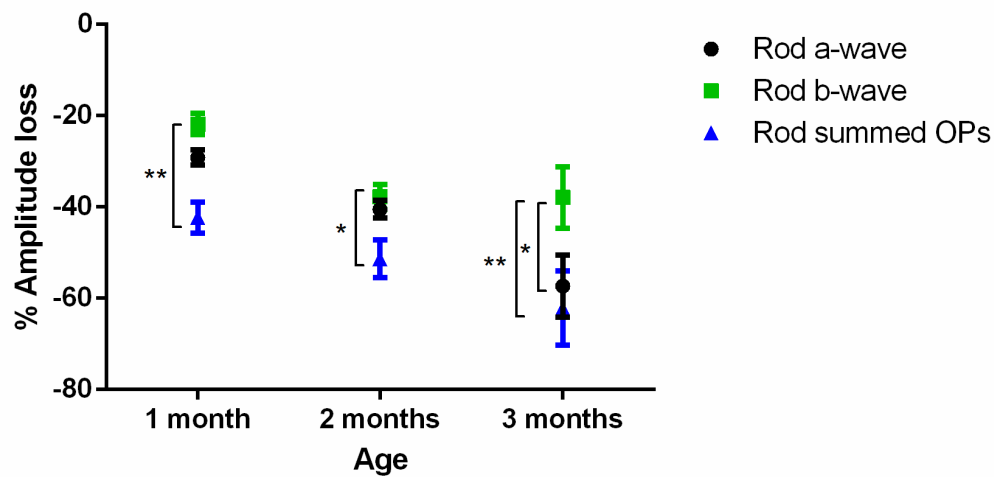


Figure 2.3: Percentage change of the rod photoreceptor and post-photoreceptor responses in P23H-3 retinae

The amplitudes of rod a-wave, b-wave, and OPs responses in P23H-3 were expressed as a percentage of the mean SD responses. At 1 and 2 months, the a-wave and b-wave responses were reduced in P23H-3 to a similar extent. However, the loss of summed OP amplitudes was significantly increased compared to those of b-wave. At 3 months, the extent of the b-wave loss was significantly less than a-wave and summed OPs. Data are expressed as means $\pm$ SEM, Two-way ANOVA, Tukey's post hoc test, * $p < 0.05$, ** $p < 0.01$, $n \geq 10$ in each group.

2.3.2. Cone pathway response is attenuated in P23H-3 transgenic rats

While cone photoreceptor function could not be evaluated in rats because of the small number of cones, the cone post-photoreceptor responses were obtained for P23H-3 and SD rats. Representative cone mediated ERG waveforms are presented in **Figure 2.4**. Apart from

P18, P23H-3 animals had generally smaller cone ERG responses compared to their age-matched controls.

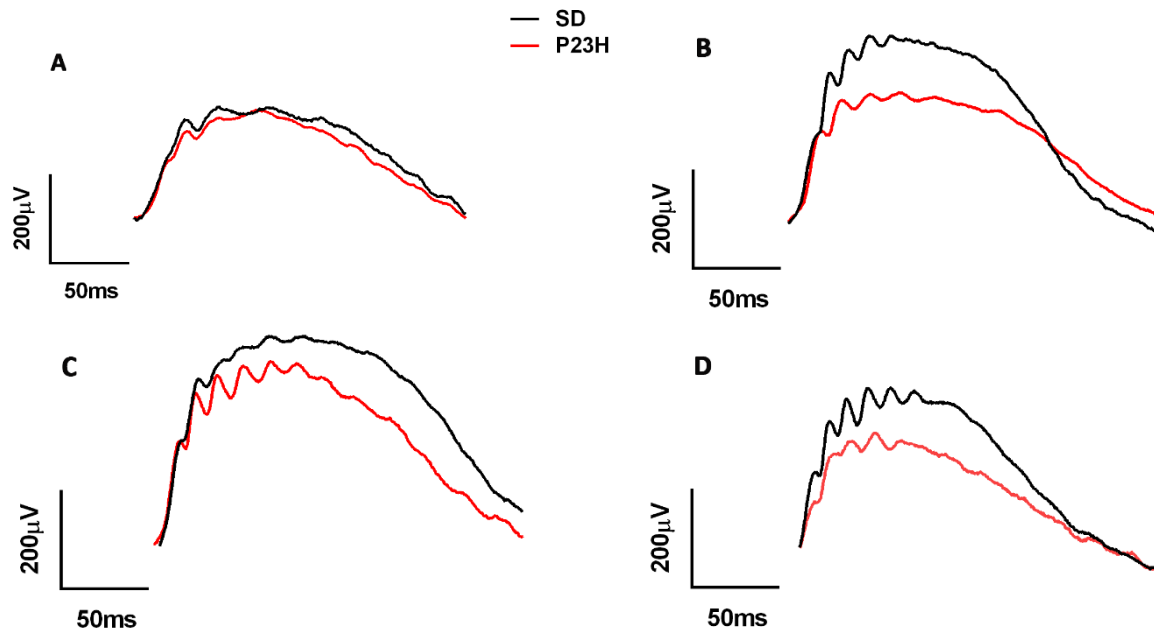


Figure 2.4: Alterations in cone responses of P23H-3 with age

Representative cone responses in SD (black) and P23H-3 (red) rats at P18 (A), 1 month (B), 2 months (C) and 3 months (D). Overall response of the cone pathway in P23H-3 is less than SD rats at 1, 2 and 3 months of age.

Modelling and quantification of the cone-related ERG response showed that cone b-wave amplitudes from P23H-3 rats were significantly less than those of SD controls at 1 (30%, $p < 0.0001$) and 2 months (21%, $p < 0.05$), but not at 3 months of age. Both P23H-3 and SD strains showed a significant increase in the cone b-wave amplitude from P18 to 1 month ($p < 0.0001$; **Figure 2.5A**).

The cone b-wave implicit time and OPs amplitudes remained similar between P23H-3 and SD animals (**Figure 2.5B-C**). However, the timing of OPs only significantly elevated in the P23H-3 compared to the SD at 1 month (**Figure 2.5D**). Moreover, while the cone OPs amplitude showed a significant increase in SD rats from P18 to 1 month ($p < 0.0001$), the cone OPs implicit time significantly decreased in both 1-month P23H-3 and SD animals compared to those at P18 ($p < 0.001$ and $p < 0.0001$ respectively; **Figure 2.5C and D**).

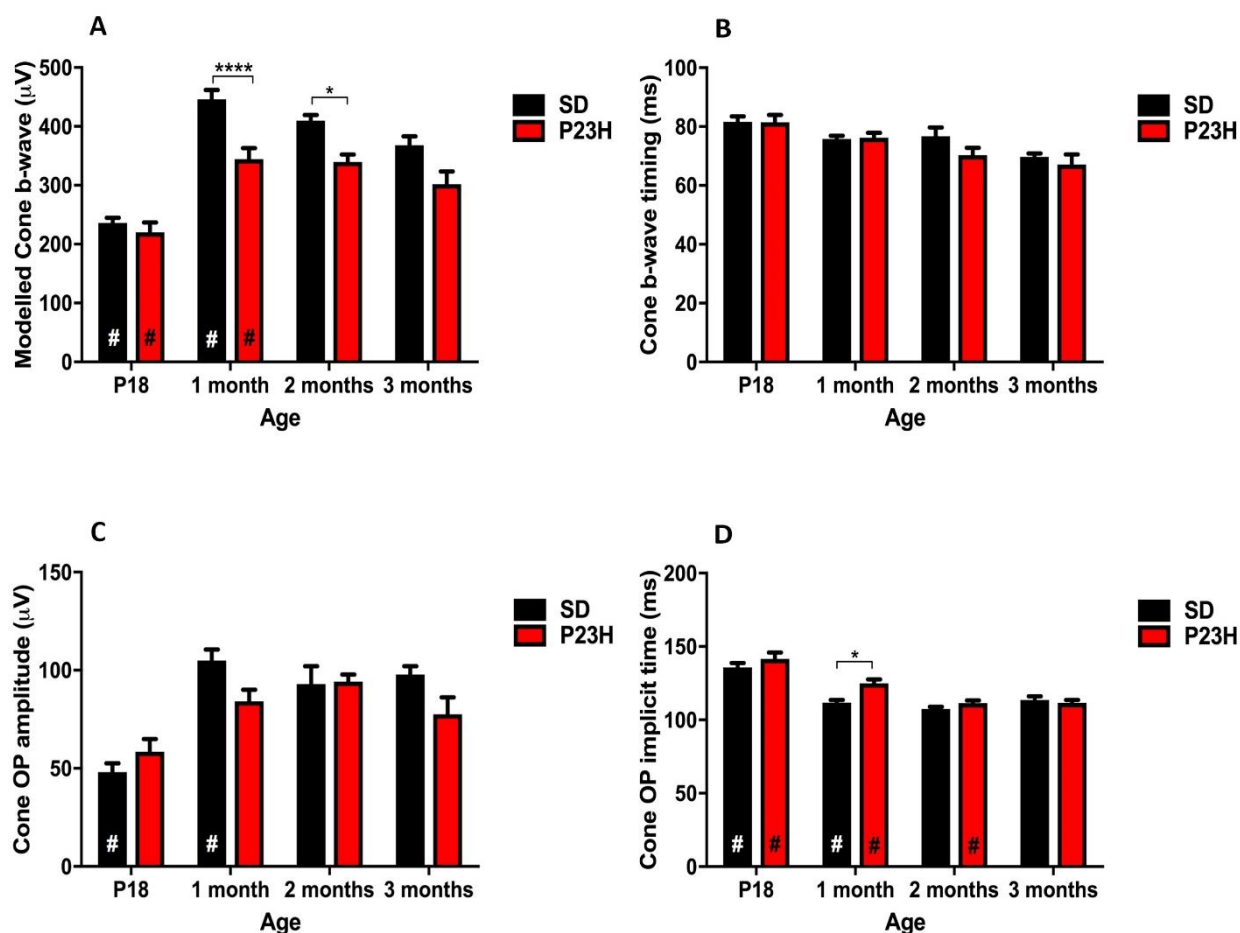


Figure 2.5: Quantitative analysis of cone functional responses in P23H-3

Quantification of cone ERG responses in SD (black) and P23H-3 (red) rats showing change in cone b-wave amplitudes (A), cone b-wave time-to peak (B), cone OP amplitude amplitudes (C), and cone OP implicit times (D). Cone b-wave amplitudes were significantly lower in P23H-3 compared to age-matched SD controls at 1 and 2 months (A). The cone b-wave timing and summed OPs amplitudes were not significantly affected in P23H-3 at any age examined (B and C). OPs timing showed a significant increase in P23H-3 compared to SD only at 1 month (D). The histograms indicate the mean $\pm$ SEM, Two-way ANOVA, Tukey's post hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, $n \geq 10$ in each group. *: significant difference for genotype, #: significant difference for age.

2.3.3. Normal *in vivo* fundus imaging of P23H-3 model of RP

Fundus photography provides a non-invasive technique of *in vivo* monitoring and/or quantitative measurement of ocular health status and is broadly used for detection and documentation of different ocular diseases such as RP (Abràmoff et al., 2010). In this study, fundus imaging was used to assess morphological alternations and progression of retinal degeneration in the retinae of P23H-3. Representative fundus images were taken from P23H-3 rats at P18, 1, 2 and 3 months and compared to age matched control SD (Figure 2.6A-H).

Images were taken with the optic nerve head at the center of the image and as can be observed there were no overt alterations or pathological features in the fundus scans of P23H-3 compared to the SD rats at any examined ages. Due to the albinism inherent in these strains, no alteration in retinal pigmentation could be observed.

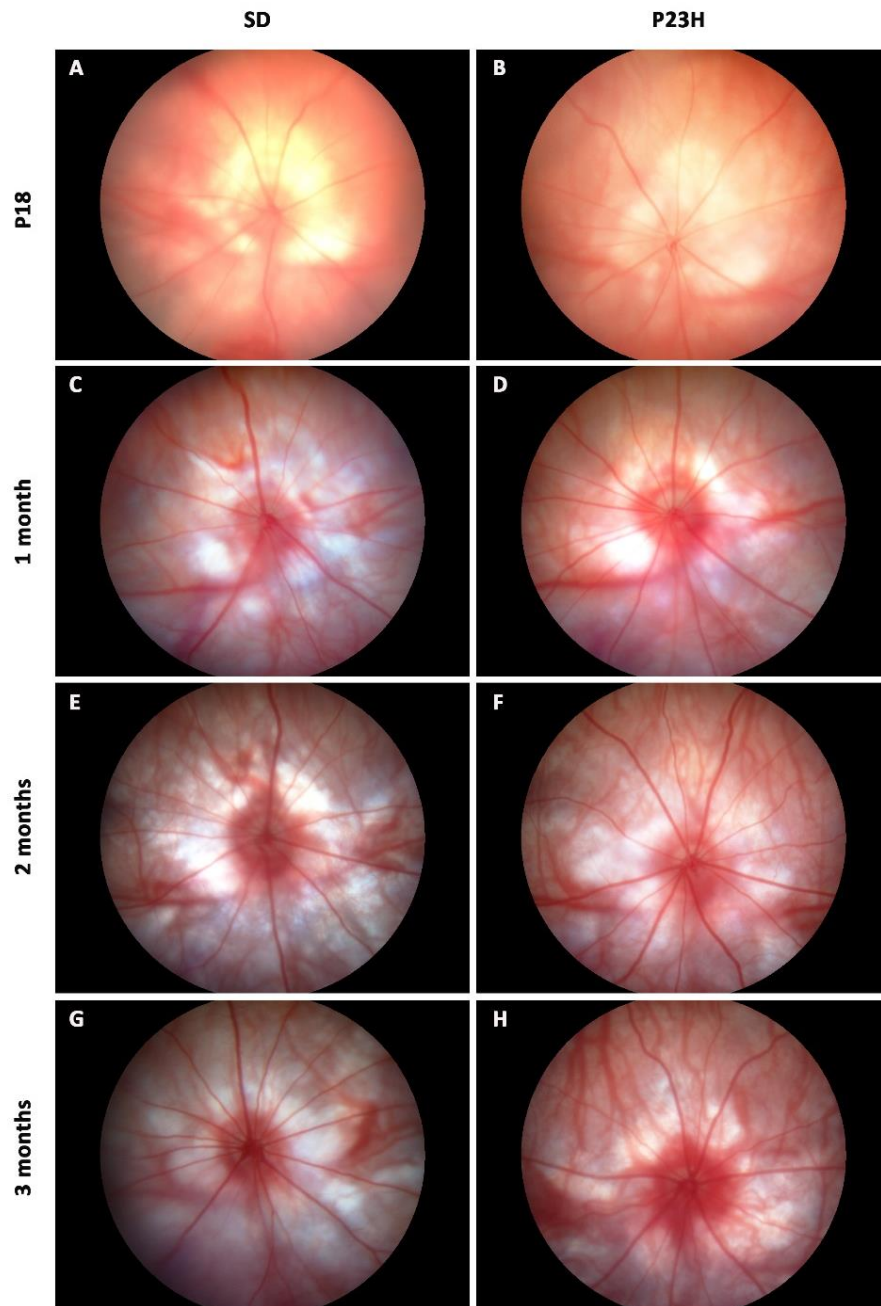


Figure 2.6: Fundus imaging of P23H-3 and SD rats with age

Representative fundus images SD (A, C, E, G) and P23H-3 (B, D, F, H) rats at P18 (A, B), 1 (C, D), 2 (E, F), and 3 (G, H) months of age. No morphological differences were apparent in fundus scans of P23H-3 compared to the age-matched SD rats.

2.3.4. Reduction in photoreceptor layer is evident at 2 months of age in P23H-3 rats

While retinal fundus showed no alteration in retinal appearance between the SD and P23H-3 strains, further *in vivo* structural analysis was performed. Optical coherence tomography (OCT) is an optical imaging method to obtain high-resolution cross-sectional view of retinal structure and is commonly used for early detection of retinal diseases and monitoring their progression as it allows quantitative measurement of retinal pathology. The thickness of retinal layers and nerve fiber layer can be quantified using this technique (Fujimoto et al., 2000). To evaluate the progression of retinal degeneration in P23H-3 rats quantitatively, OCT images were obtained non-invasively from left and right eyes of P23H-3 and SD at P18, 1, 2 and 3 months. At all ages examined (**Figure 2.7**), visualization of the retina by OCT showed normal appearance of retina with distinguishable layers. While the retinal structure of P23H-3 appeared to be similar to the SD rats at P18 (**Figure 2.7A and B**) and 1 month (**Figure 2.7C and D**), a significant loss of ONL, representing photoreceptors, was apparent in the P23H-3 retinae at 2 and 3 months of age (**Figure 2.7E-H**).

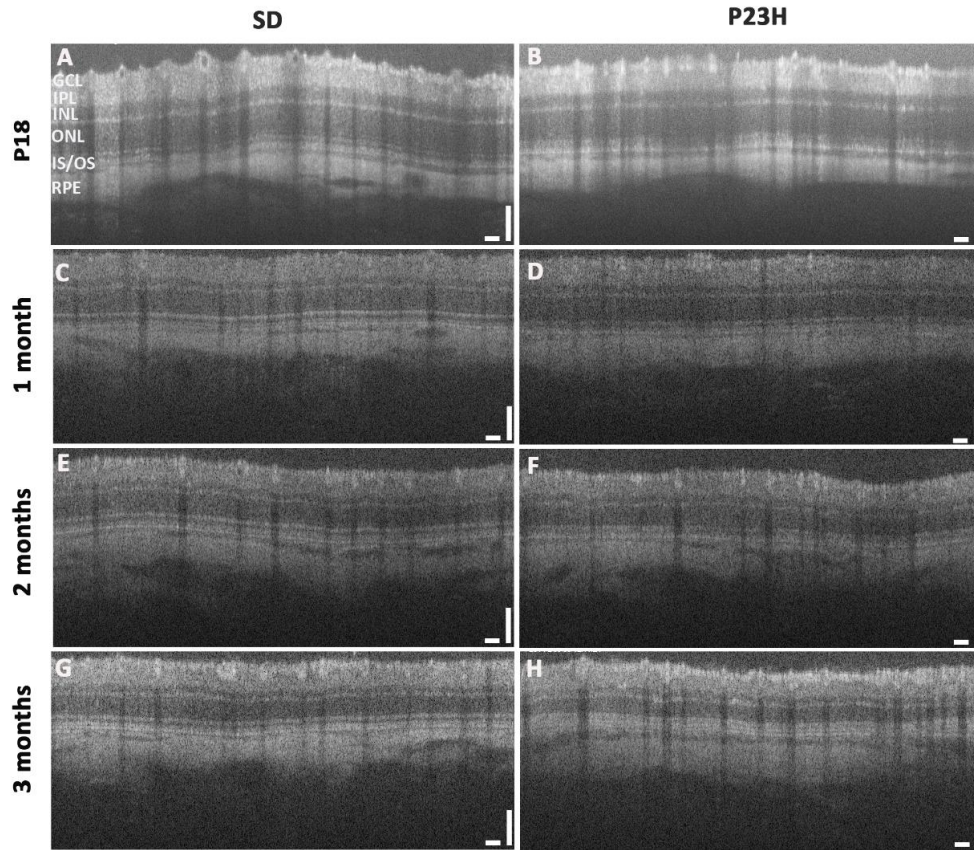


Figure 2.7: Changes in OCT images of P23H-3 and SD rats with age

Representative OCT images of the central retina in SD (A, C, E, G) and P23H (B, D, F, H) rats at P18 (A, B), 1 (C, D), 2 (E, F), and 3 (G, H) months of age. The retinal structure and lamination were normal in P23H-3 rats at all time points. The thickness of the ONL in P23H-3 showed a significant thinning from 2 months of age compared to the SD (E-H). Abbreviations: GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, ONL: outer nuclear layer, IS/OS: inner and outer segments, RPE: retinal pigment epithelium. Scale bar = 100 μ m.

Manual segmentation analysis was performed to quantify the thickness of individual retinal layers including ONL, IS/OS/RPE, INL, IPL, GCL, and total retina (**Figure 2.8A-F**). The ONL in P23H-3 was thinner than that of in SD at all ages; however, it showed statistically significant thinning from 2 months onwards (19%, $p < 0.001$ and 22%, $p < 0.001$ respectively; **Figure 2.8A**). There were no significant differences in the thicknesses of the IS/OS/RPE and INL at any age studied (**Figure 2.8B and C**). At P18, the IPL and GCL were significantly thinner in the P23H-3 than in the control SD retinae ($p < 0.05$ and $p < 0.0001$ respectively); however, this difference was not maintained in older animals (**Figure 2.8D and E**). At P18, 2 and 3 months, the total retinal thickness was significantly reduced in P23H-3 compared to the age-matched normal SD rat (**Figure 2.8F**). Also, there was a gradual decrease in retinal thickness, particularly the ONL and INL with age in both P23H-3 and SD rats (**Figure 2.8A and C**). These data suggest that structural change in P23H-3 retina becomes evident from 2 months of age.

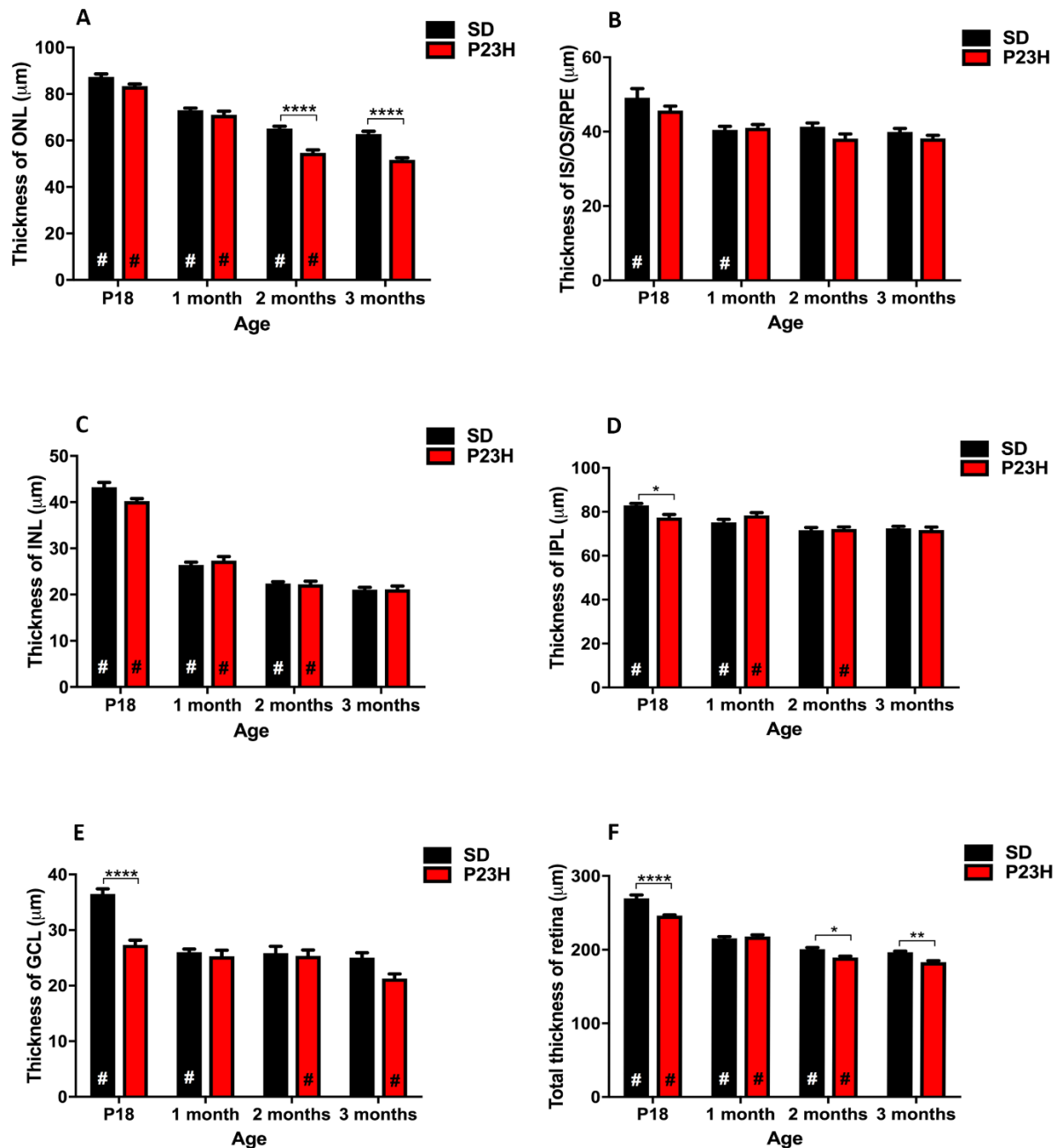


Figure 2.8: Changes in retinal thickness of P23H-3 by OCT with age

Segmentation analyses of OCT images in SD (black) and P23H-3 (red) rats showing mean thicknesses of ONL (A), IS/OS/RPE (B), INL (C), IPL (D), GCL (E) and the total retina (F). At 2 and 3 months, the ONL of P23H-3 was significantly thinner than SD (A). The IS/OS/RPE (B) and INL (C) thickness was not altered in P23H-3 at any age examined. There were no significant differences between the thickness of IPL (D) and GCL (E) in P23H-3 compared to the SD rats, except at P18 when they were significantly reduced compared to SD controls. The total retinal thickness in P23H-3 was significantly less than that of SD at P18, 2 and 3 months of age (F). The histograms indicate the mean $\pm$ SEM, Two-way ANOVA, Tukey's post hoc test, * $p < 0.05$, ** $p < 0.001$ and **** $p < 0.0001$, $n \geq 8$ in each group. #: significant difference for age. Abbreviations: ONL: outer nuclear layer, IS/OS/RPE: inner segment/outer segment/retinal pigment epithelium, INL: inner nuclear layer, IPL: inner plexiform layer GCL: ganglion cell layer.

2.3.5. Significant correlation between retinal structure and function

To confirm that retinal structural changes correlated with observed changes in retinal function, a correlation analysis was performed using structural OCT (ONL thickness) and functional ERG (rod a-wave amplitude) measures. For each animal the ONL thickness and a-wave amplitude was compared and showed a positive correlation between ONL layer thickness and retinal function in both P23H and SD (**Figure 2.9**).

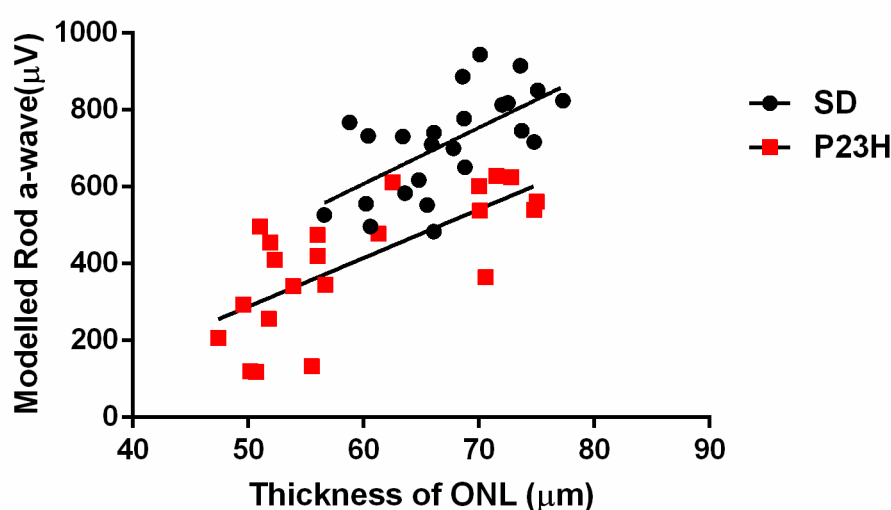


Figure 2.9: Correlation of structure and function in P23H-3 rats

Regression analysis of function (rod a-wave) versus structure (ONL thickness) showed a high positive correlation between ONL layer thickness and retinal function in both P23H-3 and SD rats. $N > 20$ for each group ($R^2 = 0.4$ and $y = 4.61 \cdot x - 268.6$ for SD and $R^2 = 0.5$ and $y = 12.65 \cdot x - 344.1$ for P23H-3).

2.3.6. Photoreceptor degeneration peaks at P18 in P23H- 3 rats

Characterization of the P23H-3 transgenic rats to date has quantified the structural and functional losses in the retinae and identified most of the degeneration is limited to the photoreceptor layer. To identify the detailed time course of photoreceptor cell death prior to structural degeneration in P23H-3 rat retina, TUNEL assay was performed on fixed frozen sections of P23H-3 and SD rats at P18, 1 month and 2 months of age. In the SD retinae, TUNEL⁺ cells were rarely observed at the ages assessed (**Figure 2.10A, C, E**). By contrast, in

the P23H-3 retinae abundant TUNEL⁺ cells were observed at P18 (**Figure 2.10B**), with progressively fewer detected at 1 month (**Figure 2.10D**) and 2 months of age (**Figure 2.10F**). The majority of TUNEL⁺ cells were in the ONL (**Figure 2.10B, D, and F**), but occasional cells were observed in the other retinal layers (**Figure 2.10B**). As observed in **Figure 2.10E** and **F**, a significant thinning was overt in the ONL of P23H-3 compared to the age-matched SD rat reflecting the previous OCT data (**Figure 2.7** and **Figure 2.8**).

Quantification of TUNEL⁺ cells at each age showed that at P18, approximately 25 TUNEL⁺ cells were detected per mm retina within the P23H-3 and this was significantly greater than that observed for age-matched SD animals ($P < 0.001$; **Figure 2.11**). The number of TUNEL⁺ cells declined to 8 TUNEL⁺ cells/mm of retina at 1 month and 1-2 TUNEL⁺ cells/mm at 2 months (**Figure 2.11A**). Despite a mean increase in detected cells at 1 month of age, this was not significantly different from SD controls. To explore whether there was an effect of retinal eccentricity on retinal degeneration, the number of TUNEL⁺ photoreceptors were quantified in central, mid-peripheral and peripheral regions of P23H-3 and SD retinae. As expected no significant numbers of TUNEL⁺ cells were detected in the SD retinae at any age or eccentricity (**Figure 2.11B-D**). At P18, uniformly high mean numbers of TUNEL⁺ cells were detected in the central, mid-peripheral and peripheral regions of the retina, with no significant difference in the number of TUNEL⁺ cells at different eccentricities (**Figure 2.11B**).

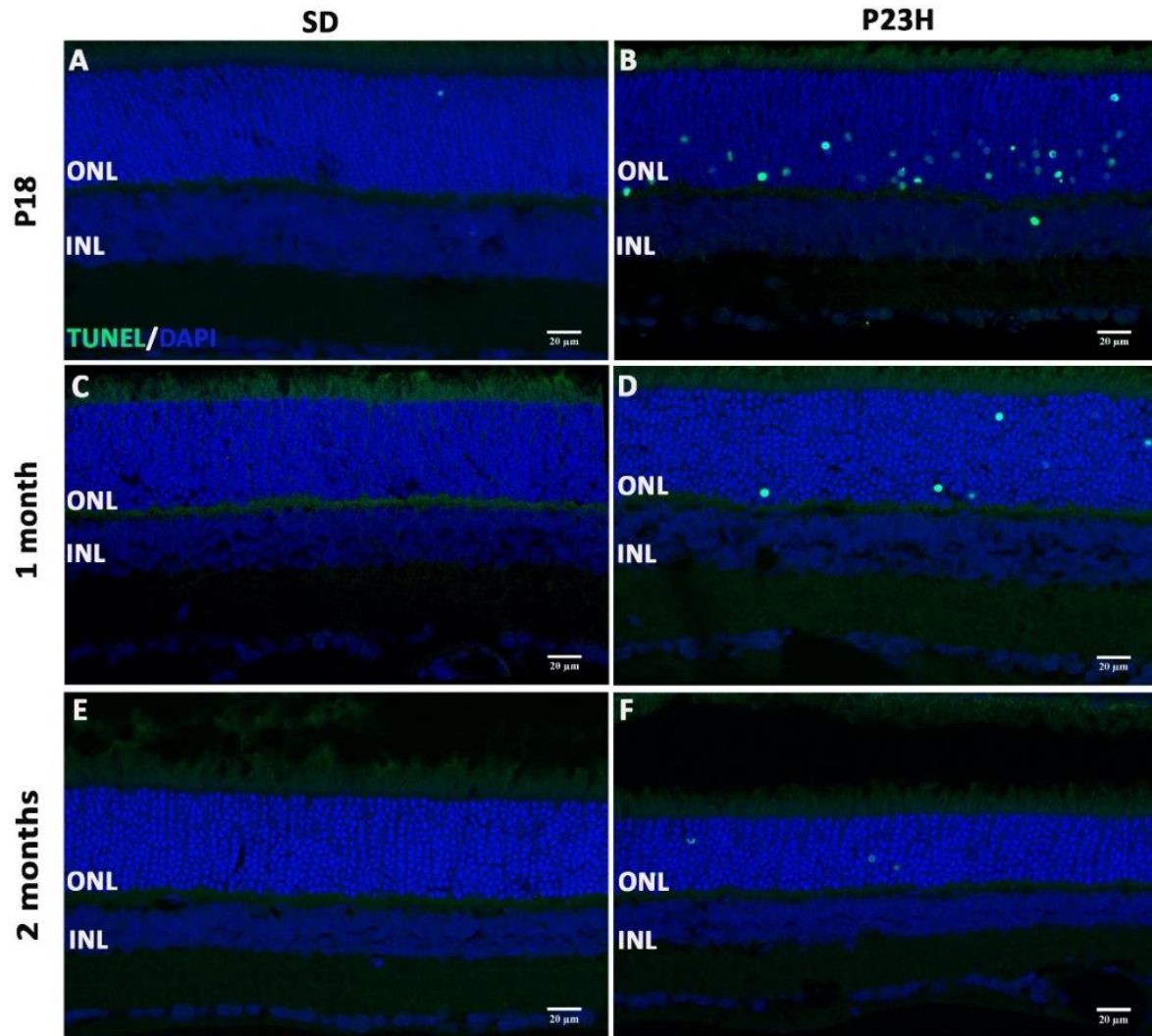


Figure 2.10: Photoreceptor cell death in P23H-3 rats with age

TUNEL (green) and nuclei (blue) staining in SD (A, C, E) and P23H-3 (B, D, F) retina at P18 (A, B), 1 month (C, D) and 2 months (E, F) of age. In P23H-3 rat retinae, TUNEL positive cells predominantly presented in the ONL across all ages. The presence of dead cells in the ONL of SD retinae was negligible. A significant loss of photoreceptors nuclei in the ONL was apparent in P23H-3 retinae at 2 months of age. Abbreviations: ONL: outer nuclear layer, INL: inner nuclear layer. Scale bar = 20 μm .

By contrast, at 1 month, the mean number of dying photoreceptor cells in the peripheral retina was significantly lower than in the central and mid-peripheral regions ($p < 0.05$, **Figure 2.11C**). This effect was transient, with no eccentricity-related effect observed at 2 months of age (**Figure 2.11D**).

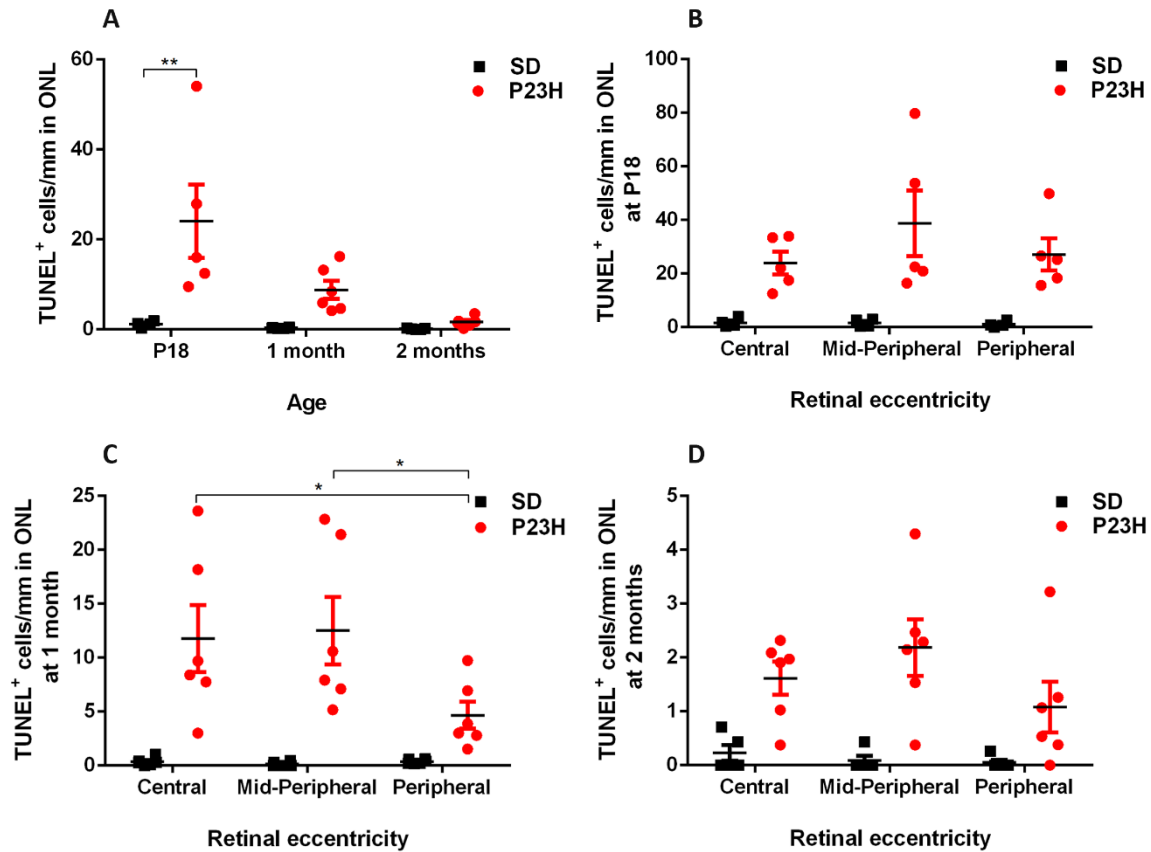


Figure 2.11: Quantification of photoreceptor cell death in P23H-3 rats and effect of retinal eccentricity

Quantification of total retinal TUNEL+ cells at each age (A) and in central to peripheral regions at P18 (B), 1 month (C) and 2 months (D). Significantly increased numbers of TUNEL+ cells were detected in the P23H-3 retina at P18, but not at 1- or 2-months compared to the SD (A). Significant differences in the number of TUNEL+ cells across retinal eccentricity were detected in the P23H-3 retina at 1-month with greater photoreceptor death in central and mid-peripheral than in peripheral regions of retina (C). No effect of retinal eccentricity was observed at P18 (B) or 2-months of age (D). Data are expressed as means $\pm$ SEM, Two-way ANOVA, Tukey's post hoc test, * $p < 0.05$ and ** $p < 0.001$, $n \geq 5$ in each group.

To corroborate the structural thickness data obtained via OCT imaging and to further assess any change relative to retinal eccentricity, the thickness of ONL (DAPI labelling) was also measured from vertical sections across the entire retina and in central to peripheral regions in P23H-3 and normal SD rats (Figure 2.12A-D). At P18 and 1 month, no significant differences were found in the mean ONL thickness of P23H-3 compared to SD at different eccentricities. However, at 2 months the mean ONL thickness in P23H-3 was significantly reduced compared to the SD retinae, similar to that found in the OCT analysis (Figure 2.12A). Comparison of the ONL thickness at different eccentricities showed no significant differences

in different regions of P23H-3 retinæ (**Figure 2.12B-D**), with reduced ONL thickness observed in the central and mid-peripheral regions of the 2-month P23H-3 retinæ ($p < 0.01$, **Figure 2.12D**).

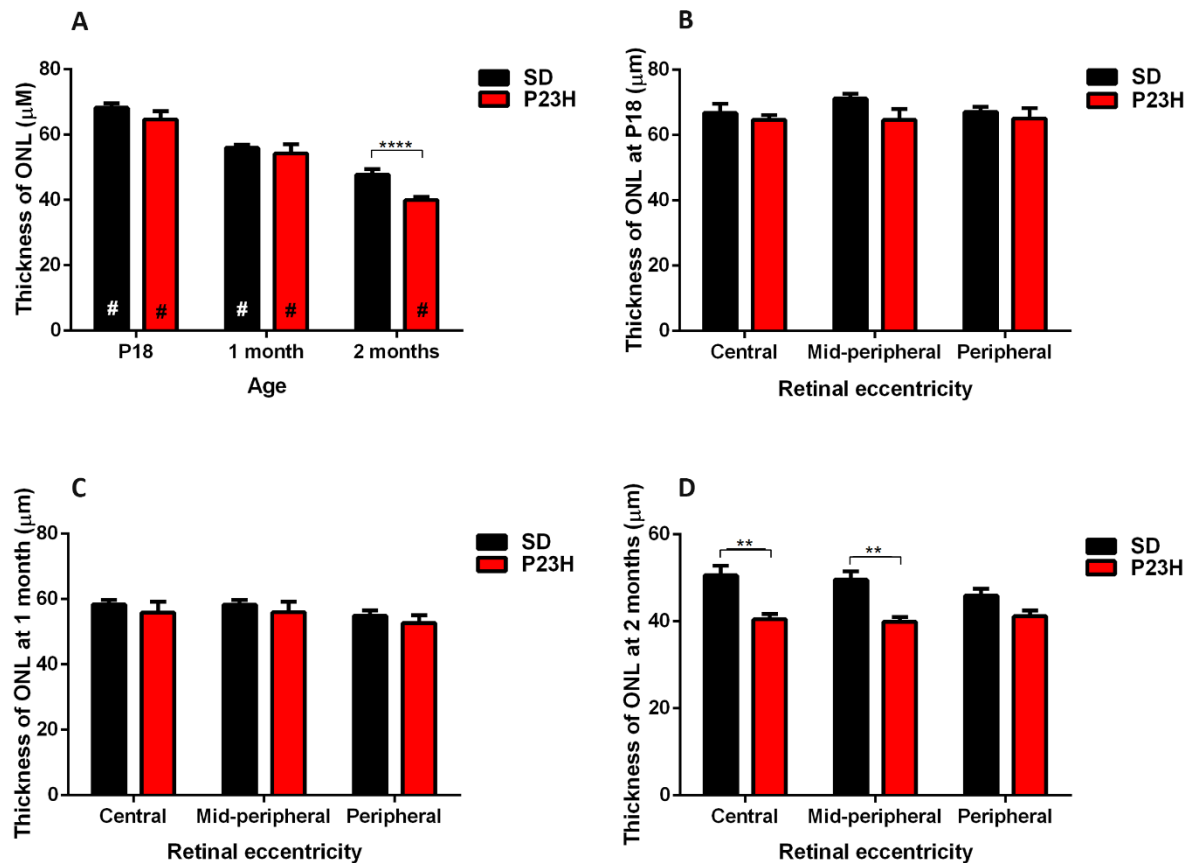


Figure 2.12: Changes in ONL thickness with age and eccentricity in P23H-3 rats

Quantification of mean ONL thickness from sections of SD and P23H-3 rats at P18, 1 and 2 months of age (**A**) and across central to peripheral eccentricity at P18 (**B**), 1 month (**C**) and 2 months (**D**). At 2 months the ONL was significantly thinned in P23H-3 compared to SD retinæ (**A** and **D**). There were no significant differences in the thickness of ONL at different regions of P23H-3 retinæ (**B-D**). All data shown as mean $\pm$ SEM, Two-way ANOVA, Tukey's post hoc test, ** $p < 0.01$, and **** $p < 0.0001$, $n \geq 5$ in each group. *: significant difference for genotype, #: significant difference for age.

2.3.7. Activation of caspase-3 and AIF in degenerating P23H-3 retina

Another aim of this study was to explore the molecular pathways of cell death involved in P23H-related photoreceptor degeneration. To assess the contribution of the caspase-dependent pathway of apoptosis, retinal immunostaining was performed for cleaved caspase-3, a key mediator of caspase-dependent apoptotic signaling pathway, at P18 when cell death was high.

Results from this labelling only showed very few photoreceptor cells in the ONL of P23H-3 were positive for activated caspase-3 at P18 (**Figure 2.13A**). While this labelling was limited, no other activated caspase-3 labelling was noted in other retinal layers. Similarly, no labelling was observed in age-mated control (SD) retinae (data not shown).

To examine whether the caspase-independent apoptosis also plays a role during the degenerative process in P23H-3 retinae, the changes in subcellular distribution of AIF was evaluated by immunocytochemistry. AIF is a mitochondrial protein cleaved upon receiving death stimuli, leaves the mitochondrion, translocates to the nucleus where it mediates apoptotic cell death independently of caspases. In the retina, AIF is mainly localized in the inner segment of photoreceptor cells, which contain mitochondria and ER and in the cytoplasm of other neuronal cells (Hisatomi et al., 2001; Sanges et al., 2006b). At P18, AIF was detected in only a few photoreceptor nuclei of P23H-3 (**Figure 2.13B**), while there was no labelling in other retinal layers or in the age-mated control animals (data not shown).

Given the limited labelling observed at the peak of photoreceptor death (P18), the data suggest that neither the caspase 3-dependent nor the AIF-mediated pathways of apoptosis are major contributors to retinal degeneration in P23H-3 transgenic rats.

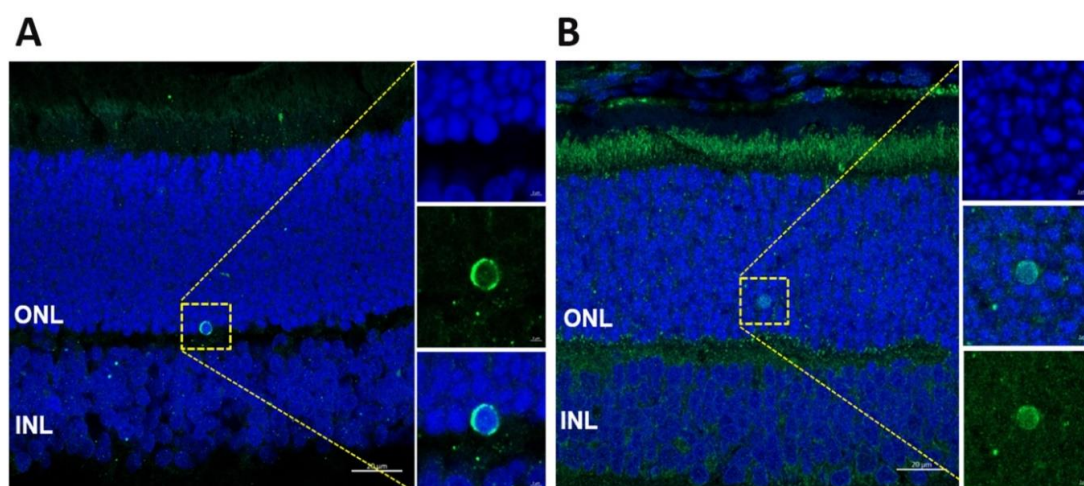


Figure 2.13: Activated caspase-3 and AIF staining in P23H retinae at P18

Confocal images of P18 retinal sections labeled with caspase-3 (**A**, green) and AIF (**B**, green). Abbreviations: ONL: outer nuclear layer, INL: inner nuclear layer. Scale bar = 20 μm.

2.3.8. Assessment of mitochondrial membrane potential in P23H-3 rats

The results of immunostaining suggested a minor participation of mitochondria-mediated cell death in photoreceptor degeneration in P23H-3. To further investigate whether this hypothesis is correct, JC-1 assay was used to evaluate the mitochondrial membrane potential in P23H-3 and SD retinæ. Mitochondrial membrane potential ($\Delta\psi_m$) is a sensitive indicator of the mitochondrial functional status, closely correlated with cell health and function. In apoptotic cells, mitochondrial depolarization occurs as a result of the formation permeability transition pores (Reers et al., 1995).

JC-1 is a membrane-permeable cationic fluorescent probe widely used to evaluate alterations in $\Delta\psi_m$ occurring during apoptosis. At low concentration, JC-1 exists as a monomer and when excited at 488 nm, emits green fluorescence. However, at higher concentrations it forms aggregates and emits an orange/red emission fluorescence. In healthy cells, JC-1 selectively and spontaneously aggregates (red) within the normal polarized mitochondria because of negative potential of the inner mitochondrial membrane (high $\Delta\psi_m$). By contrast, in apoptotic cells it cannot accumulate within the depolarized mitochondria (low $\Delta\psi_m$) and remains in the monomeric form in the cytoplasm (green). Thus, depolarization of mitochondria is indicated by a reduction in the level of red fluorescence and an increase in the level of green fluorescence (Reers et al., 1995; Salviooli et al., 1998; Smiley et al., 1991).

As the results of our TUNEL labelling suggested that cell death was high at P18 in P23H-3 transgenic rats, $\Delta\psi_m$ was assessed at this same age, as well as an earlier age coinciding with eye opening (P14). **Figure 2. 14A-F** shows representative and quantitative analysis (**M-O**) of flow cytometric results at P14. Initially, a gate was set on forward scatter (FSC) and side scatter (SSC) to eliminate debris and find single cell events for each sample (not shown). Considering physical parameters (FSC) and viability analysis (DAPI staining), three distinct subpopulations of cells were consistently detectable in all samples: small dead cells, small live

cells, and large live cells (**Figure 2. 14A, E and I**). Subsequently, cells in each subpopulation were gated to study JC-1-associated red and green fluorescence threshold (**Figure 2. 14B-D, F-H and J-L**).

To confirm that JC-1 dye was able to detect $\Delta\psi_m$ changes, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a positive control. CCCP uncouples the mitochondrial respiratory chain causing mitochondrial membrane depolarization and eventually apoptosis (Park et al., 1997). Incubating dissociated retinal cells with CCCP prior to JC-1 staining led to nearly complete elimination of JC-1 aggregates (decrease in the intensity of red fluorescence signal) and an increase in the formation of JC-1 monomers (intensity of green fluorescence signal; **Figure 2. 14B-D**, quadrant 3(Q3)).

From **Figure 2. 14**, it can be clearly seen that the profile of JC-1 staining in P23H-3 cells (**I-L**) was generally similar to that of SD cells (**E-H**) for all subpopulations at P14. In the small dead cell subpopulation, the majority of cells had depolarized mitochondria characterized by greater green fluorescence intensity (**Figure 2. 14F and J**, Q3). By contrast, in the subpopulation of small live cells, the highest percentage of cells had well-polarized mitochondria as the intensity of both green and red fluorescence were strong (**Figure 2. 14G and K**, Q2). Similarly, almost all cells in the subpopulation of large live cells showed stable levels of $\Delta\psi_m$ as was evident from formation of red mitochondrial JC-1-aggregates with negligible cytoplasmic green JC-1 monomers (**Figure 2. 14H and L**, Q2).

To quantify JC-1 data, the medians of both red and green fluorescence intensities were obtained for each subpopulation of P23H-3 and SD samples, using FlowJo software, the ratio of red/green fluorescent intensity was calculated and statistically compared. No significant differences were found between the ratio of red/green fluorescence of cells isolated from P23H-3 and SD retinæ in any of the cell subpopulations identified (**Figure 2. 14M-O**).

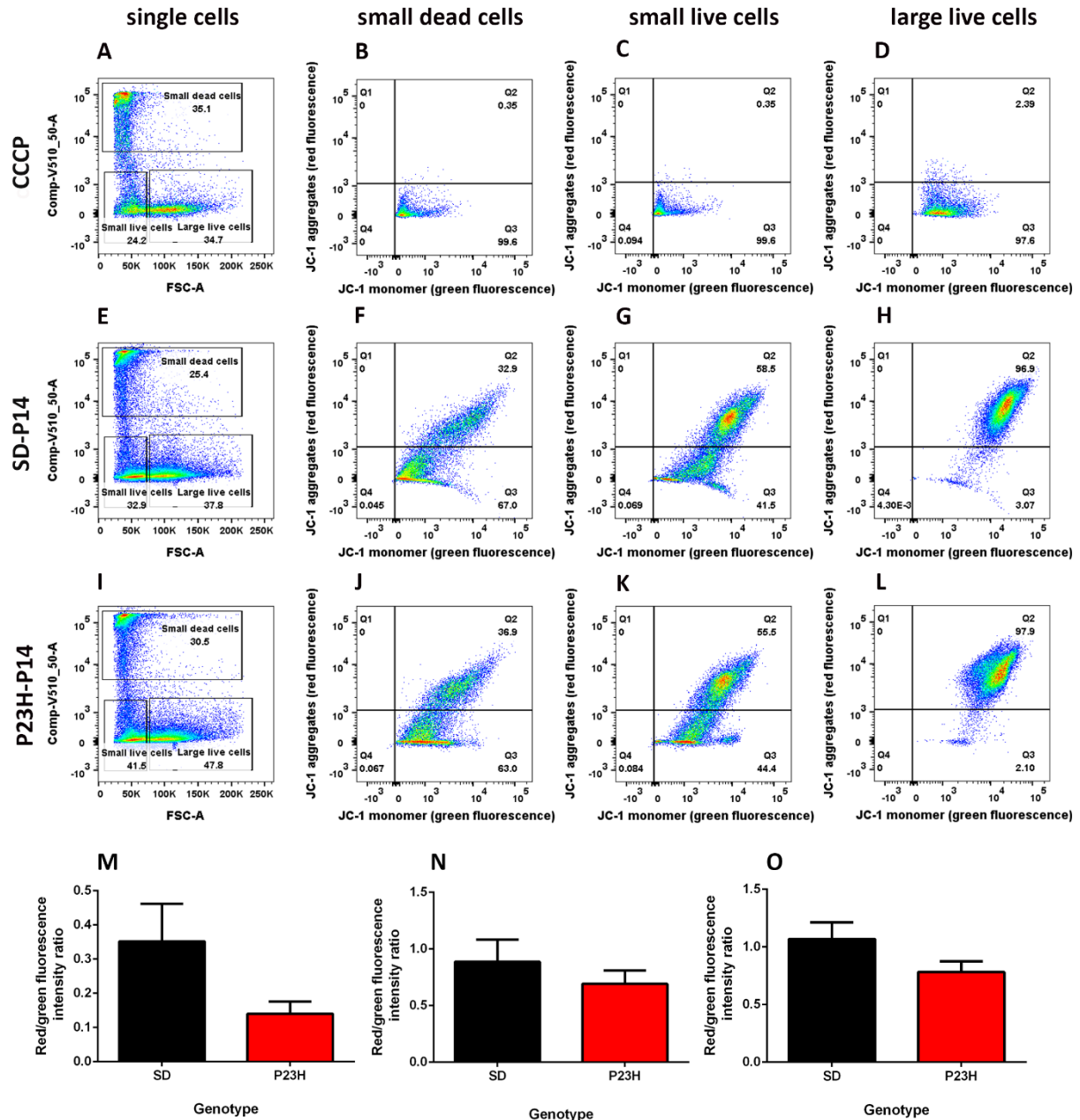


Figure 2. 14: Mitochondrial membrane potential ($\Delta\psi_m$) in P23H-3 and SD retinal cells at P14

Gating strategy for the analysis of retinal cells with different mitochondrial membrane potentials (A, E and I). Three distinct subpopulations of cells were detected in all samples: small dead cells (B-F and J), small live cells (C, G and K) and large live cells (D, H and L). Representative dot plots show ratio between polarized (red fluorescence) and depolarized (green fluorescence) mitochondria in CCCP-treated (B-D), SD (F-H) and P23H (J-L) retinal cells. Bar graphs illustrating red/green fluorescence intensity ratio indicative of mitochondrial membrane potential for small dead cell (M), small live cell (N) and large live cell (O) subpopulation of P23H-3 and SD samples. No significant differences were found between the ratio of red/green fluorescence of cells isolated from P23H-3 and SD retinæ in any of the cell subpopulations identified. Quantitative data presented as mean $\pm$ SEM, Student's t-test, $n = 6$ in each group.

A similar experiment was performed for P23H-3 and SD rats at P18 (**Figure 2.15A-L**). Again, the addition of CCCP resulted in almost complete loss of the red fluorescent signal (elimination of JC-1 aggregates) in all subpopulations of cells. Generally, a similar pattern of JC-1 staining was observed for P23H-3 and SD rats at P18 as was observed at the P14 time point (**Figure 2.15A-L**). In the subpopulation of small dead cells, nearly all cells had mitochondria with low $\Delta\psi_m$ and emitted fluorescence and almost high green no red fluorescence (**Figure 2.15F and J**, Q3). By contrast, in subpopulation of small live cells the greatest proportion of cells showed high green and red fluorescence intensity indicating the presence of healthy polarized mitochondria in these cells (**Figure 2.15G and K**, Q2). Likewise, almost all population of large live cells showed high $\Delta\psi_m$ (**Figure 2.15H and L**, Q2). Calculation of red/green fluorescence intensity ratio, as described above, did not show any statistically significant differences between P23H-3 and SD samples at P18 (**Figure 2.15M-O**). Thus, the data suggest limited contribution of the mitochondrial-mediated apoptosis pathways in the retinal cell death observed in the P23H-3 model of RP.

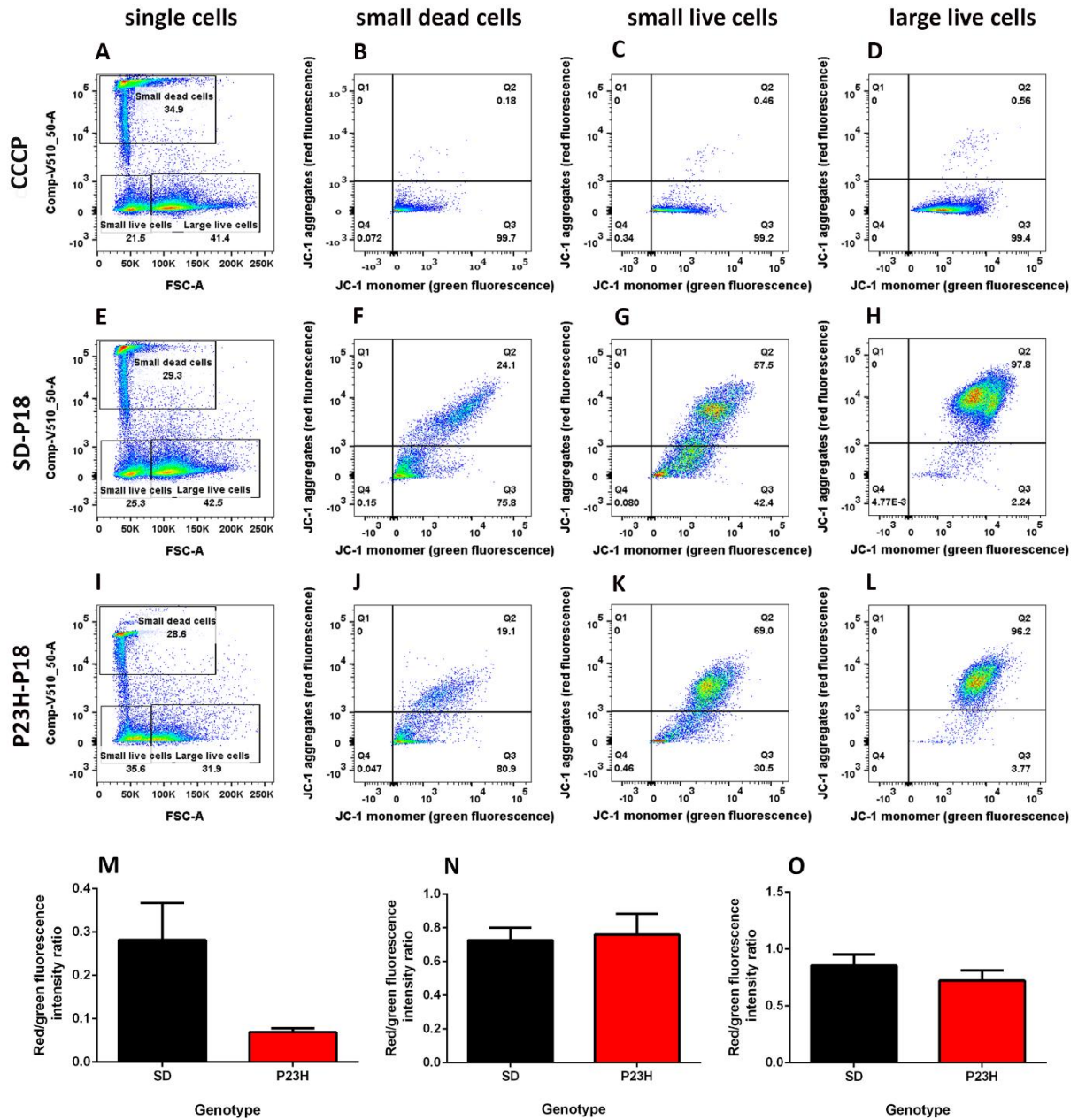


Figure 2.15: Mitochondrial membrane potential ($\Delta\psi_m$) in P23H-3 and SD retinal cells at P18

Gating strategy for the analysis of retinal cells with different mitochondrial membrane potentials (A-E and I). Three distinct subpopulations of cells were detected in all samples: small dead cells (B, F and J), small live cells (C, G and K) and large live cells (D, H and L). Representative dot plots show ratio between polarized (red fluorescence) and depolarized (green fluorescence) mitochondria in CCCP-treated (B-D), SD (F-H) and P23H (J-L) retinal cells. Bar graphs illustrating red/green fluorescence intensity ratio indicative of mitochondrial membrane potential for small dead cell (M), small live cell (N) and large live cell (O) subpopulations of P23H-3 and SD samples. No significant differences were found between the ratio of red/green fluorescence of cells isolated from P23H-3 and SD retinæ in any of the cell subpopulations identified. Quantitative data presented as mean $\pm$ SEM, Student's t-test, $n = 6$ in each group.

2.3.9. Programmed cell death related genes are altered in P23H-3 retinae

As our combined analysis of caspase-3/AIF immunostaining and mitochondrial permeability (JC-1 assay) suggested limited involvement of mitochondrion in retinal degeneration in the P23H-3 model, several cell death pathways were explored using gene expression changes. A commercial quantitative RT-PCR array system was used to quantify expression of 84 genes involved in various cell death pathways, at the two time-points characterized throughout this chapter (P14 and P18). When comparing P23H-3 with the SD samples, out of 84 genes the expression level of 13 and 25 genes were significantly altered at P14 (**Table 2.3**) and P18 (**Table 2.4**), respectively. Generally, the extent of the fold change values was limited, with only 5 genes regulated by greater than 2 fold, with all of these genes found in the P18 age group (*Fas*, *Birc3*, *Tnfrsf10b*, *Defb1*, *Galnt5*). Clustering of all the dysregulated genes into the three broad classes of cell death pathways (autophagy, apoptosis and necrosis), indicated that all three types of programmed cell death (**Figure 2.16A and B**), are involved in the photoreceptor degeneration that occurs in P23H-mediated degeneration.

Table 2.3: Regulation of programmed cell death related genes in P23H-3 retinae at P14

A commercial programmed cell death PCR array was used to screen 84 genes samples obtained from P23H-3 rats' retinae at P14. Samples were pooled, and 3 independent arrays performed. Data is relative to SD control samples and all highlighted gene alterations were statistically significant (Student's t-test, $p < 0.05$).

Gene Name	Gene Symbol	Fold change	p-value
Amyloid beta (A4) precursor protein	<i>App</i>	1.1	0.008
Huntingtin	<i>Htt</i>	1.2	0.035
Sequestosome 1	<i>Sqstm1</i>	1.3	0.004
Tumor protein p53	<i>Tp53</i>	1.4	0.022
Insulin-like growth factor 1 receptor	<i>Igf1r</i>	1.2	0.007
Myeloid cell leukemia sequence 1	<i>Mcl1</i>	1.2	0.044
Tumor necrosis factor receptor superfamily, member 11b	<i>Tnfrsf11b</i>	1.7	0.017
Caspase 3	<i>Casp3</i>	-1.2	0.047
Spermatogenesis associated 2	<i>Spata2</i>	1.4	0.026
Myelin-associated glycoprotein	<i>Mag</i>	1.2	0.028
Hspb associated protein 1	<i>Hspbap1</i>	1.3	0.014
Dihydropyrimidinase-like 4	<i>Dpysl4</i>	1.5	0.01
ATPase, H ⁺ transporting, lysosomal V1 subunit G2	<i>Atp6v1g2</i>	1.3	0.028

Table 2.4: Regulation of programmed cell death related genes in P23H-3 retinæ at P18

A commercial programmed cell death PCR array was used to screen 84 genes samples obtained from P23H-3 rats' retinæ at P18. Samples were pooled, and 3 independent arrays performed. Data is relative to SD control samples and all highlighted gene alterations were statistically significant (Student's t-test, $p < 0.05$).

Gene Name	Gene Symbol	Fold change	p-value
ATG16 autophagy related 16-like 1 (S. cerevisiae)	<i>Atg16l1</i>	1.2	0.03
ATG3 autophagy related 3 homolog (S. cerevisiae)	<i>Atg3</i>	1.2	0.01
ATG5 autophagy related 5 homolog (S. cerevisiae)	<i>Atg5</i>	1.2	0.03
Beclin 1, autophagy related	<i>Becn1</i>	1.5	0.03
Cathepsin S	<i>Ctss</i>	1.4	0.005
Mitogen-activated protein kinase 8	<i>Mapk8</i>	1.4	0.007
Sequestosome 1	<i>Sqstm1</i>	1.5	0.01
Fas (TNF receptor superfamily, member 6)	<i>Fas</i>	-2.5	0.006
B-cell leukemia/lymphoma 2 related protein A1d	<i>Bcl2a1</i>	-1.7	0.04
BCL2-like 11 (apoptosis facilitator)	<i>Bcl2l11</i>	-1.5	0.02
Baculoviral IAP repeat-containing 3	<i>Birc3</i>	-3.1	0.04
Caspase 2	<i>Casp2</i>	1.9	0.01
Caspase 6	<i>Casp6</i>	-1.2	0.03
Caspase 9	<i>Casp9</i>	1.2	0.02
DNA fragmentation factor, alpha subunit	<i>Dffa</i>	1.4	0.01
Growth arrest and DNA-damage-inducible, alpha	<i>Gadd45a</i>	1.5	0.007
Myeloid cell leukemia sequence 1	<i>Mcl1</i>	1.4	0.01
Nucleolar protein 3 (apoptosis repressor with CARD domain)	<i>Nol3</i>	1.3	0.04
Tumor necrosis factor receptor superfamily, member 10b	<i>Tnfrsf10b</i>	-2.9	0.006
X-linked inhibitor of apoptosis	<i>Xiap</i>	1.1	0.03
Defensin beta 1	<i>Defb1</i>	-19.9	0.001
Dihydropyrimidinase-like 4	<i>Dpysl4</i>	1.5	0.04
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)	<i>Galnt5</i>	-4.5	0.03
Hspb associated protein 1	<i>Hspbap1</i>	1.5	0.01
Tumor necrosis factor receptor superfamily, member 4	<i>Tnfrsf4</i>	1.2	0.04

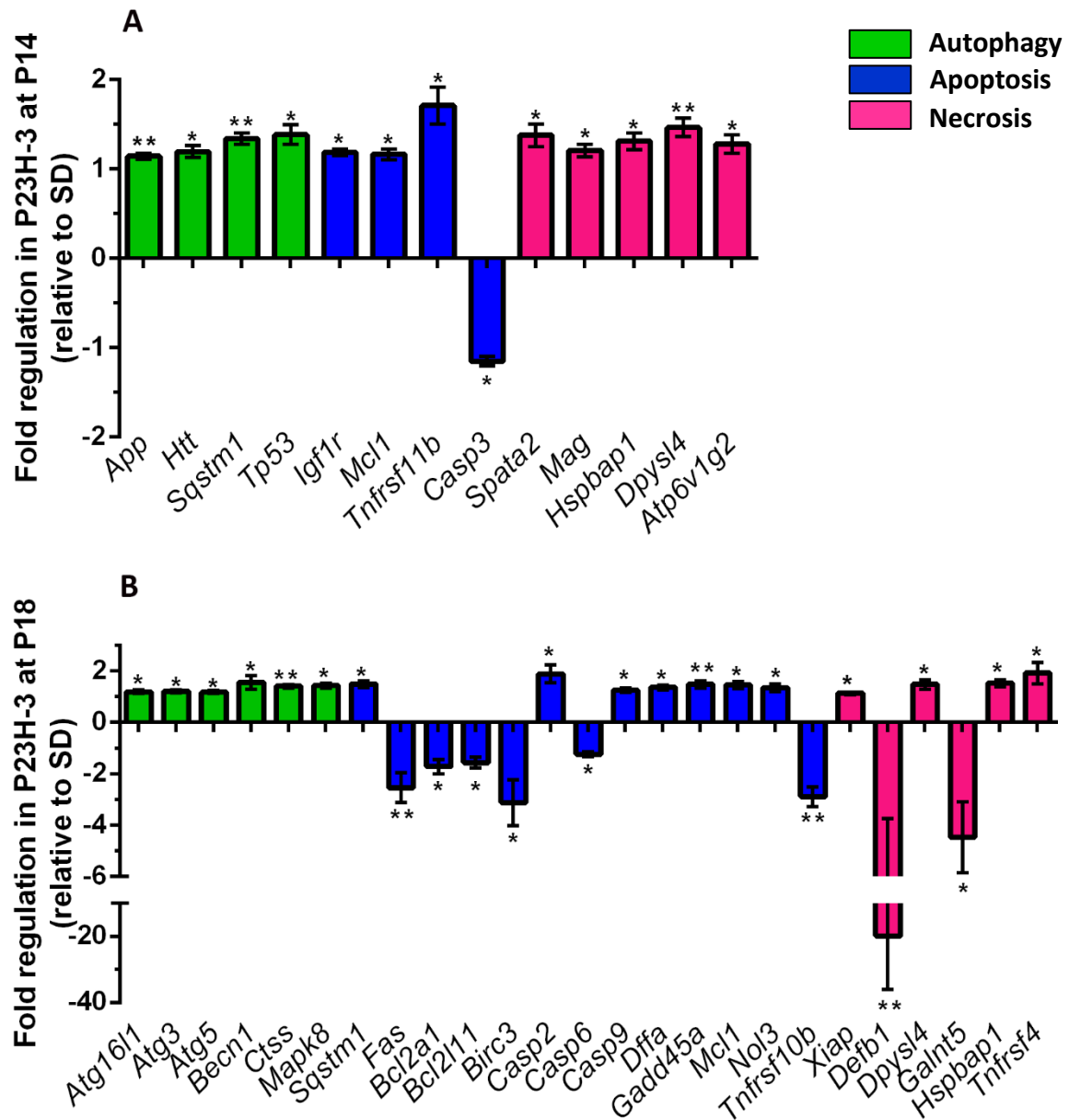


Figure 2.16: Dysregulation of programmed cell death-related genes in P23H-3 retinas
 Changes in programmed cell death related genes in P23H-3 compared to age-matched SD rats at P14(A) and P18(B). Quantitative data presented as mean $\pm$ SEM, Student's t-test, * $p < 0.05$, ** $p < 0.01$.

2.3.10. No rescue of photoreceptor death in P23H-3 rats following intravitreal injection of BAK inhibitors

Although the results of the immunofluorescence staining, mitochondrial membrane potential and death pathway gene expression suggested that mitochondrial-mediated cell death pathways weren't major contributors to photoreceptor death in the P23H-3 rat retinas, they

provided some evidence of apoptosis involvement. While the JC-1 and immunofluorescence experiments were being conducted our collaborators (Drs Ben Kile and Guillaume Lessene) developed two BAK antagonists to trial in an *in vivo* application. To establish the methods required to administer these compounds into rodent eyes with retinal degeneration we chose to develop the techniques in the P23H-3 rat eyes and establish the technical feasibility of administering these drugs *in vivo*. This experiment also provided an alternative way of assessing the role of BAK-mediated apoptosis in the retinal degeneration observed in this animal model.

BAK and BAX, pro-apoptotic members of BCL-2 family, are the critical effectors of apoptosis, which permeabilize the mitochondrial outer membrane. To assess the extent of cell death in each experiment, P23H-3 were treated with the BAK inhibitors (WEHI-1249625 and WEHI-1250993) at P16, just prior the high level of TUNEL labelling. Two days later (corresponding to P18) the number of TUNEL-labeled nuclei in the ONL of untreated (**Figure 2.17A**) and treated (**Figure 2.17B**) retinæ were quantified per mm retina in representative retinal cross sections. Data from the first inhibitor (WEHI-1249625) showed no significant difference in the number of photoreceptors undergoing cell death between drug-treated retinæ compared to the non-treated groups (**Figure 2.17C**). However, due to the uncertainty of the bioavailability of this drug, high concentrations were used *in vivo* (6 mM) and it was noted that upon isolating the eyes, there was significant precipitate in the vitreous indicating poor *in vivo* solubility of the drug.

To overcome the technical challenges that we faced using the first BAK inhibitor, another experiment was carried out, using a newer inhibitor (WEHI-1250993) that showed significantly better *in vitro* dose response characteristics (Drs Ben Kile and Guillaume Lessene, personal communication). Thus, a second experiment was performed using this more potent inhibitor allowing a lower concentration (1 μ M) to be used *in vivo*. The experimental design

was identical to the first experiment and after collection of the eyes, no evidence of drug precipitation was found in the vitreous. When quantitative analysis of cell death was performed there was no alteration in the number of TUNEL positive photoreceptors nuclei between the drug-injected and vehicle (DMSO)-injected eyes (**Figure 2.17D**). While not altering photoreceptor degeneration, these studies established the feasibility of injecting these drugs into rodent eyes at young ages. As photoreceptor death in the P23H-3 model appears not to involve changes in mitochondrial membrane permeability or involvement of the intrinsic pathway, the effectiveness of these drugs will be examined in another model of retinal degeneration (Chapter 3).

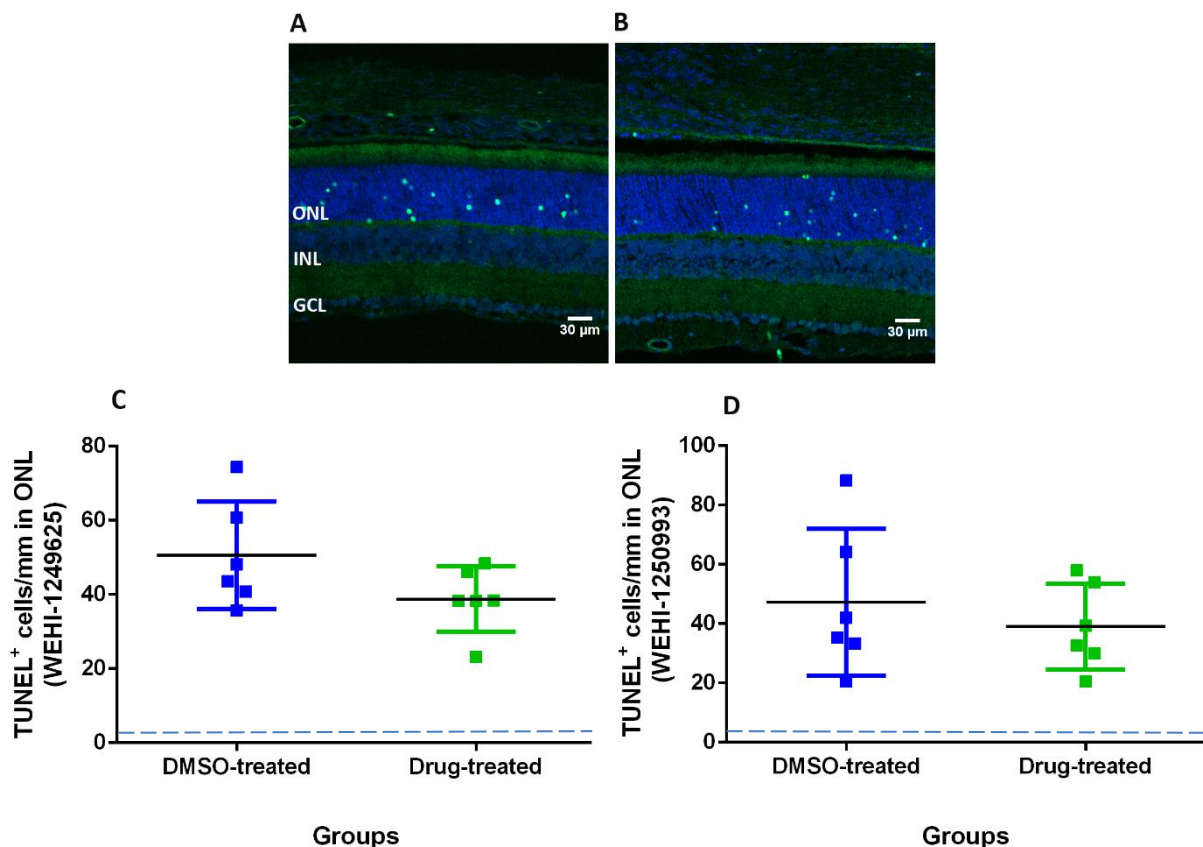


Figure 2.17: Quantification of cell death after intraocular injection of two specific inhibitors of BAK in P23H-3 rats

Sections of retinæ labeled for TUNEL (green) after 2 days in a DMSO-injected (A) and in a relative drug-injected (B) eye. Following intravitreal injection of BAK inhibitors (WEHI-1249625 and WEHI-1250993), the number of TUNEL positive photoreceptors was counted in ONL in each condition and quantified per mm (C and D). No significant differences were detected in the number of TUNEL-labeled nuclei between drug-treated and DMSO-treated retinæ in either of experiments. The dashed lines show the mean number of TUNEL⁺ cells in SD normal rats at P18, Student t-test, n = 6 in each group. Abbreviations: ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Scale bar = 30 μm.

2.4. Discussion

The P23H mutation is the most common in autosomal dominant RP (Dryja et al., 2000a; Dryja et al., 1990) and the P23H-3 rat has been an animal model for translational research and developing new therapeutic strategies to rescue photoreceptors in RP. Despite this, a definitive characterization of the degeneration that occurs in this animal model, particularly the cell death pathways involved, has until very recently been lacking. The present study provides a detailed and comprehensive phenotypic analysis of retinal function and structure of this animal model compared with SD controls during aging, as well as an assessment of cell death pathways involved. While ERG measurements exhibited functional deficits as early as 1 month of age in retinæ harboring P23H mutation, structural changes were not apparent until 2 months in these animals. This structural alteration involved cell loss, which was principally limited to the outer nuclear layer containing the retinal photoreceptors. Results of TUNEL staining suggested that the rate of photoreceptor death in P23H-3 strain is biphasic, with a more rapid initial phase peaking at P18, followed by a slower phase. Using immunostaining, only very limited activity of caspase-3 and AIF was detected in P23H-3 photoreceptors. Consistent with this, the JC1 assay showed very little evidence of changes in mitochondrial permeability in the P23H retinal cells compared to control SD cells. Moreover, analysing the gene expression profile of P23H-3 retinæ suggested the presence of multiple cell death pathways in the degenerating P23H-3 rat retina at P14 and P18. Intravitreal injection of BAK antagonists also failed to alter the extent of photoreceptor loss in the P23H-3 retina.

2.4.1. *Functional alterations in P23H-3 rat animal model of RP*

Functional characterization of albino rats heterozygous for the P23H mutation revealed both rod photoreceptor (a-wave) and post-receptoral responses (b-wave and OPs) were

significantly attenuated in amplitude compared to SD rats as early as 1 month and continually reduced by aging. From P18 to 3 months the amplitude of the rod a-, b-wave and OPs decreased by 60%, 40% and 62% respectively. Our data also showed that there was an age-related decrease in retinal function independent of genotype from 2 months onward, as have been described previously (Chaychi et al., 2015; DiLoreto et al., 1995; Nadal-Nicolás et al., 2018).

In line with these results, previous studies have described a progressive and significant decline in scotopic rod-mediated responses (a- and b-waves) of P23H-3 transgenic rats that was apparent from 1 month (Chrysostomou et al., 2009; Machida et al., 2000). Machida et al. (2000) described 74% reduction in the rod a-wave maximum amplitudes over the period of P30 to P230. In a later study, Chrysostomou et al. (2009) reported that from P16 to P120 the amplitude of the rod a- and b-wave is reduced by 45% and 20% respectively, followed by a further 60% and 50% reduction between P180 and P540. The total reduction in amplitude over 540 days of life in the P23H-3 rat was 80% for the rod a-wave and 55% for the post-receptor b-wave.

As the ERG response is serial in nature, functional deficits in the a-wave, also result in functional loss in the inner retina. In order to determine whether there was a specific inner retinal functional loss over and above the a-wave deficit, relative percentage losses were compared. When the percentage loss of rod b-wave and OPs amplitudes were compared to that of rod-a wave, rod b-wave showed similar extent of reduction at 1 and 2 months. However, at 3 months, the amplitude loss of the b-wave was significantly less than a-wave. Although no significant differences were found in the percentage loss of rod a-wave and OPs amplitudes, the loss of OPs amplitudes was significantly increased compared to those of b-wave at all time points examined. This suggests that by 3 months, inner retinal neurons remain relatively unaffected.

While the latency (implicit time) of the rod b-wave responses was slightly prolonged in P23H-3 rats compared to the SD at all ages examined, it was only statistically significant at 1 month. In addition, the phototransduction sensitivity showed no significant alterations in rhodopsin-mutant P23H-3 retinae when compared to the age-matched SD retinae, except at P18, which was significantly greater in P23H-3 retinae. Concordant with the finding of this study, Machida et al. (2000) found that the phototransduction sensitivity was not affected in P23H-3 rats at any age examined from P30 to P203.

In rats, cone photoreceptor responses could not be evaluated as cones represent only 1% of the photoreceptor population (La Vail, 1976; Szel et al., 1992); however, analysis of cone post-photoreceptor responses can be used as an indirect measure of the cone photoreceptor function. Our data indicated that P23H-3 animals had significantly lower cone b-wave amplitudes compared to SD controls at 1 and 2 months of age, while the cone b-wave implicit time, OPs amplitudes and timing remained relatively unaffected. The cone b wave decrease is unlike that reported previously for this animal model. No significant difference was found in photopic cone-related b-wave of P23H-3 rats compared to the SD between P28 and P203 (Machida et al., 2000). Similarly, Chrysostomou et al. (2009) reported that photopic cone-driven responses are preserved in P23H-3 animals up to P380.

While long-term survival of cones following loss of rods have been previously indicated in retinal degenerations caused by mutations of rod proteins (John et al., 2000; Lin et al., 2009; Lu et al., 2013; Morris et al., 2005), our data is supported by a study showing significantly lower number of L/M-cones in P23H-1 homozygous albino rat retinae when compared to SD rats at all time points studied (P30, P90 and P180). Such data suggest that in this strain, cone deterioration may initiate immediately after rod death, possibly even before P30 (Garcia-Ayuso et al., 2013). In this study, no significant age-related changes were found in the number of cones in SD normal rats. By contrast, Chrysostomou et al. (2009) suggested

that densities of L/M-cones are preserved in P23H-3 retinæ up to 1 year. They found that the cone density of P23H-3 retinæ increases between P10 and P20, followed by a subsequent reduction (50%) between P16 and P120 but not thereafter. Despite this reduction, the authors concluded that this loss of cone photoreceptors was developmental-related rather than P23H-related, as functional assessments using ERG recording showed the amplitude of the cone b-wave increased through this period and the cone density was similar between aged P23H-3 and SD retinæ (P350 and P500). However, in this study the cone density of P23H-3 was not compared with age-matched SD normal rats at earlier time points (Chrysostomou et al., 2009).

In young SD rats at P18, the rod-mediated responses were lower (lower a-wave, b-wave, and OPs amplitudes) and slower (prolonged b-wave and OPs implicit times) compared to 1-month old SD rats. This suggests that while eye opening in rats takes place between P12–P14, with all retinal layers formed by P18, the retinæ of these early post-natal animals are still developing. Postnatal development of photoreceptors in the retina is linked to elongation of the outer segment, increased expression of photopigment (opsin) and greater functional output (Fulton et al., 1995; Riki et al., 2013; Timmers et al., 1999). In rodents, the formation of synaptic interactions within the plexiform layers of retina initiates at, or slightly prior to, eye opening; however, the functional and morphological modifications of synaptic connections continue during early postnatal development (Marquardt et al., 2002; Tian, 2004; 2008). Ultimately, a combination of all these factors likely leads to the increased functional response observed by 1 month of age.

In P23H-3 strain, while rod-driven responses were similar to the SD controls (except for rod photoreceptor sensitivity) at P18, they exhibited reduced levels at 1-month old. It is possible that the loss of function observed at 1-month old P23H-3 animals is due to a “failure” of the normal functional maturation of the retina and/or the underlying P23H-3 based retinal degeneration. Support for a significant role of retinal degeneration comes from our TUNEL

analysis, which indicated an early onset of photoreceptor degeneration in retinae bearing P23H mutation with a peak at P18.

Similar to the rod-mediated pathway, the cone-driven post-receptoral responses (b-wave and OPs amplitudes) and timing (OPs implicit time) were significantly improved in SD normal rats from P18 to 1 month, confirming that retinae undergo a functional maturation during this time. Within the P23H-3 strain, the cone b-wave showed a significant increase from P18 to 1 month; however, it was still lower than that of SD controls. From 1 month onwards, the cone b-wave amplitude remained nearly unchanged in these animals for up to 3 months. These findings are consistent with those of Chrysostomou et al. (2009), who demonstrated that cone OS continue to lengthen up to P30 and then slowly shorten during late adult life. Thus, as discussed above for the rod-mediated pathway, it is hard to conclude whether the lower cone b-wave amplitudes in P23H-3 at 1 and 2 months is due to the influence(s) of P23H mutation on normal maturation of the retinae and/or an early onset of cone photoreceptor loss in this animal model.

In RP patients, the disease displays an early deterioration of rod-derived amplitude, an average reduction of 16% to 18% per year, with delayed implicit time in scotopic ERG measurements. Such impairments in retinal function are evident at early stages of RP, even when patients are asymptomatic and ocular fundus images appear normal (Berson et al., 1985; Holopigian et al., 1996; Jacobi et al., 1993; Shintani et al., 2009). Thus, the functional characteristics of P23H-3 rat animal model reasonably mimic the pathophysiological features of human RP in many aspects.

2.4.2. Structural changes and time course of cell death in P23H-3 rat model of RP

In this study, *in vivo* fundus imaging was used to monitor ocular health status of P23H-3 animals. From P18 to 3 months no obvious change or pathological features were evident in the fundus scans of P23H-3 compared to the SD rats. This is unlike the pathology observed in other animal models of RP and in those patients with the disorder who show characteristic abnormalities including bone spicule pigmentation mainly in the peripheral and/or mid-peripheral retina, attenuation of retinal vessels, and a waxy pallor of the optic nerve head (Hamel, 2006). This could be due the fact that these animals are albino, which makes it hard to visualize RD-related changes in the eye, which often rely on observation of pigmentation.

Generally, functional deficits of photoreceptors occur due to a reduction of their number and/or inner/outer segment length (Weymouth et al., 2008). To follow loss of photoreceptors over the time course of functional impairment, retinal thickness measurements were performed in the P23H-3 retinæ and compared to age-matched SD controls. The results of both OCT and histological analysis confirmed a gradual thinning of ONL and also the total retina in rats harboring P23H mutation. The difference in ONL thickness between the two strains of rat became statistically significant from 2 months onwards; reaching 22% reduction at 3 months of age. Measurement of the IS/OS using OCT showed no significant alteration in the length of the photoreceptor inner/outer segment length. These observations are consistent with previous studies indicating a steady and slow loss of retinal structure in this rat strain (Chrysostomou et al., 2009; LaVail et al., 2018; Machida et al., 2000). Machida et al. (2000) detected minimal morphological alterations in P23H-3 retinæ at P30 as was evident from a minor reduction (15%) in the number of photoreceptor cells along with slightly shortened OSs. The ROS length and ONL thickness were significantly decreased from P60 onwards compared to controls. They suggested that ONL underwent a faster degeneration between P30 and P56 and then slowed

with ongoing aging such that by P203, the ONL thickness and ROS length were half the control values (Machida et al., 2000). Photoreceptor loss proceeded steadily until 230 days when the ONL was decreased by 68% compared to SD rats (Chrysostomou et al., 2009).

Quantification of dead photoreceptors in the ONL, using TUNEL assay, indicated that in the P23H-3 rat strain photoreceptor cells die rapidly at P18, prior to maturity of the retina. The number of TUNEL-labeled photoreceptors nuclei decreased by 1 month and remained detectable as late as 2 months. These results suggest that the rate of retinal degeneration in this animal model is biphasic, with a quicker initial phase followed by a slower one. These findings are similar to previous studies, which reported that death of photoreceptors in the P23H-3 retinae initiates at about P10, even prior to eye opening (P14), with a peak occurring at approximately P17-P20 (Lee et al., 2003; Yu et al., 2004). Likewise, in P23H-1 strain retinal degeneration occurs in a comparable biphasic pattern; an early phase of intense photoreceptors death up to about P30 which peaks at around P15-P18 and a later phase of mild degeneration up to P60 (Kaur et al., 2011b; Lee et al., 2003).

The phase of rapid photoreceptor death in both strains closely overlaps with the period of naturally occurring death of photoreceptors during normal maturation of retina. Maslim et al. (1997) indicated that P15 to P22 is a critical period for rat retinal development and photoreceptor death and survival were found to be far more susceptible to levels of oxygen during this time (Maslim et al., 1997). Accordingly, Lee et al. (2003) suggested that during this period, stress-induced damage by *Rho* mutations may strongly influence survival of photoreceptors (Lee et al., 2003).

To investigate whether there is an eccentricity-dependent effect on P23H-associated retinal degeneration, TUNEL⁺ cells and ONL thickness were quantified in different regions of the retina. While at one month of age, the photoreceptors appeared to die more frequently in the mid-peripheral and central parts of the retinae compared to peripheral region, at P18 and 2

months, the degeneration proceeds relatively uniformly across the retina. Quantification of ONL thickness across the retina, as a function of eccentricity, revealed no significant differences among central, mid-peripheral and peripheral regions at any age studied. This suggests the loss of ONL thickness occurs relatively uniformly across the P23H-3 retinae. Such a result is in line with a previous study showing a similar rate of photoreceptor loss in superior and inferior hemispheres of P23H-3 and P23H-1 rat retinae at all time points examined from 4 to 29 weeks of age (Machida et al., 2000). A recent work by LaVail et al. (2018) found a higher rate of retinal degeneration in the superior hemisphere of all P23H strains than the inferior hemisphere and also reported that the loss of photoreceptors proceeds in a central-peripheral gradient (LaVail et al., 2018).

In RP patients, degeneration of rods usually initiates in the mid peripheral (equatorial) retina and progresses gradually to the macula and more peripheral regions, with loss of rod photoreceptors in the far periphery occurring at relatively advanced stages (Milam et al., 1998). Such regional distribution of retinal degeneration is also observed in humans with P23H mutations (Heckenlively et al., 1991; Stone et al., 1991), possibly related to the relative rod/cone density in different areas of human retina (Machida et al., 2000).

2.4.3. Mechanisms of photoreceptor cell death in P23H-3 rat retinae

Early studies specified apoptosis as the main pathway of photoreceptor death in RP, mainly based on finding TUNEL⁺ nuclei in the ONL (Chang et al., 1993b; Travis, 1998b). TUNEL is often considered as an indication of apoptosis even though this method also labels DNA fragments generated by non-apoptotic mechanisms of cell death, such as necrosis (Grasl et al., 1995). Classical apoptotic cell death is defined as a pathway mediated by the caspase family of proteases, caspase-dependent, in which caspase-3 has a vital role in the execution

step. In the present study several techniques were used to probe the particular cell death pathways involved in the retinal degeneration of the P23H-3 animal model.

Immunostaining results revealed limited activation of caspase-3 in photoreceptor nuclei at their peak of cell death, P18, suggesting the possible involvement of caspase-dependent pathway of apoptosis. However, only a few caspase-3 positive cells were detected in the ONL, which was inconsistent with the high number of TUNEL⁺ cells at the same stage. This finding is in agreement with a previous report showing the presence of only very small number of caspase-3 positive nuclei in the ONL of P23H-1 rat retinae at P15, which was not significantly different from age-matched *WT* control. In addition, immunostaining results showed no evidence of caspase-9 activity or cytochrome *C* leakage in P23H-1 and *WT* control (Kaur et al., 2011b). Similarly, in another study there was no evidence of caspase-3 activation in P23H rat retinae (Arango-Gonzalez et al., 2014).

The current study also showed translocation of AIF from the mitochondria-rich inner segment in only a few photoreceptor nuclei at P18. Consistent with this, western blot analysis confirmed the release of AIF and also cytochrome *C* from the mitochondria to the cytosol of P23H-3 photoreceptors at P21 (Sizova et al., 2014b). Moreover, Ozaki et al. (2013) demonstrated that topical administration of a specific peptide inhibitor of mitochondrial μ -calpain, which cleaves AIF, preserved the ONL thickness and delayed the ERG reductions in P23H-2 rats. Furthermore, activation of calpains and AIF-dependent photoreceptor cell death have been identified in several mouse and rat models of RP including *rd1* mice (Paquet-Durand et al., 2006; Paquet-Durand et al., 2010; Sanges et al., 2006b), RCS rats (Mizukoshi et al., 2010), S334ter-4 rats (Shinde et al., 2012), S334ter-3 rats (Kaur et al., 2011a), P23H-1 rats (Kaur et al., 2011b) and *Rho* mutant mice (*P23H^{Tg}*, *Rho^{P23H/-}* and *Rho^{-/-}*) (Comitato et al., 2016; Comitato et al., 2014). Therefore, AIF, as a caspase-independent mediator of apoptosis, may be another potential executioner of photoreceptor death associated with P23H mutation.

However, the number of AIF⁺ nuclei detected was not sufficient to account for the large number of TUNEL⁺ cells observed in the ONL.

There are several explanations for the discrepancy between the low number of positive cells for either caspase-3 or AIF and high number of TUNEL⁺ cells in P23H-3 retinae at P18. One possible explanation is that in the P23H retina, caspase-3 and/or AIF plays only a minor role in mediating retinal degenerating and other apoptotic and/or non-apoptotic cell death pathways may function in this process (to be addressed later). Another explanation is that, by P18 cells may actually be in the late stages of apoptosis and thus the activity of intracellular proteins such as caspase-3 and AIF has already passed, but that nuclear DNA cleavage is readily detectable by TUNEL (a measure of late stage cell death). Moreover, it is also possible that the antibodies used in this study, despite being suitable for immunohistochemical use, did not stain activated caspase-3 and AIF reliably.

In order to overcome the possible issue of using antibodies to mediators of apoptosis, this study explored mitochondrial membrane potential ($\Delta\psi_m$) is a valuable parameter manifesting cells' functional status and health that can be monitored by fluorescent probes. Assessment of $\Delta\psi_m$ in cells isolated from P23H-3 retinae using flow cytometric analysis of JC-1 assay showed no significant differences in the profile of JC-1 staining in P23H-3 and SD retinae neither at P14 nor P18. This was despite the use of CCCP as a positive control, which showed a significant change in the mitochondrial membrane potential. To our knowledge, no other studies have assessed the mitochondrial membrane potential of retinal cells in P23H strains. The data, therefore suggest that there is limited involvement of the mitochondrial-mediated apoptosis pathways in the retinal cell death observed in the P23H-3 model of RP. In addition, intravitreal administration of BAK antagonists failed to reuse photoreceptor death in the P23H-3 retina.

To gain further insight into molecular pathways that potentially mediate P23H-related photoreceptor death, a commercial quantitative RT-PCR array system was used to monitor gene expression changes associated with diverse types of programmed cell death at specific time points during degeneration (P14 and P18). At both time points, expression of relatively small number of genes exhibited significant regulation that are associated with all three types of programmed cell death.

Apoptosis has been proposed as a common mechanism of photoreceptor cell death in different animal models of RP (Jomary et al., 2001b). In the present study, several apoptosis-associated genes exhibited altered expression in degenerative P23H-3 retinæ. For example, up-regulation of *Tp53* (*p53*) was detected at early stages of retinal degeneration, P14. *Tp53* is a well-known tumor suppressor associated with not only caspase-independent pathway of apoptosis (Ranjan et al., 2016), but also other non-apoptotic mechanisms of cell death including programmed necrosis (necroptosis) (Vaseva et al., 2012). It controls apoptotic cell death by transcriptionally up-regulating AIF, directly inhibiting *Bcl-2* and *Bcl-XL* and activating *Bak* and *Bax*, leading to induction of AIF-mediated pathway of apoptosis (Ranjan et al., 2016). Thus, up-regulation of *Tp53* is consistent with our immunostaining results showing possible involvement of AIF in retinal degeneration of this animal model. Confirming the importance of *Tp53*-mediated cell death, the level of *Dpysl4* expression was also elevated at both time points analysed. *Dpysl4* has been suggested as a direct target for *Tp53* in the apoptotic response to DNA damage and its expression is induced by overexpressing *Tp53* (Junko et al., 2011). Additionally, at P14 expression levels of predominantly anti-apoptotic genes including *Mcl1*, *Igf1r* and *Tnfrsf11b* elevated that may reflect a compensatory neuro-protective effect/response initiated early during cell death in P23H-3 retinal degeneration.

At P18, mRNA levels of both anti- and pro-apoptotic genes altered. While some anti-apoptotic genes such as *Bcl2a1*, *Bcl2l11*, *Birc3* were down-regulated, others including *Mcl1*

and *Casp2* were up-regulated. Similarly, transcripts of pro-apoptotic genes either increased (*Casp9*, *Dffa*, *Gadd45a* and *Nol3*) or decreased (*Fas*, *Casp6* and *Tnfrsf10b*) at this stage. Such differential regulation may reflect a self-protective attempt by cells in retina to evade cell death and imply there is regulation of the traditional of apoptotic pathways during P23H-induced loss of photoreceptors.

Autophagy has been demonstrated to be implicated in the pathogenesis of a wide range of diseases including retinal degeneration (Kunchithapautham et al., 2007). The PCR array showed a significant up-regulation of several autophagy-associated genes (*Htt*, *Becn1*, *Atg16l*, *Atg3* and *Atg5*) in P23H-3 retinæ at both time points examined. *Htt* has a wide variety of functions including stabilizing endoplasmic reticulum (ER) (Mao et al., 2016) and regulating transcription (Zuccato et al., 2001; Zuccato et al., 2003) and vesicular transport of BDNF (Gauthier et al., 2004). The precise role of *Htt* in the retina is unclear (Ghavami et al., 2014) and various models have been proposed for how the mutant HTT affects cells (Vidal et al., 2011). The ones that appear most relevant in retinal degenerations are those involving *Htt* in ER stress, the unfolded protein response, and trafficking of BDNF vesicles. Knockdown of *Htt* results in an abnormal ER network and trafficking and it is plausible that HTT is involved in modulating ER stress (Vidal et al., 2011). Accordingly, up-regulation of *Htt* may reflect a response by the cell to ER stress due to the P23H mutation. In accordance with these findings, a previous study has indicated a dynamic expression profile of autophagy-related genes (including *Atg5*, similar to our results) in P23H-3 retinæ with the most increased levels of mRNA transcripts at P21 (Sizova et al., 2014b).

In addition to apoptosis and autophagy, necrosis has also been shown to contribute to pathogenesis of inherited retinal dystrophy (Murakami et al., 2012; Murakami et al., 2013b). Here, regulations in the expression pattern of necrosis-related genes were evident during the time course of intense death of P23H-3 photoreceptors. For instance, *Spata2* was over-

expressed at P14 and is involved in the TNF- α receptor-associated signalling complex and modulates TNF α signalling pathway. Down-regulation of this gene augments transcription of *NF κ B* and inhibits TNF α -mediated necroptosis (Schlicher et al., 2017). Thus, up-regulation of *Spata2* in the P23H-3 retinae suggests it is promoting TNF α -mediated cell death associated with necroptosis/apoptosis. Overall, the PCR array data suggest that multiple cell death mechanisms, including apoptosis, autophagy and necrosis are at play in the P23H-3 retina.

2.4.4. Other possible pathways of photoreceptor cell death in P23H retinae

Several studies have indicated that endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) is an important contributor to cell death during retinal degeneration (Gorbatyuk et al., 2010; Griciuc et al., 2011; Lin et al., 2007; Ryoo et al., 2007; Shinde et al., 2012; Wu et al., 2008; Yang et al., 2007). The P23H mutation causes misfolding of RHO and its accumulation within the ER (Kaushal et al., 1994a; Sung et al., 1993b; Sung et al., 1991b), leading to ER stress and initiation of the UPR (Comitato et al., 2016; Gorbatyuk et al., 2010; Griciuc et al., 2010; Shinde et al., 2016; Sizova et al., 2014a).

The ER is responsible for maintaining protein homeostasis, termed proteostasis, a process by which synthesis, folding, assembly, trafficking, and degradation of proteins is regulated. Failure in ER homeostasis results in accumulation of unfolded or misfolded proteins in the ER lumen, known as ER stress, which triggers UPR. ER-stress/UPR responses are mediated by three ER signal transducers, the inositol-requiring enzyme 1 (Ire1), the activating transcription factor-6 (Atf6) and the protein kinase R-like ER protein kinase (Perk). UPR aims to re-establish ER protein-folding homeostasis by transient diminution of protein translation, up-regulation of chaperones expression, activation of an ER-associated protein degradation

system and autophagy (Wang et al., 2016). If this process fails to alleviate ER stress, UPR may in turn induce a lethal stimulus, which subsequently leads to cell death.

It was proposed that sustained stimulation of UPR could induce retinal degeneration in P23H-3 rats by increasing the level of cytosolic Ca^{2+} in photoreceptors and calpain-mediated apoptosis (Shinde et al., 2016). By contrast, Comitato et al. (2016) suggested activation of calpains and ER-stress occurs independently in P23H *Rho* mutant mice (Comitato et al., 2016). In addition, a recent study revealed that the persistently activated UPR could provoke retinal degeneration in a mouse model of ADRP, generated by intraocular injection of tunicamycin in C57BL/6J mice, through the activation of pro-inflammatory cytokines such as IL-1 β (Rana et al., 2014). Other studies have supported the mechanism of Ca^{2+} -induced photoreceptor cell death and pathogenesis of ADRP (Barabas et al., 2010; Ozaki et al., 2012a; Sanges et al., 2006b). Pervious studies on cell death mediators in P23H animal models are summarised in table 2.5.

Table 2.5: Cell death pathways involved in P23H-related retinal degeneration

Activation of cell death mediators in P23H retinae	Yes	No
caspase-3	–	(Kaur et al., 2011a), (Arango-Gonzalez et al., 2014)
caspase-9	–	(Kaur et al., 2011a)
cytochrome C	(Sizova et al., 2014b)	(Kaur et al., 2011a)
AIF	(Sizova et al., 2014b), (Ozaki et al., 2013)	–
Autophagy	(Sizova et al., 2014b)	–
ER stress/UPR	(Gorbatyuk et al., 2010), (Sizova et al., 2014b), (Comitato et al., 2016), (Shinde et al., 2016)	–
calpain	(Ozaki et al., 2013), (Shinde et al., 2016), (Comitato et al., 2016)	–

2.5. Conclusion

In this chapter, the functional properties, structural phenotypic and potential cell death signalling pathways were explored in line 3 of the P23H transgenic rat. In this model, the P23H mutation in RHO causes an early onset of retinal degeneration, leading to significant functional deficits as early as 1 month and a subsequent loss of ONL neurons at 2 months of age. Consistent with other studies examining cell death in RHO mutations, the underlying molecular mechanisms of P23H-associated cell death are complex and dynamic, involving not only apoptosis, but also autophagy and necrosis. It is unlikely that targeting a single pathway will be a suitable strategy and combined targeting of all these pathways may be needed to delay or inhibit photoreceptor cell loss in retinal degenerative diseases. In an attempt to develop techniques that would allow administration of drugs in this model, intravitreal administration of a BAK antagonist was trialled. However, the lack of any effective amelioration with this single molecule highlights the difficulty in targeting a single cell death pathway.

Chapter 3.

The role of pro-apoptotic BAK and BAX in the murine *rd1* model of retinal degeneration

3.1. Introduction

Various animal models of retinitis pigmentosa (RP) are available to investigate the molecular basis of photoreceptor death and potentially establish efficient therapeutic strategies to inhibit neuronal loss. The *rd1* (*Pde6b*^{*rd1/rd1*}) mouse model bears a gene mutation in the β -subunit of phosphodiesterase 6 (*Pde6b*) and mimics pathological features of patients with autosomal recessive RP (Bowes et al., 1990; McLaughlin et al., 1993; Pittler et al., 1991). In homozygous *rd1* retinæ, a rapid degeneration of rod photoreceptors initiates from the first weeks of postnatal life, followed by a slower deterioration of cone photoreceptors at later stages (Carter-Dawson et al., 1978a).

Apoptosis is an essential physiological process for normal development of the vertebrate retina (Oppenheim, 1991) and as mentioned, is also involved in the pathogenesis of many retinal dystrophies, including RP (Chang et al., 1993a; Travis, 1998a). As mentioned in the first chapter, mitochondria are key regulators of apoptosis in that they release pro-death factors, such as cytochrome-c, from the intermembrane space to the cytosol in response to upstream lethal signals. In the cytoplasm, cytochrome-c induces activation of caspase cascade, which in turn triggers the downstream pathways to mediate dismantling and clearance of cellular components (Li et al., 1997; Tait et al., 2013; Wang et al., 2009). Furthermore, other pro-apoptotic factors released from mitochondria, such as apoptosis inducing factor (AIF) and endonuclease G, can also exert apoptotic effects in a caspase-independent manner (Wang et al., 2009).

The release of these pro-apoptotic molecules occurs following mitochondrial outer membrane permeabilization, which is tightly regulated by members of the BCL-2 family. This family of proteins are subdivided into three main categories based on their function and their structure (presence of BCL-2 Homology (BH) domains). Pro-survival members include BCL-2 and BCL-X, with four BH domains, whereas the pro-apoptotic members, including BAX and

BAK, have three BH domains and the pro-apoptotic BH3-only members include BIM, BID, and PUMA (Youle et al., 2008). Pro-apoptotic BAX and BAK are the main mediators of mitochondrial membrane disruption. Although both proteins shuttle between the mitochondrial outer membrane and the cytosol, BAX is mainly situated within the cytoplasm in healthy cells (Edlich et al., 2011; Schellenberg et al., 2013; Todt et al., 2013), whereas BAK localizes primarily at the mitochondrial outer membrane (Ferrer et al., 2012; Griffiths et al., 1999b). Upon their activation, BAX translocates to the mitochondrial outer membrane, where both BAX and BAK undergo conformational changes and oligomerization to mediate mitochondrial outer membrane permeabilization, ultimately leading to cell death (**Figure 3.1**) (Annis et al., 2005; Garcia-Saez, 2012; Westphal et al., 2014a).

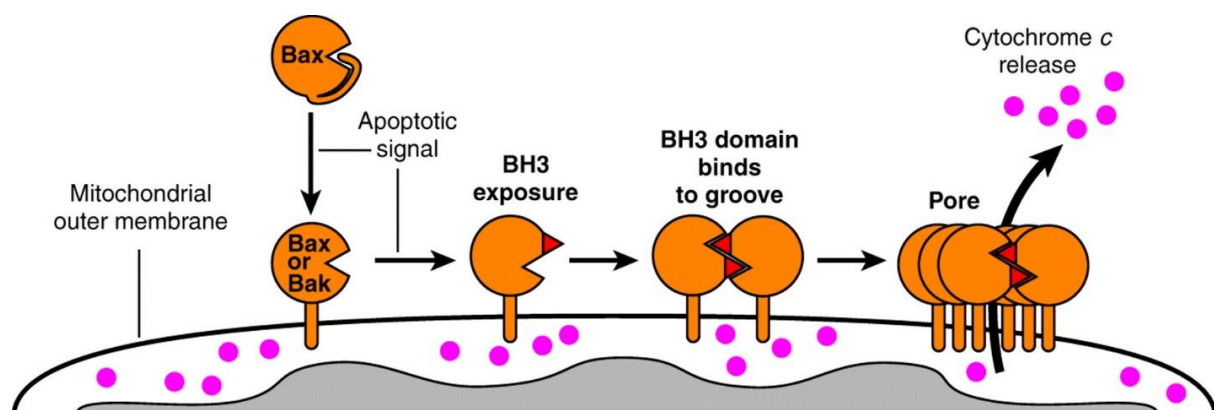


Figure 3.1: Activation and oligomerization of BAK and BAX during apoptosis

Upon receiving death signals, BAK and BAX are activated, BAX is recruited to the mitochondrial outer membrane, both BAK and BAX are oligomerized and result in the formation of pores and release of pro-apoptotic factors such as cytochrome c (Dewson et al., 2009).

Previous studies have suggested the contribution of both caspase-dependent and -independent pathways of apoptosis in photoreceptor death in the *rd1* mouse model. Using western blot analysis and immunocytochemistry, activation of caspase-3 was detected in *rd1* retinæ at the peak of retinal degeneration (Jomary et al., 2001a; Kim et al., 2002). Additionally, it was shown that prior to activation of caspase-3, cytochrome-c is released from the mitochondrial intermembrane space to the cytosol and the proapoptotic protein BID is activated. Active forms of caspase-8, which is capable of BID cleavage, were also detectable

in *rd1* retinæ during the peak time of photoreceptor death. Findings of this study suggest that both extrinsic and intrinsic pathways of apoptosis are associated with photoreceptor degeneration in this animal model and BID possibly mediates cross-talk between the two pathways (Jomary et al., 2001a). By contrast, Doonan et al. (2003) did not find activation of caspases (-9, -8, -7, -3, and -2) or translocation of cytochrome c to the cytosol in *rd1* retinæ during the peak period of retinal degeneration. They suggested that apoptotic death of photoreceptors in the *rd1*, rather than being caspase-independent, is governed by calpain-dependent mechanism(s) (Doonan et al., 2003). Later, it was demonstrated that the *rd1* mutation induces activation of both calpain and caspase-3 in the retina, implying that there is a cross-talk between these two apoptotic pathways occurring via mitochondria. A possible mechanism proposed is that activated calpain cleaves BID to form tBID which subsequently binds to BAK, leading to its oligomerization, induction of mitochondrial membrane disruption, release of pro-apoptotic factors to the cytosol, activation of effector caspases and ultimately cell death (Sharma et al., 2004). The importance of calpain in mediating photoreceptor death in *rd1* was demonstrated by another study showing that activity of calpain peaks during the rising phase of retinal degeneration (Arango-Gonzalez et al., 2014). The endogenous calpain inhibitor, calpastatin, is also down regulated coincidently with calpain activation (Paquet-Durand et al., 2006). In addition to caspases and calpain, AIF appeared to be involved in apoptotic death of photoreceptors bearing *rd1* mutation, with Sanges et al. (2006b) showing AIF and caspase-12 translocation to photoreceptor nuclei following influx of Ca^{2+} and activation of calpain (Sanges et al., 2006b). Taken together, the findings of these studies suggest that multiple mechanisms of apoptotic cell death are involved in *rd1*-related photoreceptor demise. Accordingly, pro-apoptotic BAK and BAX, which are responsible for promoting dysfunction of mitochondria during apoptosis, may have fundamental function in

mediating this process and interfering with their activity may preserve photoreceptors in *rd1* animal model.

Using immunofluorescent and western blot analysis, a recent study has reported that BAX is activated and translocated to the mitochondrial membrane of photoreceptors at early stages (P11) of retinal degeneration in *rd1* (Comitato et al., 2014). Conversely, histological assessment indicated that *Bax* deficiency (*rd1/Bax*^{-/-}) did not rescue photoreceptor loss in *rd1* retinae by P21, suggesting that *Bax* may not be involved in *rd1*-associated retinal degeneration, or if involved, its function is redundant with other mediators such as BAK (Mosinger Ogilvie et al., 1998). The idea of redundancy in the modulation of the intrinsic pathway was supported by studies investigating both developmental and pathological apoptosis in retina. Morphometric examination of *Bak*^{-/-}/*Bax*^{-/-} retinae at 2 months of age revealed that double ablation of both genes is essential to completely inhibit the developmental-related apoptotic stimuli in the rodent retina. Retinae of these mice showed increased thickness in the inner retinal layers (INL, IPL and GCL) and total retina compared to the *WT* (Hahn et al., 2003). Interestingly, there was no alteration in the outer retina, including the photoreceptor layer. Similarly, deficiency of both *Bak* and *Bax* led to prevention of photoreceptor death in a light-induced damage model of retinal degeneration. One day after light damage, less TUNEL⁺ photoreceptor nuclei were detected in *Bak*^{-/-}/*Bax*^{-/-} compared to *Bax*^{-/-} retinae. Protective effects of *Bak/Bax* elimination against loss of photoreceptors appeared to persist even one week following light injury (Hahn et al., 2004). Given the evidence of redundancy for *Bax* and *Bak* function in cell death during retinal development and in the light-induced model, it was hypothesised that BAX and BAK may play similar redundant roles in the *rd1* model.

The aims of the experiments in this chapter were to detail *Bak* and *Bax* expression during retinal degeneration in the *rd1* mouse and to assess the effect of ablating *Bak* and *Bax*

through genetic and pharmacological means on the progression of photoreceptor cell death in the *rd1* mouse model of retinal degeneration.

3.2. Materials and Methods

3.2.1. Animals

All experimental procedures followed the National Health and Medical Research Council of Australia guidelines and were in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Work was undertaken after approval from the University of Melbourne Animal Ethics committee project number 1614030. All mice used in this study were on a C57Bl7/J background. *Bax*^{-/-}, *Bak*^{-/-} and double *Bax*^{-/-}/*Bak*^{-/-} mutant mice were obtained from Dr. Ben Kile and Dr. Stefan Chappaz at the Walter & Eliza Hall Institute. The *Bax*^{-/-} mice were generated by homologous recombination using a PGK-Neo cassette to replace exons 2-4 of the *Bax* gene in 129Sv ES cells (Knudson et al., 1995). Similarly, *Bak*^{-/-} mice were generated by homologous recombination replacing exons 3-6 with a PGK-neo cassette in 129SV cells (Lindsten et al., 2000). Mixed background *Bax*^{-/-} and *Bak*^{-/-} mice were mated onto a C57Bl6/J background for at least 10 generations to generate homozygous null mutants and then crossed with homozygous *rd1* mice to generate mice that were homozygous for the *rd1* allele (*Pde6b*^{rd1/rd1}). To generate *rd1/Bax*^{-/-} *Bak*^{-/-} animals, *rd1/Bak*^{+/-} mice were crossed with *rd1/Bax*^{+/-} mice. Offspring that were *rd1/Bax*^{+/-} *Bak*^{+/-} were identified by genomic PCR and interbred. The primers used for genotyping are shown in **Table 3.1**. While the double null mutation (*Bak*^{-/-}/*Bax*^{-/-}) is generally perinatal lethal (Lindsten et al., 2000), a limited number of viable pups were obtained for this study. In all experiments, C57Bl6/J mice were used as wild-type (WT) controls and homozygous *rd1* mice (*Pde6b*^{rd1/rd1}) on C57Bl6/J background were used as positive controls for the retinal degeneration phenotype.

Table 3.1: Primer and probe sequences for ddPCR

Primers are shown relative to the mouse sequences.

Gene primer	Primer/Probe sequences in 5'-3' orientation with fluorophore	Reverse primer	size of product
<i>Bak</i> <i>Bak</i> 368 <i>Bak</i> 366 <i>Bak</i> 316	GAGCCATGAGATGTTTAGC GGTGCTCTGTGTCTGGAAATA GACCGCTTCCTCGTGCTTTA	TATTTCCAGAC ACAGAGCACC	WT allele product 911 bp mutant allele product 390 bp
<i>Bax</i> <i>BK</i> 712 <i>BK</i> 713 <i>BK</i> 714	AGCTGATCAGAACCATCATG GTTGACCAGAGTGGCGTAGG CCGCTTCCATTGCTCAGCGG	CATGATGGTTC TGATCAGCT	WT allele product 310 bp mutant allele product 507 bp

3.2.2. Histology

The gross retinal morphology of *WT*, *Bak*^{-/-}, *Bak*^{-/-}/*Bax*^{+/-}, *rd1*, *rd1/Bak*^{-/-}, *rd1/Bak*^{-/-}/*Bax*^{+/-} ($n \geq 6$ for each group) and *rd1/Bak*^{-/-}/*Bax*^{-/-} ($n = 3$) mice was investigated using hematoxylin- and eosin-stained (H&E) paraffin sections. Mice at post-natal day (P) 14 and P18 (P13 for *rd1/Bak*^{-/-}/*Bax*^{-/-} mice) were anesthetized by an intraperitoneal injection of a 5:1 mixture of ketamine (60 mg/kg; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) and xylazine (5 mg/kg; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) further diluted 1:10 in sterile saline (0.9%, Aero, Healthcare, NSW, Australia). They were then euthanized by cervical dislocation and eyes were isolated. The whole eye cups were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), containing 3% sucrose, 5% acetic acid, and 60% ethanol. They were then dehydrated by immersion in graded alcohols (70%, 95% and 100%), cleared in xylene and embedded in paraffin wax.

Serial sections of 5 μ m thickness were cut, collected on poly-L-lysine coated glass slides, and incubated overnight at 37°C. Sections were deparaffinized in xylene, rehydrated by washing in graded ethanol (100%, 90% and 70%) and finally dH₂O. Retinal tissues were stained with Mayer's hematoxylin and eosin (H&E), washed with dH₂O and then cover-slipped for imaging.

3.2.3. *Confocal microscopy and retinal layer analysis*

H&E stained sections were imaged by a laser scanning confocal microscope (Zeiss LSM510, Meta, Carl Zeiss, Oberkochen, Germany) using a x 20 air objective, at a resolution of 512 x 512 pixels, and full-tile scan mode. Three sections containing optic nerve were selected from each eye for analysis. By using a custom script of ImageJ (ImageJ 1.51, National Institutes of Health, USA), the thickness (μm) of the ganglion cell layer (GCL), inner plexiform layer (IPL) inner nuclear layer (INL), outer nuclear layer (ONL, including outer plexiform layer), inner and outer photoreceptor segments (IS\OS) and total retina was measured from each section at central and peripheral regions. The measurements from three different sections from the same eye were averaged to obtain a single value, while quantification was based upon $n \geq 6$ for each group (except $rd1/Bak^{-/-}/Bax^{-/-}$ where $n = 3$).

3.2.4. *Total RNA extraction and quality*

Retinae were collected from *WT*, *rd1*, *rd1/Bak^{-/-}* and *rd1/Bak^{-/-}/Bax^{+/-}* mice at P14 and P18 ($n \geq 5$). Tissues were immediately placed in 200 μl of RNeasyTM stabilization solution (Qiagen, VIC, Australia) and stored at -80°C until use. Total RNA was extracted using the RNeasy[®] Mini Kit (Qiagen, VIC, Australia) as per manufacturer's instructions. To remove genomic DNA contamination, samples were treated 'on column' with DNase-I (Qiagen, VIC, Australia) at room temperature for 15 min. To quantify RNA concentration and assess purity, the Agilent 2200 Tape Station (Agilent Technologies 5067-5583 D1000, Germany) was used according to manufacturer's instructions (See section 2.2.9 for details). A total of 500 ng total RNA was reverse transcribed using the SensiFASTTM cDNA Synthesis Kit (Bioline, Cat. No. BIO-65053, Australia), according to manufacturer's instructions. For each sample, 500 ng RNA was added to a mixture of 4 μl 5X TransAmp Buffer and 1 μl reverse transcriptase. The

final volume of reaction mix was brought to 20 µl by adding sterile nuclease-free water. The cDNA was amplified at the following program in a thermal cycler: 25°C for 10 min, 42°C for 15 min, and 85°C for 5 min. Samples were either used immediately or stored at -80°C prior to use.

3.2.5. Droplet digital PCR

To quantify the level of *Bax* and *Bak* mRNA in retinal samples, droplet digital PCR (ddPCR) was performed (Qx100™ Droplet Digital™ PCR system, Bio-Rad, VIC, Australia) as per the manufacturer's instructions. For each sample, 25 µl PCR reaction mixture was used containing 12.5 µl of 2x ddPCR Supermix (Bio-Rad, VIC, Australia), 1.25 µM of primers and probes (**Table 3. 2**), 1 µl of sample cDNA and 10.25 µl sterile nuclease-free water. To convert samples into droplets, each 20 µl sample was loaded into individual wells of the DG8™ cartridge (Bio-Rad, VIC, Australia), followed by 40 µl of droplet generation oil to each of the corresponding oil wells. The cartridge was then loaded into QX100™ Droplet Generator (Bio-Rad, VIC, Australia) and an emulsion of ~20,000 mono-dispersed droplets were generated. Subsequently, the droplets were transferred to a 96-well PCR plate and sealed with an aluminum sheet using a PX1™ PCR Plate Sealer (Bio-Rad, VIC, Australia). The plate was placed in a C1000 Touch™ thermal cycler (Bio-Rad, VIC, Australia) and reactions were run under the following cycling conditions: 95°C for 10 min, 40 cycles of 94°C for 30 sec and 60°C for 1 min, followed by a final 10 min enzyme deactivation step at 98°C (overall ramp rate = 2°C/s).

After PCR amplification, the plate was placed in QX100 Droplet reader (Bio-Rad, VIC, Australia), which automatically reads the droplets in each well of the plate. Data were analyzed using QuantaSoft™ software (v.1.7.4.0917, Bio-Rad). The number of positive droplets, which contain target or reference amplicon, and negative droplets, which lack target and/or reference

amplicon, were quantified for each channel, and used to calculate the concentration of the target and house-keeping genes. Counts for target genes were normalized to counts for the reference gene (hypoxanthine guanine phosphoribosyltransferase, *Hprt*) and the final concentration was expressed as copies/μl reaction.

Table 3. 2: Primer and probe sequences for ddPCR

Primers are shown relative to the mouse sequences.

Gene primer/Probe	Primer/Probe sequences in 5'-3' orientation with fluorophore	Supplier/Catalogue #
<i>Bak1</i> Fw Rev Probe	AATGGCATCTGGACAAGGAC CTTCGAAAGACCTCCTCTGTG FAM/CCCGTCCCC/ZEN/TTCTGAACAGCA/3 IABkFQ	IDT Mm.PT.58.12619952
<i>Bax</i> Fw Rev Probe	GGAGATGAACTGGACAGCAAT GCCATCAGCAAACATGTCAG FAM/CGTCAGCAA/ZEN/TCATCCTCTGCAG CT/3IABkFQ	IDT Mm.PT.58.14012210
<i>Hprt</i> Fw Rev Probe	CCCCAAAATGGTTAAGGTTGC AACAAAGTCTGGCCTGTATCC FAM/CTTGCTGGT/ZEN/GAAAAGGACCTCT CGAA/3IABkFQ	IDT Mm.PT.39a.22214828

Fw: Forward; Rev: Reverse

3.2.6. Intravitreal injection of a BAK inhibitor in *rd1/Bax^{-/-}* mice

At P16, *rd1/Bax^{-/-}* mice were anesthetized by an intraperitoneal injection of a 5:1 mixture of ketamine (60 mg/kg; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) and xylazine (5 mg/kg; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) further diluted 1:10 in sterile saline (0.9%, Aero, Healthcare, NSW, Australia). The amount of anesthetic used for each animal was calculated according to the individual body weight. The corneal reflex was anesthetized with topical administration of Alcaine (0.5%; Alcon Laboratories, Victoria, Australia) and the dilation of pupil was achieved via administration of Atropine (1%, Aspen Pharmacare, NSW, Australia) and phenylephrine hydrochloride (10%, Bausch & Lomb, NSW, Australia).

The BAK inhibitor (WEHI-1250993) was supplied by Drs. Ben Kile and Guillaume Lessene from Monash University and WEHI respectively. Prior to injection, a stock of the compound was prepared by dissolving the compound in 5% DMSO (99.9 % for molecular biology, Sigma, Australia) in sterile saline (0.9%, B. Braun Australia Pty Ltd, NSW, Australia) to achieve the intended concentration (**Table 3.3**). This concentration was based on preliminary results from Drs. Kile and Lessene indicating that this drug has activity at 100 nM *in vitro* and at 1-5 μ M in plasma. The vitreal volume of *rd1/Bax^{-/-}* mice at the age of injection was estimated at ~2 μ l in order to calculate the actual effective concentration at the retina. The vehicle-diluted compound (0.5 μ l) was injected intravitreally to the left eye using a 30-G needle attached to a 5 μ l Hamilton microsyringe (Reno, NV, USA) via a polyethylene tube. The right eye received 0.5 μ l of 5% DMSO in sterile saline as the vehicle. The animals were subsequently kept on the heat pad at 37°C to maintain body temperature and monitored during recovery. Injected animals and their eyes were monitored daily for 2 days post injection.

Table 3.3: Experimental design for intravitreal injection of BAK antagonist into *rd1/Bax^{-/-}* mice

Bak inhibitor	Molecular weight	Solvent	Estimated concentration in the retina	Number of injected animals
WEHI-1250993	541.64	5% DMSO in saline	1 μ M	8

At P18, animals were sacrificed by cervical dislocation under deep anesthesia, as described above. The eyes were isolated, and their anterior portions and lens were removed by dissection. The posterior eyecups were fixed in fresh 4% paraformaldehyde in PB for 30 min as described previously (**Sections 2.2.5.1 and 3.2.2**). To quantify dead cells in drug-injected and DMSO-injected eyes, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (DeadEndTM Fluorometric TUNEL System; Promega Corp., NSW, Australia) was performed as per manufacturer's instructions with minor modifications (**Section 2.2.5.2**).

Following TUNEL labeling, full-tile scans of the retinae from optic nerve to ciliary body were captured immediately by a Zeiss LSM800 (Carl Zeiss AG, Jena, Germany) using a x 20 air objective at resolution of 512 x 512 pixels. To quantify the number of TUNEL-positive cells, three sections (preferably with optic nerve head within the section) from each eye per animal were analyzed. The total length (mm) of each section was measured and the number of TUNEL-labeled nuclei across the ONL was quantified manually using a cell counter application (ImageJ 1.51, National Institutes of Health, USA). The values from three different sections from the same eye were averaged to obtain a single value and $n = 8$ eyes were used for drug and vehicle treatment. The total number of TUNEL positive cells was expressed per millimeter of retinal length.

3.2.7. Statistical analysis

Statistical analysis and graphing were performed using GraphPad Prism 7 (San Diego, California, USA). All data are presented as the mean $\pm$ standard error of the mean (SEM). Where appropriate, data were compared using student *t*-test, one-way ANOVA or 2-way ANOVA incorporating a Tukey's post hoc analysis for multiple comparisons. Details of individual tests used are also provided in the relevant results. Differences among the mean were considered statistically significant for $p < 0.05$ and represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ in all graphs.

3.3. Results

3.3.1. Normal development of retina in the absence of *Bak*

The main purpose of this study was to identify the functions of BAK and BAX in *rd1*-induced death of retinal photoreceptors. In order to explore the roles of these apoptotic mediators, mutant animals lacking *Bak* and *Bax* were used. As the phenotype of *rd1/Bax*^{-/-} retinae was described previously (Mosinger Ogilvie et al., 1998), the phenotype of *rd1/Bak*^{-/-}, *rd1/Bak*^{-/-}/*Bax*^{+/-} and *rd1/Bak*^{-/-}/*Bax*^{-/-} mutant mice was subsequently characterized in this study.

While this study explored the role of *Bak* and *Bak/Bax* in the *rd1* model of retinal degeneration, it was first important to determine whether loss of these apoptotic mediators altered normal retinal development. To assess whether genetic ablation of these genes altered cell death mediated refinement of normal retinal development, the gross structure of central and peripheral retinae was assessed in *Bak*^{-/-} and *Bak*^{-/-}/*Bax*^{+/-} on a wild-type (C57Bl6J) background at P14 and compared to WT (C57Bl6J) mice. In the developing rodent retina, post-natal developmental apoptosis occurs mostly during the first 2 weeks after birth (Young, 1984) and therefore P14 was chosen as that is when much of the maturation of retina has completed and the cell death-mediated refinement of the retina has subsided. The gross histology of the central retinae of the *Bak*^{-/-} (**Figure 3.2B**) and *Bak*^{-/-}/*Bax*^{+/-} (**Figure 3.2C**) mutant strains showed similar laminar retinal structure to the WT (**Figure 3.2A**), comprising the distinct nuclear layers (ONL, INL, and GCL), separated by the two plexiform layers (OPL, IPL) and there were no overt structural changes, nor obvious alterations in overall retinal thickness. When the thickness of individual retinal layers was quantified, there was no significant alteration in any of the nuclear layers (**Figure 3.2D, F, H**), or in the IPL (**Figure 3.2G**) in any of the genotypes. As IS/OS is a measure of photoreceptor health, this layer was also quantified

and showed no difference among the various genotypes (**Figure 3.2E**). Finally, total retinal thickness was also not altered compared to WT animals (**Figure 3.2I**).

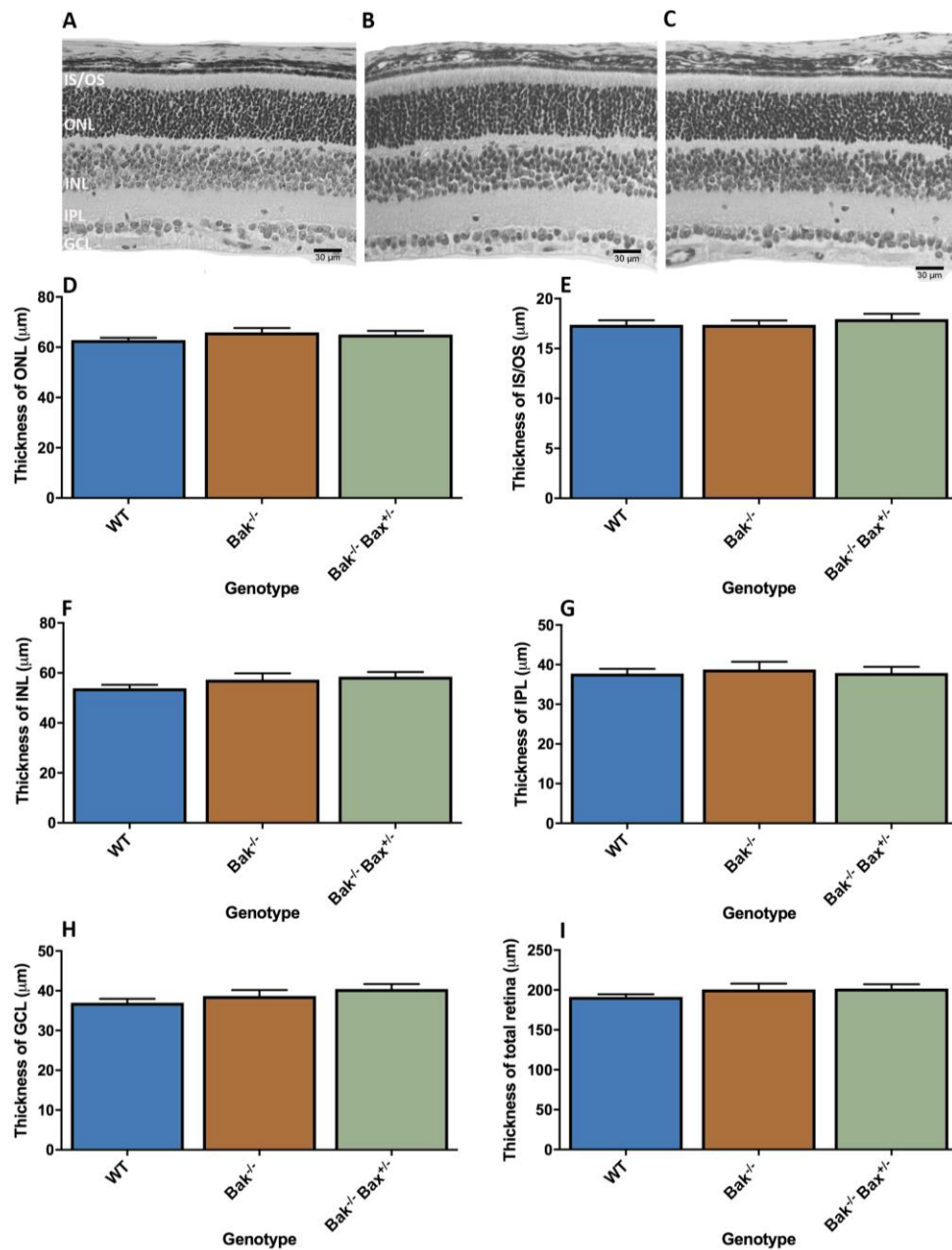


Figure 3.2: Morphology of central retina in the *Bak/Bax* mutants

Representative images showing H&E staining of WT (A), *Bak*^{-/-} (B) and *Bak*^{-/-}*Bax*^{+/-} (C) central retinal sections at P14. The thickness of ONL (D), IS/OS (E), INL (F), IPL (G), GCL (H) and total retina (I) remained unchanged in *Bak/Bax* mutant mice. The histograms indicate the mean ± SEM. One-way ANOVA, $n \geq 6$. Abbreviations: IS/OS: inner and outer segments, ONL: outer nuclear layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars = 30 μm.

Similarly, in the peripheral retina there was no evident alteration in retinal histology with all retinal layer observed (**Figure 3.3A-C**). The *Bak*^{-/-} retinae showed no change in the thickness of individual retinal layers, or the total retina compared to *WT* animals (**Figure 3.3D-I**). In the *Bak*^{-/-}/*Bax*^{+/-} mice, while the thickness of the ONL, IS/OS, IPL, GCL, layers (**Figure 3.3D, E, G and H**) and the total retinal thickness (**Figure 3.3I**) remained similar to those of *WT*, a significant increase was detected in the INL thickness (17%, $P < 0.01$; **Figure 3.3F**). These results suggest that the development of retina proceeds normally in the absence of *Bak* and in *Bak*^{-/-} mice lacking one allele of *Bax* (*Bak*^{-/-}/*Bax*^{+/-}).

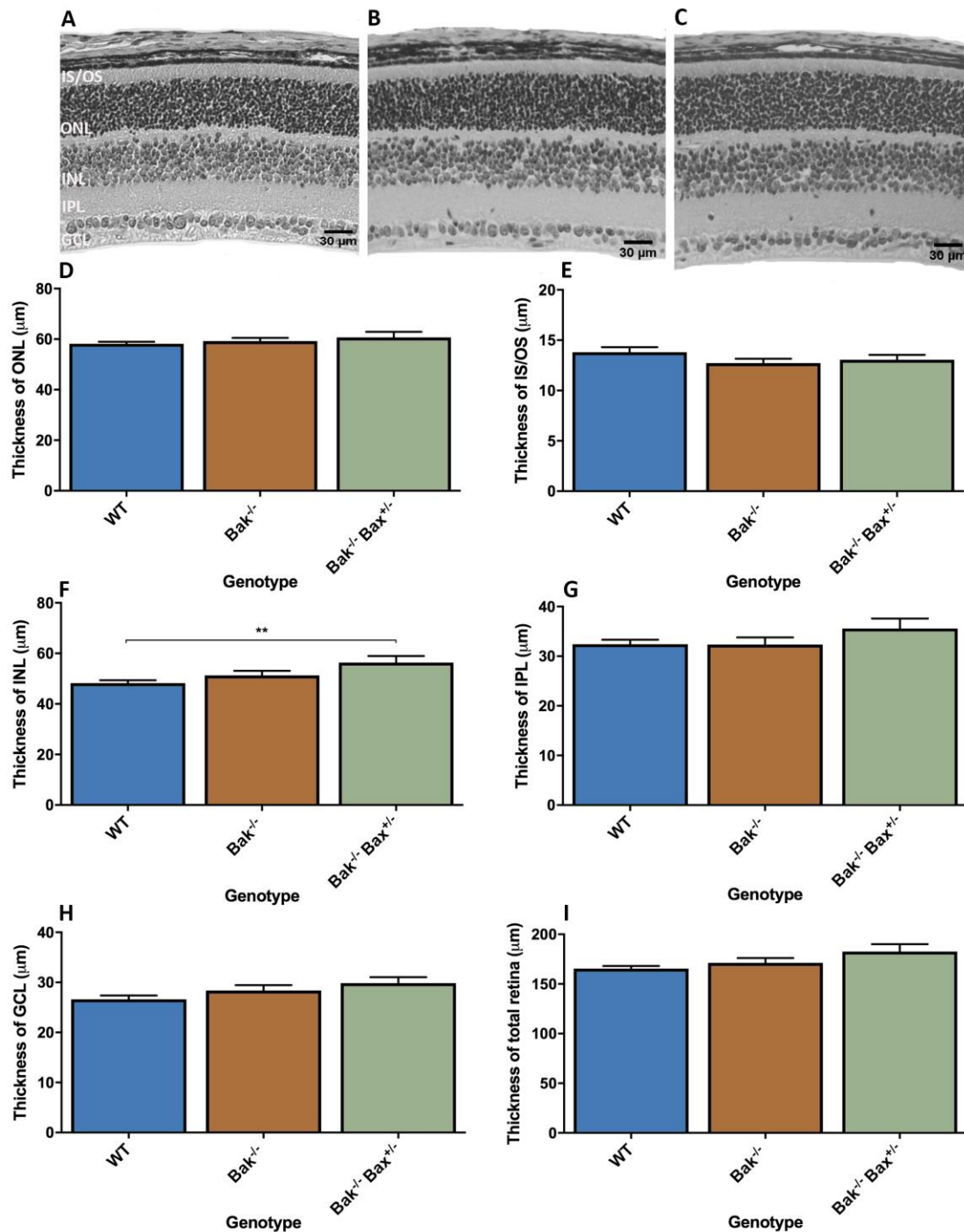


Figure 3.3: Morphology of peripheral retina in *Bak/Bax* mutants

Representative images showing H&E staining of WT (A), *Bak*^{-/-} (B) and *Bak*^{-/-}/*Bax*^{+/-} (C) retinal sections at P14. No significant differences were observed in the thickness of ONL (D), IS/OS (E), IPL (G), GCL (H) and total retina (I) in *Bak/Bax* mutant mice. While the thickness of the INL of *Bak*^{-/-} was similar to that in WT, it showed a significant increase in the *Bak*^{-/-}/*Bax*^{+/-} (F). The histograms indicate the mean ± SEM. One-way ANOVA, Tukey's post hoc test, ** *p* < 0.01, *n* ≥ 6. Abbreviations: ONL: outer nuclear layer, IS/OS: inner and outer segments, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars = 30 μm.

3.3.2. Up-regulation of *Bak*, not *Bax* expression during photoreceptor loss in the *rd1* model

Retinae of *rd1* mice undergo normal development until approximately P8, when photoreceptor degeneration initiates in a central to peripheral gradient. It proceeds rapidly, such that by P20 only a single layer of nuclei remains in the ONL. Based on this time course, we selected P14 and P18 as two key time points to carry out our experiments in *rd1* mice.

Previous work has investigated the expression of *Bak* and *Bax* during retinal degeneration in the *rd1* at P11 and showed increased *Bax* expression, while *Bak* expression was unaltered (Comitato et al., 2014). In order to determine whether this expression profile is maintained at/or after eye opening, we analyzed the pro-apoptotic *Bak* and *Bax* expression in *rd1* and *WT* retinae at P14 and P18, using RT-ddPCR. The ddPCR data showed that there were significant increases in *Bak* transcripts at both P14 (22%, $P < 0.05$) and P18 (64%, $P < 0.001$; **Figure 3.4A**). By contrast, *Bax* expression was not altered at either time point when compared to the *WT* control (**Figure 3.4B**). Also, there was a decrease in *WT* retina for both *Bak* and *Bax* from P14 to P18. The data suggest that there is differential *Bax/Bak* expression post-natally in response to *rd1*-related apoptotic stimuli.

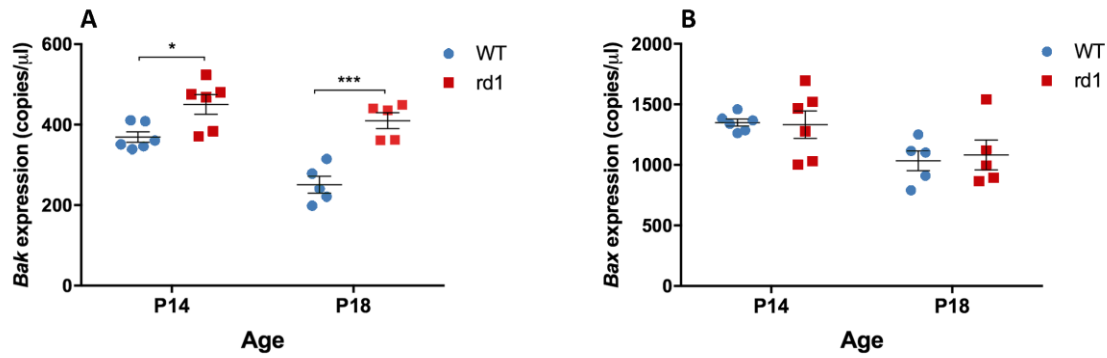


Figure 3.4: Level of *Bak* and *Bax* expression in *rd1* retinæ during retinal degeneration
mRNA expression of *Bak* (A) and *Bax* (B) was quantified in *rd1* compared to WT mice at P14 and P18 using ddPCR. Data are expressed as copies/µl and normalised to *Hprt* as the internal reference gene. For each group, individual data points are shown, and the histograms indicate the mean ± SEM. Two-way ANOVA, Tukey's post hoc test, * $p < 0.05$ and *** $p < 0.001$, $n \geq 5$.

3.3.3. Ablation of *Bak* partially rescues retinal degeneration in *rd1* at P14

To further explore the relative contributions of BAK and BAX in retinal degeneration of *rd1*, we investigated the progression of photoreceptor loss in *rd1* mice lacking *Bak* and in a separate cohort, which lacked *Bak* expression and only contained one functional *Bax* allele (*rd1/Bak*^{-/-}*Bax*^{+/-}). As previously mentioned a double knockout genotype is commonly perinatal lethal.

At P14, loss of photoreceptor nuclei in the ONL and thinning of the total retina were apparent in the central region of all retinæ bearing the *rd1* mutation (Figure 3.5B-D) compared to WT animals (Figure 3.7A). However, in *rd1/Bak*^{-/-} (Figure 3.5C) and *rd1/Bak*^{-/-}*Bax*^{+/-} (Figure 3.5D) retinæ, the ONL appeared to be thicker than that of the *rd1* control animal (Figure 3.5B). No other alterations were evident in the gross morphology of other retinal layers. When retinal layer thickness was quantified for the central retinæ, a statistically significant increase in the thickness of ONL was observed in the *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}*Bax*^{+/-} mice when compared to the *rd1* alone (32%, $p < 0.0001$ for both genotypes). Despite this increase, the ONL remained significantly thinner than WT control mice (55%, $p < 0.0001$; Figure 3.5E).

Regarding the other retinal layers, IS/OS lengths were similar in all mice carrying the *rd1* mutation (*rd1*, *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-}); however, they were all significantly shorter than their *WT* controls (69%, 63% and 64% respectively; $p < 0.0001$; **Figure 3.5F**). The thicknesses of the INL and GCL were found to be similar in *rd1*, *rd1/Bak*^{-/-}, *rd1/Bak*^{-/-}/*Bax*^{+/-} retinae compared to the *WT* mice (**Figure 3.5G and I**). While the thickness of the IPL was similar between *rd1* and *WT*, it showed a slight decrease in *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} compared to *rd1* retinae (12% and 15%; $p < 0.05$ and $p < 0.01$ respectively; **Figure 3.5H**). The total retinal thickness showed no significant differences among *rd1/Bak/Bax* mutant and *rd1* mice and all had significantly thinner retinae compared to the *WT* mice (~ 24% and $p < 0.0001$; **Figure 3.5J**).

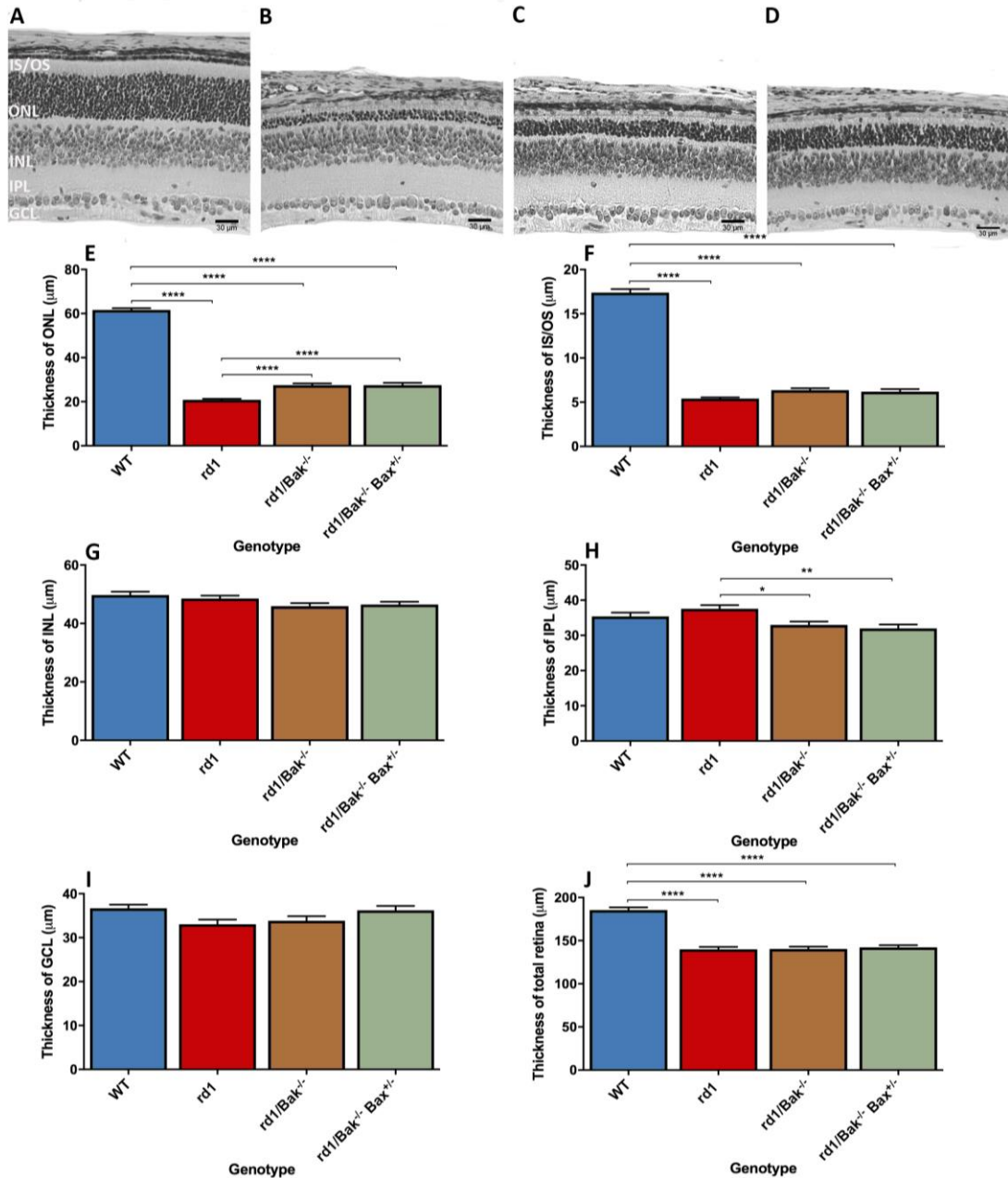


Figure 3.5: Morphology of central retina in the *rd1/Bak/Bax* mutant mice at P14

Representative images showing H&E staining of WT (A), *rd1* (B), *rd1/Bak*^{-/-} (C) and *rd1/Bak*^{-/-}/*Bax*^{+/-} (D) central retinal sections at P14. The ONL of both *rd1/Bak/Bax* mutants was significantly thickened compared to the *rd1*, but still thinner than the WT (E). The length of IS/OS were not different among *rd1* and *rd1/Bak/Bax* mutant strains and they were significantly less than of those in the WT (F). The thickness of the INL (G), GCL (I) and total retina (J) of the mice lacking Bak/Bax remained unchanged compared to the *rd1* and WT mice. The IPL slightly decreased in the *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} mice compared to the *rd1* yet were similar to the WT retinæ (H). The histograms indicate the mean ± SEM. One-way ANOVA, Tukey's post hoc test, * *p* < 0.05, ** *p* < 0.01, and **** *p* < 0.0001, *n* ≥ 6. Abbreviations: ONL: outer nuclear layer, IS/OS: inner and outer segments, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars = 30 μm.

Similar to the data in the central retina, the peripheral retina in the *rd1* mice showed a clear thinning of the ONL (**Figure 3.6B**) compared with the *WT* (**Figure 3.6A**). However, gross morphology indicated a slight thickening of the photoreceptor layer (ONL) in the *rd1* mice deficient for *Bak* with or without a mutant *Bax* allele (**Figure 3.6C and D**). These observations were verified by quantitative morphometric analysis of these retinæ, showing that the ONL was significantly thickened in the *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} mice compared to the *rd1* (29%, $p < 0.0001$), yet were still significantly thinner than the *WT* (32%, $p < 0.0001$; **Figure 3.6E**).

No significant differences were found in the IS/OS length among all mice bearing *rd1* mutation and they showed significant shortening in these mice compared to the *WT* (40%, $p < 0.0001$; **Figure 3.6F**). The thickness of INL and GCL showed no significant alterations in any of the examined mutant strains (**Figure 3.6G and I**). Although the thickness of the IPL did not change in *rd1*, it was reduced in *rd1/Bak*^{-/-} compared to the *rd1* and the *WT* (19% relative to *rd1*; $P < 0.001$ and 23% relative to *WT*; $p < 0.0001$) and in *rd1/Bak*^{-/-}/*Bax*^{+/-} retinæ compared to the *WT* (17%; $p < 0.01$; **Figure 3.6H**). The total retinal thickness remained similar among all mice carrying the *rd1* mutation and was less than that of *WT* (~ 24%, $p < 0.0001$; **Figure 3.6J**). In summary, these findings suggest that genetic elimination of *Bak*, with or without heterozygosity of *Bax*, resulted in a partial rescue of photoreceptors from *rd1*-induced degeneration in both central and peripheral retinæ at P14. This effect seemed to be limited to the outer retina, although there did appear to be a thinning of the inner plexiform layer, possibly suggesting some inner retinal effects.

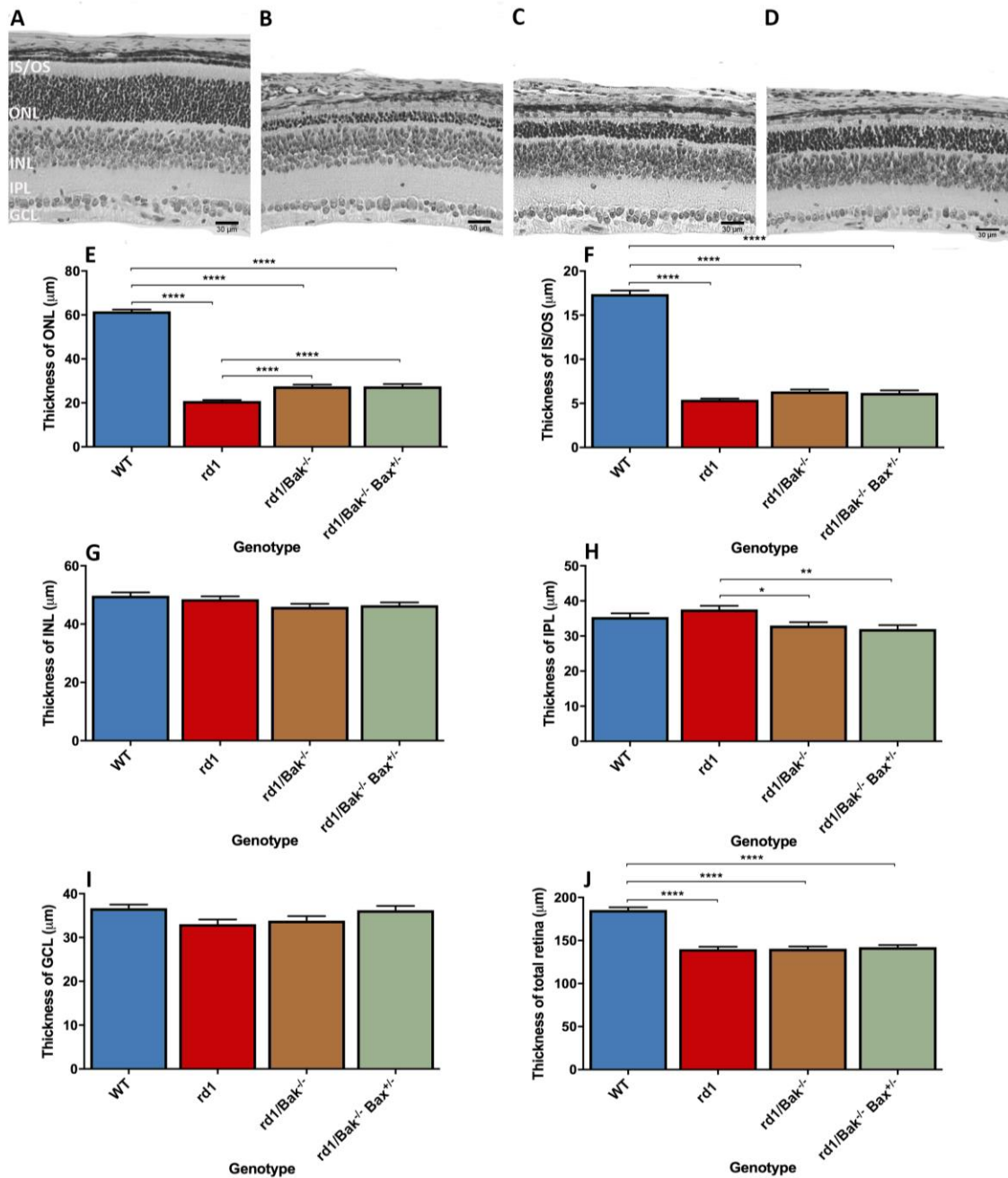


Figure 3.6: Morphology of peripheral retina in the *rd1/Bak/Bax* mutant mice at P14

Representative images showing H&E staining of WT (A), *rd1* (B), *rd1/Bak*^{-/-} (C) and *rd1/Bak*^{-/-}/*Bax*^{+/-} (D) peripheral retinal sections at P14. While there was a significant increase in the thickness of ONL in *rd1/Bak/Bax* mutant mice compared to the *rd1*, it was still less than of the WT (E). IS/OS had similar length in *rd1* and *rd1/Bak/Bax* mutant strains and they were significantly shorter than the WT (F). The thickness of the INL (G), GCL (I), and total retina (J) of the mice lacking *Bak/Bax* remained unaffected compared to the *rd1* and WT mice. The IPL thickness decreased in the *rd1/Bak*^{-/-} strain, compared to the *rd1* and WT, and in the *rd1/Bak*^{-/-}/*Bax*^{+/-} strain compared to the WT (F). The histograms indicate the mean ± SEM. One-way ANOVA, Tukey's post hoc test, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001, *n* ≥ 6. Abbreviations: ONL: outer nuclear layer, IS/OS: inner and outer segments, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars = 30 μm.

To determine whether this rescue was maintained at later stages of degeneration, histological analysis of retinæ from the different genotypes was performed at P18. Similar to findings at P14, the ONL in the central retina of *rd1/Bak/Bax* retinæ at P18 (**Figure 3.7B-D**) contained only one to two rows of nuclei compared to the *WT* retina (**Figure 3.7A**). The thickness of the ONL in *rd1/Bak*^{-/-} (**Figure 3.7C**) and *rd1/Bak*^{-/-}/*Bax*^{+/-} (**Figure 3.7D**) appeared similar to the *rd1* retinæ (**Figure 3.7B**) and total thicknesses of all these retinæ were similarly and dramatically reduced compared to *WT*. Moreover, the IS/OS were not detectable in any retinæ carrying the *rd1* mutation (**Figure 3.7B-D**). Consistent with this, quantitative measurements of individual retinal layers revealed no significant differences in the ONL thickness among *rd1* and the *rd1/Bak/Bax* mutant strains, all had significantly thinner ONL compared to the *WT* (~ 83%, $p < 0.0001$; **Figure 3.7E**). Similarly, there were significant reductions in the thicknesses of the INL (13% and $p < 0.05$ for *rd1*; 15% and $p < 0.01$ for *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-}) and GCL in *rd1* (12%, $p < 0.01$) and *rd1/Bak/Bax* mutant strains (12%, $p < 0.05$) in comparison with *WT* (**Figure 3.7F and H**); however, the thickness of the IPL remained unaffected in all genotypes (**Figure 3.7G**). The thinning in the outer and inner retinæ of *rd1*, *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} was reflected in the total retinal thicknesses, which were significantly less than that of the *WT* (~ 34%, $p < 0.0001$). Importantly, there was no difference in the *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} compared to the *rd1* retinæ (**Figure 3.7I**).

In the peripheral retinæ, morphological alterations in the ONL and total retinal thickness were similar to those found in the central retinæ. Loss of *Bak*, with or without heterozygosity of *Bax*, did not appear to protect photoreceptor nuclei in the ONL (**Figure 3.8C and D**) and all retinæ bearing *rd1* mutation (**Figure 3.8B-D**) had undergone prominent thinning compared to the *WT* (**Figure 3.8A**). Also, the IS/OS were absent in the *rd1* and *rd1/Bak/Bax* mutant retinæ (**Figure 3.8B-D**). Consistent with these observations,

morphometric quantification of the ONL thickness showed similar thinning in *rd1*, *rd1/Bak^{-/-}* and *rd1/Bak^{-/-}/Bax^{+/-}* compared to the *WT* (~ 77%, $p < 0.0001$; **Figure 3.8E**). In the inner retina, no changes were detected in the thickness of IPL and GCL among different genotypes (**Figure 3.8G and H**); however, the INL was slightly thinned in the *rd1* and *rd1/Bak^{-/-}* compared to the *WT* (~ 21%, $p < 0.05$; **Figure 3.8F**). The total thickness of the retina was significantly reduced in *rd1*, *rd1/Bak^{-/-}* and *rd1/Bak^{-/-}/Bax^{+/-}* compared to the *WT* (~ 35%, $p < 0.0001$). There were no effects of *Bak/Bax* genetic manipulation compared to the *rd1* retinal thickness (**Figure 3.8I**).

Taken together, the data suggest that deficiency in *Bak*, with one or two normal alleles of *Bax*, only delays the progression of retinal degeneration and is unable to exert a prolonged protection against *rd1*-related intrinsic apoptotic stimuli in photoreceptors.

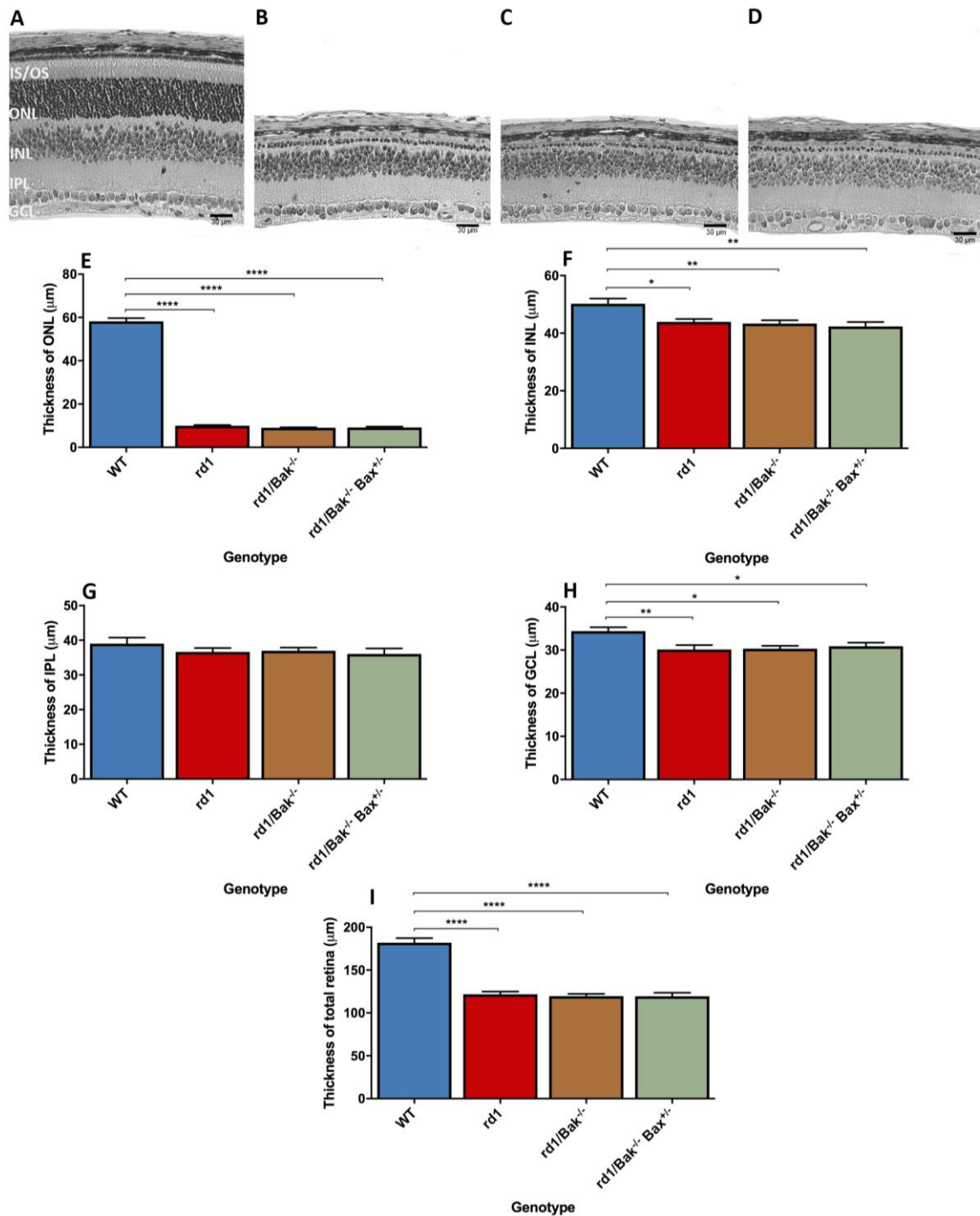


Figure 3.7: Morphology of central retina in the *Bak/Bax* mutant mice at P18

Representative images showing H&E staining of WT (A), *rd1* (B), *rd1/Bak*^{-/-} (C) and *rd1/Bak*^{-/-}/*Bax*^{+/-} (D) central retinal sections at P18. The ONL thickness of the *rd1/Bak/Bax* mutant mice was not different from *rd1* (E). IS/OS was absent in *rd1*, *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} retinæ. The thickness of INL (F), GCL (H), and total retina (I) were similar in all mice bearing *rd1* mutation and they were significantly less than WT. The IPL thickness remained unchanged in all genotypes (G). The histograms indicate the mean ± SEM. One-way ANOVA, Tukey's post hoc test, * *p* < 0.05, ** *p* < 0.01, and **** *p* < 0.0001, *n* ≥ 6. Scale bars = 30 µm. Abbreviations: ONL: outer nuclear layer, IS/OS: inner and outer segments, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

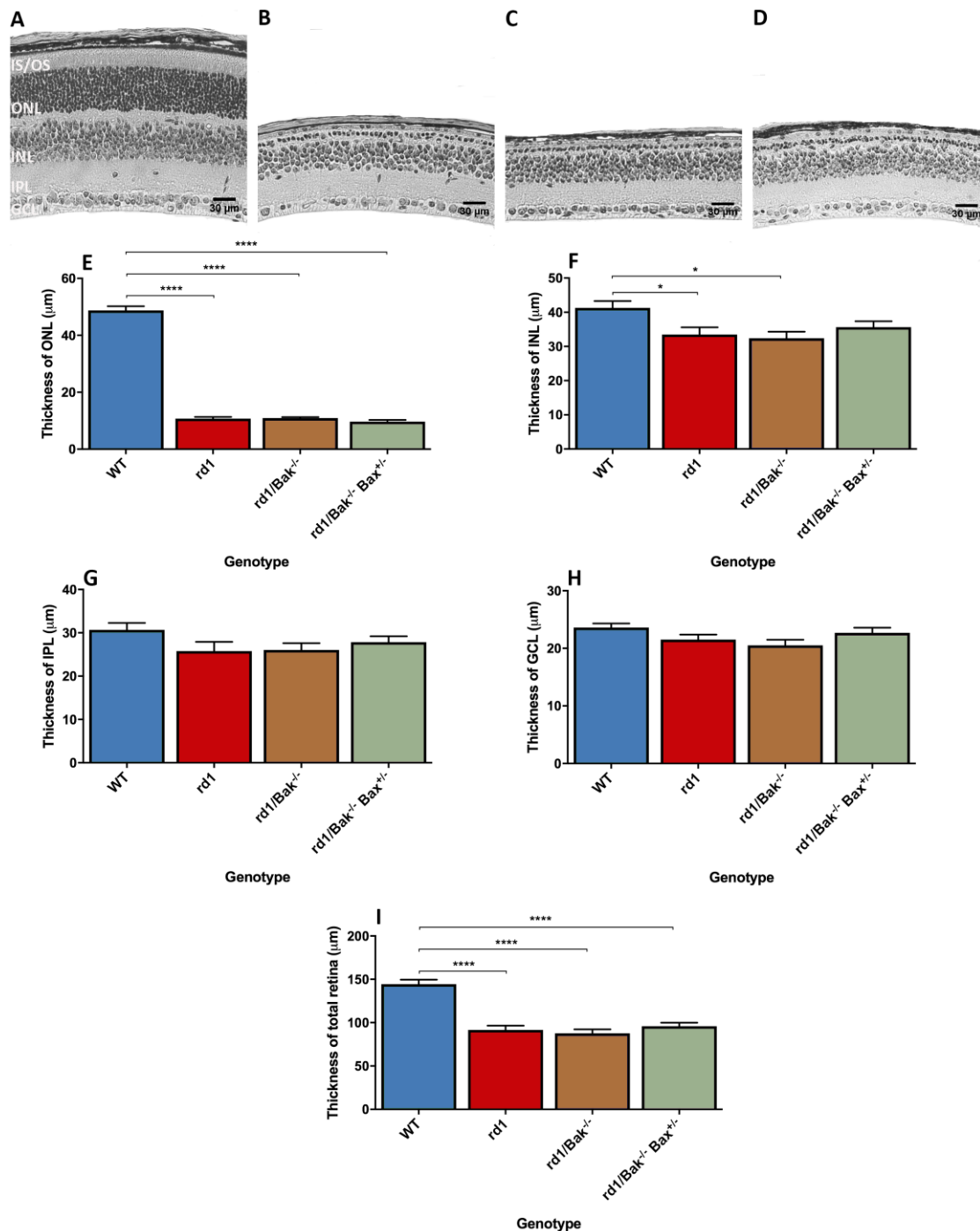


Figure 3.8: Morphology of peripheral retina in the *rd1/Bak/Bax* mutant mice at P18

Representative images showing H&E staining of WT (A), *rd1* (B), *rd1/Bak^{-/-}* (C) and *rd1/Bak^{-/-}Bax^{+/-}* (D) peripheral retinal sections at P18. The ONL thickness of the *rd1/Bak/Bax* mutant mice was not different from *rd1* (E). IS/OS was no longer present in *rd1*, *rd1/Bak^{-/-}* and *rd1/Bak^{-/-}Bax^{+/-}* retinæ. The thickness of IPL (G) and GCL (H) remained unchanged in all genotypes. The thickness of INL in *rd1/Bak^{-/-}* and *rd1* was smaller than WT (F). The total retina was thinner in all mice with *rd1* mutation compared to the WT (I). The histograms indicate the mean $\pm$ SEM. One-way ANOVA, Tukey's post hoc test, * $p < 0.05$, and **** $p < 0.0001$, $n \geq 6$. Abbreviations: ONL: outer nuclear layer, IS/OS: inner and outer segments, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars = 30 μ m.

3.3.4. Ablation of *Bak* does not change the level of *Bax* mRNA in *rd1* retinae

The results in the previous section indicate that loss of *Bak* alone resulted in a partial and transient rescue of photoreceptors loss. The data and those of others (Mosinger Ogilvie et al., 1998) suggest that BAK and BAX play overlapping or redundant roles in *rd1*-induced apoptosis and that *Bak* deficiency may be compensated by increased expression of *Bax*. This redundancy would help explain the transient nature of photoreceptor rescue observed in **Figure 3.5** to **Figure 3.8**. To examine whether there was any compensatory increase in the expression of *Bax* in the absence of *Bak*, we conducted ddPCR to measure the levels of *Bax* mRNA in the *rd1/Bak^{-/-}* retinae at P14 and P18. The ddPCR results showed that inactivation of *Bak* in *rd1/Bak^{-/-}* mice did not alter the expression level of *Bax* at P14 (**Figure 3.9A**) or at P18 (**Figure 3.9B**). Interestingly, retinae with only one allele of *Bax* (*rd1/Bak^{-/-}/Bax^{+/-}*) exhibited approximately half the level of *Bax* expression than that observed in the retinae with two copies of *Bax* (*rd1/Bak^{-/-}/Bax^{+/-}*; **Figure 3.9A** and **B**), highlighting the sensitivity of the ddPCR technique. The data indicate that loss of *Bak* does not cause any compensatory alteration in the *Bax* expression and that reduced expression of *Bax* mRNA by reducing gene dosage is sufficient to mediate apoptotic pathways in the *rd1* retina.

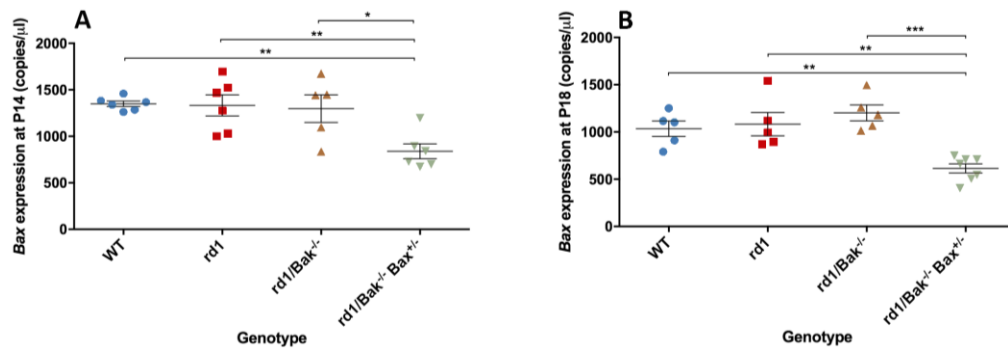


Figure 3.9: Level of *Bax* expression in *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} retinæ

Retinæ from *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} were isolated and ddPCR performed at P14 (A) and P18 (B). Data show that the expression of *Bax* does not change in *rd1* retinæ lacking *Bak*. However, deletion of one allele of *Bax* resulted in significantly lower level of *Bax* mRNA in *rd1/Bak*^{-/-}/*Bax*^{+/-} retinæ compared to the other genotypes at both time points examined. Expression was expressed as copies/μl and normalized to *Hprt* as the internal reference gene. Individual data points are plotted along with their mean ± SEM. One-way ANOVA, Tukey's post hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n \geq 5$.

3.3.5. Ablation of both *Bak* and *Bax* inhibits retinal cell death

The current results suggest that elimination of *Bak*, with or without loss of a single *Bax* allele, is not sufficient to halt photoreceptor loss in *rd1* rodent model of RP. Deletion of both *Bak* and *Bax* has been associated with perinatal lethality and only ~10% of *Bak*^{-/-}/*Bax*^{-/-} mice survive to adulthood (Lindsten et al., 2000). In our study, no double mutant animals were identified at weaning or at P14. Genotyping of mice soon after birth identified several double mutant mice, which commonly did not survive beyond P5. However, three *rd1/Bak*^{-/-}/*Bax*^{-/-} mice did survive until P13, permitting analyses of these retinæ.

As can be observed in **Figure 3.10**, the central retinæ of the double knock out mutant mice (**Figure 3.10C**) demonstrated substantial thickening in the inner retinal layers including INL, IPL and GCL compared to both *rd1* and WT (**Figure 3.10B** and **A** respectively). Interestingly, while the ONL of *rd1* retinæ (**Figure 3.10B**) had profound thinning, it appeared to remain unaffected in *rd1/Bak*^{-/-}/*Bax*^{-/-} mice (**Figure 3.10C**). Quantitative morphometry of the central retinæ revealed that while the ONL was thinned in the *rd1* retinæ, it showed normal thickness in *rd1* mice lacking *Bak/Bax* compared to the WT (114% increase relative to *rd1*, $p < 0.0001$; **Figure 3.10D**). The length of IS/OS was significantly greater in the *rd1/Bak*^{-/-}

/Bax^{-/-} retinæ compared to the *rd1* (72%, $p < 0.0001$); however, they were still shortened compared to the *WT* (39%, $p < 0.0001$; **Figure 3.10E**). In the double *Bak/Bax* mutant mice, significant increases were also detected in the thickness of all inner retinal layers including INL, IPL and GCL, not only compared to the *rd1* (114%, 62% and 58% respectively, $p < 0.0001$) but also the *WT* (88%, 42%, and 40% respectively, $p < 0.0001$; **Figure 3.10F-H**). These layer thickness changes were reflected in the overall retinal thickness, with *rd1/Bak^{-/-}/Bax^{-/-}* animals exhibiting a significant increase (88% relative to *rd1* and 40% relative to *WT*, $p < 0.0001$; **Figure 3.10I**).

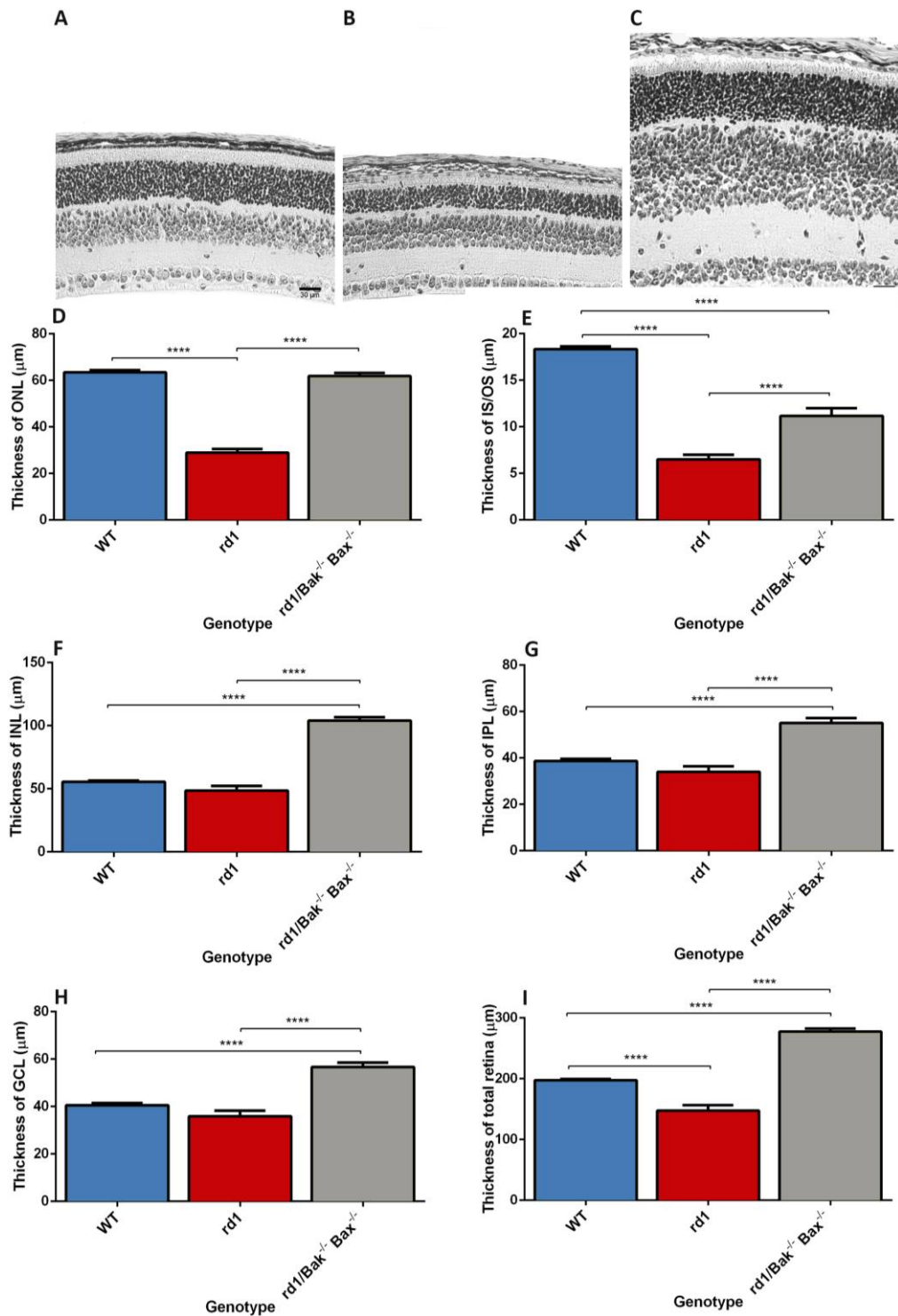


Figure 3.10: Morphology of central retina in the *rd1/Bak/Bax* mutant mice at P13

Representative images showing H&E staining of WT (A), *rd1* (B) and *rd1/Bak^{-/-}/Bax^{-/-}* (C) central retinal sections at P13. The ONL thickness of the *rd1/Bak^{-/-}/Bax^{-/-}* mutant strain was significantly greater compared to the *rd1* and remained similar to that of WT (D). IS/OS showed significant preservation in the *rd1/Bak^{-/-}/Bax^{-/-}* retinae compared to the *rd1* (E). The thickness of INL (F), IPL (G) and GCL (H) and total retina (I) significantly increased in mice deficient for *Bak/Bax* compared to the *rd1* and normal mice. The histograms indicate the mean $\pm$ SEM. One-way ANOVA, Tukey's post hoc test, **** $p < 0.0001$, $n = 3$. Abbreviations: ONL: outer nuclear layer, IS/OS: inner and outer segments, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars = 30 μm .

In the peripheral retina, morphological changes were consistent with those in the central retina. Loss of *Bak/Bax* in *rd1* mice (**Figure 3.11C**) resulted in a significant preservation of photoreceptors in the ONL, such that the thickness of the ONL was comparable to the *WT* (**Figure 3.11A**). The INL, IPL, GCL and total retina were clearly thicker in *rd1/Bak^{-/-}/Bax^{-/-}* mice (**Figure 3.11C**) compared to both *rd1* and *WT* (**Figure 3.11B** and **A** respectively). Such changes in the morphology of *Bak/Bax* double mutant retinæ were also confirmed by quantitative morphometric analyses. The ONL thickness in the *rd1* mice lacking *Bak/Bax* was significantly greater *rd1* animals (70%, $p < 0.0001$) and similar to that of the *WT* (**Figure 3.11D**). The IS/OS were also significantly longer in the *rd1/Bak^{-/-}/Bax^{-/-}* retinæ compared to the *rd1* (47%, $p < 0.0001$); however, they remained shorter than that observed in the *WT* retinæ (26%, $p < 0.0001$; **Figure 3.11E**). The thicknesses of inner retinal layers (INL, IPL and GCL) were significantly increased in the *rd1/Bak^{-/-}/Bax^{-/-}* retinæ compared to both *rd1* (99%, 55% and 90%, $p < 0.0001$) and *WT* (89%, 51% and 77% respectively, $p < 0.0001$; **Figure 3.11F-H**). As a result, the total retina was also thicker in the *Bak/Bax* double mutant retinæ compared to the *rd1* (80%, $p < 0.0001$) and *WT* (51%, $p < 0.0001$; **Figure 3.11I**). Overall the data indicate that loss of both *Bak* and *Bax* led to a complete rescue of photoreceptors from degeneration in *rd1* mice, implying that these two pro-apoptotic proteins play a vital role in the implementation of photoreceptor death. In addition, the dramatic increases in inner retinal layer thicknesses indicate amelioration of developmental cell death in these layers.

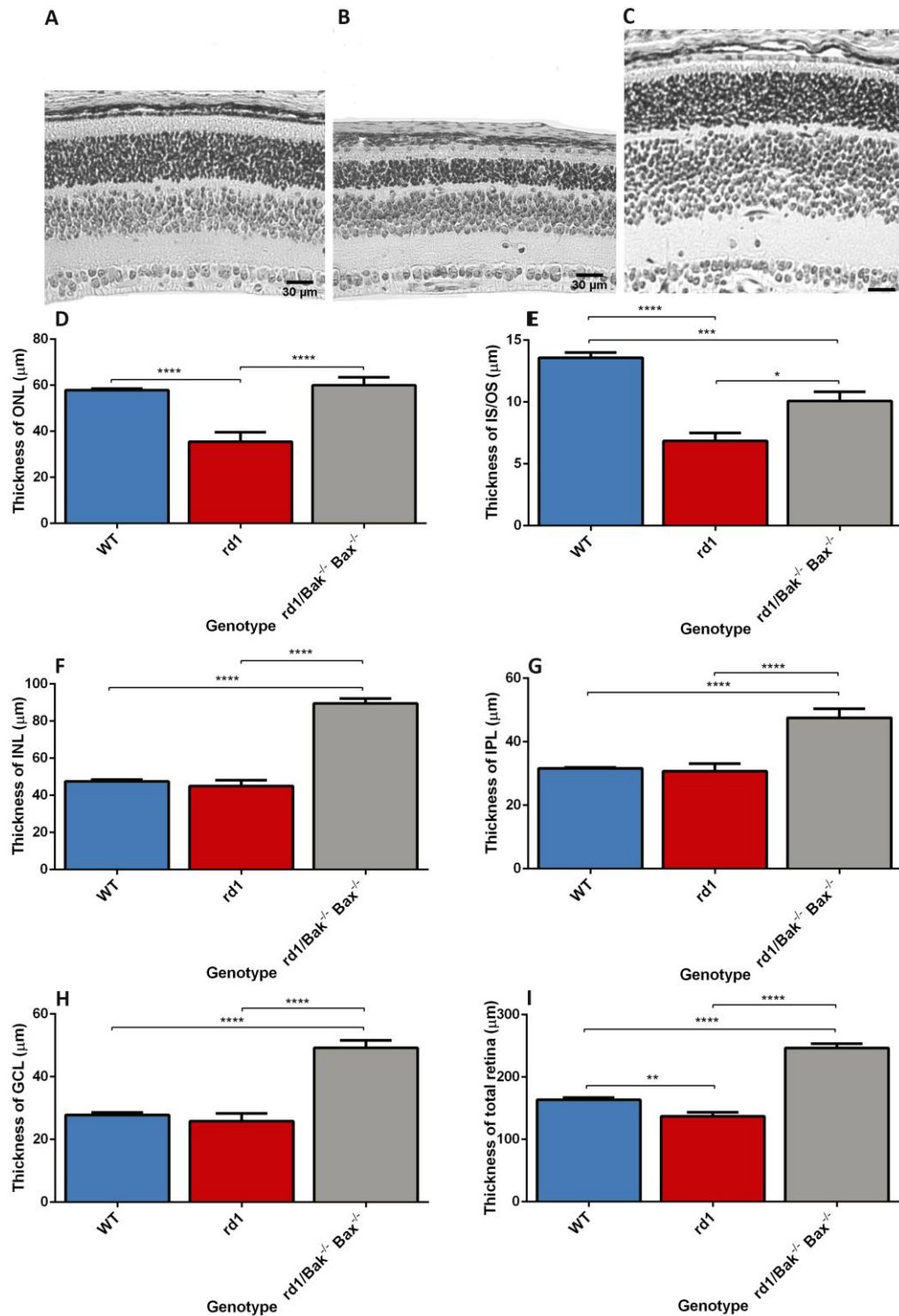


Figure 3.11: Morphology of peripheral retina in the *rd1/Bak/Bax* mutant mice at P13

Representative images showing H&E staining of WT (A), *rd1* (B) and *rd1/Bak^{-/-}/Bax^{-/-}* (C) peripheral retinal sections at P13. The ONL in *rd1/Bak^{-/-}/Bax^{-/-}* mutant strain was significantly preserved compared to the *rd1* and showed similar thickness to that of WT (D). IS/OS showed increase in their lengths in the *rd1/Bak^{-/-}/Bax^{-/-}* retinae compared to the *rd1* (E). The INL (F), IPL (G) and GCL (H) and total retina (I) showed significant thickening in mice lacking *Bak/Bax* compared to the *rd1* and WT. The histograms indicate the mean $\pm$ SEM. One-way ANOVA, Tukey's post hoc test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, **** $p < 0.0001$, $n = 3$. Abbreviations: ONL: outer nuclear layer, IS/OS: inner and outer segments, INL: inner nuclear layer, IPL: inner plexiform layer GCL: ganglion cell layer. Scale bars = 30 μm .

3.3.6. Efficacy of a BAK inhibitor to ameliorate photoreceptor death

The results in the *Bax/Bak* double mutant mice demonstrated that BAK and BAX are major contributors of photoreceptor degeneration in *rd1* animal model. This suggests use of BAK and BAX antagonists to inhibit apoptosis could be potentially beneficial for forms of RP that involve *PDE6* mutations. Since a pharmacological antagonist of BAK was available, it was used in combination with animals harboring the *rd1/Bax* mutations. P16 *rd1/Bax*^{-/-} mice underwent intravitreal injection of the specific inhibitor (or vehicle) at P16 and retinæ were isolated at P18 to assess the level of cell death using TUNEL. **Figure 3.12** shows vertical sections of *rd1/Bax*^{-/-} retinæ from DMSO-injected (**Figure 3.12A**) and drug-injected (**Figure 3.12B**) eyes labeled for TUNEL. To determine whether the extent of cell death varied between drug- and DMSO-injected eyes, TUNEL-positive nuclei in the ONL were quantified per millimeter of retinal section (**Figure 3.12C**). Data showed there was a significant reduction in the number of dying photoreceptors in drug-treated retinæ compared to the vehicle-treated eyes (21%, * $P < 0.05$; **Figure 3.12C**). There were few TUNEL-labeled cells in other layers of both drug- and DMSO-injected eyes (**Figure 3.12A and B**). These findings show that the antagonist can be used *in vivo* to inhibit the activity of BAK and this opens the way for using a combinatorial regime of BAK and BAX antagonists to ameliorate cell death in the *rd1* model. This finding shows the efficiency of the BAK antagonist to inhibit the activity of *in vivo* and opens the way for using a combinatorial regime of BAK and BAX antagonists to ameliorate cell death in the *rd1* model.

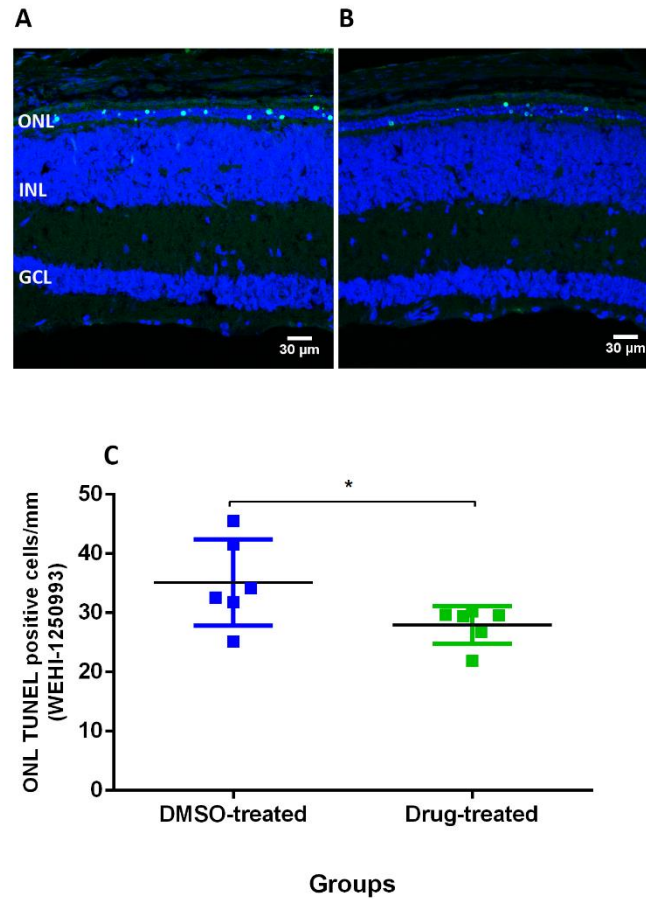


Figure 3.12: Intraocular injection of a BAK inhibitor in *rd1/Bax*^{-/-} mice

Sections of retinæ from P18 animals were labeled for TUNEL (green) 2 days after intravitreal injection of vehicle (DMSO; **A**) and a specific Bak inhibitor (WEHI-1250993; **B**). In each condition, the number of TUNEL-positive photoreceptors was counted in ONL and quantified per mm (**C**). The number of TUNEL-labeled nuclei was significantly reduced in drug-treated retinæ compared to DMSO-treated retinæ. Student t-test, * $p < 0.05$, $n = 6$. Abbreviations: ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Scale bar = 30µm.

3.4. Discussion

Apoptotic cell death is known to mediate elimination of excessive and non-functional neural cells during normal development of the retina (Buss et al., 2004; Oppenheim, 1991; Penfold et al., 1986). In addition to this prominent role in development, apoptosis is also implicated in the pathologic cell death in a variety of retinal degeneration diseases including RP (Chang et al., 1993b; Portera-Cailliau et al., 1994; Travis, 1998b). Apoptosis is tightly regulated by members of several protein families, among which the BCL-2 family play a pivotal role via a balance of its anti- and pro-apoptotic members. Two key members of this family are the multidomain proapoptotic proteins BAK and BAX, which promote mitochondrial outer membrane permeabilization, an irreversible execution step of apoptosis (Westphal et al., 2014b). In this chapter, the roles of BAK and BAX, proapoptotic members of the BCL-2 family of proteins, were explored in the developmental and pathological apoptosis of the rodent retina, specifically during retinal degeneration in the *rd1* model of RP. The results of this study firstly demonstrated that in the absence of *Bak*, the architecture and lamination of retina remained normal and similar to the wild-type (WT) at P14, even in the presence of at least one copy of its apoptosis regulating partner, *Bax*.

Unlike the P23H data presented in Chapter 2, the *rd1* model exhibited direct evidence for the involvement of the intrinsic apoptosis pathway. Specifically, the level of *Bak* transcripts were significantly elevated at both P14 and P18 in the *rd1* model, while *Bax* expression was not altered at either time points when compared to the WT control. Reflecting this importance, genetic elimination of *Bak*, with or without heterozygosity of *Bax*, led to a significant, but transient, rescue of the ONL at early stages (P14) of retinal degeneration, which was not maintained at P18. By contrast, combined elimination of *Bak* and *Bax* (*rd1/Bak*^{-/-}/*Bax*^{-/-}) in a limited number of animals resulted in preservation of the ONL thickness at the WT level, despite the presence of *rd1* mutation. In these animals, inner retinal layers including INL, IPL

and GCL were also considerably thickened, not only compared to the age-matched *rd1*, but also to *WT* control.

3.4.1. *Bak* and *Bax* are key regulatory components of retinal development

Histological comparison of retinae from mice lacking *Bak* (*Bak*^{-/-} and *Bak*^{-/-}/*Bax*^{+/-}), showed that retinal architecture and lamination were similar to the *WT*, with the thickness of all retinal layers other than the INL remaining unchanged in both central and peripheral regions. Consistent with the data, Hahn et al. (2003) reported conserved trilaminar retinal architecture in *Bak*^{-/-} retinae, with no quantitative differences in the thickness of individual retinal layers at 2 months of age.

While loss of *Bak* did not alter the normal retinal development, an increase in INL thickness was observed in the peripheral retina for the *Bak*^{-/-}/*Bax*^{+/-} animals. This result raises the possibility that developmental cell death in this layer may be delayed and/or halted due to heterozygosity of *Bax*. However, the identity of those neuronal cells in the INL, in which apoptosis was impaired remains to be determined.

While ablation of both *Bak* and *Bax* was on the *rd1* background (*rd1/Bak*^{-/-}/*Bax*^{-/-}), the data provide evidence that development of retina was aberrant in these mice by P13. Although all retinal layers were present, the inner retinal layers (INL, IPL and GCL) displayed abnormal thickening compared to the *WT*, suggesting a fundamental function for the intrinsic apoptotic pathway and thus BAK and BAX, in elimination of neuronal cells during development. Indeed, a previous work showed that deletion of both *Bak* and *Bax* results in the inhibition of physiological cell death at P7, when apoptosis normally peaks in rodent retina (Hahn et al., 2003). The importance of the mitochondria-mediated pathway of apoptosis in postnatal retinal development was also confirmed by another study in which active form of caspase-9, the initiator caspase of intrinsic pathway which activates downstream effector caspases, was

detected in retinae undergoing maturation (Donovan et al., 2006). Further evidence for the involvement of BAK and/or BAX in postnatal retinal development was provided by a study indicating that in the mouse retina, down-regulation of these pro-apoptotic proteins initiates at about P10 when developmental apoptosis has completed (Donovan et al., 2006). The current data showing inner retinal thickness changes are consistent with previous studies showing that deletion of *Bax* (*Bax*^{-/-} and *Bak*^{-/-}/*Bax*^{-/-}), resulted in reduced apoptosis and a subsequent increase in the number of cells in the GCL and INL, as well as thicker IPL, with a more prominent effect in *Bak*^{-/-}/*Bax*^{-/-} retinae at 2 months of age (Hahn et al., 2003). Comparable findings have been described in transgenic mice overexpressing *Bcl-2*, a main BAX antagonist. In these mice, similar to *Bak*^{-/-}/*Bax*^{-/-} retinae, the inner retinal thickness was doubled, and total retinal thickness was increased by approximately 50% (Strettoi et al., 2002b).

Highlighting the complexity of the apoptotic pathway, caspase-3 null mice show a temporary thickening in the INL for approximately 2 weeks and the GCL remain unaffected. As activation of executioner caspases, such as caspase-3, is downstream of mitochondrial membrane disruption induced by BAK and BAX activation, one would expect the persistent retinal abnormalities in *Bax/Bak* null mice and *Bcl-2* transgenic mutants to be reflected in the caspase-3 null retinae. As the phenotypes are disparate, this implies that caspase-3 is only one of several downstream components responsible for exerting the pro-apoptotic activity of BAX and BAK and its deficiency may lead to activation of other effector caspases and/or apoptotic pathways to resume normal development of the retina (Zeiss et al., 2004b).

Overall, our data combined with other studies, indicate that BAK and BAX are principal mediators of developmental retinal cell death. The normal development of retina that we observed in *Bak*^{-/-} mice, along with the finding from other studies (Hahn et al., 2003; Mosinger Ogilvie et al., 1998; Pequignot et al., 2003) showing that *Bax*^{-/-} retinae have abnormal thickening in the inner retinal layers, suggest that *Bax* function may be more prominent in the

physiological cell death of the inner retina. The fact that only combined ablation of both *Bak* and *Bax* induced complete resistance to developmental apoptosis suggests that BAK is also involved in this process, though its importance may be less than BAX and *Bax* can compensate for *Bak*-deficiency.

3.4.2. Combined ablation of Bak and Bax is required to prevent photoreceptor death

Our results demonstrated that *Bak* expression was elevated at P14 and P18 in the *rd1* mouse model of RP, while the level of *Bax* transcripts remained unchanged. Interestingly, the data are unlike those of a recent study which, reported up-regulation of *Bax* at both mRNA and protein levels accompanied by its localization to the mitochondrial membrane at an earlier stage (P11) of retinal degeneration of *rd1* (Comitato et al., 2014). However, this study reported no change in *Bak* gene expression. A possible reason for these discrepancies is that while BAK and BAX are involved in mediating *rd1*-related demise of photoreceptors, their expression is regulated differently at different stages of the photoreceptor degeneration. The functional significance for this differential regulation at different stages of retinal degeneration remains to be determined.

Based on the increased *Bak* expression at P14 and P18 in the *rd1* model, the extent of photoreceptor cell death in *rd1* mice deficient for *Bak*, with or without heterozygosity of *Bax*, was investigated. Our morphometric analysis of *rd1/Bak*^{-/-} and *rd1/Bak*^{-/-}/*Bax*^{+/-} retinæ indicated that genetic elimination of *Bak*, partially and only transiently protected photoreceptors in the early stages of retinal degeneration, at P14. The additional reduction in *Bax* expression (due to loss of one copy) showed no additive effect on the inhibition of ONL degeneration. However, by P18, the thickness of ONL was no longer different between *rd1*

and the two *rd1/Bak/Bax* mutants. Similarly, a previous work on *rd1/Bax*^{-/-} mutant mice demonstrated that single deletion of *Bax* failed to prevent photoreceptor degeneration in *rd1* mice (Mosinger Ogilvie et al., 1998). However, in this study the histological appearance of retinae was assessed at P21, which based on our findings would have been too late to detect any transient rescue. Together, these raise the possibility of *Bak* and *Bax* redundancy in mediating *rd1*-related cell death and/or activation of other cell death pathways as a result of apoptosis suppression.

The most striking finding of this study was that combined deficiency of both *Bak* and *Bax* highly protected photoreceptors from degeneration caused by *rd1* mutation and/or developmental-related cell death. At P13 of age, the thickness of the ONL in *rd1/Bak*^{-/-}/*Bax*^{-/-} retinae was similar to the *WT* and almost double that in the *rd1* retina. Developmental death of photoreceptors in the C57Bl/6 mouse initiates at about P12 and lasts till P30 (Mervin et al., 2002). The overlap between time course of retinal degeneration in *rd1* and naturally occurring death of photoreceptors during normal development of retina makes it hard to conclude whether *Bak* and *Bax* ablation suppressed the *rd1*-associated degeneration and/or developmental apoptosis. However, in earlier studies, the outer retina of C57Bl/6 mouse, OPL and ONL, appeared unaffected in the absence of *Bax* and/or *Bak* expression up to 2 months. But, there is evidence of photoreceptor effects after the ablation of *Bax* and *Bak*, with data showing ectopic rod photoreceptors residing within the INL, suggesting these cells are unable to migrate successfully to the ONL during development, yet survive into adulthood, due to the removal of apoptotic functions (Hahn et al., 2003; Mosinger Ogilvie et al., 1998; Pequignot et al., 2003). Given the findings of these studies, it is likely that combined inactivation of *Bak* and *Bax* vastly rescued photoreceptors from *rd1*-related deterioration. Similar to this study, Hahn et al. (2004) demonstrated genetic removal of *Bak* and *Bax* provided more effective protection against acute light-induced damage when compared to the *Bax* deficiency alone (Hahn et al., 2004).

Taken together these findings clearly reveal that apoptotic cell death mediated by activation of BAK and BAX is one of the major pathways of photoreceptor loss in *rd1* animal model of RP. That the loss of *Bak*, or *Bax* alone (Mosinger Ogilvie et al., 1998) were insufficient to provide a protection of photoreceptors from *rd1*-related degeneration but that double ablation of *Bak/Bax* provided higher level of protection, indicate that they probably serve largely overlapping and/or compensatory roles in regulating apoptotic cell death of photoreceptors. Nevertheless, this functional compensation between BAK and BAX is not reflected in their relative transcripts as we didn't detect any compensatory increase in the expression of *Bax* in the absence of *Bak* at the mRNA level.

It is worth noting that current evidence regarding the contribution of the anti-apoptotic members of BCL-2 family responsible for inhibiting BAK and BAX, in retinal degeneration are contradictory. While overexpression of *Bcl-2* transiently improved photoreceptor survival in *rd1* retinæ, between P14 and P40, in one study (Chen et al., 1996a), it did not appear to rescue photoreceptors from *rd1*-associated degeneration in another study (Joseph et al., 1996b). Additionally, high levels of BCL-2 were found to have a dose-dependent toxicity on *WT* photoreceptors (Chen et al., 1996b). Given our finding that BAK and BAX are main regulators of photoreceptor death in *rd1*, additional studies will be needed to elucidate more clearly the potential role of anti-apoptotic members of BCL-2 family in retinal degeneration.

3.4.3. Targeting the apoptotic pathway in *rd1* with a BAK inhibitor

Modulating apoptosis by small molecules suppressing pro-apoptotic members of BCL-2 family, BAK and BAX, provides a potential to develop new treatments for those diseases associated with cell death such as retinal degeneration. These therapeutic strategies mainly aim at targeting interfaces between these proteins through which they interact with each other. However, generally the biochemical properties of the interfaces of BCL-2 family

members make the development of reliable inhibitors extremely difficult. In addition, structural characteristics of the interactions between BAX and BAK and other members of BCL-2 family, as well as formation of BAX and BAK homoligomers, by which mitochondrial outer membrane permeabilization is initiated, have not been fully characterized. Accordingly, pharmaceutical development of compounds targeting BAK and BAX has been tremendously challenging and is yet to deliver a compound that can be used reliably *in vivo* (Roy et al., 2014; Wells et al., 2007).

In spite of these difficulties, Dr. Guillaume Lessene and his colleagues have designed a wide range of BAK inhibitors able to suppress apoptotic cell death efficiently *in vitro* (unpublished data). The central role of BAK and BAX in mediating *rd1*-induced degeneration opened the door to use this animal model as an *in vivo* system to evaluate the possibility of pharmacologically targeting the MOMP to prevent cell death. Here, administration of a specific inhibitor of BAK (WEHI-1250993) via intravitreal injection, along with genetic elimination of *Bax* resulted in a fewer TUNEL-positive cells in the ONL of *rd1* mice. This study provides the first evidence that such BAK antagonists may also have effective anti-apoptotic activity *in vivo* and opens the possibility of using them to prevent photoreceptor cell death therapeutically.

3.4.4. Conclusion

In summary, BAK and BAX are central regulators of developmental apoptotic cell death in retina. This current study provides strong evidence that these two pro-apoptotic factors similarly play a vital role in mediating *rd1*-related apoptosis during retinal degeneration. Our combination of pharmacological inhibition and genetic ablation directly identifies both BAK and BAX in the apoptosis that accompanies retinal degeneration. The advent of a novel and

effective BAK antagonist increases the potential feasibility of using pharmacological approaches to target both BAK and BAX as a therapeutic strategy to inhibit photoreceptor death in retinal degenerative diseases.

Chapter 4.

Conclusion and future directions

4.1. Introduction

In RP patients and animal models, while clinical symptoms share similar features regardless of the initial causative mutation, the molecular mechanisms by which cell death cascade(s) are triggered in photoreceptor cells may be unique (Comitato et al., 2016). Understanding the detailed regulatory network of cell death processes related to different genes and/or mutations are extremely important to not only provide novel insights into the pathogenesis of the retinal degenerative diseases, but also to evolve effective mutation-independent therapeutic strategies.

The overarching aim of the current study was to discover the role of apoptosis in retinal degeneration of RP using two different animal models: P23H-3 rat, a model for autosomal dominant RP, and *rd1* mouse, a model of autosomal recessive RP. However, since the original description of the P23H rat model (Steinberg et al., 1996), the level of information about the timetable of cell death and loss of visual function in strain 3 of this model was fragmented and incomplete. Therefore, prior to investigating the cell death signaling pathways that contribute to photoreceptor degeneration in this model, a more detailed phenotypic characterization was conducted.

Comparing the results obtained in this thesis, while in P23H-3 rat multiple apoptotic and non-apoptotic pathways of programmed cell death likely mediate retinal degeneration, in the *rd1* mouse, cell death is highly dependent on the activity of the intrinsic apoptotic pathway, specifically BAK and BAX. These results suggest that the causative genetic defect associated with RP may potentially trigger distinctive pathway(s) of cell death in retina.

4.2. Functional and structural alterations in P23H-3 rat

The P23H mutant rhodopsin transgenic rat displays similar properties to human autosomal dominant RP (Steinberg et al., 1996). In P23H-3, while level of cell death was high at P18 with lower levels at 1 and 2 months, a significant loss of ONL structure was not evident until 2 months. Correlating with early onset of photoreceptor degeneration, P23H-3 rat exhibited deficits in both rod- and cone-mediated pathways as early as 1 month and these became more extensive and pronounced with age. Therefore, the death of photoreceptors precedes the functional alterations, which occur prior to the actual detectable loss of retinal structure. To discuss it in more details, at 1 month, the a-wave amplitude was already reduced by ~ 30%; however, quantitative measurements by both OCT and histology showed no change in the thickness of ONL. A significant thinning in the ONL of P23H-3 was evident at 2 months of age, when there was ~ 40% reduction in rod a-wave.

These findings prove the sensitivity and efficacy of functional assessments (ERG) for monitoring retinopathies of animal models, which can detect abnormalities in retina earlier than apparent structural changes. In addition, this study applied both *in vivo* OCT imaging and histology to quantify ONL thickness. Although measured values for ONL thickness using frozen sections were lower than those obtained from OCT, due to fixation procedures, both techniques exhibited comparable trend of ONL loss, suggesting anatomical alterations can be effectively assessed by OCT. Similarly, difference between OCT and histology quantifications have been shown for P23H-1 animals, but with a good correlation in the decreasing values (Cuenca et al., 2014a; Orhan et al., 2015b).

4.3. Activation of multiple cell death pathways in P23H-3 retina

Despite the prevalence of TUNEL⁺ nuclei at P18, activation of caspase-3 and nuclear translocation of apoptosis inducing factor (AIF) were detected rarely in ONL at P18, suggesting that caspase-dependent and AIF-mediated pathways of apoptosis may not be major contributors to photoreceptor cell death in this model. Consistent with this assumption, mitochondrial membrane potential ($\Delta\psi_m$) assessment using flow cytometric analysis of JC-1 fluorescent assay indicated very little evidence of changes in mitochondrial permeability in the P23H retinal cells compared to control SD cells. As the data suggested other apoptotic and/or non-apoptotic cell death pathways may function in P23H-3 retinae, the transcripts levels of 86 genes associated with different types of cell death were analyzed. This revealed significant up–or down–regulation in the expression levels of 13 and 25 genes at P14 and P18 respectively involved in mediating apoptosis, autophagy and necrosis.

Together, these findings indicate that P23H-associated retinal degeneration is mediated by a combination of apoptotic and non-apoptotic mechanisms of cell death. While it remains to be determined what sequence and network of molecular events lead to the activation of these alternate cell death pathways, the study highlights the difficulty of attempting to prevent photoreceptor cell death by targeting a single pathway. It is likely that multiple pathways may need to be targeted to effectively inhibit neuronal death.

4.4. *Bak* and *Bax*, two key regulatory genes of neuronal cell death in the mouse retina

The *rd1* mouse model carries a gene mutation in the β -subunit of phosphodiesterase 6 (*Pde6b*) and mimics pathological features of patients with autosomal recessive RP (Bowes et

al., 1990; McLaughlin et al., 1993; Pittler et al., 1991). In *rd1*, combined deficiency of *Bak* and *Bax* (*rd1/Bak^{-/-}/Bax^{-/-}*) resulted in inhibition of apoptosis-induced retinal degeneration and thereby highly protected photoreceptors. Intriguingly, single deletion of *Bak*, with one or two alleles of *Bax*, partially and only transiently delayed the progression of retinal degeneration, implying that *Bak* deficiency is insufficient to rescue photoreceptors in this animal model of RP. These findings are consistent with previous studies showing while single mutation of *Bax* failed to inhibit photoreceptor degeneration in *rd1* (Mosinger Ogilvie et al., 1998) and in a mouse model of acute light-induced cell death (Hahn et al., 2004); double mutations of *Bak* and *Bax* rescued photoreceptors from light damage (Hahn et al., 2004).

In C57Bl7/J mice, structure and lamination of retina remained unaffected in mice lacking *Bak* (*Bak^{-/-}* and *Bak^{-/-}/Bax^{+/-}*); however, elimination of both *Bak* and *Bax* on the *rd1* background (*rd1/Bak^{-/-}/Bax^{-/-}*) led to aberrant maturation of retina evidenced by substantial thickening of inner retinal layers (INL, IPL and GCL). Similarly, previous works have reported combined inactivation of *Bak* and *Bax* (*Bak^{-/-}/Bax^{-/-}*) led to an increase in inner retinal layers thickness, whereas genetic removal of *Bak* alone had no effects on retinal structure (Hahn et al., 2003; Mosinger Ogilvie et al., 1998).

Overall, these findings suggest *Bak* and *Bax* are two regulatory genes of neuronal cell death in normal maturation of mouse retina as well as *rd1*-dependent death of photoreceptors. BAK and BAX act redundantly and that absence of one could be compensated functionally by the other.

4.5. Comparison of cell death pathways in P23H-3 and *rd1*

One important aspect of the current study was to characterize cell death pathways in two different animal models of RP: P23H-3 transgenic rat, and *rd1* mouse. The results imply that expression of the dominant P23H RHO mutant protein in the rat retina triggers several

apoptotic and non-apoptotic signaling pathways in photoreceptors. However, in *rd1*, BAK and BAX appeared to be central in governing photoreceptor death. Following activation of these two pro-apoptotic proteins and induction of mitochondrial outer membrane disruption different pathways of apoptosis including intrinsic, AIF- and calpain-mediated may be activated. Interestingly, this study suggests regardless of the type of cell death mechanisms associated with *rd1* recessive mutation, the events that occur at the mitochondrion are the main determinants of cell death.

These findings concerning the P23H-3 model of RP highlight the importance of non-apoptotic mechanisms of cell death in mediating inherited retinal dystrophies and suggest that while different mutations may stimulate common down-stream pathways of cell death in photoreceptors, the particular causative gene and/or mutation may specifically dictate the activation of distinct pathway(s). Reports from previous studies have also raised the possibility that cell death pathways may be distinctively triggered in degenerating photoreceptors in a mutation-dependent manner (Comitato et al., 2016; Kaur et al., 2011b). Comitato et al. (2016) suggested that dominant and recessive mutations in the *Rho* may activate different mechanisms of cell death in photoreceptors (Comitato et al., 2016). They demonstrated that in the *Rho* knock-out mouse (*Rho*^{-/-}), a model for autosomal recessive RP, functional loss of RHO did not trigger ER-stress pathways and photoreceptor death was mediated through by calpains and AIF. However, in retinæ carrying the P23H dominant mutation (*P23H*^{Tg}), while calpains and AIF appeared to be activated, ER stress was found to play a vital role.

The genetic basis and mutations leading to RP are highly complicated and heterogeneous and thus make their treatment difficult and challenging. However, photoreceptor death is a common characteristic feature of all RP patients and animal models. Accordingly, modulating cell death pathways using pharmacological agents offers the potential to therapeutically retard or even arrest the loss of photoreceptors. Precise understanding of

signal transduction pathways regulating photoreceptor cell death is critical to assist in the development of such therapies.

The findings of the current study provide novel insights into developing future treatments aimed at manipulating cell death. Firstly, the implication of alternative mechanisms of cell death, rather than apoptosis, in photoreceptor degeneration may introduce several new targets, such as *Tp53* and *Spata2*, for neuroprotective treatments. Secondly, combined targeting of different molecules and/or signaling pathways of cell death may be required to effectively interfere with photoreceptor death. Considering the functional cross-talk among different pathways of cell death, targeting critical convergence points of all these mechanisms might be the best way for creating more powerful treatment strategies.

The finding that of BAK and BAX have a central function in driving *rd1*-associated cell death provided the opportunity to use this animal model as an *in vivo* system to test the feasibility and efficiency of a specific inhibitor of BAK (WEHI-1250993) in suppressing photoreceptor death. A single intravitreal administration of the BAK antagonist together with genetic removal of *Bax* significantly reduced, but did not eliminate, cell death in *rd1* retina after 2 days. This indicates that under the conditions used in the experiment, the BAK inhibitor was not completely effective. One possible explanation is that the intravitreal injection was not an ideal delivery system. Another explanation is that the dosage of the drug was not optimal and/or its biological half life was not long enough. Accordingly, the bioavailable level of the drug in the photoreceptor layer was not adequate to exert complete preventive effect. Considering the poor aqueous solubility of the drug, repeated intravitreal injections may be required to improve the amount of drug delivered to photoreceptors. Further work is needed to optimize the concentration and administration timing of the drug to reach maximum preservation of photoreceptors.

Overall, this study confirms the potential utility of cell death inhibitors to combat retinal degenerative diseases and introduces BAK and BAX as pivotal therapeutic points for RP patients carrying *PDE6B* mutation.

4.6. Future directions

Determining the underlying mechanisms of photoreceptor death associated with retinal degenerative diseases, including RP, remains a main challenge due to the complex nature of cell death pathways. This study has made contributions to understanding how photoreceptors die in the retina using P23H-3 transgenic rat and *rd1* mouse, two rodent models used commonly in retinal research.

In P23H-3 rats, gene expression profiling provided evidence for multiple mechanisms of cell death in retinal degeneration; however, further studies are required to unravel the regulatory components of each pathway as well as their potential functional redundancy and interplay in mediating photoreceptor death. PCR array data showed relatively small number of cell death-related genes are differentially expressed in P23H-3 retina and alterations in expression levels were small but significant. This suggests regulatory mechanisms of cell death may occur at the protein levels via post-transcriptional mechanisms, including protein-protein interactions, post-translational modifications (e.g. phosphorylation, proteolytic cleavage, glycosylation), and changes of their subcellular localization. Because cDNA assays are not able to detect such changes, proteomics-based studies would be of interest in future studies to better understand details of cell death pathway activations. In addition, retina contains different types of cell, but retinal degeneration specifically occurs in photoreceptor cells. Isolating photoreceptor cells for single-cell gene/protein analysis may be another important approach to characterize cell death pathways specifically in these cells.

This study presented BAK and BAX as key players of *rd1*-induced retinal degeneration. This important finding mainly obtained by analysing *Bak/Bax* double knock out mice. However, this study was limited by poor viability of *Bak^{-/-}/Bax^{-/-}* mice due to perinatal death. Conditional deletion of *Bak* and *Bax* in the photoreceptors via *Cre/LoxP* recombination approach would be an ideal system for analysing long-term effects of their inactivation on retinal degeneration of *rd1*. In addition, it would be important to identify how protective effects of *Bak/Bax* mutations on photoreceptor death are reflected in retinal function. Thereby, functional ERG assessments would be also of interest in future studies.

The current study provided a proof of concept for the application of cell death inhibitors as a therapeutic option for retinal degenerative diseases. Detailed therapeutic validation of these approaches remains to be established specially to determine the long-term effectiveness of them.

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