Mechanism of Photoreceptor Cell Death in Rhodopsin Mutant Rat Models (S334ter-3 and P23H-1) of Autosomal Dominant Retinitis Pigmentosa

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Summary

Retinitis pigmentosa (RP) denotes a group of genetically heterogeneous retinal degenerative disorders characterized by initial loss of rod photoreceptors followed by secondary degeneration of cones, which causes irreversible vision loss in humans. No promising cure is available for this devastating eye condition as yet. Mutations in rod pigment gene 'Rhodopsin' account for approximately 25–30% of human autosomal dominant RP cases. S334ter and P23H are two such mutations, which result in C-terminus truncation and misfolding of rhodopsin protein respectively. Despite the knowledge about genetic causes, the mechanisms leading to photoreceptor degeneration in RP remain poorly understood till date. Previous research has often linked photoreceptor cell death to apoptosis, but recent studies indicate the involvement of alternative non-apoptotic cell death markers during retinal degeneration in RP. Since S334ter and P23H mutants reportedly have delayed recovery during phototransduction, calcium dysregulation is expected in these mutants, and it was hypothesized that calcium dependent processes might be involved in the retinal degenerative events in these mutants. Accordingly, photoreceptor degeneration in S334ter and P23H transgenic rats was analysed with an aim to investigate the role of calcium sensitive calpain proteases and poly(ADP-ribose) polymerase (PARP) enzyme. The study unequivocally showed that calpain and PARP alongwith high oxidative stress play significant role during photoreceptor cell death in these mutants, reflecting earlier similar findings of non apoptotic events during retinal degeneration in PDE6 mutant *rd1* mice. Further, specific inhibitors of calpain and PARP seemed neuroprotective under special paradigms of *in-vitro* treatment in case of P23H and S334ter cultured retinas. Classical apoptotic events were observed only in the S334ter mutant. Additionally, both mutant retinas showed accumulation of cGMP, a factor considered common during photoreceptor cell death in different models of RP. Retinal guanylyl cyclase and other phototransduction proteins also showed alterations in the expression patterns suggesting downstream consequences of rhodopsin mutations on visual transduction cascade, which might affect the calcium levels, and hence the related processes ultimately leading to cell death.

Zusammenfassung

Retinitis pigmentosa (RP) bezeichnet eine Gruppe von genetisch heterogenen degenerativen Netzhauterkrankungen. RP ist charakterisiert durch den anfänglichen Verlust von Stäbchen-Photorezeptoren, gefolgt von einer sekundäre Degeneration der Zapfen, was zum irreversiblen Verlust der Sehkraft beim Menschen führt. Bisher gibt es kein Heilmittel für diese fatale Augenkrankheit. Mutationen im Gen für das Stab-Pigment "Rhodopsin" machen etwa 25-30% der autosomal dominanten RP Fälle aus. S334ter und P23H, zwei dieser Mutationen, führen zu C-terminalen Abbruch und Fehlfaltung von Rhodopsin. Die Mechanismen, die zur Degeneration in RP führen, sind bis heute schlecht verstanden. Frühere Untersuchungen haben oft den Zelltod mit Apoptose verbunden, aber neuere Studien zeigen die Beteiligung von alternativen nicht-apoptotischen Zelltod-Markern. Da S334ter und P23H-Mutanten eine verzögerte Erholung während der Phototransduktion haben sollen, ist in diesen Mutanten eine Kalzium-Fehlregulation zu erwarten. Es wurde die Hypothese aufgestellt, dass Kalzium-abhängige Prozesse in den degenerativen Abläufen dieser Mutanten beteiligt sein könnten. Daher wurde die Photorezeptor-Degeneration in S334ter und P23H transgenen Ratten mit dem Ziel analysiert, die Rolle der kalziumsensitiven Calpaine und des Poly (ADP-Ribose) Polymerase (PARP) Enzyms zu untersuchen. Die Studie zeigte eindeutig, dass Calpain und PARP zusammen mit hohem oxidativem Stress signifikant beteiligt sind am Zelltod dieser Mutanten, was frühere Studien von nicht-apoptotischen Abläufen während der Degeneration in PDE6 *rd1* mutanten Mäusen wiederspiegelt. Darüber hinaus schienen spezifische Inhibitoren von Calpain und PARP unter bestimmten Bedingungen in P23H und S334ter Mutanten neuroprotektiv zu sein. Klassische apoptotische Abläufe wurden nur in der S334ter Mutante beobachtet. Zusätzlich zeigten beide Mutanten akkumuliertes cGMP, ein Faktor, der häufig während des Zelltods in verschiedenen Modellen der RP auftreten soll. Die retinale Guanylylzyklase und andere Phototransduktions-Proteine zeigten ebenfalls veränderte Expressions-Muster, was auf Konsequenzen der Rhodopsin Mutationen auf die visuelle Transduktions-Kaskade hindeutet, den Kalzium-Spiegel und die damit verbundenen Prozesse beeinflussen könnte, letztendlich zum Zelltod führend.

Contents

Chapter I. General Introduction

1. Vertebrate Retina and Photo-transduction

Among all the primary senses, vision is the most important and beautiful sense, which enables us to orient, and to perceive the world around us. Human visual system is highly developed and marvelously coordinated, and consists of two main components; eye and brain. First step of vision takes place in the photoreceptor cells of the eye, where visual pigment 'Rhodopsin' on absorption of photons of light starts a chain of biochemical events known as photo-transduction that culminates with nerve impulses being sent through the optic nerve to a part of the brain called visual cortex. After processing and interpreting these impulses, brain gives the information about what is being seen (Luo et al., 2008).

A successful and uninterrupted phototransduction is of utmost importance for the normal vision (Burns and Baylor, 2001). Owing to the need of continuous renewal of visual pigment for uninterrupted visual transduction, and due to a large number of various proteins engaged in this cascade, the photoreceptors carry a remarkably huge metabolic burden. Therefore, even the mildest alterations in the structural and/or functional organisation of theses cells due to mutations in the genes encoding these proteins cause retinal degenerations by stimulating the photoreceptor cell death (Phelan and Bok, 2000; Shastry, 1997). Retinitis Pigmentosa (RP) is one of such heterogeneous retinopathies, for which no promising cure is available as yet (Hartong et al., 2006; Pagon and Daiger, 1993).

A plethora of different Rhodopsin (RHO) mutations have been identified to be responsible for a significant number of patients suffering from RP, which account for 25–30% of autosomal dominant RP (ADRP) (Berson et al., 2001; Sohocki et al., 2001). Genetic mutations results in abnormal functioning of RHO molecule leading to photoreceptor cell death (Manyosa et al., 2003; Mendes et al., 2005; Stojanovic and Hwa, 2002).

This PhD dissertation was aimed to disentangle the possible mechanism of photoreceptor degeneration in two transgenic rats (S334ter and P23H) of ADRP. The study evidently showed that up-regulation of calpain and PARP together with high oxidative stress contributes to the cell death in these mutant rat models. The

overlapping of observed cell death mechanism in RHO transgenic rats to other mutants *viz.,* PDE6 mutant *rd1*mouse model, may be useful during therapeutic intervention aimed at the development of mutation independent strategies to protect the degenerating retina by targeting and blocking the common denominators of cell death. Therefore, additional goal of the project was to work towards a mutation independent approach for the neuroprotection of the degenerating retina by using specific calpain and PARP inhibitors. The study demonstrated that specific inhibitors of calpain and PARP, when used in special paradigms render a protective effect to the photoreceptors in P23H and S334ter organotypic retinal cultures. Further investigation about the status of various phototransduction components revealed the accumulation of cGMP in these mutants, which is considered a common factor in different models of retinal degenerations.

To understand the intricate pathways of disease pathology in retinal degenerations, it is pertinent to understand the normal structural and functional organization of visual system. Following section of this chapter deals with a brief introduction to the structure of vertebrate retina followed by explanation of visual process with emphasis on visual transduction cascade mechanism and various proteins involved in this signalling pathway.

1.1 Vertebrate Retina

Pioneering studies during 17th century showed that 'light detector' in the eye was the retina. More than 150 years ago, when retina was observed microscopically, retinal components responsible for light capturing were not clear. Later, Max Schultze (1825-1874) demonstrated that rods and cones were responsible for scotopic (night) and photopic (colour) vision respectively (http://www.accessexcellence.org/AE/AEC/CC/vision_background.php; Sterling and Salthouse, 1981; Wassle and Boycott, 1991).

More than a century ago, in a significant development in the field of retinal research, Santiago Ramón y Cajal (1893) described in detail the cellular organization of the retina and depicted diagrammatically the route of signals propagation from the photoreceptors cells of the retina to the brain (Wade, 2008).

Since then, the retina has been extensively studied, which made its neuro-anatomical structure very clear until now (Masland, 2001). The retina is a complex tissue specialized to perform the task of transforming light energy into the neuronal impulses (Kolb, 1995d).

1.1.1 Structure of the Retina

Vertebrate retina is a thin (~25-mm) layer of neural tissue forming most of the posterior part of the eye. During embryonic development, the retina and the optic nerve evaginate directly from the developing brain; therefore, it is an integral part of the central nervous system (CNS) (Kolb, 1995a; Kolb, 1995b; Wassle and Boycott, 1991). Retina has orderly laminated structure composed of five main categories of neurons (i.e., photoreceptor cells, bipolar cells, amacrine cells, horizontal cells and ganglion cells, which are arranged in three nuclear/cellular layers and two plexiform layers (Kolb, 1995d) (Fig. 1.1) The three cellular (nuclear) layers include:

1) Outer nuclear layer (ONL) comprising of cell bodies of photoreceptors (rods and cones)

2) Inner nuclear layer (INL) consists of cell bodies of the bipolar cells, amacrine cells, horizontal cells and Müller glial cells;

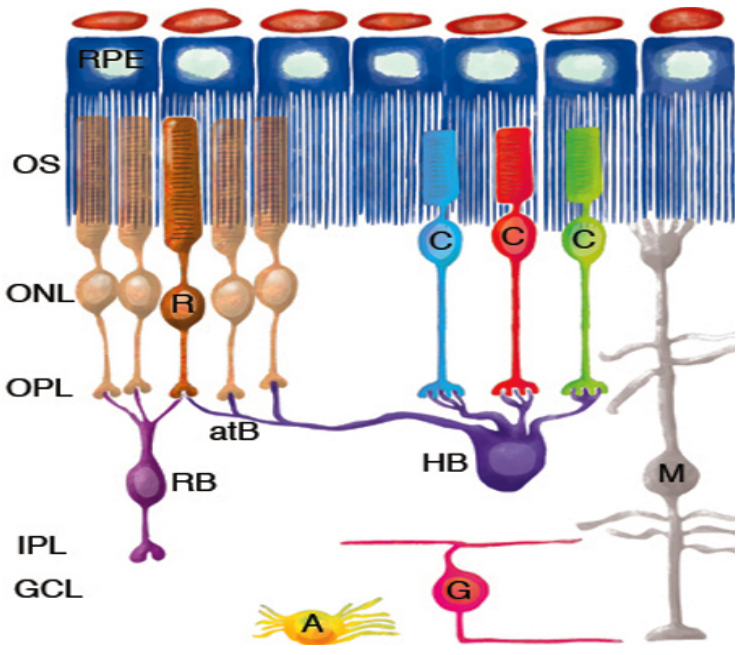

Figure 1.1: A drawing of the retina showing relevant retinal cell types. The retina consists of three cellular/nuclear layers (outer, inner and ganglion cell layers). Nuclear layers of the retina are separated by two plexiform layers (outer and inner plexiform layers). All nuclear layers and plexiform layers are depicted in the picture in abbreviated forms along with individual cell types. *Abbreviations*: RP= retinal pigmented epithelium, OS =photoreceptor outer segments; R= rod, C = cone photoreceptors; RPE = retinal pigmented epithelium; ONL = outer nuclear layer, OPL= outer plexiform layer, RB= Rod bipolar cells atB = axon terminal of B-type horizontal cell (HB), IPL = inner plexiform layer, GCL= ganglion cell layer, M= Muller cells, A = astrocytes. *Picture source*: (Fisher et al., 1995).

3) Ganglion cell layer (GCL) is formed by the cell bodies of ganglion cells and displaced amacrine cells.

Above mentioned three nuclear layers are separated by two synaptic (plexiform) layers;

1) Outer plexiform layer (OPL) containing the processes and synaptic terminals of photoreceptors, horizontal cells, and bipolar cells

2) Inner plexiform layer (IPL) containing the processes and terminals of bipolar cells, amacrine cells, and ganglion cells.

Additionally, there is one major type of glial cells, called Müller cell in the retina. Müller glial cells span the retina vertically, having their cell bodies placed in INL and the processes filling all space in the retina, which remain unoccupied by other retinal neurons. They are thought to be involved in the protection and/or repair of retinal neurons (Sung and Chuang, 2010). Besides these retinal neuronal cells, a monolayer of retinal pigment epithelial (RPE) cells is present between the retina and choroids (vascularized and pigmented connective tissue). Due to its origin from neuro-ectoderm, RPE is considered to be a part of the retina (Simo et al., 2010; Strauss, 2005).

1.1.2 Photoreceptor Cells

Retinal neurons that are sensitive to light are called photoreceptor (Photon=light, Greek) cells. They are located in the outermost region of the retina that is located farthest from the incoming light. Light crosses the transparent inner retinal layers before reaching the photoreceptors. Photoreceptors are of two types: the rods and the cones. Rod photoreceptors mediate vision in dim light and are responsible for night (scotopic) vision, while, cones are functional in bright light, therefore, they are responsible for daytime (photopic) vision and colour perception (Kolb, 1995c).

Both rod and cone photoreceptor cells are polarized neurons with similar basic structures consisting of four major parts; 1) an outer segment (OS); 2) an inner segment (IS) that are connected by a thin rudimentary cilium; 3) a cell body accommodating the nucleus and; 4) a synaptic region (axonal tip bearing synaptic vesicles at the terminals (Fig1.1). The OS is equipped with the molecular components for light absorption and generation of electrical signals *via* the process of phototransduction, while the IS houses the cellular machinery for protein synthesis and energy production (Kolb, 1995c; Murray et al., 2009). After their synthesis in the endoplasmic reticulum (ER) and post-translational processing in golgi bodies, opsins are transported to the base of the OS where the formation of new disks and plasma membrane occurs (Hargrave and McDowell, 1992b; Hargrave et al., 1983; Hargrave et al., 1984; Murray et al., 2009). Rod photoreceptors are the predominant cell type in the retina, and are extremely differentiated neurons having highly specialized membranous sacs filled with thousands of orderly arranged flattened membrane discs. Slender filaments join the adjacent discs and disc rims to the nearby plasma membrane. Visual pigment RHO is the predominant protein of rods, as it forms ~30% of the entire protein content of photoreceptors and ~ 90% of total protein content of OS of rods and is localized mainly to the disc and plasma membrane of Rod OS in highly dense form (Hargrave, 2001; Papermaster and Dreyer, 1974; Smith et al., 1975). A single human rod cell contains about $4x10^7$ rhodopsin molecules. Due to this high density of RHO molecule coupled with ordered alignment of disc membranes within the OS rod cell efficiency increases greatly to absorb the incident photon (Roof and Heuser, 1982; Sung and Tai, 2000).

Cone photoreceptors exist in three different forms depending on the sensitivities of their photo pigment (cone opsin) towards light of various wavelengths of visible spectrum. Cones that are sensitive to short wavelengths, medium wavelengths and long wavelengths are called S or blue cones, M or green and L or red cones respectively. Most mammals possess two types of cones, i.e., S and M cones and that is why, have dichromatic vision, however, human retina has three types of cone opsins (S, M and L), and hence have trichromatic vision (http//:conesimage.wistatutor.com/content/nervous-coordination). Human retina

has about 130 million photoreceptors, 5 million bipolar cells, and 1 million ganglion cells. The relative rod to cone ratio is 90% to 10% respectively with rods being distributed throughout the retina with the exception of the macula, which is the cone rich region of human retina. Nocturnal animals like rodents possess a rod dominated retina with average rod to cone ratio of about 97% to 3% (Cook and Desplan, 2001; Sung and Chuang, 2010).

1.1.3 Rod Visual Pigment Rhodopsin

Rod visual pigment 'Rhodopsin' (RHO) consists of two parts; a protein part called 'opsin' and a chromophore or ligand part called 'retinal'. RHO belongs to the superfamily of seven-helix, G-protein–coupled receptor (GPCR) proteins (Palczewski, 2006; Sakmar, 1998). The chromophore 11-*cis*-retinal (derivative of vitamin A) is covalently bound to the protein opsin by a protonated Schiff base at a lysine residue 296 to form the final functional RHO molecule (Hargrave and McDowell, 1992a).

Similar to other membrane proteins, RHO is synthesized inside the ER, where the nascent opsin polypeptide undergoes multiple post-translational modifications such as disulfide bond formation and glycosylation followed by folding of protein aided by molecular chaperones (Fukuda et al., 1979; Kaushal et al., 1994). After proper folding, RHO leaves the ER and enters the golgi apparatus, where it undergoes further glycosylation modifications before being transported to the OS (Liang et al., 1979). Unlike other GPCRs, which are activated by binding of a biochemical ligand to them, the 11-*cis* retinal acts as a strong antagonist and keeps RHO completely inactive in the dark. RHO gets activated only after the photo-isomerization of *cis*-11-retinal to the all-trans configuration upon absorption of a light photon (Lamb and Pugh, 2006; Palczewski, 2006).

Opsin protein of rod cells is a single polypeptide chain consisting of 348 amino acids. Like a typical GPCR protein, the opsin polypeptide chain crosses the disc membrane seven times as shown in Fig. 1.2, with its N-terminal tail facing the intradiscal (extracellular) space and the C-terminal tail exposed to the cytoplasm. Seven transmembrane helical segments are linked together by three extracellular (E1, E2 and E3) and three cytoplasmic loops (C1, C2 and C3). The two adjacent

cysteines Cys 322 and Cys 323 towards the C-terminal of opsin are palmitoylated. These neighbouring palmitoyl-cysteines (322 and 333) are dipped into the lipid bilayer and forms fourth cytoplasmic loop, the 'C4'. Both C-terminus and the cytoplasmic loop region are accessible to various phototransduction cascade proteins like transducin, rhodopsin kinase (GRK1) and arrestin (Jindrova and Detwiler, 1998; Sakmar, 2002).

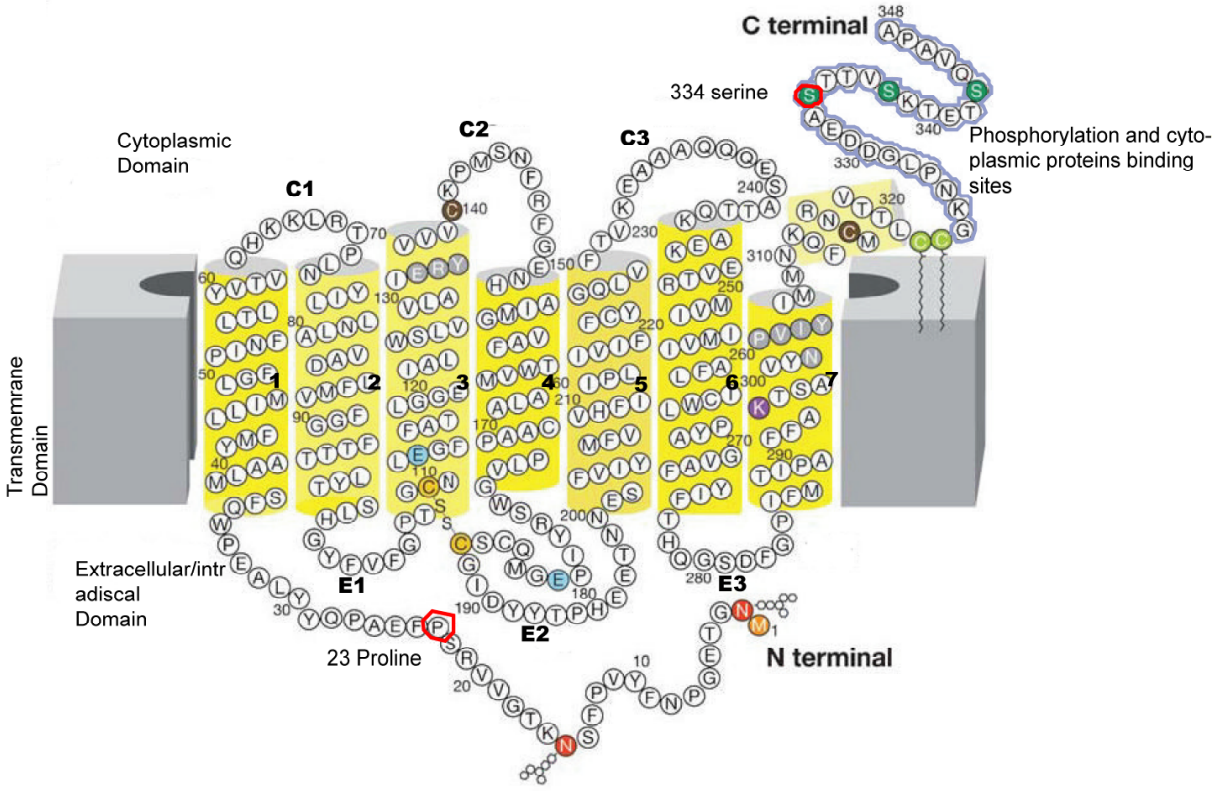

Figure 1.2. Structure of Rhodopsin molecule: Two-dimensional model. The opsin protein passes through the membrane seven times, with its C-terminus and N-terminus towards cytoplasmic and extracellular (Intradiscal) sides. C1, C2, and C3 correspond to the cytoplasmic loops, and E1, E2, and E3 correspond to extracellular loops. The transmembrane domain is α-helical (yellow cylinders). Cys110-Cys187 bridge (depicted in dark yellow) increases the helix stability. Chromophore, 11-cis-retinal, is attached to Lys296 (dark purple) via a protonated Schiff base. The positive charge of the base is neutralized by counter ion Glu113 (blue). Asn2 and Asn15 (red) are sites of glycosylation, and Met1 (orange) is acetylated. Cys322 and Cys323 (light green) are palmitoylated. Ser334, Ser338, and Ser343 (green) are the predominant phosphorylation sites. *Picture modified from* (Palczewski, 2006).

Rhodopsin has four highly specialized domains namely; cytoplasmic, intradiscal, ligand-binding and transmembrane domains (Fig. 1.2) (Dominguez et al., 2006; Mirzadegan et al., 2003; Murray et al., 2009). Each domain has special function. The cytoplasmic domain towards C-terminal is essential in regulating both the trafficking of opsin from IS to OS and in forming proper interactions with other

proteins in the phototransduction cascade. This domain is the major phosphorylation site, which helps in rapid deactivation after light absorption (Deretic, 1998; MacKenzie et al., 1984; Palczewski et al., 1989). Retinal binding domain, which is localized within the 7th transmembrane portion of the opsin molecule, is the site for binding to11-cis- retinal for the formation of fully functional RHO molecule. A correctly placed retinal absorbs the light photon and initiates the phototransduction cascade (Anukanth and Khorana, 1994; Khorana, 1992; Kim et al., 2004; Park et al., 2008; Zhukovsky et al., 1991). Intradiscal and transmembrane domains are essential to maintain a proper structure and correct folding of RHO to have a fully functional structure (Braiman et al., 1988; Davidson et al., 1994; Karnik and Khorana, 1990; Murray et al., 2009).

During phototransduction, absorption of light photon causes photo-isomerization of 11-*cis* retinal to all-trans retinal. Photo-isomerised retinal causes bleaching of the photopigment RHO and disables it to absorb more photons of light. Therefore, the regeneration and maintenance of visual pigment is extremely important. For this, 11-*cis* retinal is reproduced from all trans retinal *via* an enzymatically controlled pathway called 'visual cycle' occurring in photoreceptors and RPE (Golczak et al., 2008; Hargrave and McDowell, 1992a; McBee et al., 2001; Wright et al., 2010).

1.2 Photo-transduction

Phototransduction is the process by which light photons captured by visual pigment molecule generates an electrical impulse through a chain of biochemical reactions occurring in the disc membranes of OS of photoreceptor cell and is effected by G-Protein Cascade (Arshavsky et al., 2002).

1.2.1 Inactive state of RHO under dark conditions

Under dark conditions, RHO remains inactive in the disc membrane of rod OS, which have unusually high levels of cGMP, maintained by its continuous formation from GTP and retinal guanylyl cyclase (retGC). High affinity of cGMP for cGMP gated cation channels (CNGC) located in the plasma membrane cause them to

remain open. There is a continuous efflux of Na^+ out of the cell and an active transport of Na^+ and Ca^{2+} ions into the cell ("dark current") through the open CNG channels, resulting in high intracellular Ca^{2+} levels. This state of membrane is called 'depolarized' and is due to non-selective ion channels that admit Na^+ and Ca^{2+}, as well K^+. High Ca^{2+} concentrations trigger the depolarized rods to secrete neurotransmitter (gtutamate) constantly. Consequently, the neurons, to which the rod cells are synapsed, are continuously stimulated (Arshavsky et al., 2002; Baehr et al., 1979; Hargrave and McDowell, 1992a; Hargrave et al., 1984; Lamb and Pugh, 2006).

1.2.2. Activation Phase

Hitting of RHO by light photons results in photo-isomerisation of 1-*cis*-retinal to all-trans-retinal, forming activated RHO also called meta-Rhodopsin-II. The activated RHO undergoes a conformational change and acts as guanine effector protein (GEF), which binds and subsequently activates the heterotrimeric G protein transducin (having three subunits: αβγ) by catalyzing the exchange of GDP for GTP detaching α-subunit from the βγ-complex. The dissociated α-subunit of transducin (Gαt-GTP) in turn activates cGMP- phosphodiesterase (PDE) by binding to the two inhibitory γ-subunits of PDE, releasing the active catalytic α and β subunits. Activated PDE hydrolyzes cGMP (cyclic guanosine monophosphate) to GMP causing a decrease in the cGMP concentration that result in closure of CNG channels. The CNG channels are very important for Ca^{2+} regulation, as they are the only source for Ca^{2+} influx into the OS. CNG channels balance the Ca^{2+} influx by Ca^{2+} efflux through a Na^+/Ca^{2+}-K^+ exchange (NCKX) mechanism (McNaughton, 1990; Pugh and Lamb, 1990). When the CNG channels are closed in light, the balance between Ca^{2+} entry and Ca^{2+} extrusion is disturbed as the exchanger continues to efflux Ca^{2+} from the cytosol but the entry of Ca^{2+} stops (Kaupp and Seifert, 2002). This causes a fall in Na^+ and Ca^{2+} concentration inside the cell leading to hyperpolarization of the entire cell membrane. Since Ca^{2+} causes glutamate-containing vesicles to fuse with cell membrane and release their contents at the synaptic terminals of photoreceptor cells to second order neurons, therefore, low Ca^{2+} concentration and membrane hyperpolarization results in

decreased glutamate release to the inner retinal neurons (Hargrave and McDowell, 1992a).

1.2.3. Deactivation and Recovery Phase

Light stimulated decrease in the cytoplasmic Ca^{2+} concentration initiates processes for the deactivation of transduction, and recovery of photoactivated RHO from the light response by enhancing the synthesis of new cGMP and by light adaptation (adjusting the sensitivity of the transduction machinery) (Burns and Baylor, 2001). The deactivation of phototransduction is a complex process (Burns and Arshavsky, 2005; Yau and Hardie, 2009), and refers to inactivation of all the activated intermediates rapidly, restoring the system to its basal state of dark conditions ready for subsequent signalling. For this, all the three protein intermediates; activated RHO (metarhdopsinII), Gαt-GTP, and activated PDE must be inactivated, and cGMP levels must be restored to its basal level by RetGC (Arshavsky, 2002a). To inactivate the activated RHO, decreased Ca^{2+} concentration provides a negative feedback mechanism that controls three biochemical processes as follows:

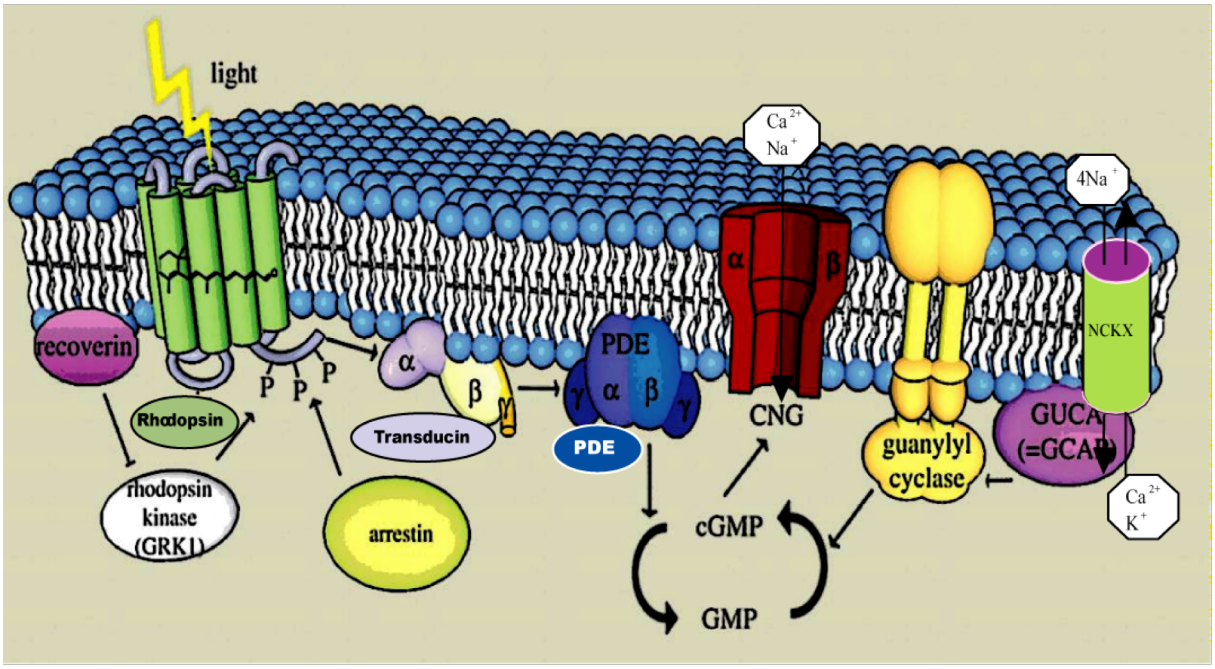

Figure 1.3 Mechanism of phototransduction cascade in rod photoreceptors: Under dark conditions, ions enter the cells through the open cGMP-gated (CNG) channels causing depolarization of the cell. On absorption of light by rhodopsin,

retinal changes conformation-causing actvation of rhodopsin, which activates heteromeric G-Protein transducin whereby the α-subunit detaches from the βγ-complex. Gα-transducin activates cGMP-phosphodiesterase (PDE) by detaching the two γ-subunits from the αβ-complex of PDE. Activated PDE hydrolyses cGMP and a decrease in the cGMP levels in the photoreceptors closes the cGMP-gated channels, which results in hyperpolarization of the cell. Because of the continued activity of the Na^{+}–Ca^{2+} exchanger (NCKX), the Ca^{2+} concentration decreases and this decrease in the Ca^{2+} level activates recoverin and causes release of Ca^{2+} from it. Recoverin subsequently activates Rhodopsin-kinase (GRK1), which phosphorylates (P) the activated rhodopsin. Arrestin binding to activated RHO returns it to the inactive state. The lower Ca^{2+} concentration also triggers the release of Ca^{2+} from guanylate-cyclase-activating protein (GCAP=GUCA), which in turn activates RetGC to synthesize cGMP. Increased cGMP concentration opens the cGMP-gated channels, enabling transport of ions into the cell, thereby depolarising the cell and returning it to the dark state. *Picture adapted from* (Larhammar et al., 2009).

1) Decreased Ca^{2+} levels stimulate the activity of retinal guanylyl cyclase (RetGC) that synthesizes new cGMP from GTP supplied by guanine nucleotide cycle consisting of guanylate kinase (GK) and nucleoside diphosphate kinase (NDPK) (Ridge et al., 2003). Ca^{2+} sensitivity is relayed to GC by a Ca^{2+}-binding protein called GC-activating proteins (GCAP). In dark, the GCAPs remain in their inactive form with Ca^{2+} bound to them, when the Ca^{2+} concentration is about 300–500 nM. However, in light, when Ca^{2+} levels are lowered to 50–100 nM, GCAPs release the Ca^{2+}, which then stimulates RetGC activity (Koch and Stryer, 1988). After the restoration of cGMP levels, CNG channels are opened, enabling influx of Ca^{2+} along with other ions, thereby depolarising the cell and returning it to the dark state (Smith et al., 2009).

2) Rhodopsin kinase (GRK1), a member of a class of GPCR kinases, phosphorylates the activated RHO mediated by another small Ca^{2+}-binding protein, recoverin (Koch and Stryer, 1988) to quench the activated RHO. Opsin molecule has three main serine phosphorylation sites on C-terminal. Cytosolic protein β-arrestin binds to the phosphorylated serine residues on C-terminal leading to complete inactivation of RHO, which further blocks the formation of Gαt-GTP complex (Arshavsky, 2002b; Zhao et al., 1995).

3) With decrease in Ca^{2+} level, ligand sensitivity of the CNG channel increases. This regulation of ligand sensitivity by Ca^{2+} is mediated by a third Ca^{2+}-dependent protein, (CaM) Calmodulin (Hsu and Molday, 1993). All three reactions help to restore the dark state and to adjust the light sensitivity of the cell.

2. Retinitis Pigmentosa: Disease pathology and Genetics

2.1 Retinitis Pigmentosa (RP)

Retinitis pigmentosa is the most prevalent and best characterized among all the inherited retinopathies, which represent a large spectrum of eye diseases usually culminating in the progressive and irreversible vision loss (Bird, 1992; Gregory-Evans and Bhattacharya, 1998; Hartong et al., 2006; Humphries, 1993). It is a genetically and clinically heterogeneous condition characterized by initial symptoms of night blindness (nyctalopia) and peripheral vision loss (tunnel vision) due to rod degeneration. As the disease progresses, central vision also gets compromised owing to secondary degeneration of cones leading to complete blindness (Phelan and Bok, 2000). The age-at-onset is highly variable ranging from adolescence to early adulthood; however, severe visual impairment occurs by the age 40 to 50 years (Hartong et al., 2006).

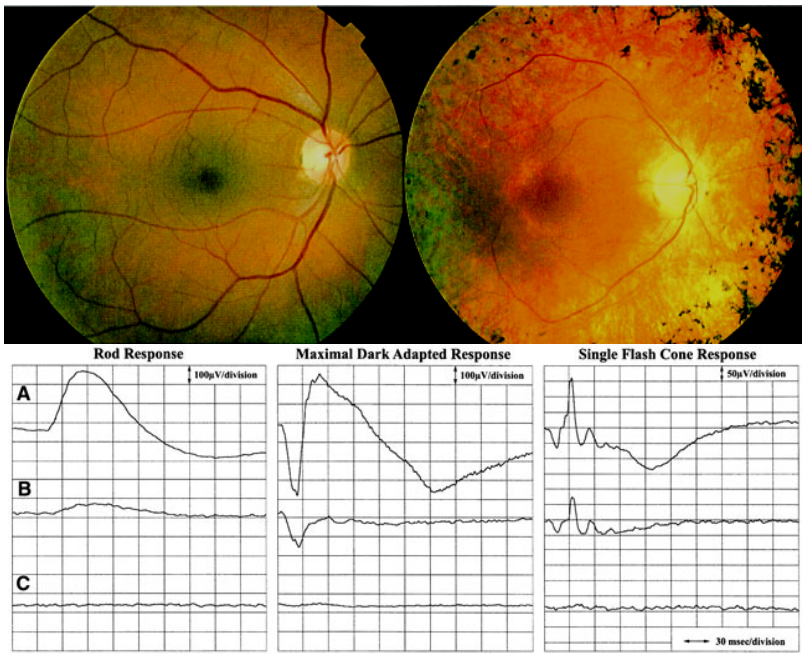

Figure 1.4 Photographs of a normal (left) and RP retina (right): typical RP features are evident in affected retina (upper right): marked pigment epithelial thinning, retinal vascular attenuation, classical 'bone spicule' and intraretinal pigmentary deposits Combined rod and cone responses to the maximal intensity flash presented in the dark-adapted state and single flash light-adapted cone responses are shown in (A) a normal individual, (B) a patient with moderately advanced RP and (C) a patient with advanced RP; *Picture and explanation from* (Farrar et al., 2002).

2.1.1 Clinical Features of RP

Clinically, retinal changes include abnormal fundus showing the presence of attenuated vasculature, and intraretinal pigmentary deposits (that is why name pigmentosa), which may have the appearance of bone spicules (Fig.1.4) (Pagon and Daiger, 1993; Weleber et al., 1993). Electroretinographic (ERG) responses are an early indicator of loss of rod and cone function in RP and diminution of ERG responses can be evident within the first few years of life, even though symptoms may appear much later. RP patients have decreased or undetectable rod and cone responses, typically with a greater loss of rod than cone ERG responses (Farrar et al., 2002).

2.1.2 Non-Syndromic and Syndromic forms of RP

RP can be classified as nonsyndromic or "simple" (mainly confined to the eye; without affecting other organs or tissues) and syndromic (associated with abnormalities in other organ systems (Shintani et al., 2009) or systemic (affecting multiple tissues) (Daiger et al., 2007). Above described type of RP is nonsyndromic. The major forms of syndromic RP include Usher's syndrome, Bardet-Biedl (Laurence-Moon) syndrome, Leber congenital amaurosis (LCA), Stargadt's disease, Refsum syndrome and achromatopsia (Boughman et al., 1983; Daiger et al., 2007).

2.2 Prevalence and Genetics of RP

It is estimated that about 1.5 million people could be affected with RP all over the world (Gregory-Evans and Bhattacharya, 1998). This disease has a worldwide prevalence of 1 case per 3000 persons to 1 case per 7000 persons (Haim, 2002). Table1.1 shows the Prevalence of nonsyndromic and syndromic RP Types (Haim, 2002).

Table1.1: Prevalence and estimated percentages of Retinitis Pigmentosa types

Category	Type	Approximate % of RP types
Nonsyndromic RP	Autosomal dominant RP	20
	Autosomal recessive RP	13

	X-linked RP	8
	Digenic RP	Very rare
	Subtotal	65
Syndromic and systemic RP	Usher syndrome	10
	Bardet-Biedl syndrome	5
	Leber congenital amaurosis (LCA)	4
	Unknown: Simplex or Isolated or RP	20
	Other	10
	Subtotal	25
Other or unknown types of RP		10
Total*		100

*The total prevalence is 1 case per 3100 persons (range, 1 case per 3000 persons to 1 case per 7000 persons), or 32.2 cases per 100 000 persons: adapted from (Haim, 2002).

Fifty per cent of the reported cases are sporadic. For remainder cases, Nonsyndromic and nonsystemic RP encompass 65% of all cases (Farrar et al., 2002).

2.2.1 Inheritance Patterns

There are different inheritance patterns observed in RP such as autosomal dominant (only one copy of gene having the mutation, 15-20% of cases), autosomal recessive (affects men and women equally, however, both copies of the relevant gene must have the mutation, 20-25%), X-linked (presence of an abnormal gene on an X chromosome, 10% to 15% cases), and mitochondrial or digenic (rare cases). Digenic RP is inherited in a pseudo-dominant pattern (1/4 risk) (Bunker et al., 1984; Hamel, 2006; Hartong et al., 2006; Kajiwara et al., 1994; Rivolta et al., 2002). Sporadic cases of RP are called “simplex” or “isolated, and they represent 10-40% of all individuals. Cause of such simplex RP cases may be a *de novo* mutation, which could be inherited in ADRP or X-linked mode (Pagon and Daiger, 2005).

2.2.3 Genes Involved in RP Pathology

Molecular genetics of RP is very complex, which could be attributed to the genetic heterogeneity of the disease as different genes may cause the overlapping pathology and conversely, different mutations in the same gene may cause different forms of the diseases (Daiger et al., 2007; Hims et al., 2003; Rivolta et al., 2002). For most RP genes studied to date, many different mutations responsible for disease manifestation have been identified (Daiger et al., 2007). Presently, approximately 60 genes harbouring different mutations have been identified to be responsible for RP (RetNet; http://sph.uth.tmc.edu/Ret-Net/ November, 2011). Genes responsible for RP are generally known to be expressed in the photoreceptor cells (Rivolta et al., 2002). However, there is remarkable functional diversity in the types of the genes implicated in RP as they encode proteins involved in important functional mechanisms such as phototransduction, visual cycle and photoreceptor structure (al-Maghtheh et al., 1993; van Soest et al., 1999). Additionally, individuals with same mutations frequently show different phenotype and pathogenesis of disease, which could probably due to the impact of interplay between genetic and/or environmental factors (Daiger et al., 2007).

2.3 Rhodopsin gene mutations and RP

RHO is the single most important gene responsible for approximately 25–30% of human ADRP cases (Berson et al., 2001; Colley et al., 1995; Sohocki et al., 2001; Wilson and Wensel, 2003). Since RHO is both a crucial component of phototransduction as well as the structural protein of the photoreceptor outer segments, each RHO mutations cause a specific pathogenesis (Mendes et al., 2005). The gene locus for RHO in humans is 3q21.4 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=RHO). Till date, more than 100 different mutations have been documented in RHO gene, which are mainly responsible for ADRP. However, recessive RP and congenital stationary night blindness are also caused by some mutations in RHO gene (www.sph.uth.tmc.edu/RetNet/). These mutations have been found to occur throughout the RHO molecule. Owing to the unique structure of RHO to have

distinct regions specialized for various function, it is obvious that the phenotype depends upon the location of the mutation in different domains of the molecule (Bessant et al., 2001; Dryja et al., 1990; Humphries, 1993; Wright, 1992).

Based on their properties to express in cultured epithelial cells, RHO mutations were initially classified into two categories; class I and class II (Sung et al., 1993). Class I mutants (15%) resemble wild-type RHO in expression level. They fold correctly, and form functional RHO molecule. These mutations are predominantly found near the carboxyl terminus of the protein. It has been reported that class I mutant RHO molecules have defects in their polarized OS distribution (Sung et al., 1993; Sung et al., 1991; Sung and Tai, 2000). Class II (85%) mutants exhibit low expression and stability, variable ability to regenerate chromophore, and inefficient transport to the plasma membrane. These mutants are reported to be misfolded and are retained in the endoplasmic reticulum (ER) causing the ER stress evoking the unfolded protein response (UPR). P23H mutation belongs to class II of RHO mutation (Sung et al., 1993; Sung et al., 1991).

Mendes et al. (2005) extended the RHO mutation classification further depending on their location in the molecule together with their biochemical properties (Mendes et al., 2005) (see table 1.2)

Table 1.2: Rhodopsin mutation classification (Mendes et al., 2005)

RHO mutant Class	Property	Mutation site
Class I	Fold correctly but not sorted to OS	L328, T342, **Q344**, V345, A346, P347,
Class II	Don't fold correctly and are retained within ER	T17, **P23H**, G51, T58, V87, G89, G106, C110, L125, A164, C167, P171, Y178, E181, G182, C187, G188, D190, H211, C222, P267, S270, K296
Class III	Endocytosis defect	R135
Class IV	Do not manifest folding defect necessarily but the RHO stability is affected	T4
Class V	Enhanced transducin activation rate by mutants	M44, V137
Class VI	Constitutive activation of opsin protein even in the absence of	G90, T94, A292

	retinal	
Unclassified	Defect not observed or yet to be studied	N15, Q28, L40, F45, L46, P53, G109, G114, S127, L131, C140, E150, P170, G174, P180, Q184, S186, T193, M207, V209, P215, M216, F220, E249, G284, T289, S287, E341

Recently, Rakoczy and Kiel et al. provided even wider classification of RHO mutations based on the structural, functional and energetic analysis. In this classification, a new class of mutants has been devised (class VII: mutants affecting binding to partner proteins), along with sub classification of Class II into IIa (destabilising), IIb (retinal binding) and IIc (residues facing lipids in the membrane). In addition, class V has been eliminated completely (Rakoczy et al., 2011).

2.4. ADRP caused by P23H and S334ter mutations in RHO

Both P23H and S334ter are “gain of function” mutations and cause ADRP (Mendes and Cheetham, 2008; Mendes et al., 2005). P23H mutations have been observed in approximately 12% of ADRP patients and are responsible for the majority of RHO-related RP cases.

2.4.1. S334ter Mutation

This class I mutation is of missense category due to which, last 15 amino acids (from Serine at 334 residue viz., S334 to A348) from the C-terminal of RHO molecule are deleted. The C-terminal tail of RHO is responsible for quenching of activated RHO during deactivation and recovery phase of phototransduction. The shutdown of phototransduction cascade occurs by phosphorylation of serine and threonine residues by rhodopsin kinase (GRK1) within the last 15 amino acids of the C-terminal tail. Subsequent binding of arrestin to phosphorylated RHO blocks further interaction of activated RHO with G protein-transducin (G-alfa) leading to the deactivation of the protein after light absorption (Green et al., 2000). Various studies in the past have revealed that light-dependent phosphorylation of RHO occurs at one of three serine (Ser^{334}, Ser^{338} and Ser^{343}) and one threonine (Thr^{336}) residues at the C-terminal segment (McDowell et al., 1993; Ohguro et al., 1993; Ohguro et al., 1995; Papac et al., 1993). Therefore, C-terminal truncation at residue 334 has very severe phenotype (Green et al., 2000; Liu et al., 1999). As such S334ter mutation is not found in humans, however, a similar mutation

Q334ter (last 5 amino acid chain deletion towards C- terminal) has been reported to cause a severe form of ADRP in humans in RP patients. S334ter and Q334ter mutations exhibit similar phenotypic effects as the RHO molecule due to the reason that both of them result in truncated RHO (Deretic et al., 1998; Green et al., 2000; Sung et al., 1994; Tam et al., 2006).

Secondly, C-terminus has been shown to be involved in the sorting of RHO from the photoreceptor IS to OS (Deretic et al., 1998). Other C-terminal mutations such as Gln344ter truncation mutations and Pro347Ser substitution mutation have also been shown to exhibit abnormal sorting of the protein. Failure of truncated S334ter RHO to sort properly to OS together with its prolonged activation has been postulated to cause cell death in RP (Alfinito and Townes-Anderson, 2002; Deretic, 1998; Deretic, 2000; Deretic, 2006; Deretic et al., 2005).

2.4.2 P23H mutation

It was the first mutation discovered in the RHO gene. P23H is a missense point mutation, which causes transversion of nucleotide C to A in the codon 23, consequently changing the codon CCC for proline (Pro) to CAC for histidine (His) (Dryja et al., 1990; http://www.sph.uth.tmc.edu/RetNet; Sung et al., 1991). Crystal structure of RHO shows the location of proline residue at 23rd position of opsin molecule is in the N-terminal intradiscal tail, which form the N-terminal plug of the molecule (Palczewski et al., 2000). The N-terminal plug is important for correct positioning and binding of 11-cis-retinal to retinal attachment site for the formation of fully functional P23H mutation could lead to N-terminal plug's misfolding, consequently, disabling the mutant P23H to bind correctly to the 11-cis-retinal (Kaushal et al., 1994; Lin and Lavail, 2010).

Studies on P23H mutations in the past have shown that this substitution mutation belongs to the class II RHO mutants that are characterized by protein misfolding and oligomeric aggregates formation retained in ER (Lin and Lavail, 2010; Sung et al., 1993). Additionally, P23H is not properly glycosylated as compared to wild-type RHO, and forms complexes with ER-resident chaperones (Anukanth and Khorana, 1994; Liu et al., 1996; Noorwez et al., 2004), as a result,

mutant P23H RHO stays in the ER and golgi instead of translocating to the photoreceptor OS (Frederick et al., 2001; Saliba et al., 2002; Tam et al., 2006). Therefore, it is postulated that the ER overload or other cellular stress pathways cause the photoreceptor cell death by evoking the UPR (unfolded protein response) signalling (Abdulaev et al., 1997; Griciuc et al., 2010; Lin and Lavail, 2010; Lin et al., 2007).

3. Animal Models of RP

Animal models showing disease conditions similar to human patients are precious research tools to investigate the mechanisms of disease pathology and to explore the treatment strategies. Since the functional and structural architecture of vertebrate retina is conserved across the species, it is easier to study animal models of retinal degenerations belonging to different species. Several animal models of inherited retinal diseases are available either occurring naturally or created transgenically, which are valuable research tools to understand the genetic and biochemical defects as well as to explore the treatment options for retinopathies (Baehr and Frederick, 2009; Chader, 2002; Dowling, 1977).

3.1 Advantages of Using Animal Models

Retinal degeneration research depends tremendously on the use of animal models as they provide a variety of candidate genes implicated in RP. Understanding of the mechanism by which a mutated gene's products or functions lead to the diseases condition paves the way to develop the therapy to partially or even completely cure the disease progression. Furthermore, pre-clinical safety in humans requires the identification of safe starting doses, selection of relevant parameters for clinical monitoring, and to the identification of the population that could be "at more risk" to exclude it from a clinical trial. All these concerns can be easily resolved by demonstrating the safety and efficacy of a therapy in animal species, before approaching human subjects for clinical trials (Flannery, 1999; Kohler et al., 1997; Rivas and Vecino, 2009).

3.2 Naturally occurring and Transgenic Animal Models of RP

Both naturally occurring and genetically engineered animal models of RP are available (Table 1.3). Naturally occurring models of RP represent vast number of different mutations implicated in RP (Baehr and Frederick, 2009) and phylogenetically they range from invertebrates to vertebrates (Chader, 2002). However, most of the natural animal models express recessive forms of RP. As a vast number of patients represent ADRP and lack of appropriate ADRP animal model becomes an obstacle to investigate the disease mechanism and develop the therapy for all sorts of RP. To this end, bioengineered animal models have been developed. Such animal models, better known as transgenic models are created by inserting the mutant transgenes in the genome of a wild animal that mimic the human disease patterns (Hong et al., 2000; Naash et al., 1993; Petters et al., 1997).

Table1.3: Various animal models of RP; *Source:* (Foundation Fighting Blindness; Rivas and Vecino, 2009).

Animal	Inheritance	Genetic Defect
Rodent Models (Natural and Transgenic)		
Rat Models (Natural)		
RCS	ARRP	Deletion in Mertk gene, the major expression site of which in rat retina is RPE
Rat Models (Transgenic)		
P23H	**ADRP**	**Mutant mouse opsin gene for P23H mutation**
S334ter	**ADRP**	**Mutant opsin carries stop codon at residue S334 resulting in lack of last 15 amino acids in RHO towards C-terminus.**
Mouse Models (Natural)		
Rd-1	ARRP	Mutation in β subunit of the rod PDE gene
Rd-4	ADRP	Gnb1 Gene mutation, which codes for the transducin ß-1-subunit (Tß1) protein
Rd-8	RP, LCA	Single base deletion in the Crb1 gene, resulting in truncation of the transmembrane and cytoplasmic domain of CRB1
Rd-10	ARRP	Mutation in β subunit of the rod PDE gene (Pde6ß *rd1*0)

Rd-12	ARRP, LCA	Nonsense mutation in the Rpe65 gene
Rd-16		Mutation in Centrosomal protein, CEP290, which is localized in the connecting cilium of photoreceptors
Peripherin/rds also rd2	ADRP	Frameshift mutation in Peripherin2 gene, resulting in 117C-terminal removal
Mouse Models (Transgenic)		
I-255/256	ADRP	RHO mutation (3bp deletion at codon 255/256 (Iso-leucine) located in 6th transmembrane domain
RPE65 KO		RPE65-/- mouse over accumulate retinyl esters in RPE and lacks 11 cis-retinal
P347S	ADRP	RHO C-terminal P347 mutation
Q344ter	ADRP	RHO C-terminal truncation of last five amino acids
Peripherin rds 307		Heterozygous and homozygous for codon 307 for Periperin
RHO KO		Non-functional RHO gene
VPP mouse	ADRP	Triple point mutations of RHO V20G, P23H, P27L at N-terminus
Canine Models (Natural)		
Cone degeneration (Cd)	ARRP	Disease mapped to chromosal region homologous to human 8q (homolog for human achromotopsia); Gene not identified yet
Progressive rod-cone degeneration (prcd)	ARRP	Disease has been mapped to CFA9, which is homologous to human 17q
Early retinal degeneration (Erd)	ARRP	Locus for Erd mapped to canine chr27 corresponding to HSA12p in humans. Canine SHARP1 and STK38L (NDR2) considered potential candidate genes
Rod-Cone dysplasia type 1 (rcd1)	ARRP	Mutation in cGMP-PDEβ
Rod-cone dysplasia type 2 (rcd2)	ARRP	Genetic defect unknown (Pathology similar to the Irish setter with elevated levels of cGMP, but PDE subunits are normal)
Rod-cone dysplasia type 3 (rcd3)	ARRP	Mutation in cGMP-PDEα
Photoreceptor dysplasia (Pd)	ARRP	Genetic defect unknown
X-linked progressive retinal atrophy (XLPRA)	X-linked recessive	Genetic defect unknown
Retinal Degeneration (RPE-65)	ARRP	Frameshift mutation in RPE-65 gene causing premature stop codon and truncation the protein

Feline Models (Natural)		
Abyssinian cat- rdAc	ARRP	SNP in an intronic region of CEP290 gene
Abyssinian cat- Rdy	ADRP	The gene Rdy has been adopted to name this cat model
Porcine Models (Transgenic)		
Pigs (P347L)	ADRP	Mutations P347L and P347S in RHO gene
Chicken Models (Natural)		
Chickens Rd	ARRP	Null mutation in GC1 gene causing low level of cGMP
Chicken Rdd (retinal dysplasia and degeneration)	ARRP	PDE6A mutations which cause ARRP in humans

3.3 Rhodopsin mutant rat models of RP

A large number of RHO mutant animal models belonging to different species are available with highly variable onset and progression of RP, and majority of them are transgenic. They have been created for the purpose of investigating the disease mechanisms and testing the therapeutic strategies (Lem et al., 1999; Lewin et al., 1998; Mendes et al., 2005).

The features which make the rats as ideal models to study the RP include: i) availability of ample knowledge about the basic cell and molecular biology of the normal rodent retina (particularly rats), which makes it easier to compare the diseased retina to healthy retina, ii) rat and human retina show a great degree of similarity in pattern of retinal disease, iii) larger size of the rat eye than that of a mouse simplifies the surgical manipulations, iv) fast breeding of rats provide a large number of litters in a short gestation period (Flannery, 1999; Rivas and Vecino, 2009).

Genetically engineered RHO mutant P23H and S334ter rat models have been created by Dr. Mathew LaVail and co-workers (University of California, San Fransisco, USA) that emulate the expression pattern of the retinal disease in humans. These rat models are valuable research tools to investigatethe possible disease mechanisms and development of therapy (Rivas and Vecino, 2009; Steinberg, 1996).

3.3.1 S334ter Transgenic Rats

In S334ter transgenic rats, the opsin transgene contains a termination codon at residue 334, resulting in the expression of truncated RHO lacking the last 15 amino acids at C-terminal, required for phosphorylation of photo-activated RHO. Five lines of S334ter transgenic rats have been created which differ in retinal degeneration rates, proportional to the transgene copy number and the resultant level of expression of the mutant mouse opsin (Lee et al., 2003). Line-3 of S334ter rats is fastest degenerating line (Liu et al., 1999). Heterozygous S334ter line-3 rats never develop rod outer segments (Li et al., 2010b).

3.3.2 P23H Transgenic Rats

P23H transgenic rats contain a mutant mouse opsin gene (transgene with histidine substitution at the proline 23 position), in addition to the endogenous normal opsin genes (Lewin et al., 1998; Machida et al., 2000). P23H rat retina shows photoreceptor degeneration course similar to human ADRP patients with P23H mutation (Berson et al., 2002). There are three lines of P23H transgenic rats differing in their rates of photoreceptor degeneration. Line-1 of P23H transgenic rats shows the fastest loss of photoreceptors, as there is approximately 40% cell loss in the ONL and 40% shortening in rod outer segments at 4 weeks of age. Overall 70% to 90% photoreceptors are lost by two months of age (Machida et al., 2000; Organisciak et al., 2003).

Chapter II. Mechanism of photoreceptor cell death in S334ter-3 and P23H-1 rat models of ADRP

1. Introduction

A thorough understanding of cell death and cell survival mechanisms is crucial for developing successful therapies aiming at preventing the diseases including RP. To this end, modulation of cell death mechanisms targeting specific key players of cell destruction could pave a way towards promising therapeutic approach. However, until now, the photoreceptor cell death mechanisms in inherited retinal degenerations are not completely understood (Sancho-Pelluz et al., 2008). Photoreceptors cell death in RP appears to be executed by common key players irrespective of primary genetic defect (Wong, 1994). The presumption that different genetic mutations could ultimately trigger similar cell death mechanisms in RP yields the possibility of treating RP caused by different genetic defects/mutations by using similar pharmacological interventions (Chang et al., 1993; Doonan et al., 2005; Marigo, 2007). Blocking the cell death by mutation independent approach refers to the modulation of cell death pathway in order to stop the disease manifestation by targeting common core regulators of cell death mechanisms.

1.1 Cell death mechanisms

Most of the past studies have frequently indicated the involvement of apoptotic pathways in neuronal cell death (Leist and Jaattela, 2001). Apoptosis is a form of programmed cell death (PCD), which is crucial event during the development, immunity and homeostasis of organisms (Galluzzi et al., 2007). However, despite being important and beneficial as developmental cell death, apoptosis has been implicated in various forms of the neurodegenerative diseases including RP (Jimenez del Rio and Velez-Pardo, 2001; Offen et al., 2000; Tatton et al., 1997). Previous studies on photoreceptor degeneration suggested the caspase-mediated apoptosis as the final common pathway in retinal degeneration (Chang et al., 1993). Nevertheless, recent studies conducted on PDE6 mutant *rd1* mouse model of RP, point towards the involvement of other alternative caspase-independent, and hence, non-apoptotic routes to the photoreceptor cell demise (Paquet-Durand et al., 2007; Sancho-Pelluz et al., 2008) (Lohr et al., 2006; Rohrer et al., 2004).

Obviously, greater knowledge about these neurodegenerative mechanisms induced by different mutations is crucial for developing the rational therapy.

1.1.1 Apoptotic Cell Death Mechanism

Apoptosis affects isolated dysfunctional cells, which actively mediate their own demise through intrinsically stimulated mechanisms without damaging adjacent healthy neighbouring cells (Kerr et al., 1972; Leist and Jaattela, 2001; Lockshin and Zakeri, 2004a). Apoptotic cells present certain morphological features like cell shrinkage, membrane blebbing, disruption of mitochondrial function, chromatin condensation, and fragmentation into 'apoptotic bodies' that are engulfed by macrophages without triggering inflammatory response (Saraste and Pulkki, 2000). All of these processes are usually mediated by caspases, therefore, caspase activity is considered central to apoptotic cell death (Nicotera, 2002; Samali et al., 1999; Zeiss, 2003). Nevertheless, caspase-independent apoptosis has also been observed in neuronal and photoreceptor cell death models (Borner and Monney, 1999; Carmody and Cotter, 2000; Donovan and Cotter, 2002; Okuno et al., 1998; Selznick et al., 2000).

Caspases are a family of cysteine-aspartyl-specific proteases comprising at least 14 members, which are synthesized as inactive precursors and get activated during apotosis by undergoing proteolytic cleavage/processing (Cryns and Yuan, 1998; Degterev et al., 2003; Earnshaw, 1999; Nicholson, 1999; Thornberry and Lazebnik, 1998). Caspases can be divided into two sub-groups; firstly, caspase-1 like proteases that help in cytokine processing and secondly, all the other caspases that participate in cell death (Creagh et al., 2003). Caspases mediating the cell death may be further divided into two categories: activators/initiators (Caspases-8,-10 and -12) and executioners/effectors (caspase-3,-6,-7) of cell death. In response to the death stimuli, the activator caspases are activated, which subsequently activate the executioner/effector caspases (Slee et al., 1999a). Once activated, caspases are able to proteolyse additional caspases which degrade crucial structural components and regulatory proteins required for cell survival in a highly orchestered manner and (Wolf and Green, 1999). Activation of caspase-3 results in degradation of many important

cellular proteins (Slee et al., 2001).

Underlying pathways responsible for caspase activation may be extrinsic or intrinsic. Extrinsic pathway also called “the receptor-mediated pathway” mediates via ligand binding to cell surface death receptors (e.g., Fas and tumor necrosis factor receptor1 (TNFR1), receptor oligomerisation followed by the recruitment and activation of caspase-8 (Ashkenazi and Dixit, 1998; Muzio et al., 1996). In the intrinsic or mitochondria-initiated pathway, caspase activation is initiated by the release of cytochrome-c (cyto c) from the mitochondrial intermembrane space. Cyto-c attaches to Apaf-1 (apoptosis activating factor-1) in ATP dependent manner in order to form cyto-c/apaf-1 complex, which activates caspase-9. Activated caspase-9 in turn cleaves and activates the executioner caspases-3 and -7 (Green and Amarante-Mendes, 1998; Kroemer and Reed, 2000; Slee et al., 1999b). Importantly, the initial event in this pathway remains mitochondrial outer membrane pemeabilization (MOMP), which can be triggered by various death stimuli and help to release cyto-c and other proapoptotic factors. MOMP is controlled by Bcl2 family of proteins, which has both pro- as well as anti-apoptotic members (Gross et al., 1999; van Loo et al., 2002).

Existence of different pathways for the activation of executor caspases shows the complexity of apoptotic cell death pathway. Despite a defined mechanism and the fact that this mode of cell death has been repeatedly implicated in neurodegenerative diseases, the attempts to prevent the cell death by using caspase inhibitors have not always given the desired outcomes, which indicates the need to explore alternate cell death pathways that might be operational during cell death possibly independent of caspases or conventional apoptotic markers. The question as to why have these alternative cell death pathways attracted less attention than caspases-mediated program of cell death lies in the fact that possibly several types of proteases/proteins are activated during cell death, and impact of some may dominate the others, which makes some of the biochemical changes more difficult to detect.

1.1.2 Non-Apoptotic Cell death Pathway involving Calpain and PARP

In addition to the conventionally defined cell-death mechanisms, other forms of cell death programs have emerged recently in neurodegenerative and particularly retinal degenerations, which are unique in the sense that they use common death machinery in specially defined programs. In fact, in the recent past a number of studies have shown the involvement of calcium sensitive calpain proteases (Paquet-Durand et al., 2006; Trifunovic et al., 2010) and Poly(ADP-ribose) polymerase 1 (PARP-1) in the cell death in many neurodegenerations as well as in retinal degenerations (Paquet-Durand et al., 2007; Sahaboglu et al., 2010).

1.1.2.1 Calpain in neuronal and inherited Retinal Degeneration

High levels of Ca^{2+} have often been suggested to be a crucial factor in neuronal and photoreceptor cell death (Fox et al., 1999; Kristian and Siesjo, 1998; Nicotera and Orrenius, 1992; Takano et al., 2005). Also, over activation of Ca^{2+}-dependent enzymes (*i.e.*, calpain proteases) has been linked to the degeneration of photoreceptors in inherited as well as non-inherited models of RP (Azarian and Williams, 1995; Azuma et al., 2004; Azuma and Shearer, 2008; Paquet-Durand et al., 2006).

Calpain (EC 3.4.22.17) proteases are a family of calcium-activated non-lysosomal cysteine proteases, which are expressed in all cells of the body along-with their endogenous inhibitor, calpastatin (Goll et al., 2003; Hood et al., 2004; Suzuki et al., 2004). With 15 known isoforms, calpains exist as dimers of 80kDa “catalytic” subunit and 30kDa “regulatory” subunit. There are two major calpain isoforms reported in mammals, calpain-1 (μ-form) and calpain-2 (m-form), and both of them are expressed in all tissues of the body. They differ in requirement of Ca^{2+} for their activation (~50 μM for calpain-1 and ~500 μM for calpain-2) and possess several calcium-binding sites, which affects their enzymatic activity allosterically (Goll et al., 2003). Subunit 80-kDa is unique for each isoform, whereas the 30 kDa is common to both calpains 1 and 2. Calpain-3, another isoform was first described as a skeletal muscle-specific calpain isoform. However, subsequent studies showed its location in several other tissues also including retina (Azuma and Shearer, 2008).

Substrates of calpains within the cells include a wide variety of proteins including regulatory, cytoskeletal and receptor proteins, transcription factors and signal transduction enzymes. Additionally, calpains can also interfere with the proteolytic activities of caspases (Chan and Mattson, 1999; Croall and DeMartino, 1991). Generally, calpain proteases do not act as harmful enzymes. As their activity is dependent on intracellular Ca^{2+} levels, therefore, at physiologically normal calcium levels (<0.05 µM), calpains act as biomodulators of calcium-regulated processes like signal transduction, cell proliferation, cell cycle progression, arrestin processing and platelet activation (Azuma and Shearer, 2008; Goll et al., 2003). However, elevated levels of cellular Ca^{2+} lead to their persistent and pathologic over-activation, which has been implicated in various neurodegenerative pathologies (such as Parkinson, Huntington and Alzheimer diseases) including the retinal dystrophies (Doonan et al., 2005; Dufty et al., 2007; Gomez-Vicente et al., 2005; Grynspan et al., 1997; Kim and Kim, 2001; Nixon et al., 1994; Vosler et al., 2008). High Ca^{2+} levels and dissociation of calpastatin from calpains causes neuronal cell death by the cleavage of neuronal substrates leading to inhibition of essential neuronal survival mechanisms (Hood et al., 2004; Suzuki et al., 2004; Vosler et al., 2008). A down-regulation of calpastatin has been shown to be related to neurodegeneration including retinal degeneration (Paquet-Durand et al., 2006), whereas, the over-expression of calpastatin correlates to decreased neuronal cell death (Camins et al., 2006; Higuchi et al., 2005; Wingrave et al., 2004). Conversely, various studies conducted on animal models of inherited retinal degeneration have shown the association of hyper-activated calpains with photoreceptor cell death (Doonan et al., 2005; Gomez-Vicente et al., 2005; Paquet-Durand et al., 2006; Sanges et al., 2006; Trifunovic et al., 2010) and ganglion cell degeneration (Araujo et al., 2004; Das et al., 2006; Oka et al., 2006).

1.1.2.2 PARP (Poly (ADP-ribose) Polymerase) in neuronal and inherited retinal degeneration

PARP-1 (EC 2.4.2.30, 116kDa) is the prototypic member of PARP (Poly-(ADP-ribose) polymerase) family of enzymes, which are mainly nuclear, and play very important role in DNA repair processes (Hassa and Hottiger, 2008). PARP-1 gets activated in response to DNA damage particularly single strand breaks, which

might develop as a result of pathological signals and/or oxidative stress (Jagtap and Szabo, 2005; Skaper, 2003). To repair damaged DNA, PARP-1 uses β-NAD+ to generate poly-ADP-ribose (PAR) polymers onto itself or other acceptor proteins, e.g., histones or other DNA repair proteins, at the expense of ATP consumption (Herceg and Wang, 2001; Jagtap and Szabo, 2005).

Although the basal activity of PARP-1 enzyme is beneficial for maintenance of genomic integrity, a hyper-activity of the same in response to excessive DNA damge could lead to cell death (Boulu et al., 2001; Koh et al., 2005; Paquet-Durand et al., 2007). Thus, PARP transforms DNA damage into intracellular signals, which can either activate DNA repair processes or can lead to cell death routes (Decker and Muller, 2002). It has been suggested by various studies that PARP over activation can induce cell death either by AIF release from mitochondria or simply due to neurotoxicity caused by PAR accumulation (Wang et al., 2009). Since, a large proportion of cellular NAD^+, the substrate for PARP activity lies within the mitochondria (Rossi et al., 2009), and over-activation of PARP-1 in the nucleus act as a nuclear signal that reaches to mitochondria, where NAD consumption, coupled with ATP depletion causes the energetic instability within the cell that in turn causes increased oxidative stress. One route to cell death *via* PARP mediation could be through all these events as they cause mitochondrial membrane depolarization and open the mitochondrial permeability transition pores (MPTP) (Du et al., 2003; Sims et al., 1983). Opened MPTP may in turn lead to a leakage of mitochondrial proteins (e.g., AIF) into the cytosol, which might further translocate to the nucleus, and induce peripheral chromatin condensation and large-scale fragmentation of DNA (Boujrad et al., 2007; Hong et al., 2004) (Wang et al., 2009). Excessive generation of PAR might be the alternative route to death when PARP activity exceeds its basal limits (Andrabi et al., 2008).

The importance of PARP1 hyperactivity and its correlation to photoreceptor cell death has been accomplished in *rd1* and PARP1 KO mouse models. The *rd1* photoreceptors showed an increase activity of PARP1, which was shown to decrease after PARP inhibition (Paquet-Durand et al., 2007). Corroborating these findings, cGMP-induced photoreceptor death was shown to decrease strongly in

PARP-1 KO retina as compared to wild type, suggesting that PARP-1 plays a key role in photoreceptor death (Sahaboglu et al., 2010).

Additionally, high oxidative stress has been reported to be a causative factor of cell death in certain neurodegenerative conditions (Marcum et al., 2005; Pignol et al., 2006). Also in inherited photoreceptor degeneration, oxidative stress has been shown to be related to photoreceptor cell death in the *rd1* mouse model of RP (Komeima et al., 2006), and use of antioxidants has been reported to be useful in such cases (Komeima et al., 2006; Sasaki et al., 2009).

The present study was designed on the hypothesis that RHO mutant S334ter and P23H rat retinas might show an up-regulation of these proteins during the course of photoreceptor degeneration, as these mutants have reportedly slow recovery during phototransduction, which may cause calcium ion imbalance in the cells. Therefore, high calcium level related processes such as activation of calpains, oxidative stress and PARP activation might be involved in the photoreceptor cell death in these mutants of RHO. Moreover, a similarity of cell death pathway in different mutations might prove very useful while developing a mutation independent therapy.

2. Aim

To examine PARP and calpain expression and activity alongwith the measurement of oxidative DNA damage in order to investigate the possible mechanisms involved in the photoreceptor cell death in the S334ter and P23H rat models of RP.

3. Results

3.1 Cell Death Markers

3.1.1 General Morphology – TUNEL staining

Analysis of P23H and the S334ter retinal cross-sections during the first postnatal (PN) month showed a progressive reduction in outer nuclear layer (ONL) thickness compared to wild type CD rats as described previously (Liu et al., 1999; Machida et al., 2000). TUNEL assay was used to identify the photoreceptors undergoing cell death, which showed cell death starting before PN10 in both the mutants,

reaching to peak at PN12 and PN15 in S334ter and P23H retinas respectively, followed by regression in cell death (Fig 1.2). In S334ter rat retinas, a significant elevation of photoreceptor cell death was evident as early as PN8, however, the highest percentage of TUNEL-positive ONL cells was found at PN12 (S334ter: 6.1 % ± 1.1 SD, n=3; CD: 0.02 % ± 0.01 SD, n=3, p<0.001) (Fig. 2.2 A1 and A2). At PN15, the number of dying cells in ONL, still significantly higher than CD, was reduced as by then most of the photoreceptors had already disappeared (S334ter PN15: 2.2 % ± 0.8 SD, n=3, p<0.001). In case of P23H retina, high number of TUNEL positive cells were seen as compared to age matched CD retina during the first postnatal month. Peak of cell death was seen at PN15 (P23H: 2.7 % ± 0.8 SD, n=3; CD: 0.02 % ± 0.01 CD, n=3, p<0.001) (Fig. 2.2 B1 and B2). In wild type (wt) CD controls, TUNEL-positive cells were detected only very occasionally in the ONL (Fig. 2.2). Overall, TUNEL positive cells remained detectable as late as PN30 in both mutants.

Quantification of photoreceptor cell death during the first postnatal month showed that TUNEL-positive cells were significantly elevated from PN10 onwards, increased in number until PN12 and decreased subsequently in S334ter retinas (Fig. 2.1A). In P23H rat retina, TUNEL stained dying cells were detectable as early as PN10, showing a peak of cell death at PN15 (Fig. 2.1B). For a detailed further analysis of other cell death markers, PN ages showing the peak of cell death were chosen, i.e. PN12 for S334ter and PN15 for P23H mutants respectively.

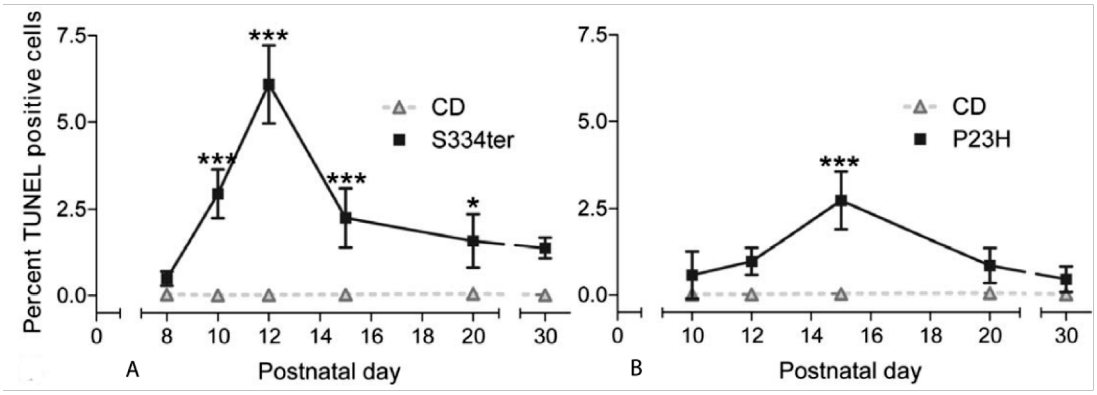

Figure 2.1 Quantification of photoreceptor cell death during the first postnatal month: (A) In S334ter, TUNEL-positive cells were significantly elevated from PN10 on, increased in number until PN12 and decreased subsequently. (B) Increased numbers of

TUNEL stained, dying cells in P23H rats, showing a peak of cell death at PN15. Values are indicated as Means and SD and representatve of three different animals; * p <0.05; ** p <0.01; *** p <0.001 ***(Kaur et al., 2011: PLoS One)***.

3.1.2 Apoptotic Cell death markers

In an attempt to identify the photoreceptor cell death pathway potentially involved in photoreceptor degeneration, first of all, investigation about involvement of classical apoptotic markers was done in S334ter-3 and P23H-1 rat retinas. The apoptotic cell death markers caspase-3, caspase-9 and cyto-c were studied by immunostaining method.

3.1.2.1 Activation of caspase-3

Since, cleavage of caspase-3 is the hallmark feature of apoptotic mode of cell death, therefore, immunostaining was performed for cleaved caspase-3 (cc-3) to study its possible involvement in photoreceptor cell death. Immunolabeling revealed activated caspase-3 immunoreactivity was significantly increased in in S334ter (S334ter PN12: 5.1 % ± 0.1 SD, n=3) compared to wt CD or P23H (CD PN12: 0.01 % ± 0.00 SD, n=3, p<0.001; P23H PN12: 0.12 % ± 0.06 SD, n=3, p>0.05) (Fig. 2.2C1-C2; Table 2.1) as described earlier (Liu *et al.* 1999b). On the other hand however, very few positive cells were seen in the ONL of P23H rat retina, (0.1 % ± 0.04 SD, n=3) and wild-type (wt) CD retinas at PN15 (CD: 0.03 % ± 0.02 SD, n=4, p>0.05) (Fig. 2.2 D1-D2; Table 2.1).

3.1.2.2 Caspase-9

To investigate further the involvement of other classical apoptotic markers of 'mitochondrial pathway of caspase activation' during photoreceptor degeneration in S334ter and P23H retinas, immunostaining for caspase-9 cleaved at Asp353 was performed. Caspase-9 immunostaining labelled a relatively large number of photoreceptors in S334ter retina (Fig 2.2 E1-E2), but showed no labelling in P23H and wt control rat retinas (Fig 2.2 F1-F2).

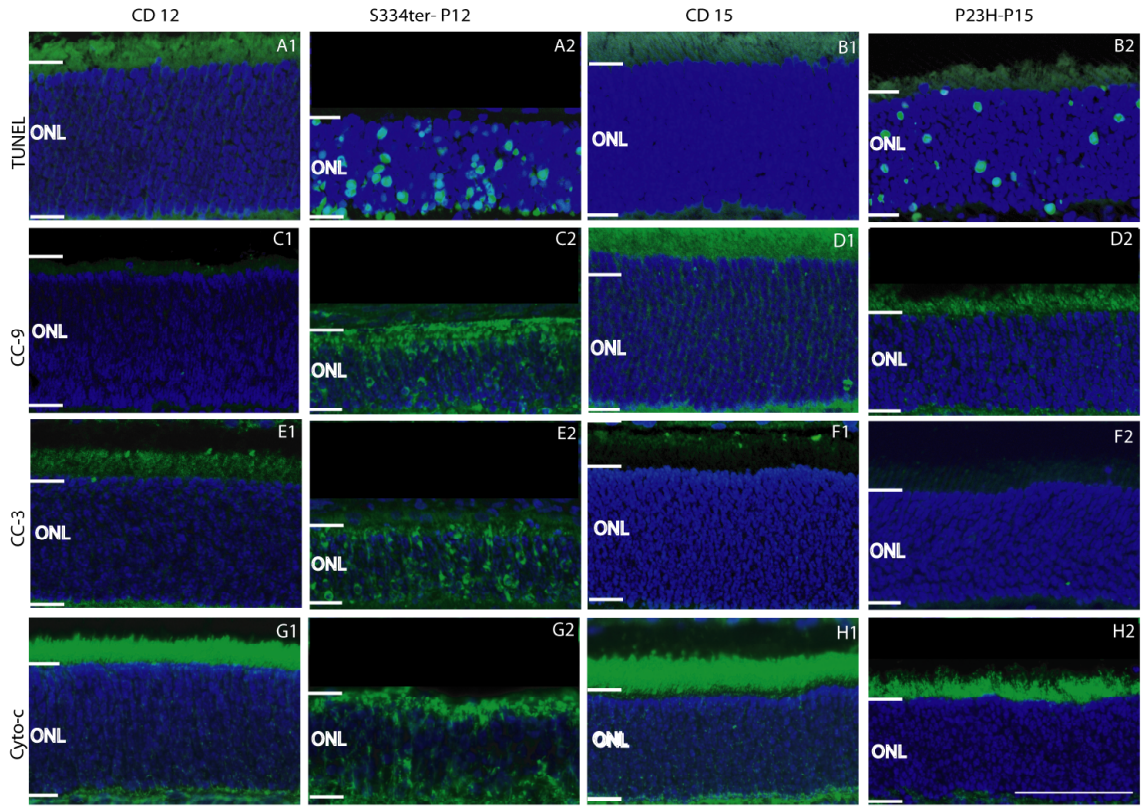

Figure 2.2 Differential regulation of apoptotic cell death markers in (1) wt (CD) and (2) rhodopsin transgenic rats. (A–B) TUNEL assay for dying cells, (C–D) caspase-9 immunostaining, (E–F) Caspase-3 immunostaining, (G–H)Cytochrome-c immunostaining, TUNEL staining is evident in both S334ter (A2) and P23H (B2). All other markers(cc-9, cc-3, cyto c) clearly upregulated in S334ter (panels (C, E, G) compared to age matched wt CD rats. P23H shows only very occasional staining for cc-9, cc-3, and cyto c (panel D,F,H), **Abbreviations**: cc-9: cleaved caspase-9, cc-3: cleaved caspase-3: cyto-c: cytochrome c), (**Labels**: Blue; DAPI nuclear staining, Green; TUNEL, cc-9, cc3, cyto.c), Scale bar = 50 µm ***(Kaur et al., 2011: PLoS One)***.

3.1.2.3 Cytochrome-c leakage

Cytochrome-c (cyto-c) acts upstream caspase-9 during activation of caspases. Immunostaining for cyto-c showed positive staining in the photoreceptors of S334ter only. Since normally cyto-c is localised inside the mitochondia, which are present in the inner segments, therefore, wild type rat retina showed inner segments staining against cyto-c bodies both at PN12 and PN15 (Fig. 2.2 G1, H1). However, in apoptotic condition, cyto-c leaks to the cytoplasm from mitochondrial intermembrane space. Accordingly, a number of photoreceptors (cell bodies) labelled by cyto-c were seen in S334ter retina at PN12 (Fig. 2.2 G2). However, no cyto-c positve cells were seen in P23H retinas except for inner segment staining (Fig. 2.2 H2).

3.1.3 Non-Apoptotic Cell death Markers

After observing classical apoptotic cell death markers (caspase-3, caspase-9 and cyto-c) only in S334ter retina, and their evident lack of involvement in P23H retinal degeneration, study was conducted to look the alternative non-apoptotic possible cell death route in the P23H mutant retina. Additionally, as caspase-3 dependent cell death and partial rescue of retina in S334ter rat was reported already (Liu et al., 1999), the study was extended to look for other non-apoptotic cell death markers in S334ter retina as well. For this, calpain and PARP expression and activity was checked in both transgenic rat retinas.

3.1.3.1 Expression and activity of calpains

Activation of ubiquitously expressed calpain-type proteases has been often connected to alternative cell death mechanisms (Vosler et al., 2008; Vosler and Chen, 2009). To study a potential participation of calpains in rat retinal degeneration, expression of the three major calpain isoforms, calpains 1, 2 and 3 was investigated by means of immnostaining and western blotting. Additionally, an *in-situ* enzymatic assay was used to study calpain activity directly at the cellular level (Paquet-Durand et al., 2006).

3.1.3.1.1 Calpain Expression

Expression pattern for calpain isoforms 1 and 2 was not seen to differ in transgenic retinas as compared to the wild type CD retina. Neither immunostaining nor WB analysis showed any marked differences between S334ter or P23H mutants and their corresponding wild-type controls. Immunostaining showed that in transgenic as well as CD retinas, calpains-1 and -2 were evenly distributed throughout all retinal layers as described previously (data not shown for isoform 1 and 2 of calpains). Interestingly, calpain-3 immunolabelling in mutant rats was seen to be more intense, with S334ter retina showing the strongest labelling (Fig. 2.3). Although calpain-3 immunostaining was also present in the entire retina, with a more intense labelling observed in a subpopulation of INL and GCL cells and in sublayers in the IPL in both wild type CD and mutant rats, however the staining

was stronger in both the mutants (Fig. 2.3 A2-B2). This staining showed the outline of photoreceptor cells, reflecting a membranous distribution of calpain-3. Nevertheless, WB for calpain-3 failed to detect a significant up-regulation in mutant retina.

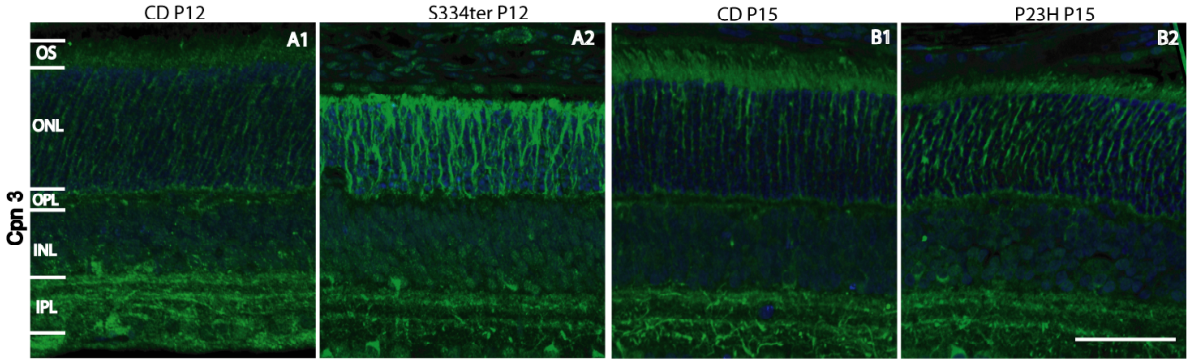

Figure 2.3 Calpain expression in rhodosin mutant rat retinas. Calpain-3 (cpn3) expression is clearly increased in mutants, specially in S334ter (A2), as all the layers of retina show stronger labelling of cpn3 in mutants (A2,B2), although wt (CD) retina also shows a weaker staining for cpn3 in all layers (A1, B1) (**Labels**: Blue; DAPI nuclear staining, Green; calpain3), Scale bar=50 µm ***(Kaur et al., 2011, PLoS One)***.

3.1.3.1.2 Calpain Activity

Since elevated calpain activity would not necessarily require its increased expression, therefore, the calpain activity was examined at cellular level by using an *in-situ* enzymatic assay for calpain proteolysis. In both mutant retinas, numerous photoreceptors in the ONL were brightly labelled, whereas in wild-type animals very few positive cells were detected (Fig. 2.4,A-B). S334ter retina showed comparatively higher number of calpain positive cells (Fig. 2.4, A1-A2) as compared to the P23H retina (Fig. 2.4, B1-B2). Comparison at various postnatal ages showed that in S334ter animals, calpain activity appeared early in the development, already at PN8 and increased progressively reaching a peak value at PN12 (5.2 % ± 0.8 SD, n=4; CD: 0.1 % ± 0.02 SD, n=3, $p<0.001$) and then showing regression with increased postnatal age. In P23H rats, increase in photoreceptor calpain activity was first detected at PN10 that became statistically significant at PN15 (2.3 % ± 0.4 SD, n=4; CD: 0.04 % ± 0.02 SD, n=3, $p<0.001$), with a later decrease at PN30 (Table 2.1 and Fig. 2.8).

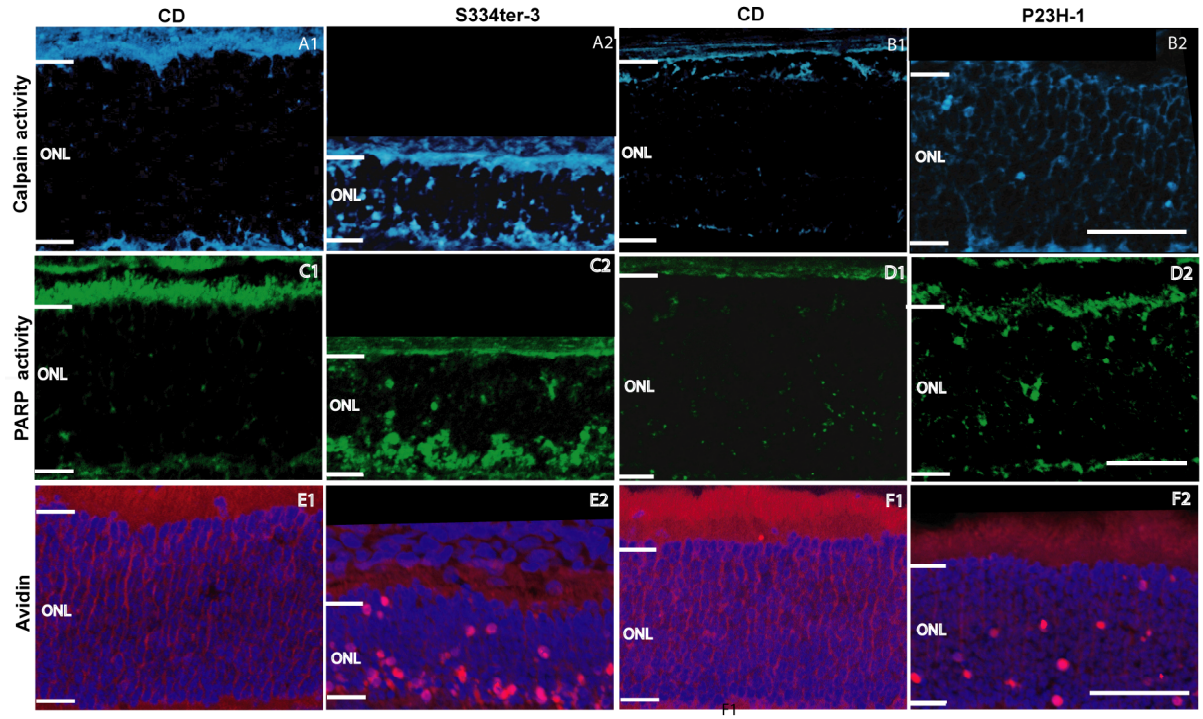

Figure 2.4 Differential regulations of caspase independent cell death markers in wild type and rhodopsin transgenic rats. Calpain *in-situ* actvity assay, (A-B), PARP *in-situ* activity assay (C-D), avidin staining (E-F). Calpain activity, PARP activity and avidin staining is upregulated in mutants in comarison to age matched wild type (CD) retinas. However, number of photoreceptors positive for all these markers was more in S334ter (A2, C2, E2) as compared to P23H (B2, D2, F2). Scale bar = 50µm ***(Kaur et al., 2011: PLoS One)***.

3.1.3.2 Calpastatin expression

As the activity of calpains is regulated by its endogenous inhibitor calpastatin (Maki et al., 1987), therefore WB and immunostaining was performed to assess its expression in the S334ter and P23H retinas. Although, predicted molecular weight of calpastatin is ~77 kDa, however, calpastatin WB is known to produce several bands with apparent molecular weights ranging from 17 to 110 kDa and may show considerable variation between different tissues and species (Croall and DeMartino, 1991; Goll et al., 2003; Hood et al., 2004). Four major bands corresponding to 52, 60, 65, and 76 kDa were identified for calpastatin WB of rat retinas (Fig. 2.5A). Quantification was performed for two major calpastatin bands of 76 kDa and 52 kDa of mutant retinas in comparison with CD retina, which showed a statistically significant decrease for both bands in P23H retina (76 kDa: $p<0.05$, 52kDa: $p<0.01$, n=3) (Fig. 2.5B), and for the 52 kDa band in S334ter retina (76 kDa: $p>0.05$, 52 kDa: $p<0.05$, n=3) (Fig. 2.5C).

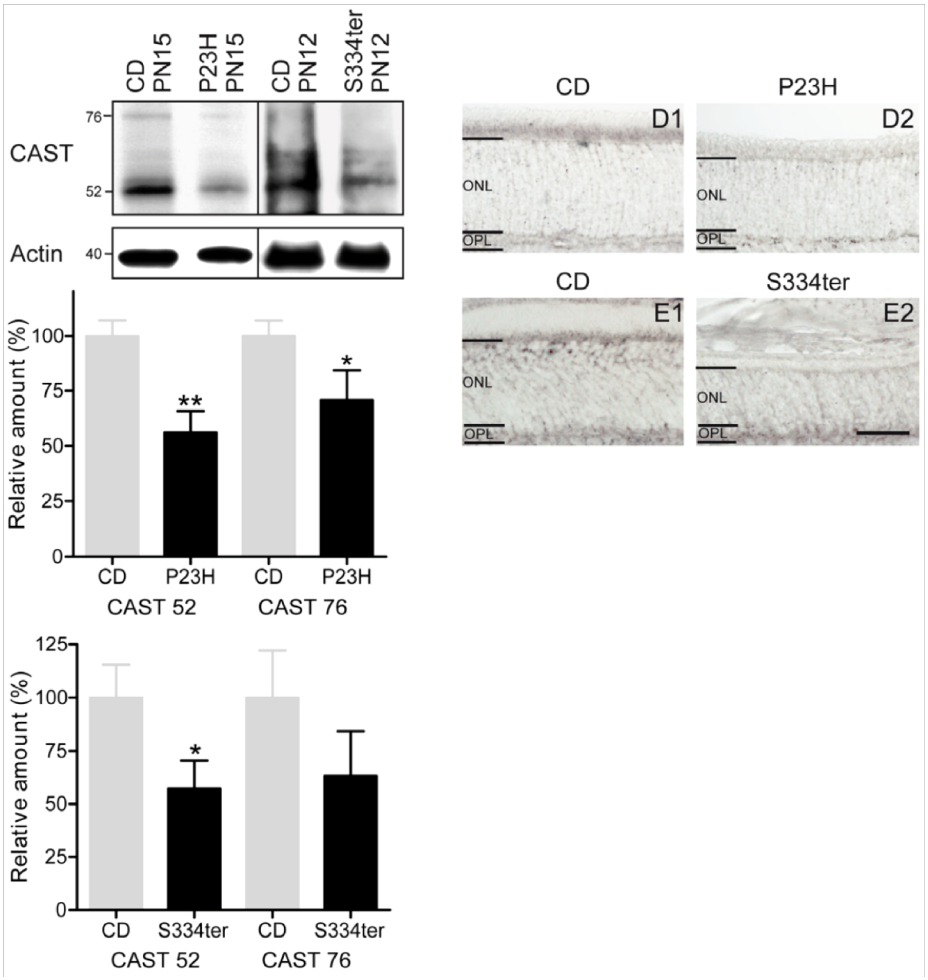

Figure 2.5 Expression of calpastatin in Rhodopsin transgenic rat retinas (A) Immunoblotting for calpastatin showed decreased levels in both, P23H and S334ter rats. (B–C) Quantification of the main calpastatin bands at 76 and 52 kDa showed decrease expression in both mutant retinas. This decrease in expression was statistically significant for both bands in (B) P23H retina and (C) for the 52 kDa band in S334ter retina. Values are mean ±SD from three different experiments each containing retinas from 6 animals. (D–E) Calpastatin staining was less intense in inner segments in both RHO transgenic rats * *p*,0.05; ** *p*,0.01., Scale bar = 50 µm ***(Kaur et al., 2011: PLoS One)***.

Simultaneously, calpastatin immunostaining was performed to test whether the observed decrease in expression at the tissue level was localized to photoreceptors. As described in other species (Paquet-Durand et al., 2006; Persson et al., 1993), in wild-type retinas, calpastatin antibody showed a weak labelling of most parts of the retina including cellular (ONL and INL) and synaptic layers (OPL and IPL), and more strongly labelled photoreceptor inner segments. In both transgenic rats, calpastatin staining was less intense, especially in the inner segments, confirming the WB results (Fig. 2.5, D-E).

3.1.3.3 PARP and PAR

To investigate the involvement of PARP as a potential cell death marker in RHO transgenic rats, two different approaches were used. Firstly, PARP activity at cellular level was examined using an *in-situ* enzyme activity assay that detects the incorporation of biotin labelled NAD^+ (Paquet-Durand et al., 2007). Only negligible number of PARP activity labelled photoreceptor cells were detected in wild type CD retina, while in P23H (PN15; 0.8 % ± 0.3 SD, n=5; CD: 0.01 % ± 0.003 SD, n=3, $p<0.001$) and especially in S334ter rats (PN12; 1.6 % ± 0.5 SD, n=3; CD: 0.004 % ± 0.004 SD, n=3, $p<0.001$), many photoreceptor nuclei were labelled (Fig. 2.4, C-D). Quantification of PARP positive cells at various PN ages showed more PARP positive cells in mutants as compared to wild type CD retinas (Table2.1 and Fig. 2.8).

Secondly, WB was performed to assess the expression level of PARP-1 protein, which identified the characteristic 116kDa PARP-1 band. More intense band was observed in mutants. PARP-1 knock out negative control did not show this 116kDa band (Fig. 2.6C). Quantification of WB bands showed a statistically significant increase of PARP-1 in S334ter. In P23H, although the band was more intense in comparison to CD, however, it did not show significantly increased protein expression (P23H: $p>0.05$, n=5; S334ter: $p<0.0001$, n=4) (Fig. 2.6D). As PARP activity generates PAR polymers, therefore, PAR immunostaining and WB was performed to its levels in retinal tissues to indirectly confirm the PARP activity. In line with PARP activity assay results, PAR immunohistochemistry showed numerous PAR positive cells in transgenic rat ONL (Fig.2.6A-B) (P23H PN15: 1.2 % ± 0.6 SD, n=3, $p<0.01$; S334ter PN12: 2.9 % ± 1.2 SD, n=3, $p<0.001$). S334ter retina again showed the highest numbers of positive cells characteristically clustered towards OPL. Only very occasional PAR-positive cells were detected in wild type CD retina (CD PN15: 0.0 % ± 0.0 SD, n=3; CD PN12: 0.01 % ± 0.01 SD, n=3) (Table 2.1).

PAR WB on retinal samples from P23H, S334ter and wt CD rat retinas showed a strong labelling corresponding to molecular weights ranging from 100 – 240 kDa (Fig. 2.6C). Interestingly, in *wt* CD retina, the poly-ADP-ribosylation of high molecular weight proteins appeared to be decreasing with post-natal age.

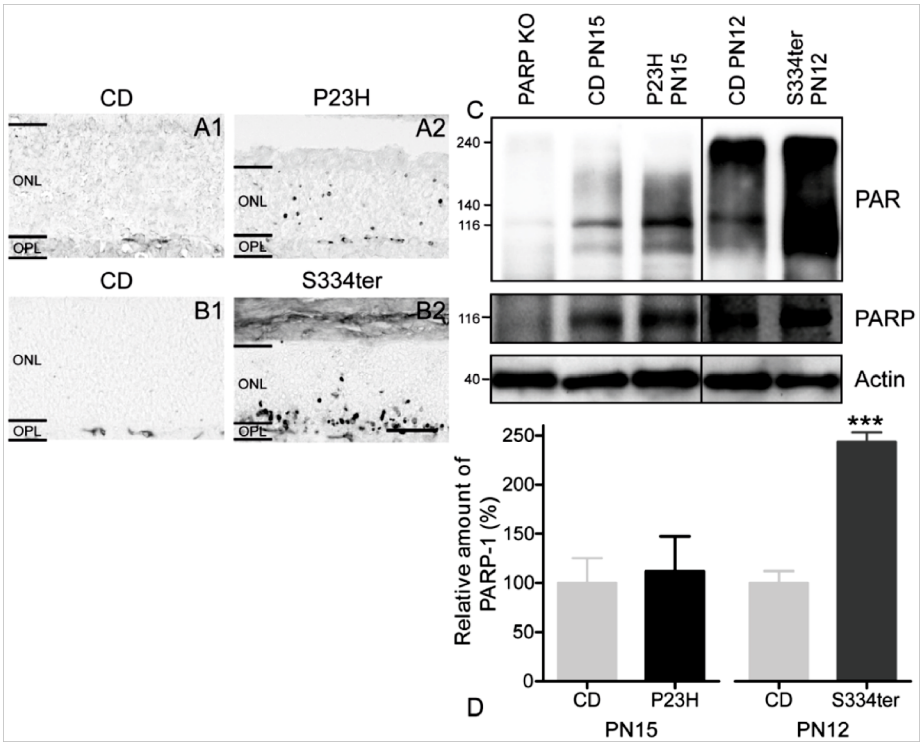

Figure 2.6 PAR accumulation and PARP-1 expression in Rhodopsin transgenic rats. (A–B). Accumulation of PAR was found in many cells in P23H and especially in S334ter retinas. (C) PAR WB identified a band at 116 kDa, which most likely represented PARP-1 itself and additionally strong labelling of proteins ranging from 100 to 240 kDa, suggesting poly(ADP-ribosyl)ation. Retinal PAR levels were increased in both transgenic animals. (C–D) PARP-1 protein expression was similar in wt and P23H retina, but significantly increased in S334ter. PARP-1 knock-out retina was used as negative control. Values are mean ± SD from four different experiments each containing retinas from 6 animals. ****p*,0.001. Scale bar = 50 µm ***(Kaur et al., 2011: PLoS One)***.

WB also recognized an approximately 116 kDa band which was absent in PARP-1 knock-out mouse samples, suggesting that this band likely indicated poly(ADP-ribosyl)ation of PARP-1 (Mendoza-Alvarez and Alvarez-Gonzalez, 1993).

3.1.3.4 Oxidative DNA damage

Cellular oxidative DNA damage was examined by staining with fluorescently conjugated avidin. Many avidin-positive cells were observed in the ONL of both RHO mutants, with PN12 S334ter retina showing the highest levels of oxidative stress (S334ter PN12: 3.2 % ± 0.8 SD, n=3, $p<0.001$; P23H PN15: 0.9 % ± 0.2 SD, n=3, $p<0.001$) (Table 2.1). In *wt*, avidin-positive cells were observed only very occasionally (CD PN15: 0.01% ±0.01 SD, n=3; PN12: 0.001% ±0.002 SD, n=3) (Fig. 2.3 G-H).

3.1.3.5 Differential co-localization of TUNEL with other metabolic markers

To determine the percentage of dying cells labelled for the different biochemical markers, co-labelling with TUNEL assay (as an indicator towards final stages of cell death) was performed. In both mutants, calpain and PARP activity co-localized to a large extent with TUNEL staining (~30-40 %), while avidin labeling for oxidative DNA damage co-localized only in 12-13 % of ONL cells (Fig. 2.7, A-C; E-G). Interestingly, caspase-3 co-localization was observed in almost 47 % of TUNEL-positive cells in S334ter animals, while in P23H retina, it occurred only in 4 % of TUNEL-positive cells (Fig. 2.7, D, H). These results suggest that photoreceptor cell death in both mutants was highly dependent on calpain and PARP activity, with an additional involvement of caspase activity in S334ter, but not in P23H retina.

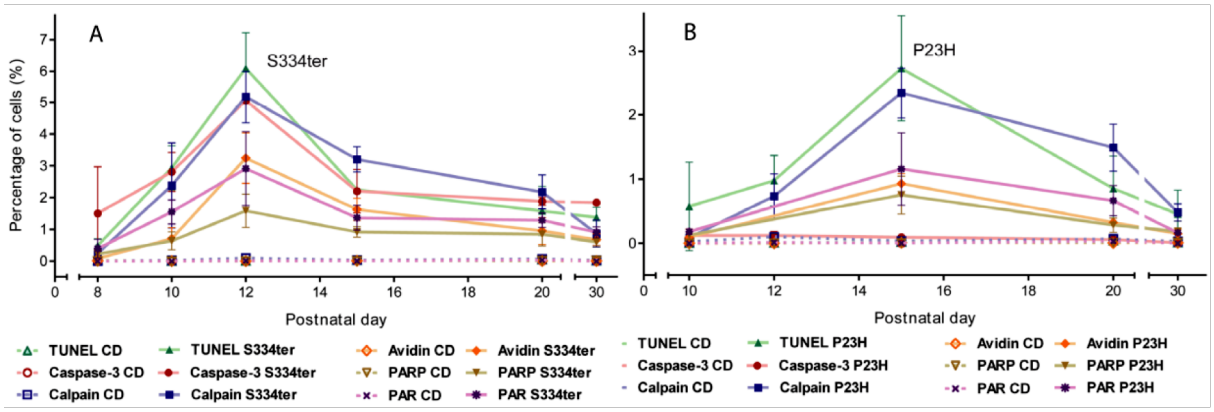

Figure 2.7 Progression of metabolic cell death markers during 1st postnatal month. Percentage of labelled ONL cells in (A) S334terand (B) P23Htransgenic rats. In both RP animal models, all markers analysed peaked together with cell death as shown in the graph, however, activation of caspase-3 was absent in P23H retina, but present in S334ter retina. In both mutants, calpain activity showed a delayed decline after the peak of cell death. Values are mean ±SD from at least three different animals. All mean ±SD and *p* values are shown in table 2.1. ***(Kaur et al., 2011: PLoS One)***.

3.1.3.5 Temporal progression of TUNEL and other cell death markers

A comprehensive analysis of progression of various cell death markers was done to see their correlation to the TUNEL assay at various PN stages until PN30 for both the mutants. All the markers (cc3, calpain activity, PARP activity, avidin staining and PAR) showed a high degree of correlation of their activity or upregulation, with cell death (Fig. 2.8 and Table 2.1).

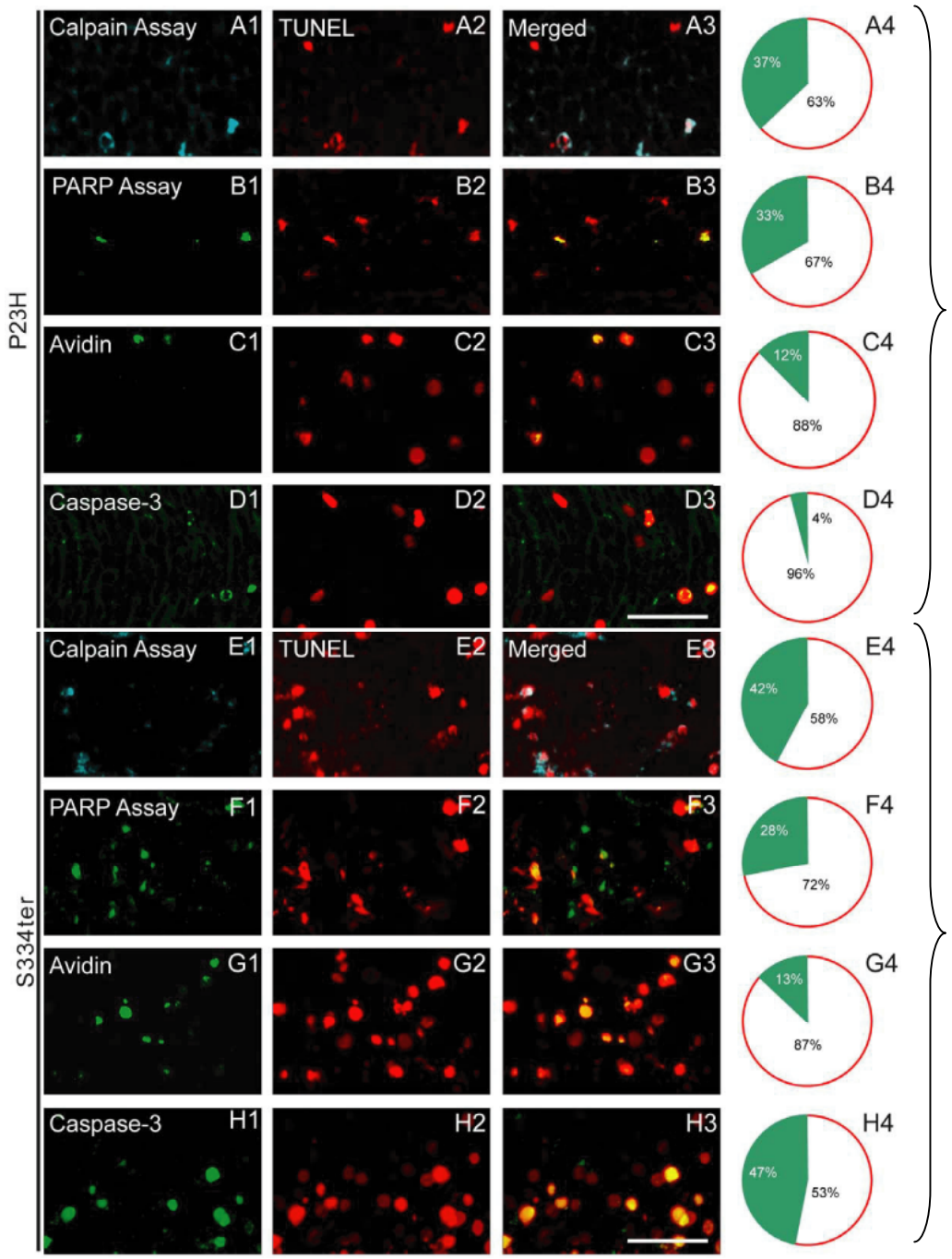

Figure 2.7 Fluorescence pictures demonstrating the co-labelling of TUNEL with other cell death markers. (A2–H2) Co- labelling of TUNEL with: (A1, E1) calpain assay, (B1, F1) PARP assay, (C1, G1) avidin and (D1, H1) caspase-3 in (A–D) P23H retina at PN15 and (E–H). S334ter retina at PN12 transgenic rats (A3–H3) Merged pictures. (A4–H4) Right panel indicates the percentages of co-labelled cells in the ONL. In both mutants calpain and PARP activity co-localized with TUNEL in 30–40% of cells, while avidin-binding co-localized in 12–13% with TUNEL. Caspase-3 co-localization was observed in almost 47% of TUNEL-positive cells in S334ter but only in 4% in P23H retinas. Scale bar = 25 µm ***(Kaur et al., 2011: PLoS One).***

Table 2.1. Quantification of various cell death markers (Supplementary information*: PLoS One; Kaur et al., 2011*)

Age	Animal	TUNEL	Caspase-3	Calpain assay	Avidin	PARP	PAR
		Mean ± SD *p*	Mean ± SD *p*	Mean ± SD *p*	Mean ± SD *p*	Mean ± SD *p*	Mean ± SD *p*
PN8	CD	0,026 ± 0,044	0,014 ± 0,006	0,003 ± 0,004	0,009 ± 0,008	0,004 ± 0,006	0,000 ± 0,000
	S334ter	0,493 ± 0,209 >0.05	1,504 ± 1,469 <0.01	0,245 ± 0,132 >0.05	0,083 ± 0,116 >0.05	0,216 ± 0,103 >0.05	0,394 ± 0,294 >0.05
PN10	CD	0,004 ± 0,003	0,003 ± 0,002	0,025 ± 0,013	0,000 ± 0,000	0,001 ± 0,002	0,000 ± 0,000
	P23H	0,573 ± 0,687 >0.05	0,121 ± 0,102 >0.05	0,088 ± 0,007 >0.05	0,095 ± 0,057 >0.05	0,125 ± 0,059 >0.05	0,181 ± 0,053 >0.05
	S334ter	2,939 ± 0,698 <0.001	2,811 ± 0,614 <0.001	2,381 ± 1,339 <0.001	0,710 ± 0,347 <0.05	0,639 ± 0,280 <0.01	1,549 ± 0,379 <0.001
PN12	CD	0,015 ± 0,012	0,007 ± 0,004	0,101 ± 0,015	0,001 ± 0,002	0,004 ± 0,004	0,010 ± 0,011
	P23H	0,974 ± 0,392 >0.05	0,121 ± 0,065 >0.05	-	-	-	-
	S334ter	6,088 ± 1,132 <0.001	5,056 ± 0,115 <0.001	5,176 ± 0,818 <0.001	3,245 ± 0,793 <0.001	1,585 ± 0,523 <0.001	2,910 ± 1,161 <0.001
PN15	CD	0,023 ± 0,004	0,031 ± 0,016	0,036 ± 0,024	0,009 ± 0,007	0,011 ± 0,003	0,000 ± 0,000
	P23H	2,729 ± 0,824 <0.001	0,094 ± 0,044 >0.05	2,342 ± 0,392 <0.001	0,928 ± 0,158 <0.001	0,751 ± 0,293 <0.001	1,155 ± 0,563 <0.01
	S334ter	2,245 ± 0,846 <0.001	2,205 ± 0,683 <0.001	3,205 ± 0,393 <0.001	1,628 ± 0,347 <0.001	0,921 ± 0,165 <0.001	1,365 ± 0,393 <0.001
PN20	CD	0,045 ± 0,035	0,011 ± 0,010	0,070 ± 0,068	0,005 ± 0,004	0,014 ± 0,005	0,033 ± 0,056
	P23H	0,850 ± 0,508 >0.05	0,059 ± 0,070 >0.05	1,488 ± 0,368 <0.01	0,324 ± 0,025 >0.05	0,282 ± 0,110 >0.05	0,663 ± 0,231 >0.05
	S334ter	1,583 ± 0,771 <0.05	1,881 ± 0,126 <0.001	2,175 ± 0,536 <0.001	0,952 ± 0,422 <0.001	0,846 ± 0,366 <0.001	1,292 ± 0,228 <0.01
PN30	CD	0,013 ± 0,022	0,000 ± 0,000	0,030 ± 0,046	0,006 ± 0,011	0,017 ± 0,017	0,000 ± 0,000
	P23H	0,453 ± 0,372 >0.05	0,007 ± 0,000 >0.05	0,479 ± 0,133 >0.05	0,149 ± 0,072 >0.05	0,186 ± 0,022 >0.05	0,163 ± 0,024 >0.05
	S334ter	1,382 ± 0,303 >0.05	1,840 ± 0,000 <0.01	0,869 ± 0,424 >0.05	0,677 ± 0,186 <0.05	0,604 ± 0,075 <0.01	0,908 ± 0,173 >0.05

3.1.3.6 Apoptosis inducing Factor (AIF)

AIF is a flavoprotein residing in mitochondrial intermembrane space, where it is thought to be acting as an oxidoreductase in mitochondrial electron transport (van Gurp et al., 2003). In recent years, AIF has been implicated in cell death across the studies in PARP-1 mediated cell death pathway (Wang et al., 2010; Wang et al., 2009). With an aim to look for the possibility of AIF's involvement in photoreceptor degeneration in RHO mutant rats, IHC was performed on paraffin sections. Interestingly, S334ter-3 retina showed a positive staining for AIF by depicting it in subpopulations of cells in the ONL, however, neither P23H-1 nor CD retina showed any staining for AIF (Fig2.9, A-B).

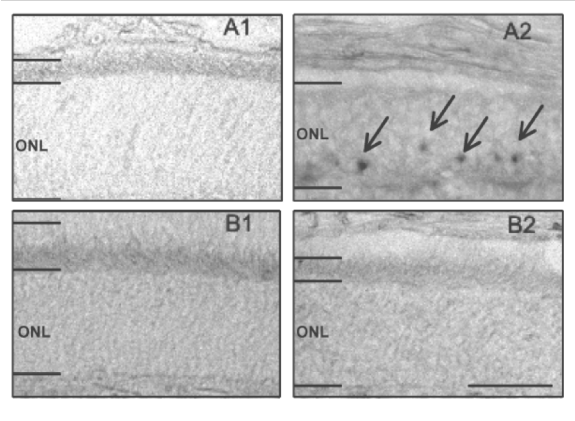

Figure 2.9. Apoptotic inducing factor (AIF) immunostaing. S334ter retina showed a subpopulation of photoreceptors positive for AIF immuno-labeling (A2). No AIF positive cells are seen in either wt CD (A1, A2) or P23H retinas (B2). Scale bar = 50 µm.

4. Discussion

The neurodegenerative mechanisms governing photoreceptor cell death in RP are not completely understood till date. A number of early publications suggested apoptosis to be the sole mode of photoreceptor cell death, however, present study has revealed that conventional apoptotic cell death markers (i.e. activated caspase-3, caspase-9, cytochrome c leakage) played a role only under specific circumstances in addition to highlighting the significant presence of other metabolic cell death markers for non-apoptotic cell death in two RHO mutant rat models. Moreover, these results suggested that down-stream cell death mechanisms triggered by different genetic mutations, across various species, share a number of key components. Since, RP is genetically heterogeneous disease, therefore, the discovery that different mutations share the biochemical parameters provide a window of opportunity for the development of mutation-independent RP therapies.

4.1 Caspase-3 and 9 activity and Cytochrome c leakage (classical cell death markers)

Photoreceptor cell death in retinal regenerations has often been referred to as apoptosis (Chang et al., 1993; Reme et al., 1998). TUNEL method, which was earlier used as apoptotic marker, detects non-apoptotic DNA fragmentation as well (Gavrieli et al., 1992). Therefore, other classical apoptotic makers were also investigated in the present study. Classical apoptosis mediates *via* activity of caspase-type proteases (Clarke and Clarke, 1996), with caspase-3 as the major player and executioner of apoptotic cell death (Jellinger, 2001). In mitochondrial pathway, caspase activation starts by the release of cyto-c from the mitochondrial

inter-membrane space, which on attaching to Apaf-1 activates caspase-9. Activated caspase-9 further cleaves and activates the executioner caspases-3 and -7 (Green and Amarante-Mendes, 1998; Kroemer and Reed, 2000; Slee et al., 1999b). Previous studies reported caspase-3 activation in the S334ter model (Liu et al., 1999). Present study confirmed these results with two important considerations: (i) in S334ter retina, caspase-3 and caspase-9 activity along with cyto-c leakage appear to occur concomitant with calpain and PARP activity, indicating that these pathways of cell death are executed side by side; (ii) in P23H rat, caspase-3 activity apparently was not significantly higher than in age-matched wild type rats, suggesting that P23H caspase-3 activity relates only to developmental but not to mutation induced cell death. This intriguing discrepancy found in the two RP models reveals how location of mutations in the RHO might affect the phenotype leading to caspase-independent cell death in one model and /or to degeneration that involves caspase activation in the other. The activity of caspase-3 independent pathways in both rat mutants and in *rd1* mice (Paquet-Durand et al., 2006; Paquet-Durand et al., 2010) may explain why previous attempts of caspase inhibition provided no or only partial protection to the photoreceptor (Liu et al., 1999; Yoshizawa et al., 2002).

4.2 Calpain activity and Calpastatin expression

In the studied RHO transgenic rats, no detectable change in expression of calpains was found at the tissue level; nevertheless, an increased signal of calpain-3 in immunofluorescence may refer to changes in expression, localization, and/or activation within photoreceptors. Importantly, a strong activity of calpains together with significant decrease in the expression of the endogenous inhibitor calpastatin was observed in the RHO transgenic rats as explained previously (Maki et al., 1989; Rao et al., 2008). These results in RHO mutant retinas are in agreement with earlier studies on *rd1* retina where calpastatin down-regulation corresponds to a strong elevation of calpain activity (Paquet-Durand et al., 2006). These results strengthen the argument that calpain activation is an important step during retinal neurodegeneration resulting from various genetic mutations (Paquet-Durand et al., 2010; Sancho-Pelluz et al., 2008; Sharma and Rohrer, 2007).

Therefore, targeting calpain activity may address a wider spectrum of RP-causing mutations and hence, might be more effective than inhibiting caspase activity alone.

4.3 DNA damage and PARP activity

Oxidative stress has been suggested to be an important factor during inherited retinal degenerations (Komeima et al., 2006). Although, it is not clear whether the oxidative stress is a causative factor involved in primary retinal degeneration (i.e. rod degeneration) or it is a secondary phenomenon contributing to the mutation-independent death of cones and second order retinal neurons (Sancho-Pelluz et al., 2008). Due to various reasons, an excessive mitochondrial metabolism results in reactive oxygen species generation (Halliwell, 2006), which creates characteristic oxidized compounds, such as 8-oxo-guanosine, the main oxidation product in the DNA (Oka et al., 2008). The accumulation of 8-oxo-guanosine observed in photoreceptors of S334ter and P23H rat retina corresponds to previous findings in *rd1* retina (Paquet-Durand et al., 2007; Sanz et al., 2007). In both mutant rats and *rd1* mice, oxidative DNA damage may be a crucial event to induce the PARP activation (Oka et al., 2008), a ubiquitously expressed nuclear protein, which is activated by DNA damage and helps to repair DNA (Schreiber et al., 2006). An excessive activation of PARP and the production of high amounts of neurotoxic PAR polymer (Andrabi et al., 2006; Vosler et al., 2009; Yu et al., 2006), have also been linked with cell death, particularly in the context of neurodegenerative diseases, where PARP has been proposed to be the main player of a novel form of caspase-independent cell death, also termed as PARthanatos (Wang et al., 2009).

The present study evidently showed PARP hyper-activation in degenerating photoreceptors of S334ter and P23H rats, along with an accumulation of PAR polymers, corresponding to earlier similar findings in the *rd1* mouse (Paquet-Durand et al., 2007). Although in P23H rats and *rd1* mice, strong increase in PARP activity was not matched by increased PARP expression, however, in S334ter retina up-regulation PARP activity was clearly matched with

PARP-1 expression. The reasons for stronger PARP up-regulation exclusively in S334ter retina are unclear, but could be linked to the more severe form of degeneration in this model. At the same time, it does not appear to be a compensatory PARP upregulation due to cleavage by the activated caspase-3, since no signs of increased PARP cleavage product were seen in S334ter tissue. This data clearly shows PARP activation as a key event in photoreceptor degeneration regardless of the causative genetic mutation and suggests PARP as a target for neuroprotective treatments.

4.4 Temporal progression of metabolic cell death markers

Both RHO transgenic models are characterized by an early death of photoreceptors, which start degenerating earlier than the age (PN15) at which the rat retina gets functionally mature in both the studied models of RP (Weidman and Kuwabara, 1968). Therefore, mutation induced cell death observed in these rats overlapped temporally with developmental cell death to some extent (Young, 1984), importantly however, all the cell death markers studied here were seen strongly upregulated only in RP mutants, implying that developmental cell death events had only negligible effect on the overall cell death. The P23H retina showed a comparatively slower progression of photoreceptor cell death than S334ter, in line with earlier studies (Liu et al., 1999; Machida et al., 2000). In S334ter rat, caspase-3 activation was increased, however, it coincided numerically and temporally with calpain activation during the second PN week, indicating that both of these proteolytic patways are activated and executed in parallel in these mutants. This co-activation points to some sort of cross-talk between caspases and calpains (Gomez-Vicente et al., 2005) and may explain at least partially, the extremely fast progression of degeneration in this model. Alternatively, it would also be possible that different cell death mechanisms get induced because of the faster progression of S334ter degeneration (possibly linked to a stronger genetic insult). However, *rd1* mouse retina inspite of displaying essentially the same rate of cell death, reportedly shows only the non-apoptotic markers activated in it (Chang et al., 1993), suggesting that the speed of degeneration apparently was not the deciding factor for a particular cell death pathways to be operational.

In P23H retina, caspase-3 activity seemingly plays only a minor, if any, role, while the calpain activity is very prominent. This model shows extreme resemblance to *rd1* mouse model in displaying the presense and/or absense of certain cell death markers. Therefore, implications for a therapy of RP in this model with respect to *rd1* are important since these results suggest that targeting exclusively the caspase cascade – as was previously proposed (Yoshizawa et al., 2002) is unlikely to yield positive results while, targeting non-apoptotic events such as calpain or PARP activation may provide beneficial effects (Paquet-Durand et al., 2010; Sahaboglu et al., 2010). Nevertheless, identification of all associated and interacting proteins involved in photoreceptor cell death is fundamental to work towards neuroprotective strategy, because blocking single enzyme may not be enough, given the fact that as the cell may still be able to activate/continue other mechanisms or routes to die (Gomez-Vicente et al., 2005; Lockshin and Zakeri, 2004b).

Collectively, all analysed markers (*i.e*. caspase-3 activation, calpain and PARP activity, PAR accumulation, oxidative DNA damage, TUNEL) coincided during their progression and followed a similar pattern. The metabolic cell death markers not only correlated temporally, but also co-localized with TUNEL staining, indicating that these events occurr relatively late during degenerative processes. Alternatively, this close correlation may also be explained by assuming that cell death, once triggered, is executed very rapidly. Remarkably, in both transgenic models, after the peak of degeneration, the number of cells showing calpain activity exceeded the number of TUNEL-positive cells. This could be due to a higher detection sensitivity of the calpain assay, but may also indicate that proteolytic activity of calpains persists at times when the nuclear DNA has already disintegrated.

4.5 Apoptosis inducing factor (AIF) release

Translocation of AIF from mitochondria to nucleus is considered to cause DNA fragmentation resulting in cell death (Krantic et al., 2007). Some studies suggest that AIF acts as a mediator of PARP-induced apoptosis (Cipriani et al., 2005;

Culmsee et al., 2005; Yu et al., 2006), while others point out that AIF release is induced by PARP activation and synthesis of free PAR polymers during caspase independent cell death mechanism (Hong et al., 2004; Wang et al., 2009). Sanges et al. earlier showed that AIF translocates from mitochondria to nuclei in *rd1* photoreceptor cells (Sanges et al., 2006). Subsequent study involving *rd1* photoreceptors proposed that its release from mitochondria was triggered/aided by PARP activation (Paquet-Durand et al., 2007). Present study showed AIF staining only in S334ter retina. It is noteworthy that only a subpopulation of photoreceptors was positive for this protein in S334ter retina, which makes it difficult to conclude as to what causes its release. The assumption about AIF's triggering by PARP seems speculative as we did not see any AIF positive cells in P23H retina, which otherwise shows upregulation of PARP. Whether AIF release depends on excess PARP activity and consumption of NAD (Du et al., 2003; Sims et al., 1983) and/or by PAR polymers, needs further investigation in S334ter retina.

6. Conclusion

Present study provides a comprehensive overview of several important cell death markers associated with photoreceptor cell death in two rat models of RP bearing RHO mutations. Additionally, the study highlights that there are mechanistic similarities in the cell death mode between the two studied models, which also shows homology to other rodent models of RP having different mutations. While in S334ter retina, cell death was associated with both apoptotic and non-apoptotic markers, whereas, P23H mutant appeared to be degenerating essentially by non-apoptotic mechanisms. Interestingly, in both mutants, cell death was clearly related to the activation of calpain and PARP, as well as accumulation of PAR and oxidatively damaged DNA, similar to what has previously been reported in *rd1* mouse retina. Taken together, these findings suggest that non-apoptotic cell death plays an important role in inherited photoreceptor degenerations. Nevertheless, apoptotic events may occur additionally in certain mutations such as RHO C-terminal truncation S334ter mutation. This finding may have important implications for the development of therapy in the future because the study helped to improve the understanding of cell death pathways that alternative cell death mechanisms

along with the apoptotic cell death pathways are implicated in retinal degeneration, which in turn may yield a number of novel targets for neuroprotective treatments. Additionally, a homology of cell death pathways was observed in different mutations, which strongly improves the perspectives for a mutation-independent neuroprotective treatment of RP.

Chapter III. Neuroprotection of S334ter3 and P23H-1 retinas by using specific inhibitors of calpains and PARP

1. Introduction

Major goal of RP research is to develop effective therapy against this yet incurable eye disease. Presently, no generally effective treatment is available for RP. So far, evidences generated from animal and/or cell culture based studies have offered the optimistic scenario for RP treatment only to limited extent. Broadly, RP treatment options can be divided into two categories: replacement (when photoreceptors are dead and need to be replaced) and the treatments aimed to prolong the life of photoreceptors (when some of them are still alive and functional) (Abegg et al., 2000; Delyfer et al., 2004; Rivas and Vecino, 2009; Shintani et al., 2009).

1.1 Replacement treatment strategy

The treatment strategy involving replacement can further be divided into two categories: transplantation (photoreceptor or stem cell transplantation) and retinal prosthetic devices.

1.1.1 Transplantation treatment Strategies

1.1.1.1 Retinal cell transplantation

The idea of replacing dead retinal neurons (e.g., photoreceptor cells) with healthy, donor cells by transplantation has been worked over for the past several years (West et al., 2009). This method refers to the placement of sheets of developing retina or retinal pigment epithelial (RPE) cells into the sub-retinal space (Delyfer et al., 2004; Tao et al., 2007). In the past, several studies have been carried out to transplant photoreceptors or even entire retinas to replace the dead photoreceptors (Aramant and Seiler, 2004; McGill et al., 2007; Zhang et al., 2003), however, generally the transplanted tissue fail to integrate properly into the structural organization of the host retina. Nevertheless, lately, implanting the fetal retina with RPE has been reported to be effective in RP patients (Radtke et al., 2008). Second approach is the transplantation of photoreceptor precursor cells.

Although initial studies did not show successful transplantation of photoreceptor precursors, however, post-mitotic rod precursor cells taken from the mouse retina at PN1 have been shown to integrate in the host tissue and develop into rod photoreceptors (MacLaren et al., 2006). Another study showed that human embryonic stem cells kept in special medium differentiate into photoreceptor progenitors, which are able to integrate into degenerated mouse retinas (Lamba et al., 2006). Recently, S334ter-3 rats have been reported to restore visual function following retinal progenitor layer transplants (Seiler et al., 2010).

1.1.1.2 Stem Cell transplantation

A very exciting and comparatively new area of research is the development of stem cell transplantation-based therapies for a wide spectrum of diseases. Stem cells have the potential to multiply and differentiate into almost any type of cell in the body. Theoretically, they can be transplanted into the retinal space, and on providing appropriate biological signals; transplanted stem cells could develop into mature, functional photoreceptor cells (Baker and Brown, 2009; Huang et al., 2011). Stem cell transplantation in retinal degenerations using pluripotent embryonic stem cells (ESCs) (Amabile and Meissner, 2009; Baker and Brown, 2009) did not yield very promising results till date as the success of differentiation of ESCs into functional retinal cells has reportedly been less (Vugler et al., 2007). In recent times, adult stem cells (McKay, 1997; Momma et al., 2000; Tropepe et al., 2000) have also been used as therapeutic option in retinal and other neurodegenerative disorders (Delyfer et al., 2004), however, use of adult neuronal stem cells or retinal stem cells in the retina could not bring favourable results (Sakaguchi et al., 2004; Van Hoffelen et al., 2003). Nevertheless, RPE cells taken from human ESCs when transplanted into rats with retinal degenerative disease have been shown to improve the vision of treated rats over untreated controls (Lund et al., 2006). Taken together, it may be possible to transplant the degenerated retina with stem cells isolated from adult tissue or from ESCs or even the iPSCs (induced pluripotent stem cells). However, in spite of high hopes from stem cell based therapies for various human diseases, the ethical issues remain unresolved as to whether the embryos should be used for such research.

1.1.2 Retinal prosthetic devices

A retinal prosthetic device may help in restoration of vision by means of a device implanted on the retina, which uses electrical signals to bypass dysfunctional or dead photoreceptors and stimulate the residual viable, non-photoreceptor cells of the retina (Weiland et al., 2005). Presently, efforts are being made by various research groups engaged in RP research to develop electrical retinal implants that can be attached to the retina of patients to restore the vision (Zrenner, 2002). Two types of retinal implants being developed include the subretinal implants (Behrend et al., 2011; Zrenner et al., 2011), and the epiretinal implants (Hayes et al., 2003; Rizzo, 2011).

Technical advancements in retinal implant devices continue to provide the hope of vision for patients with end-stage disease, albeit some critical issues regarding the long-term stability and biological compatibility of such devices need to be addressed (Besch and Zrenner, 2003; Musarella and Macdonald, 2011).

1.2 Treatments to prolong the life of Photoreceptors

1.2.1 Gene therapy

Gene therapy is the replacement of a defective (mutated) gene with healthy gene, so that an important protein is again synthesized for normal functioning in a cell (Delyfer et al., 2004; Kohno et al., 2005). Starting in the 1990, several research groups showed that the defective genes could be replaced in animal models of RP and visual function could be restored partially (Li et al., 2010a; Liu et al., 2011). In 2001, a study reported good restoration of visual function in a dog model for Leber's congenital amaurosis (LCA), which can be caused by a mutation in the RPE65 gene. A study reported in 2008 that vision of one LCA patient was improved on introduction of viral vectors subretinally carrying healthy copies of RPE65 genes (Bainbridge and Ali, 2008). However, despite being popular and preferred therapeutic option, gene therapy faces certain challenges; for instance, high degree of genetic heterogeneity in RP poses a tough task of tailor designing of gene therapy for each particular mutation in patients. Moreover, for successful gene therapy, the cells should be healthy, however, by the time a human patient

shows RP symptoms, the cells carrying the original mutation are no longer viable and become unfit for gene therapy (Smith et al., 2009).

Above-mentioned replacement therapeutic options (transplantation or prosthesis) are usually possible only after all the photoreceptors are lost, and gene therapy needs to be designed individually for every patient. Therefore, a therapy that can be applied at any stage of the disease progression, and which is independent of primary genetic insult is much sought for the treatment of RP. Neuroprotection is the perfect answer for such a mutation-independent therapeutic option, which holds the promise to cater larger number of patients affected with different genetic mutations.

1.2.2 Neuroprotection

Neuroprotection refers to the mechanisms and strategies aimed to block or delay the neuronal cell death in various forms of neurodegenerations. This therapeutic option is aimed at preventing the photoreceptor loss by either blocking cell death and/or enhancing the pro-survival pathways (Trifunovic et al., 2012). Various neuroprotective strategies, which have shown beneficial effects on the photoreceptor survival, include the application of neurotrophic growth factors (CNTF, BDNF, FGF) (Azadi et al., 2007; Chaum, 2003; Chen and Li, 2011; Li et al., 2010b), antioxidants such as lutein or zeaxantin (Sanz et al., 2007; Sasaki et al., 2009), Vitamin A (Berson et al., 1993) and insulin (Valenciano et al., 2006).

Understanding of the mechanisms governing the photoreceptor cell death in RP makes it possible to develop a neuroprotective therapy by using pharmacological approaches that directly target various molecules implicated in cell death pathways (Okoye et al., 2003). Earlier, photoreceptor cell death in RP was considered to be apoptotic (Chang et al., 1993; Delyfer et al., 2004; Portera-Cailliau et al., 1994), and it was believed that targeting the apoptotic routes might stop the progression of disease. However, recent findings including our own (Kaur et al., 2011) pointed out various cell death markers *viz*., calpain and PARP other than the typical apoptotic markers linking to photoreceptor degeneration (Paquet-Durand et al., 2006; Paquet-Durand et al., 2010; Paquet-Durand et al., 2007; Sahaboglu et al., 2010).

These comparatively newly identified cell death markers have not only shed a light about alternative mechanisms of cell death being operative in retinal degenerations, but also have opened up new avenues for neuroprotective therapeutic strategies (Dragana Trifunović, Accepted December, 2011). Furthermore, in recent times, some studies have claimed to rescue the dying photoreceptors by the application of PARP and/or calpain inhibitors in neurodegenerations in general, and retinal degenerations in particular (Azuma and Shearer, 2008; Paquet-Durand et al., 2010; Paquet-Durand et al., 2007; Wang and Yuen, 1994).

All these studies lend strong support to the idea that the inhibitors of calpain proteases and PARP could be useful agents for neuroprotective treatment in RP. The present study was designed to; i) test the efficacy of selective calpain and PARP inhibitors to rescue the degenerating photoreceptors, ii) determine whether the rescue, if any, is dose dependent, and whether the treatment paradigm consisting of single or combined treatments differ in the neuroprotective potential.

2. Aim

To test the efficacy of specific Calpain and PARP inhibitors in order to determine their therapeutic potential in the neuroprotection of the degenerating retina by using the organotypic retinal culture system for S334ter-3 and P23H-1 rat retinas.

3. Results

Having shown that calpain proteases and PARP-1 are activated and play a significant role during photoreceptor cell death in RHO mutant S3334ter and P23H rat models of RP, we wanted to determine the effect of inhibition of these enzymes in the respective rat models. In the present study, two different calpain inhibitors [calpain inhibitor VI (*N*-(4-Fluorophenylsulfonyl)-L-valyl-L-leucinal**;** also called SJA6017) and calpain inhibitor XI (Z-l-Abu-CONH $(CH2)_3$-morpholine; also referred to as CX295)] (Calbiochem, Darmstadt, Germany) and one PARP inhibitor (N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N dimethylacetamide.HCl;

also referred as PJ34) (Alexis Biochem, Lausen, Switzerland), were tested in order to ascertain the efficacy of these inhibitors in curbing the degeneration of photoreceptors in S334ter and P23H retina. Both calpain inhibitors have been reported to reduce calpain activity during photoreceptor cell death (Paquet-Durand et al., 2006; Paquet-Durand et al., 2010; Sharma and Rohrer, 2004). Similarly, PARP inhibitor PJ34 has been shown to be neuroprotective in *rd1* retina that shows PARP dependent cell death (Paquet-Durand et al., 2007).

Organotypic retinal cultures system is an easily accessible tool to observe the biological processes of complete tissues in highly controlled *in-vitro* conditions, and is employed to study a wide range of neurobiological processes including cell death and neuroprotection (Bull et al., 2011). For *in-vitro* inhibitor treatment experiments, S334ter and P23H animals were sacrificed at PN5 and PN8 respectively as the cell degeneration has been observed to start before PN8 in S334ter-3 and PN10 in P23H-1 rat retinas, when the normal developmental cell death is still occurring (Kaur et al., 2011). Organotypic retinal explants were prepared as described earlier (Caffe et al., 1989; Pinzon-Duarte et al., 2000), and were kept in R16 serum free medium (see chapter VI: Materials and Methods). Before being treated with inhibitors, the explants were allowed to adjust to new culturing conditions for 2 days. The inhibitors were dissolved in the R16 complete medium before being applied. Untreated cultures received only vehicle (DMSO or ddH_2O). Different treatment paradigms included: i) single treatment with calpain and PARP inhibitors (calpain inhibitor VI and calpain inhibitor XI at 100µM and 5µM and PJ34 6µM) ii) combined treatment (mixture of calpain and PARP inhibitors: calpain inhibitor XI (5µM) and PJ34 6µM or calpain inhibitor VI (5µM) and PJ34 6µM) see table 3.1. The concentrations to be applied to the retinal explants were selected according to previous literature (Paquet-Durand et al., 2006; Paquet-Durand et al., 2010; Paquet-Durand et al., 2007; Sancho-Pelluz et al., 2010; Sharma and Rohrer, 2004). The cultures received treatments for 5 days in case of S334ter cultures and 10 days in case of P23H cultures. The retinas remained in cultures for 7 and 12 days for S334ter and P23H retinas respectively.

Table 3.1 Inhibitor treatments of S334ter and P23H retinal cultures

Culturing	Inhibitors	Concentration	Day of	DIV

day (PN)			Application	(days *in vitro*)
Single Inhibitor Applications				
S334ter: PN5 P23H: PN8	Calpain Inhibitor VI/SJA6017	100µM and 5µM	DIV2	S334ter DIV7 P23H DIV12
S334ter: PN5 P23H: PN8	Calpain Inhibitor XI/CX295	100µM, 5µM	DIV2	S334ter DIV7 P23H DIV12
S334ter: PN5 P23H: PN8	PARP inhibitor PJ34	6µM	DIV2	S334ter DIV7 P23H DIV12
Combined Inhibitor Applications				
S334ter: PN5 P23H: PN8	Calpain Inhibitors (VI or XI) and PJ34	5µM and 6µM	DIV2	S334ter DIV7 P23H DIV12

3.1 Effect of single inhibitor treatment

3.1.1 Calpain Inhibitors treatment at 100µM

Treatment of both S334ter and P23H retinal explants with either calpain inhibitor XI (CIXI) or calpain inhibitor VI (CIVI) at 100µM resulted in very large number of cells undergoing degeneration as shown by TUNEL assay, as compared to untreated cultures. A massive degeneration of cells was seen in INL and OPL with the majority of cells dying in ONL, implying that these concentrations were injurious to the retina (Fig. 3.1A-a,b.f and 3.1B; Fig. 3.2A-a,b,f; 3.2B).

CD (wt) retinal cultures prepared as a control for either S334ter or P23H (P5DIV7 and P8DIV12, respectively) also showed a high degree of cell death when they were treated with calpian inhibitors at 100µM (Fig. 3.3).

3.1.2 Calpain Inhibitors treatment at low concentration

After observing the degeneration of photoreceptors at a massive scale post treatment according to previous paradigms in mutants as well as wt retinal cultures, a low concentration (5µM) of each calpain inhibitor was tested according to previous studies pointing to beneficial effects of calpain inhibitors at lower concentrations (Paquet-Durand et al., 2010). Both S334ter and P23H explants

treated with low concentrations (5μM), of both calpain inhibitor VI and calpain inhibitor XI revealed that TUNEL positive cells decreased tremendously as compared to 100μM concentration treatment paradigm, however still the effect was not protective as dying cells exceeded in treated than in untreated cultures (untreated: 4.117 ± 0.2700 n=7; P23H P8DIV12 treated (CIVI, 5μM): 4.340 ± 0.5666, n=6, *p* >0.05; P23H P8DIV12 treated (CIVI, 5μM): 5.446 ± 1.197 n=4, *p* >0.05). In both the mutant retinal cultures, CIXI showed more TUNEL positive cells as compared to CIVI (Fig. 3.1A-a,b,f and 3.1B; Fig. 3.2A-a,b,f and 3.2B). Additionally, number of photoreceptors undergoing degeneration was always more in case of S334ter as compared to P23H retinal cultures**.** Lowering of concentration of inhibitors decreased the cell death as compared to higher concentrations.

3.1.3 PJ34 treatment reduces cell death in P23H retina *in-vitro*

For application of PJ34, earlier reported literature was surveyed and 6μM concentration was selected for the present study (Paquet-Durand et al., 2007; Sancho-Pelluz et al., 2010). Treatment with PARP inhibitor PJ34 at 6μM was observed to be beneficial as number of TUNEL positive cells decreased in treated cells greatly in P23H retinal cultures. Quantification of TUNEL positive cells revealed that application of PJ34 resulted in photoreceptor cell survival (Untreated: 4.320% ± 0.2109 SEM, n=6; PJ34 (6μM): 2.422 ± 0.4969 n=3, *p*< 0.01) (Fig. 3.1A a,e; 3.1B).

S334ter P5DIV7 explants treated with PARP inhibitor PJ34 at 6μM concentration again showed a large number of TUNEL positive cells and appeared non-protective. Quantification values also showed PJ34 treatment in S334ter cultured retinas to be unprotectve, nevertheless, this effect was statistically nonsignificant (Fig. 3.2A a,e; 3.2B) (Untreated vs. PJ34: Untreated: 6.836% ± 0.9266 SEM, n=10; PJ34 (6μM): 9.484% ± 1.604 SEM, n=7; *p*>0.05).

A. P23H-1 P8DIV12: Effects of treatment with inhibitors of calpain (XI and VI) and PARP (PJ34)

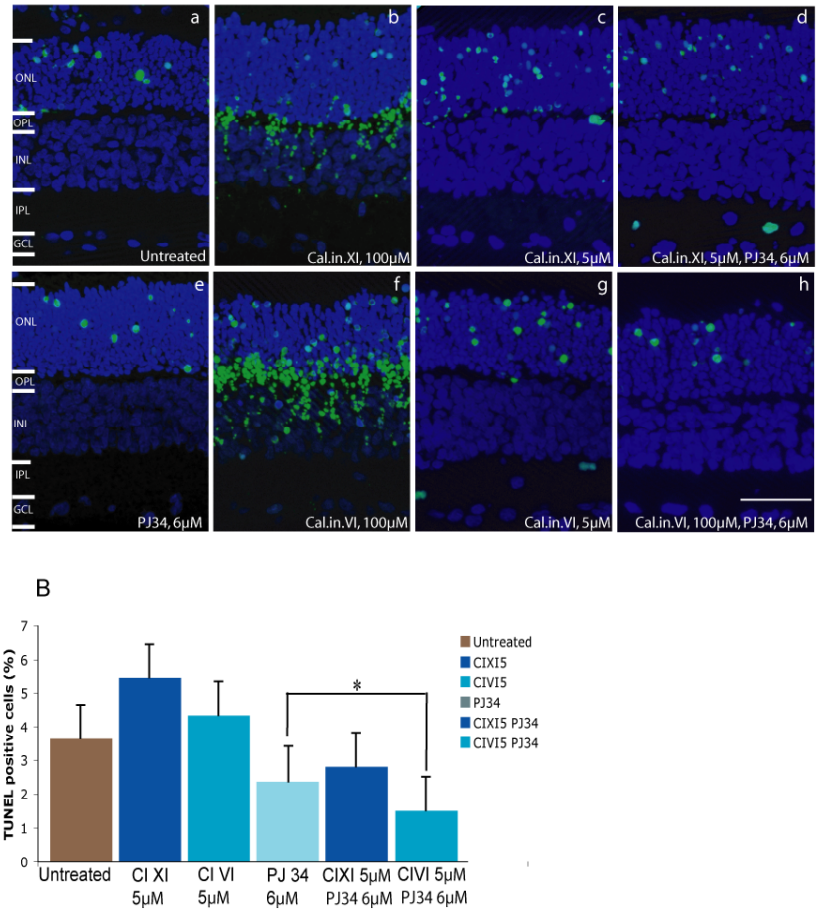

Figure 3.1 Inhibition of calpain and PARP in P23H-1 P8DIV12 retinal cultures. A. Lower doses of inhibitors of calpains and PARP rescues photoreceptors while higher doses induce cell death. a) Untreated retinal explants b) large number of dying cells observed as displayed by TUNEL assay after treatment with Cal. inh. XI100 μM and Cal. inh.VI 100μM, (b, f). As the concentration of calpain inhibitors was decreased to 5μM, cell death decreased to large extent (c,g), which was more pronounced after treatment with Cal. inh. VI (g). Treatment with PARP inhibitor PJ34 at concentration 6μM reduces cell death (e). Combined treatment with both either calpain inhibitor at 5μM and PJ34 at 6μM, greatly reduced the cell death and hence, provided survival, (d,h). The strongest protective effect was observed for cal. inh. VI (5μM) andPJ34 (6μM) (B), Significance levels were: *p< 0.05, Scale bar=25μm.

A. S334ter-3 P5DIV7: Effects of treatment with inhibitors of calpain (XI and VI) and PARP (PJ34)

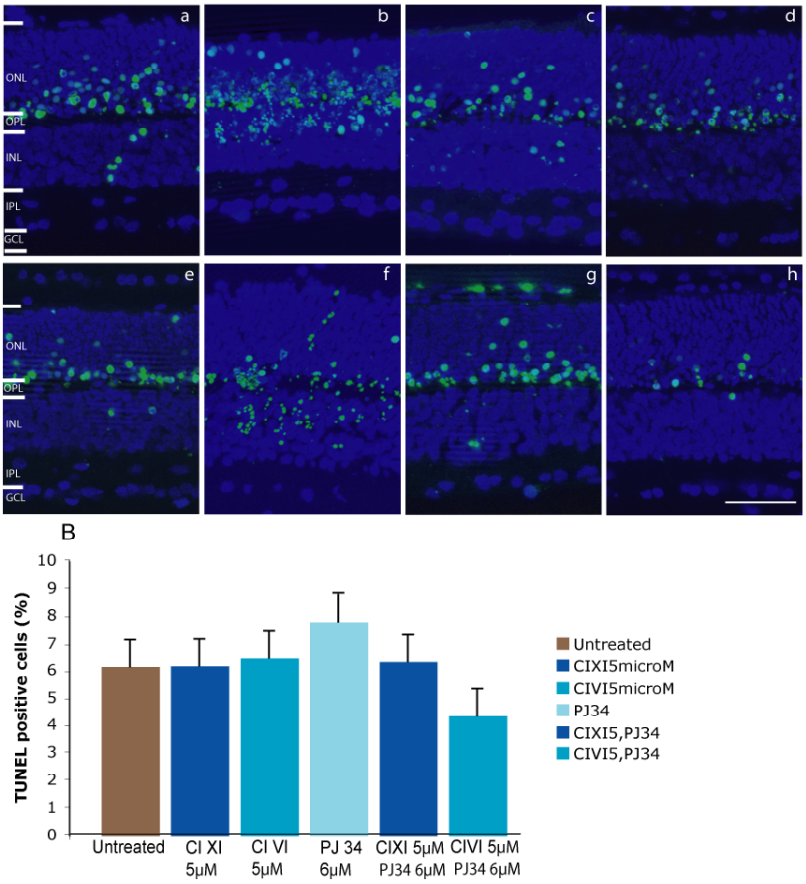

Figure 3.2 Inhibition of calpain and PARP in S334ter3 P5DIV7 retinal cultures. A. Lower doses of inhibitors of calpains and PARP rescues photoreceptors while higher doses induce cell death. a) Untreated retinal explants b) large number of dying cells observed after treatment with Cal. inh.XI100 μM and Cal. inh.VI100μM as displayed by TUNEL assay (b, f). As the concentration of calpain inhibitors was decreased to 5μM, cell death decreased, however, still the effect was not protective (c, g). Treatment with PARP inhibitor PJ34 at concentration 6μM was not beneficial (e). Combined treatment with calpain inhibitor XI at 5μM and PJ34 at 6μM was not protective, however, a combination of Cal. Inh. VI (5μM) andPJ34 (6μM) seemed to reduced the cell death (d,h). (B), Significance levels were: n.s. p>0.05, Scale bar=25μm.

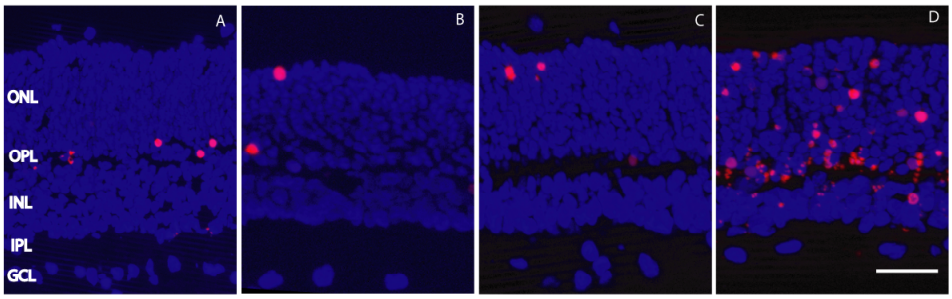

Figure 3.3 Calpain inhibitors induce cell death in wild type CD retinal cultures. A) and B) represent P5DIV7 and P10DIV10 untreated retinal cultures displaying occasional TUNEL positive (red staining) cells, C) represents P10DIV10 wt

retinal explants treated with PJ34 at 6μM and D) represents P10DIV10 wt retinal culture treated with cal. inh. XI at 100μM, Scale bar (in D) 25μm.

3.2 Combined calpain and PARP inhibitor treatment enhances cell survival

After having observed beneficial effect of PJ34 application in P23H retinal cultures, and a reduction in cell death after calpain inhibition at low concentration in comparison to higher concentration application, albeit not protective yet, we tested combined treatment paradigms, where combination of calpain inhibitor VI or XI at 5μM and PJ34 at 6μM were prepared, and applied to the retinal explant cultures.

In P23H, a protective effect was observed in cultures treated a combination of calpain inhibitor XI or calpain inhibitor VI and PJ34 as compared to the untreated cultures as number of TUNEL positive cells decreased. Quantification of treated cultures showed that combinations of either calpain inhibitor VI or calpain inhibitor XI and PJ34 significantly enhanced the cell survival (Untreated: 3.903%±0.3173 SEM, n=8; CIVI+PJ34: 2.672% ± 0.4513 SEM, n=7; $p< 0.05$) and (Untreated: 3.903% ± 0.3173, n=8; CIXI+PJ34: 2.740% ± 0.2835, n=8; $p< 0.05$). It is noteworthy that although both calpain inhibitors provided rescue, however, calpain inhibitor VI was more protective as compared to calpain inhibitor XI (Fig. 3.1A-d,h, and 3.1B)

In case of S334ter, combination of CIVI and PJ34 appeared to be protective, however, the effect was statistically nonsignificant (Untreated: 6.836% ± 0.9266 SEM n=10); CIVI+PJ34 (4.358% ± 1.139 SEM, n=4, $p> 0.05$). On the other hand, treatment with CIXI and PJ34 failed to show any protective effect in S334ter cultures (Untreated: (6.836% ± 0.9266 SEM, n=10) CIXI+PJ34 (7.138% ± 2.248 SEM, n=4, $p> 0.05$) (Fig. 3.2A- d,h and 3.2B).

Table 3.2: Quantification values of TUNEL positive after treatment with calpain and PARP inhibitors in S334ter and P23H retinal explants

		% TUNEL positive cells in ONL				
		PJ34 (6μM)	CIVI (SJA6017) 5μM	CIXI (CX295) 5μM	CIVI(5μM)+ PJ34 (6μM	CIXI(5μM)+ PJ34 (6μM
		Mean ± SEM *p*	Mean ±SEM P	Mean±SEM *p*	Mean ± SEM *p*	Mean ± SEM *p*
S 3	Untreated	6.836%±0.9266	6.836%±0.9266	6.836%±0.9266	6.836%±0.9266	6.836%±0.9266

	Treated	9.484%±1.604 p >0.05	--	--	4.358%±1.139, p> 0.05	7.138%±2.248p > 0.05
P23H P8DIV12	Untreated	4.320%±0.2109	4.117 ± 0.2700	4.117 ± 0.2700	3.903%±0.3173	3.903%±0.3173
	Treated	2.422±0.4969, p < 0.01	4.340 ± 0.5666 p >0.05	5.446 ± 1.197 p >0.05	2.672%±0.4513 p < 0.05	2.740%±0.2835 < 0.01

4. Discussion

Implication of calpain and PARP hyperactivation in photoreceptor cell death provides a positive insight about use of inhibitors of these enzymes as a neuroprotective strategy in retinal degenerations. Until date, only few studies investigated the therapeutic potential of calpain and PARP inhibitors on inherited photoreceptor degeneration. The present study was undertaken to strengthen the argument that pharmacological inhibition of calpains and PARP hold the therapeutic potential in photoreceptor degenerations. The study showed that *in-vitro* treatment of S334ter-3 and P23H-1 transgenic rat retinas with selective inhibitors of calpains and PARP renders a protective effect to the dying photoreceptors under specifically defined regimens.

4.1 Implication of calpain proteases and PARP during photoreceptor degeneration in RP

In recent years, pathological significance of Ca^{2+} overload in the nervous system has incited a huge interest to investigate its possible role in various forms of neurodegenerative disorders. High intracellular Ca^{2+}-concentrations are known to occur in many neorodegenerative processes including the degenerating *rd1* rod photoreceptors (Sanges et al., 2006). Alterations in Ca^{2+} homeostasis cause the activation of Ca^{2+} sensitive non-lysosomal calpain proteases (Bartoli and Richard, 2005; Goll et al., 2003; Tompa et al., 2004), which under otherwise physiologically normal calcium levels are biomodulators of many Ca^{2+} regulated processes. Under pathological conditions, localized influx of Ca^{2+} into the cell activates of calpains, which then carry on the controlled proteolysis of its substrate proteins causing the cell death (Azuma and Shearer, 2008).

Calpains have been shown to play vital role in caspase-independent photoreceptor cell death pathways in various models of inherited retinal degenerations, e.g., *rd1* mice (Paquet-Durand et al., 2010; Pearce-Kelling et al., 2001; Sanges et al., 2006), and Royal College of Surgeons (RCS) rats (Mizukoshi et al., 2010). Additionally, they are also reported to be involved in calcium-induced cell death in a murine photoreceptor-derived cell line (Gomez-Vicente et al., 2005; Sanvicens et al., 2004). Importantly, we found a strong correlation between photoreceptor cell death and calpain hyperactivation together with a downregulation of calpastatin in P23H and S334ter RHO mutant rat models of RP (see chapter II) and (Kaur et al., 2011). All these studies unequivocally highlight the involvement of calpains in photoreceptor degeneration across various mutants and points strongly towards working on the development of mutation independent therapy aimed at neuroprotection of the retina by applying the inhibitors pharmacologically.

Another key player of cell death, which has increasingly been recognised in various neurodegenerative diseases is DNA repairing enzyme PARP-1, which on hyperactivation has been shown to play crucial roles in various neurodegenerative disorders (Jagtap and Szabo, 2005; Yokoyama et al., 2010), including the photoreceptor degeneration in PDE6 mutant *rd1* mouse model of RP (Paquet-Durand et al., 2007). Recently, PARP-/- mice were reported to show protective effect to photoreceptor degeneration (Sahaboglu et al., 2010). Interestingly, we have found a correlation between PARP activity and photoreceptor cell death in RHO mutant S334ter and P23H rat modes (Kaur et al., 2011). These findings have evidently provided the clue to use PARP inhibitors in a bid to develop mutation independent neuroprotective therapy.

While PARP inhibitors have been reported to provide beneficial effects in experimental systems (Goebel and Winkler, 2006; Szabadfi et al., 2010; Uehara et al., 2006), and in inherited retinal degeneration (Paquet-Durand et al., 2007), however, the outcome of applying calpain inhibitors has been inconsistent among various studies, nevertheless, some experimental and inherited retinal degenerations have shown protective effects of calpain inhibition (Doonan et al., 2005; Paquet-Durand et al., 2010; Sharma and Rohrer, 2007).

4.2. Detrimental effects of calpain inhibitors applied individually

Both S334ter (P5DIV7) and P23H (P8DIV12), treated with calpain inhibitor VI or calpain inhibitor XI at concentrations of 100μM presented a detrimental effect on photoreceptor cells, which was particularly severe in case of calpain inhibitor XI. Interestingly, when *wt* CD retinal cultures were given the treatment under similar paradigms, the photoreceptors were found to be degenerating at large scale similar to mutant retinal cultures, nevertheless, some TUNEL staining was seen in *wt* untreated cultures, which may arise due to culturing conditions. The detrimental effects of calpain inhibitors at higher concentration could be due to the fact that calpains are ubiquitously present in the organisms, which clearly state the importance of their functions for the cell survival. At physiologically normal calcium levels, calpains regulates calcium-regulated processes like signal transduction, cell proliferation, cell cycle progression and arrestin processing (Azuma and Shearer, 2008; Goll et al., 2003), and all the calpains are not activated at once to start destructing their target proteins, infact, a local population of calpains get activated first of all by transient calcium influx and these activated proteases then pass on the signal to other calpains (Goll et al., 2003). Therefore, sometimes the pharmacological interference with the calpain system may cause unwanted side effects and could counter-balance the beneficial effects of calpain systems. In the present study, higher concentrations of calpain inhibitors during systemic application might have outbalanced their pro-survival effects in the sense that application of more than necessary amount of these inhibitors may result in the loss of activities of some calpains, which are otherwise required for the normal functioning of the cells (Paquet-Durand, 2010).

4.3 Treatment with PARP inhibitor PJ34

Treatment with PARP inhibitor PJ34 in P23H at 6μM concentration showed a significant protective effect. However, in S334ter retinal explants treated with PJ34, no rescue was observed. P23H retina shows a great level of overlapping of mechanistic way of degeneration with *rd1* mouse model of RP, which also displays PARP dependent degeneration of photoreceptors (Sahaboglu et al., 2010), and

attenuation of PARylation and cell death after *in-vitro* application of PARP inhibitor PJ34 to *rd1* retinal explants (Paquet-Durand et al., 2007).

The significant decrease in cell death post PJ34 application in P23H explants could be attributed to the reason that PARP-1 have a bearing on Ca^{2+} regulation via PAR mediated alterations in mitochondrial Ca^{2+} *via* an unknown pathway (Vosler et al., 2009), and calpain activity has been reported to occur downstream the PARP-1 activation (Moubarak et al., 2007), therefore, possibly blocking the PARP itself is somehow effective to arrest the degeneration to some extent as the PARP-1 would no longer transmit the death signal to calpains.

In case of S334ter retina, failure of PJ34 at halting/decreasing photoreceptor cell death could be due to involvement of multiple cell death pathways involving capase mediated as well as alternative cell demise mechanisms involving calpains, PARP and AIF (Kaur et al., 2011; Liu et al., 1999) (chapter II) and/or to the high number of dying photoreceptors simultanously (Kaur et al., 2011; Liu et al., 1999). Therefore, it appears obvious that blocking of one cell death agent may not be enough, as the other routes to cell death remain open. Alternately, a combination of caspase, calpain and PARP inhibitors might be the answer to rescue the photoreceptors in this case.

4.3 Protective effect of combined treatment

As the inhibitor concentration was decreased to 5µM, the number of TUNEL positive cells decreased greatly in cultured retinas of both P23H and S334ter rats, but still the effect was not protective in both the mutants with both the inhibitors. Therefore, a combined application strategy was designed, which consisted of either calpain inhibitor in combination with PARP inhibitor at lower concentrations.

A protective effect was observed for the combined treatment. It is noteworthy, that the effect was more pronounced for calpain inhibitor VI and PJ34 combined than the combination of calpain inhibitor XI and PJ34 in case of P23H retinal cultures, while, In case of S334ter condition, only a combination of calpain inhibitor VI and PJ34 appeared to provide rescue, and no protective effect was

seen for calpain inhibitor XI and PJ34 combination. The difference shown in response to the treatments could be due to the fact that P23H retina appears to degenerate by calpain and PARP dependent pathway exclusively (Kaur et al., 2011), therefore, inhibition of both the calpain and PARP reasonably provide the protection reinforcably.

This study highlights that photoreceptor cell viability depends on specifically optimized paradigms of the inhibitor concentration and the treatment methods (separate or combined) used during pharmacological inhibition of calpains and PARP. The observation that the calpain inhibitor VI was more beneficial than calpain inhibitor XI points out the importance of specificity of certain calpain isoforms for survival of the photoreceptors as blocking them either enhanced the number of dying photoreceptor cells or imparted no protective effect at all (Paquet-Durand, 2010). Additionally, specificities of various calpain inhibitors vary considerably (Azuma and Shearer, 2008), while trying to inhibit the calpains, there could be cross-inhibition of other cysteine-type proteases (Donkor, 2000; Gomez-Vicente et al., 2005; Ravid et al., 1994) that may be providing survival effects to the photoreceptors and other retinal cells. Moreover, calpain system is vital for the organisms (Carafoli and Molinari, 1998). Therefore, a systemic pharmacological treatment of the calpain system may in some cases cause severe side effects and could prove to be harmful rather than providing any beneficial effects.

Taken together, the present study provides an optimistic scenario about working towards a neuroprotective mutation independent therapy for RP as our results are in agreement to previously published neuroprotective effects of calpain and PARP inhibitors used in this study. Precisely, calpain inhibitor VI also called SJA6017, a di-peptidyl aldehyde has been reported to protect retinal neurons against ischaemia-induced damage (Sakamoto et al., 2000). In the photoreceptor context, it has been shown to completely arrest the cGMP-dependent cell death in 661W murine photoreceptor-like cell line (Sharma and Rohrer, 2007). Calpain Inhibitor XI or CX295 has been shown to protect cortical neurones from anoxia–ischaemia-induced damage (Blomgren et al., 2001). Recently, CX295 was shown to render protection to *rd1* photoreceptors under special paradigm of treatment (Paquet-Durand et al., 2010). Both SJA6017 and CX295 have been reported to

reduce calpain activity in *rd1* retina *ex-vivo* with CX295 imparting more effect (Paquet-Durand et al., 2006). Interestingly, calpain inhibitor CX295 reportedly is more effective in PDE6 mutant *rd1* mouse model, while it the same was not as effective as SJA60 in RHO mutants. This discrepancy could be due to species specificity of calpains. The potential of PJ34 to provide neuroprotection has been reported in *rd1* model of RP (Paquet-Durand et al., 2007). Importantly, no study was conducted to ascertain the neuroprotective capacities of combined application of these calpain and PARP inhibitors in RP. Present study is the of this kind and has hinted strongly towards the thrapeutic potential of these inhibitors in halting or atleast slowing down the pace of cell degeneration in retinal degenerations.

5. Conclusion

The present study suggests that calpain and PARP inhibitors appear to offer reasonable candidates as pharmacotherapeutic agents to prevent photoreceptor degeneration due to different genetic mutations. Additionally, the study also highlights that at certain concentrations the calpain inhibition yield detrimental effects. Also, a combined application of calpain and PARP inhibitors is more beneficial in providing the rescue, as RP models P23H and S334ter rats may have concomitant activation of PARP and calpain, and blocking one pathway may not be enough to prevent the cell death. Calpain and PARP targeting is a feasible approach for the treatment of inherited retinal degeneration, however, in the future, carefully targeted and highly selective calpain inhibitors might further improve this. Briefly, our study supports the argument that PARP and calpain inhibitors could provide new avenues as neuroprotective therapy to at least partially arrest the photoreceptor degeneration in inherited retinal degeneration. The immediate challenges, however, include the development of methods to deliver these inhibitors to specific cells of the retina and to test their efficacy *in-vivo.*

Chapter IV: cGMP regulation in Rhodopsin mutant S334ter and P23H rat retinas

1. Introduction

cGMP signaling is important for the photoreceptors to evoke effective photoresponse (Pugh and Lamb, 1990). Light stimulates the hydrolysis of cGMP by PDE resulting in closure of cGMP-gated ion channels. This event leads to decrease in the free intracellular Ca^{2+} concentration, which results in abrupt hyperpolarization of the photoreceptor membrane. Additionally, decrease in the Ca^{2+} levels provides feedback to the processes responsible for photoreceptor recovery and adaptation to background light (Arshavsky et al., 2002; Lamb and Pugh, 2006). PDE mutant *rd1* mouse model of RP are characterized by accumulation of cGMP in the photoreceptors due to their inefficient hydrolysis (Farber et al., 1988). Interestingly, different animal models with mutations in different genes (related to phototransduction) also have shown elevated cGMP levels in their photoreceptors (Mendes et al., 2005). Apparently, alterations in physiological cGMP levels are associated with retinal degenerations (Ramamurthy et al., 2004; Sahaboglu et al., 2010; Sharma and Rohrer, 2007; Trifunovic et al., 2010; Tucker et al., 2004; Ulshafer et al., 1980).

RHO mutations are accountable for more than 25% of the ADRP cases. Modulations in the protein structure and/or function due to mutations may have downstream consequences on the functioning of other transduction proteins leading to retinal degenerations (Rosenfeld et al., 1992; Shastry, 1994; Sohocki et al., 2001). Since, S334ter truncated RHO molecule fail to phosphorylate owing to lack of phosphorylation sites and arrestin binding sites, it continue to be in prolonged activation state, thus, there are alterations in the phototransduction cascade during recovery phase (Green et al., 2000; Lee et al., 2003). Similarly, P23H mutations with defect in folding are reported to possess abnormal disc morphology leading to impaired retinal function (Liu et al., 1997). Patients with ADRP caused by P23H mutation were reported to show impaired activation and slow recovery of the phototransduction cascade (Birch et al., 1995). Similar pattern of delayed phororesponse recovery was later echoed in studies conducted on

P23H mouse models (Goto et al., 1996). Moreover, RHO phosphorylation misregulation was also reported in P23H rats that might affect the retinal degeneration in these rats (Saito et al., 2008). Since, Ca^{2+} ions play a key role in recovery of the photo-response (Hsu and Molday, 1993; Koch and Stryer, 1988), a slower recovery could result from abnormal Ca^{2+} levels. Moreover, Ca^{2+} ions are implicated in the cell death (Nicotera and Orrenius, 1992). Additionally, we have reported Ca^{2+}sensitive calpain type proteases to be active in rhodopsin mutant P23H-1 and S334ter-3 rat models of RP, therefore, cGMP is the obvious target to be investigated in these rat models, since they regulate the CNG channels, and hence the Ca^{2+} levels in the photoreceptors (Abe et al., 2008). To this end, P23H-1 and S334ter-3 rat models provide the excellent opportunity to investigate expression of cGMP and other transduction proteins to see the possible defects in their expression levels and to know what role, if any, they might play in the degenerative process of photoreceptors. Identification of abnormalities in the expression pattern of transduction proteins may represent specific signatures of P23H and S334ter mutations that could depict the events related to pathologic changes in photoreceptor function.

2. Aim

To investigate the levels of cGMP and expression of phototransduction proteins in order to ascertain their possible impact during photoreceptor cell death in Rhodopsin mutant S334ter-3 and P23H-1 rat retinas.

3. Results

In the present study, status of various phototransduction components were examined including rhodopsin, transducin, PDE6, cGMP and arrestin by means of basic histological immunostaining and WB method by using specific antibodies (see chapter VI, table 5.1 and 5.2) in S334ter and P23H rat retinas.

3.1 Expression of Rhodopsin

First of all immunostaining was performed by using an antibody against RHO (Chemicon/ MAB 5316) to assess the RHO distribution in the photoreceptors of

S334ter-3 and P23H-1 rat models, which revealed mis-localization in S334ter-3 mutant retinas at PN12 as revealed by strong labelling of the cell bodies and the inner segments in comparison to age matched controlled CD retina. Outer segments of rod photoreceptors appear very tiny and underdeveloped as compared to the corresponding layers of wild type retina (Fig.4.1A) confirming earlier reports (Martinez-Navarrete et al., 2011; Ray et al., 2010). In P23H-1 mutant retina, at PN15 RHO was seen to be present in OS and weakly in cell bodies (Fig. 4.2A), however, at later stage (PN30) the labeling was observed all over ONL and cell bodies (Kaur, 2010).

Secondly, WB was performed to assess the expression levels of RHO in S334ter and P23H rat retinas at various PN ages (S334ter: PN5, PN 12 and PN 32; P23H: PN15, PN20 and PN32 alongwith corresponding ages of wild type CD retina). Predicted molecular weight of RHO is 35kDa. Wild-type rhodopsin exhibits itself primarily as a monomer (approximately 35 kDa) and, to a lesser extent, as dimers (approximately 70 kDa) and tetramers (approximately 120 kDa) (Tam and Moritz, 2006). A decreased level of RHO expression was observed at 35kDa in S334ter retinal tissue at all PN ages. PN5 did not show any band at this molecular weight. At PN12 the expression of RHO was evidently less as compared to wt retina. Also, at PN32 no band was observed owing to the fact that most of the rods have fully degenerated at this time point (Fig 4.4). P23H-1 retina also revealed less RHO level as compared to wt retina from PN15 in to PN 32 in WB analysis. P23H retinal tissue also produced di- and trimers like wild type RHO, however the level of expression in this case was less than the wild type at all ages, as previously shown (Tam and Moritz, 2007). Another anti-rhodopsin antibody Rho1D4 (Chemicon/ MAB5356), directed against C-terminus, which recognises last 9 amino acids, was used to examine the distribution and expression level of RHO in S334ter retinal tissue. Since, S334ter lacks the last 15 amino acids, therefore, in heterozygous rat retinal tissue, only wild type rhodopsin (*wt*) is recognised by this antibody. Immunolabeling showed RHO protein contained within the cell body (Fig. 4.1B). Rho1D4 antibody was also used to investigate the levels of rhodopsin by immunoblots prepared from P12 *wt* and S334ter retinas. S334ter rhodopsin was observed to be less than the wt at PN12 (Fig. 4.4).

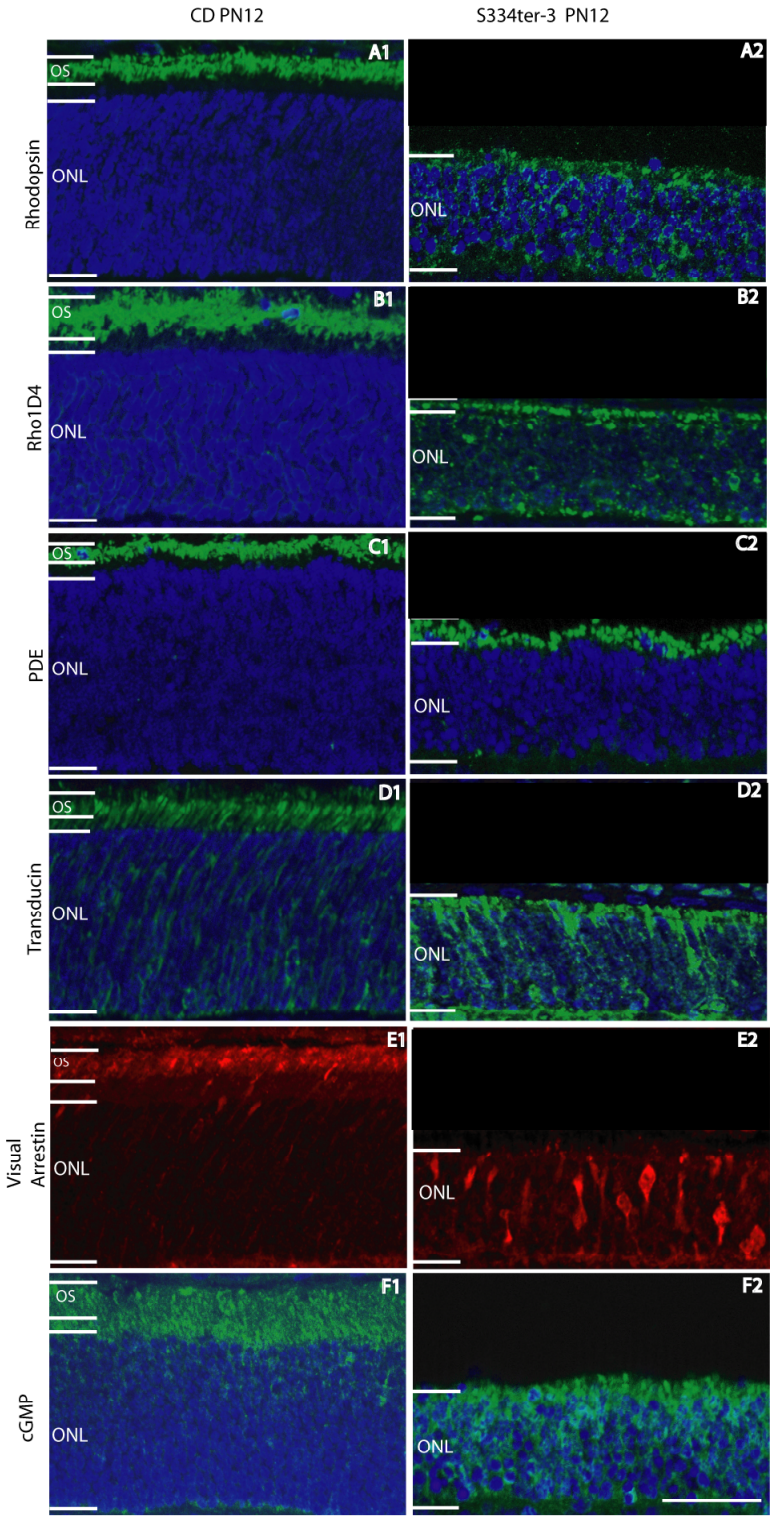

Figure 4.1. Differential expression of various phototransduction components in Rhodopsin mutant S334ter and wild type CD rat retinas. Rhodopsin is present in the outer segments as evident by immunostaining in case of CD rat retina (A1). Intense labeling of photoreceptor cell bodies is seen in S334ter retina. No deveoped outer segments are seen in S33ter retina. Rho14D antibody which specifically recognises wild type rhodopsin at C-terminus (last 9 amino acids) also shows strong labeling of photoreceptor cell bodies, inner segments and poorly developed outer segments (B1, B2). PDE6 is seen located to the outer segments in CD, and in incompletely developed much shorter outer segments in case of S334ter retinas (C1,C2). Transducin expression (in outer segments, inner segments and cell bodies) is similar in wt CD and S334ter retina, however, staining intensity is stronger in case of S334ter and transducin appears localised to the cell bodies. No outer segment labeling was seen for transducin in S334ter retina. (D1,D2). Visual arrestin is loclalised to outer segments in the CD retinas (E1), but in case of S334ter retinas, arrestin clearly shows its localisation in the cell bodies cell bodies (D2). A strong accumulation of cGMP is seen in the S334ter retinas as compared to CD retinas (F1,F2) (**Labels**: Blue; DAPI nuclear staining) Scale bar =50µm.

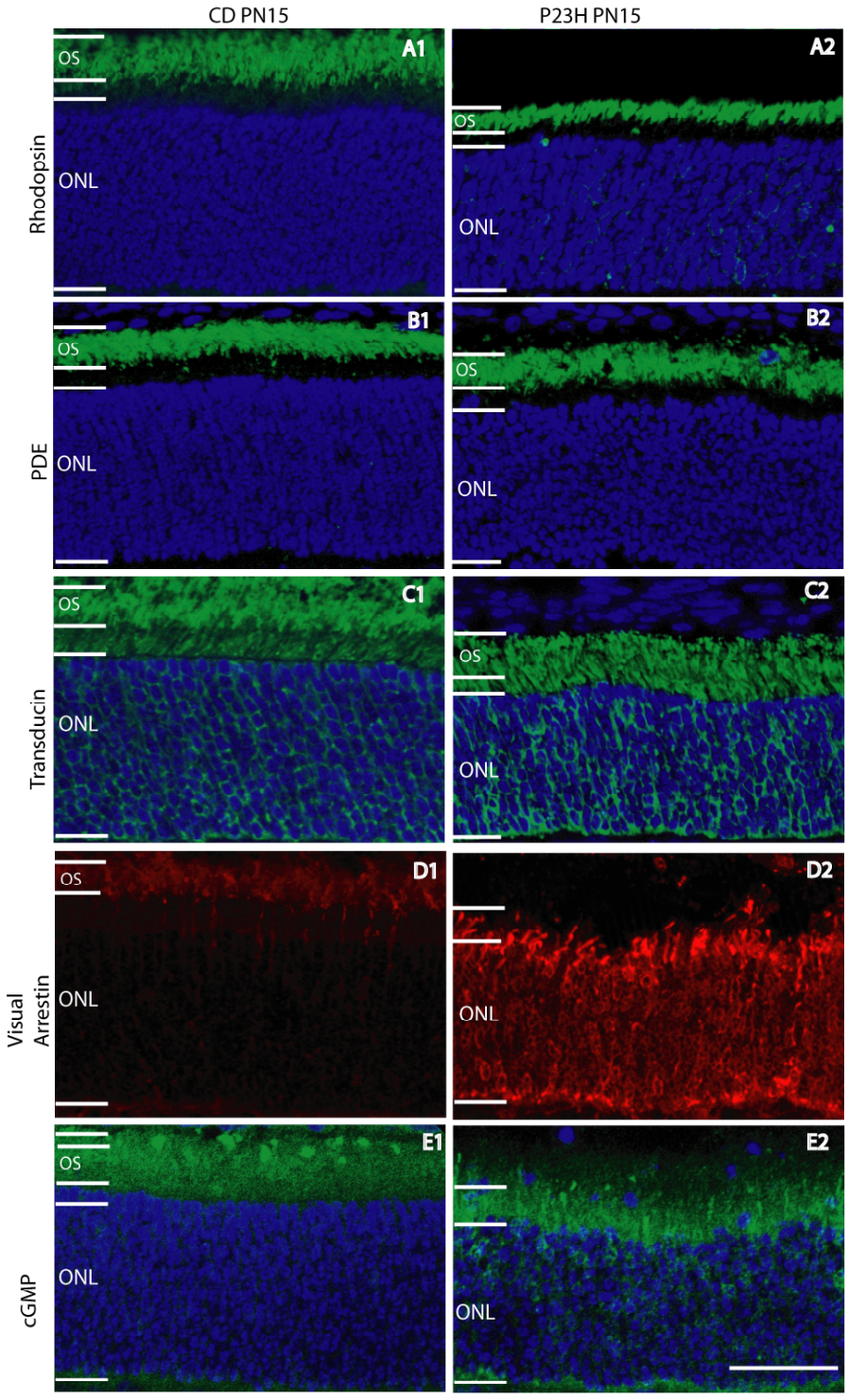

Figure 4.2. Differential expression of various phototransduction components in Rhodopsin mutant P23H and wild type CD rat retinas. Rhodopsin is present in the outer segments as evident by immunostaining of the same in both CD and P23H retinas. Some photoreceptor cell bodies also depict rhodopsin localised to them in P23H retina. A1, A2) PDE (in the outer segments) and transducin expression (in outer segments, inner segments and cell bodies) is similar in wt CD and P23H retinas (B, C). Visual arrestin is localised to outer segments in the CD retinas (D1), but in case of P23H retinas, arrestin clearly shows ist presence in the inner segments and cell bodies (D2). An accumulation of cGMP is seen in the P23H retinas as compared to CD retinas (E1,E2).) (**Labels**: Blue; DAPI nuclear staining) Scale bar =50µm.

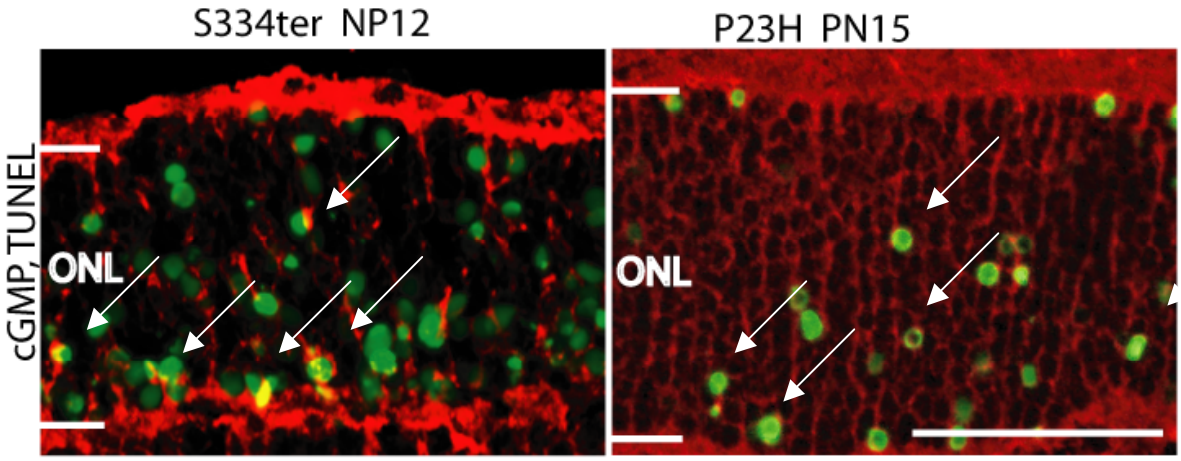

Figure 4.3 Fluorescent pictures showing co-labelling of cGMP with TUNEL staining in rhodopsin mutant rat retinas. Many cells were co-stained for cGMP (red) and TUNEL (green) in S334ter and P23H rat retinas at PN12 and PN15 respectively, Scale bar=50µm.

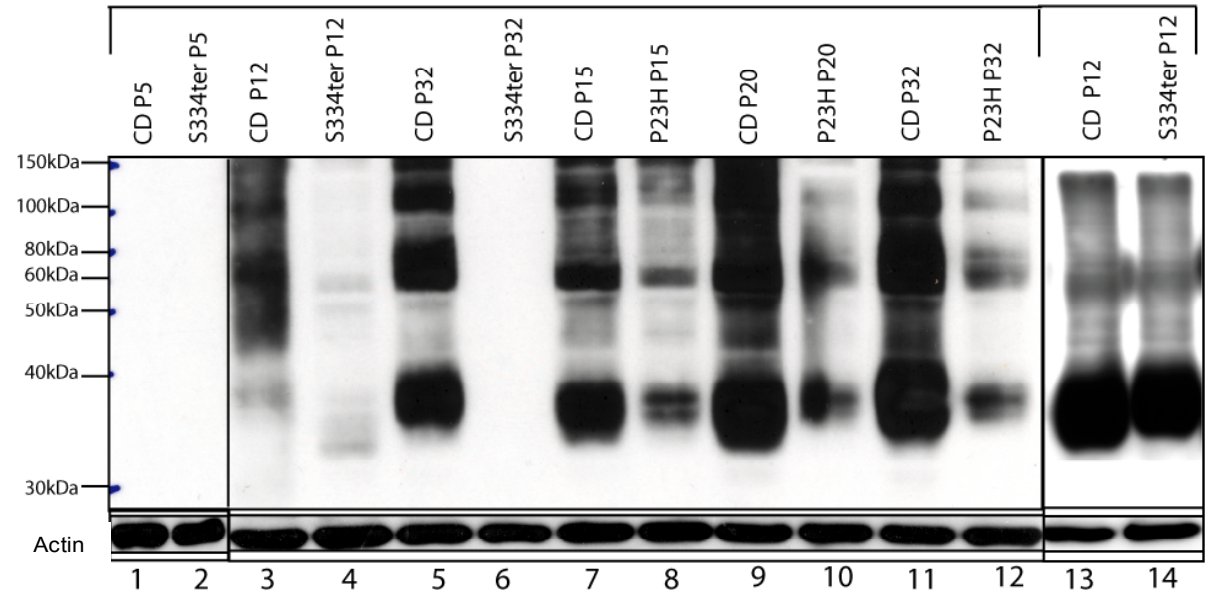

Figure 4.4. Western blots showing Rhodopsin expression in S334ter and P23H retinal tissue. Lane 1 and 2 depict wt CD and S334ter rhodopsin at PN5 (no rhodopsin expression at this age). S334ter retina shows less expression at PN12 (lane 2-4) and no expression in adult retina at PN32 (lane 5-6) as most of the rod photoreceptors are already lost at this age. P23H retina shows less rhodopsin expression from PN 15 to PN32 (lane 8, 10, 12) in comparison to wt rhodopsin expression. Lane 13 and 14 show the expression of rhodopsin. Wild-type rhodopsin appears primarily as a monomer (approximately 35 kDa), and also to lesser extent as dimers (approximately 70 kDa) and tetramers (approximately 120 kDa). Lane 13 and 14 display rhodopsin expressin agianst antibody Rho14D, which recognizes last 9 amino acids towards C-terminus of rhodopsin molecule. S334ter retinal tissue showed a lesser expression at PN12 as evident from band width at about 35kDa as compared to wt CD retinal tissue at the same age.

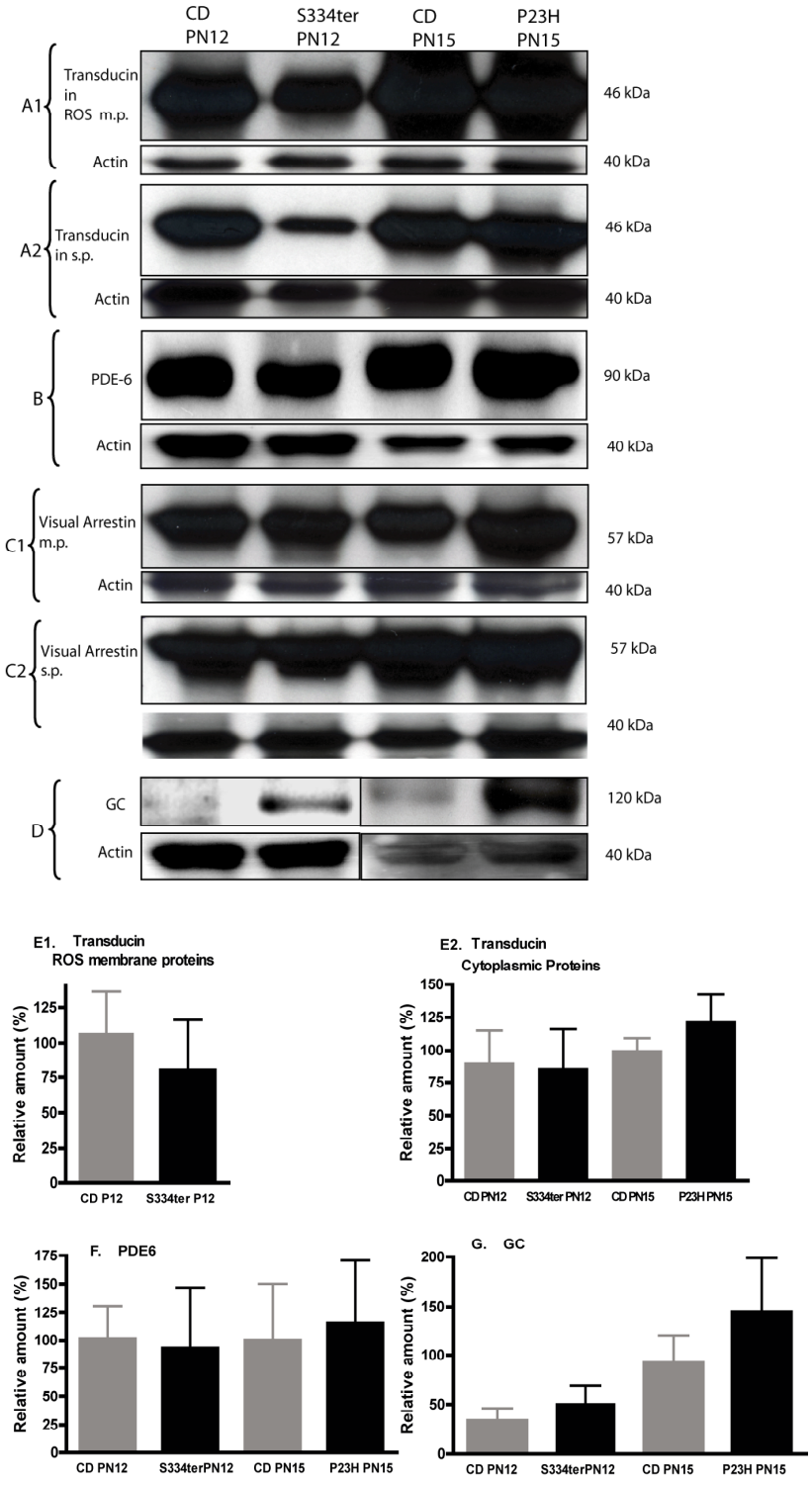

Figure 4.5. Expression of phototransduction proteins in Rhodopsin mutant S33ter and P23H rat retinas as deicted by western blot method. Transducin expresses at lesser level in in rod outer segment membrane proteins (m.p.) and cytoplasmic soluble proteins (s.p.) in S334ter retina. P23H shows more expression of soluble proteins (s.p.), while its level in m.p. was same like the wild type transducin (A1,A2,E1,E2). PDE6 expression in S334ter retina is less than wt CD PDE6 at PN12. P23H retina exhibits PDE6 nearly same level as wt CD retina (B, F). Visual arrestin displays more expression in P23H in the ouer segment membrane proteins, while S334ter expresses same level of PDE6 as that of CD retina. In cytoplamic soluble proteins, no change in expression was seen in mutants than the wt CD retinal tissues (C1,C2). Both S334ter and P23H retinal tissue show increased GC in comparisn to wt CD condition (D,G) Quantification (graphic) values are mean ± SD from three or four different experiments each containing retinas from 6 animals: Significance level; $p>0.05$.

3.2 Levels of cGMP

As S334ter and P23H mutants have been reporteted to show impaired recovery during phototransduction, and both show upregulation of high Ca^{2+} ion related processes, for instance, oxidative stress and calpain activation, therefore status of cGMP was sought in these mutants. The present study investigated the level of cGMP in P23H and S334ter rat retina by immunolabelling. The staining was performed using antibody against cGMP (gifted by Jan de Vente, Maastricht University, Netherlands). Interestingly, accumulation of cGMP was observed in both the rat retinas as evident from a strong staining of photoreceptor cells in the ONL of both the mutants as compared to wt CD retina at PN12 (S334ter) and PN15 (P23H) (Fig. 4.1F and Fig. 4.2E). The S334ter retina, nevertheless showed more photoreceptors stained by cGMP as compared to P23H retina. (Fig.4.1F). A co-labelling of the cGMP with TUNEL assay displayed many cells co-stained by cGMP and TUNEL assay, suggesting a correlation of cGMP accumulation to photoreceptor cell death (Fig. 4.3).

3.3 Expression of retinal guanylyl cyclase

The synthesis of cGMP is regulated by retGC in the retina. During recovery phase of phototransduction, fall in Ca^{2+} concentration induces the release of retGC from GCAP for synthesis of cGMP to restore its basal levels (Gurevich and Benovic, 1995). In order to investigate the potential source of observed elevated cGMP levels in the RHO mutant retinas, the expression level of retGC was examined by means of WB using antibody against GC (generously provided by Prof. Dr. Karl-Wilhelm Koch, University of Oldenburg, Germany). The WB of GC identifies a band at 120 kDa (Mihelec et al., 2011). Both S334ter and P23H retinas showed an increase in GC protein in the WB as compared to wt retina, which was more prominent in case of P23H retina (Fig. 4.5 D,G). A statistical analysis also depicted an increase in GC expression in P23H retinal tissue. Quantification of WB also revealed more GC expression in mutants than in their wt counterparts (Fig. 4.2 F,L), however, it was statistically non-significant (S334ter: $p>0.05$, n=4; S334ter: $p>0.05$, n=4: P23H: $p>0.05$, n=4; S334ter: $p>0.05$, n=4). Since, the antibody

available was specific for WB, no immunolabelling could be performed on retinal tissue in rat retina.

3.4 Expression of Phosphodiesterase-6 (PDE6)

As phosphodiesterase-6 (PDE6) is responsible for hydrolysis of cGMP during phototransduction, therefore, its expression was examined both by immunostaining and WB by using specific antibody against PDE6ß (Dianova/ PA1-722). The immunolabling showed normal distribution of PDE6 in the OS of photoreceptors in mutants (P23H-1 and S334ter-3) and wt retina (Fig. 4.1C and Fig. 4.2B), nevertheless the OS in S334ter showing immunoreactivity to PDE6 were shorter than those of wt and P23H (Fig. 4.1C).

Secondly, WB was performed to assess the expression level of PDE6ß protein, which identified the characteristic 90kDa. WB analysis showed decreased level of expression of PDE6 in case of S334ter retina as compared to wt retina (S334ter: $p>0.05$, n=3). PDE level in P23H retinal tissues were not different from age matched control wt retinas at PN15 (P23H: $p>0.05$, n=3) (Fig. 4.5 B, F).

3.5 Expression of G Protein Transducin

Transducin, the heterotrimeric G protein of the rod phototransduction cascade, is activated by interacting with activated RHO. During recovery phase of visual transduction, phosphorylation of RHO by GRK1 weakens the interaction between transducin and rhodopsin, and the binding of arrestin to phosphorylated RHO prevents further interaction between RHO and transducin (Lamb and Pugh, 2006).

G-protein transducin shows different distribution within the photoreceptors in light and dark. In dark it is localized mainly to the OS, whereas in light conditions, it translocates to inner compartments including IS, cell bodies and synaptic terminals (Lamb and Pugh, 2006). Approximately 90% of transducin translocates from the rod OS to the inner compartments in bright light (Sokolov et al., 2002). Transducin's distribution in the photoreceptors was checked by means of immunostaining by using the antibody specific for Gα subunit (Santa Cruz/ sc-389). In both P23H, immunostaining showed transducin distribution in photo-

receptor OS, IS and the cell bodies like the wt retina (Fig. 4.1D; Fig. 4.2C). However, in S334ter retina, the transducin was seen clearly localised to cell bodies with intense staining, which could be due to incomplete development of the outer segment in his case.

Simultaneously, western blotting was used to assess the levels of transducin in retinas of both transgenic rats. Since transducin protein shifts from OS to inner compartments of cell upon illumination, its expression in rod outer segment membrane and cytoplasmic proteins was checked separately. For this, a different method was used to homogenize the retinal tissue, and to separate the rod outer segment proteins from the cytoplasmic protein content. WB of transducin (Gαt1) identifies band at 46kDa, and there was no obvious difference between the mutant P23H and CD retina in rod OS membrane protein (m.p.) fraction at PN15, however, cytoplasmic protein fraction showed a slight increase in transducin band thickness at 46kDa in P23H retinal tissue at PN15 as compared to age matched wt retinal tissue (P23H: (rod outer segment m.p.): $p>0.05$, n=3; (cytoplasmic protein portion): $p>0.05$, n=3). S334ter-3 retina showed a decreased expression of transducin as compared to the CD at PN12 in the rod OS membrane protein content (Fig. 4.5A1) Quantification also showed a decrease of transducin in S334ter in both rod outer segment and cytoplasmic protein portions, however, it was statistically non-significant (Fig. 4.5E1) (S334ter: (rod outer segment m.p.): $p>0.05$, n=3; (cytoplasmic protein portion): $p>0.05$, n=3) (Fig. 4.5A2; Fig. 4.5E2).

3.6 Visual Arrestin

Arrestins are cytoplasmic proteins, which bind to phosphorylated RHO, and help to prevent further interaction of activated RHO with transducin, a key event in turning off the phototransduction cascade (Zhao et al., 1995). During light conditions, both transducin and arrestin migrate in opposite directions and get re-distributed within the photoreceptors (Whelan and McGinnis, 1988). Stable binding of arrestin to phosphorylated RHO is a key step, as it reduces soluble arrestin in the outer segment, which may possibly creates a gradient triggering redistribution of arrestin from the inner segment to the outer segment. Employing immunostaining and WB techniques, expression of arrestin was checked in S334ter and P23H retinas by

using antibody against visual arrestin (Thermo scientific/ PA1-731).

As normally arrestin shows reverse distribution as compared to transducin, it is localized in the inner segments in the dark, but is translocated to OS in light conditions (Lamb and Pugh, 2006). In the present study, experiments were performed using retina adapted in light conditions. S334ter rat retina showed arrestin localization primarily to the cell bodies as compared to age matched wt retina (Fig. 4.1E). Similarly, P23H retina at PN15 depicted arrestin to be localized to cell bodies (Fig. 4.2D). WB showed no apparent change in expression of visual arrestin in outer segment membrane protein fraction, as well as in the cytoplasmic portion of proteins in both S334ter and CD retinal tissues at predicted molecular weight of arrestin (57kDa). In case of P23H retinal tissue, a slight increased expression of arrestin was seen in rod outer segment protein component, however, cytoplasmic protein part did not show any obvious change in the band thickness for arrestin in comparison to wt CD retinas (Fig. 4.5C).

4. Discussion

Second messenger cGMP plays pivotal role in intracellular signalling, including the regulation of cell survival or cell degenerative pathways (Fiscus et al., 2002). In the past, studies on different RP animal models have clearly established connection between disturbances in cGMP homeostasis and photoreceptor cell death. PDE6 mutant *rd1* mouse retina shows high cGMP due to its insufficient hydrolysis by mutant PDE enzyme (Farber et al., 1988). Interestingly, elevated levels of cGMP are found in other animal models of RP too, which are characterized by mutations in different genes, e.g., Rhodopsin (Mendes et al., 2005), cyclic-nucleotide-gated channel-a3 (CNGa3) (Michalakis et al., 2010), aryl hydrocarbon receptor-interacting protein-like (AIPL1) (Ramamurthy et al., 2004), GC1 and GC-2 (Karan et al., 2008), or guanylyl cyclase activating protein (GCAP) (Nishiguchi et al., 2004; Olshevskaya et al., 2002), implying that high cGMP could be common phenomenon in various models of retinal degenerations.

RHO mutations can be broadly categorized into two groups: loss-of-function or gain-of-function (Mendes and Cheetham, 2008; Mendes et al., 2010). Gain-of-function mutations may cause an increased or sometimes constitutive

phototransduction activity, and loss-of-function mutations may reduce phototransduction activity. Both defects somehow cause irregularities in cGMP levels. Since both S334ter and P23H are gain of function ADRP mutations (Mendes et al., 2005; Robinson et al., 1994), and phototransduction abnormalities are reported for both these mutations specifically during recovery phase (Birch et al., 1995; Goto et al., 1995; Green et al., 2001; Lee et al., 2003), therefore, irregularities in Ca^{2+} and cGMP levels are anticipated in these mutations.

Present study investigated the status of cGMP, alongwith the investigation of other phototransduction proteins in RHO mutant rat models and found that cGMP levels are upregulated in S334ter-3 and P23H-1 rat retinas. Further, retGC, which synthesises cGMP, is upregulated in P23H-1 and S334ter-3 rat retinas. Simultaneously, level of PDE-6 expression was observed to be less in S334ter-3 retina but not in P23H-1 retina. Other phototransduction such as RHO, transducin and arrestin also showed some alterations in expression patterns.

4.1. cGMP and retGC

Accumulation of cGMP in the photoreceptors of both S334ter and P23H retinas was observed confirming an earlier study, which also showed elevated cGMP levels in the photoreceptors of P23H mouse retina (Roof et al., 1994). Since, in retinal photoreceptors, synthesis and hydrolysis of cGMP is directly regulated by retGC and phosphodiesterase-6 (PDE6) respectively (Cote, 2004; Peshenko et al., 2011), a defect in either of these components could affect the relative rates of synthesis and hydrolysis of cGMP, which may result in an irregular cycle leading to either low or high cGMP level. Expression of retGC in these mutants seemed more as compared to wild type CD retina. RetGC is regulated by guanylate cyclase activating protein (GCAP) in mammalian rod photoreceptors (Dizhoor and Hurley, 1999; Palczewski et al., 2004; Peshenko and Dizhoor, 2007). After photoexcitation, recovery of the cell occurs through the inactivation of opsin, shutoff of the phototransduction cascade, and resynthesis of cGMP by the activation of retGCs via GCAP (Kaupp and Seifert, 2002). Ca^{2+} feedback is of utmost importance for controlling the recovery and light adaptation. At low intracellular Ca^{2+} levels (during light), GCAPs activate retGC to restore basal cGMP levels.

High Ca^{2+} concentrations (above 500 nM) typical for dark-adapted photoreceptors turn the GCAPs into inhibitors of retGC. A high retGC expression would mean constitutive retGC activity, which may be responsible for the observed cGMP accumulation in RHO mutant rats (Sokal et al., 1998).

Accumulation of cGMP in photoreceptors may cause constitutive activation of cyclic nucleotide gated channels (CNGC), which may further affect Ca^{2+}-dependent processes within the cells. Although, intracellular calcium level have not been directly assessed during retinal degenerations in P23H or S334ter mutants, and calcium-channel blocker D-*cis*-diltiazem failed to impart any protective effect in P23H-1 rat retina (Bush et al., 2000). However, we have reported an upregulation of calcium sensitive calpain proteases in the degenerating retinas of P23H and S334ter (Kaur et al., 2011). Therefore, high cGMP level apparently comply with Ca^{2+} dysregulation, which could occur by its continued influx through open CNG channels from extracellular sources. Although other souces may also contribute to the pathological Ca^{2+} overload in the cells, for instance, cGMP regulated protein kinase G (PKG) may cause Ca^{2+}-influx within the cell from intracellular stores (Frasson et al., 1999; Wagner et al., 2003) (Traverso et al., 2002). Although no study about PKG activity is available for S334ter and P23H mutants, however, *rd1* mice, which apparently share several key components during photoreceptor degeneration with RHO mutant rats, have been reported to have PKG as well as (Pilz and Broderick, 2005) calpain activation (Paquet-Durand et al., 2006). Interestingly, activation of calpains via cGMP-PKG route has been shown in osteoclasts (Yaroslavskiy et al., 2007), and this point raises the possibility of similar pattern of events in case of *rd1* and RHO mutant retinas as it shows cGMP upregulation alongwith calpain activation. Interestingly, calpains have been reported to cleave some $Na^{+}/Ca^{2+},K^{+}$ exchangers (NCKX) in neuronal cells with excitotoxicity, and may also account for Ca^{2+} overload (Bano et al., 2005). The impact of Ca^{2+} overload, on the one hand, results in processes sensitive to their presense, e.g., activation of calpains, on the other hand, it also inhibits GCAP, GC activity and thus cGMP production. Theoretically this negative feedback mechanism should prevent intracellular cGMP levels to rise, however, for reasons yet unknown, this feedback mechanism appear nonfunctional or

ineffective in degenerating P23H and S334ter retina similar to rd1 situation (Paquet-Durand, 2010) as high cGMP level apparently co-exists alongwith constitutive GC activation and other processes, which are witnessed only by Ca^{2+} overload in the cells. A plausible explanation to the inefficiency of Ca^{2+} feedback mechanism in case of RHO mutant rat retinas remain elusive as yet.

4.2 Phototransduction proteins

A large number of studies in the past have investigated the expression level and distribution pattern of rhodopsin along with some other phototransduction proteins in rhodopsin mutant S334ter and P23H mutants (Green et al., 2000; Lee and Flannery, 2007; Tam and Moritz, 2006; Tam et al., 2006), it was sought to investigate the major phototransduction components in our experimental system to examine their expression and distribution patterns. Since, cGMP hydrolysis is regulated mainly by cGMP-PDE6, which in turn may be modulated by the G-protein transducin and Rhodopsin and GRK1/phosphatase system, any change encountered by any of these components could account for the altered PDE6 activity in mutant retinas, which may be responsible for cGMP accumulation.

4.2.1 Rhodopsin

As described previously, S334ter RHO is present all over the rod photoreceptors, including cell bodies and the inner segments in addition to outer segments, and their level in the overall retinal tissue, as revealed by WB, was less in mutant than the wt CD retinas. PN5 showed no band in both CD and mutant retina as no RHO is expressed at this age in rat retina (Morrow et al., 1998). Also, adult S334ter retinal tissue did not show RHO bands corresponding to age matched wt retinal tissue as most of the rod cells are already degenerated by this time in S334ter retina. A less rhodopsin at PN12 may be attributed to decreased number of rod photoreceptor cells at this stage as 50% of rods undergo degeneration between PN11 to PN12 (Liu et al., 1999).

Various studies in the past have described the mislocalization of C-truncated

RHO due to mis-sorting in rod cell bodies along with prolonged photo-response of mutant RHO in absence of phosphorylation sites and inability to shut off by arrestin binding in S334ter mutants, and a mislocalised protein alongwith constitutively activated phototransduction cascade has been postulated to kill the photoreceptors by switching the molecular pathways leading to cell degeneration (Birch et al., 1995; Goto et al., 1995; Green et al., 2001; Lee et al., 2003). It has been suggested that S334ter RHO after folding and processing in the ER and golgi, is packaged in the vesicles to be transported. However, their final destination is not properly defined, since the outer segments are under-developed (Hombrebueno et al., 2010; Martinez-Navarrete et al., 2011). Localization of RHO in the cell bodies may be due to the presence of RHO-containing vesicles in the cytoplasm, where they might interfere with the normal vesicular transport, normal cellular processes leading to cell death. Simultaneously, a prolonged activation of mislocalised S334ter opsin may induce functional abnormalities, outer segment shortening and eventual photoreceptor death (Green et al., 2000; Lee and Flannery, 2007).

P23H-1 retina also revealed less RHO level as compared to wt retina from PN15 in to PN 32 in WB analysis. WB of RHO produced di- and trimers bands like wild type RHO supporting the accumulation of P23H in misfolded state in the IS, however the level of expression in this case was less than the wild type retinal tissue at all ages (Tam and Moritz, 2006). In P23H-1 mutant retina, RHO was seen to be distributed to the OS, IS and some amount was seen to be localized to cell bodies (Kaur, 2010) as reported earlier (Tam et al., 2006). Both S334ter and P23H RHO mislocalise, former due to sorting defect and latter due to folding defect. As the experimental animals in the present study were heterozygous, which have normal gene in presence of mutant gene, therefore, RHO was observed to be localised in the OS as well (Tam and Moritz, 2006).

4.2.2 Phosphodiesterase-6, G-Transducin and Arrestin

Both S334ter and P23H retinas showed normal distribution of PDE6 in the OS of retina like the wt PDE6 localisation. However, expression level of PDE6 in S334ter

retinal tissue was less than wild type at PN12, whereas, P23H revealed unaltered expression PDE6 levels. One reason of observed down regulation of PDE6 in S334ter3 rat retinas could be incomplete development of rod OS (Li et al., 2010b; Liu et al., 1999), and and so may be the protein content in them.

G-protein transducin and arrestin translocate within photoreceptors under light conditions in opposite directions. Approximately 90% of transducin translocates from the rod OS to the inner compartments and the majority of arrestin redistributes to rod outer segments upon illumination (Lamb and Pugh, 2006; Sokolov et al., 2002). Redistribution of transducin and arrestin within the photoreceptors plays an important role in light adaptation as well as cell survival mechanisms to prevent exagerated signaling in extremely bright light conditions (Arshavsky, 2002b; Frechter et al., 2007; Lee et al., 2004). Since, RHO shutoff is not complete in S334ter mutants; therefore, it may be probable that RHO and transducin remain in prolonged association, which may result in decreased expression of Gα in photoreceptors, which may in turn be accountable for less PDE expression resulting in incomplete cGMP hydrolysis. The expression level of arrestin in wild-type and mutant retinas was not affected. These results indicate that translocation of arrestin in mutant retinas seems to occur independent of downstream signaling events in phototransduction, and that the light-dependent redistribution of arrestin does not require the phosphorylation of activated rhodopsin (Mendez et al., 2003). Interestingly, despite seemingly unaltered expression levels of arrestin in S334ter and P23H rats, the immunostaining revealed mislocalised arrestin in these mutant rats. It is interesting to note that even if the protein levels appear same, the distribution of it might be abnormal. Previously, it has been proposed that upon degeneration of OS, the proteins normally meant to be routed towards the OS may just stop their movement, for short of their final destination, filling up the remaining photoreceptor cell cytoplasm. Alternately, there could be passive concomitant misrouting of phototransduction proteins alongwith rhodopsin in these mutants, as these transduction proteins are suggested to be tightly associated with rhodopsin much before they reach the OS (Roof et al., 1994). An abnormal localization of proteins may cause abnormal functioning of phototransduction, particularly during recovery

phase as reported in earlier studies, leading to induction of cell death (Azarian et al., 1995). Another important consideration is that arrestins are cleaved by calpains, and because, these rats have a high activity of these enzymes in the retinas, the arrestins possibly are proteolysed in them, therefore, earlier reported functional defects in recovery could be attributed to mislocalization and cleavage of arrestins by calpains. Therefore, all these events would affect the functionality of phototransduction cascade many fold. Lack of phosphorylation site in S33ter RHO, and misfolded P23H RHO's retention inside the ER, arrestin mislocalisation and probably its cleavage by calpains, together with abnormal expression of other components may explain the severity of degeneration in theses mutants.

Taken together, it may be inferred that mutations in rhodopsin may cause alterations in the expression of other photoreceptor components, which in turn may affect the functions of these components causing abnormal functioning of phototransduction process that leads to degeneration of photoreceptors.

5. Conclusion

The present study revealed that S334ter and P23H rat models show an accumulation of cGMP in their photoreceptors. Further, both rat models showed high expression of retGC enzyme. Among other phototransduction proteins, RHO is mislocalised in the photoreceptors, which was more evident in S334ter. The expression of RHO is also less in both the mutants. PDE6 and transducin appear unaltered in P23H retinas, however, S334ter retina showed less PDE6, and transducin. Arrestin looks normal in the expression level, but is mislocalised in both S334ter and P23H retinas. Taken together, abnormal and/or mislocalised expression of various phototransduction components may explain abnormal recovery response of phototransduction, which could have a bearing on calcium homeostasis that would start a whole battery of events of pathological significance related to calcium ion dysregulations as observed in these rats. Accumulated cGMP levels indicate that it could be a therapeutic target as it has been reported to be upregulated not only in *rd1* but other mutants too and an exogenous regulation of the same could prevent the downstream processes leading the cell to degeneration. It will have implication in mutation independent approach for

neuroprotection of the retina. Whatever may be the reason for high cGMP levels in the rhodopsin mutants, it certainly explains that high cGMP would cause high cytoplasmic calcium levels, which could be responsible for the activation of calcium sensitive protease, *viz.*, calpains in these mutant models.

Chapter V: General Discussion and Conclusion

1. Mechanism of photoreceptor cell death in S334ter-3 and P23H-1 transgenic rat models of ADRP

A deeper understanding of the molecular pathways involved in cell death and cell survival, is crucial to develop effective therapies for diseases including RP, and identification of key players of cell death is the first step in this endeavour. However, until date, the mechanisms of photoreceptor cell death in inherited retinal degenerations have not been completely understood (Sancho-Pelluz et al., 2008). While the previous studies mostly suggested the caspase-mediated apoptosis as the final cell death pathway in retinal degenerative diseases (Chang et al., 1993), but the recent studies point towards caspase independent alternative pathways involved during photoreceptor degeneration. Present study showed that the conventional apoptotic cell death agents (i.e. activated caspase-3, caspase-9, cytochrome c leakage) played a role only under specific circumstances in certain mutants and highlighted the involvement of alternative “caspase-independent”, and hence, non-apoptotic routes to the photoreceptor cell demise in S334ter and P23H rat models of ADRP.

1.1 Role of classical apoptotic cell death agents during photoreceptor degeneration of S334ter-3 and P23H-1

Majority of the earlier studies related to photoreceptor cell death suggested apotosis (Chang et al., 1993; Reme et al., 1998) as the exclusive mode of cell death in retinal degenerations, being executed by caspase-type proteases (Jellinger, 2001). Caspase mediated cell death occurs by mitochondria-initiated pathway, in which cytochrome-c (cyto c) upon release from the mitochondrial intermembrane space attaches to Apaf-1 (apoptosis activating factor-1) and forms cyto-c/apaf-1 complex, which activates caspase-9. Activated caspase-9 in turn cleaves and activates the executioner caspases-3 and -7 (Green and Amarante-Mendes, 1998; Kroemer and Reed, 2000; Slee et al., 1999b). All of these markers (cyto c, caspase-9 and caspase-3) appear to play a role in cell death only in S334ter-3 and not in P23H. A previous study also found photoreceptor cell death in S334ter rats (one of the studied model here) to be mediated by apoptotic cell

death player -caspase-3 (Liu et al., 1999). Present study confirmed these results, but with two important considerations: (i) in S334ter retina, caspase-3, caspase-9 and cyto c leakage appear concomitant with other alternate cell death markers (i.e., calpain and PARP activity), indicating that these mechanisms are executed simultaneously; (ii) in P23H rat retina, caspase-3 activity was not significantly higher than in age-matched wt retinas suggesting that any caspase-3 activity seen in P23H relates only to developmental, and not to mutation induced cell death. This intriguing discrepancy between the two RP models shows how location of mutation in rhodopsin affects the phenotype leading to caspase-independent cell death in one case and induction of caspase independent as well as caspase dependent cell death pathways in the other that may well explain the severity of mutation in the latter. Furthermore, activity of caspase independent pathways in both the rhodopsin mutants and in *rd1* mice (Paquet-Durand et al., 2006; Paquet-Durand et al., 2010) could explain why previous attempts of using caspase inhibition provided none or only partial protection to dying photoreceptors (Liu et al., 1999; Yoshizawa et al., 2002).

1.2 Alternative cell death routes involving Calpians and PARP

S334ter mutants have been reported to show prolonged activation in absence of phosphorylation sites, meant for arrestin binding to quench the activated RHO (Green et al., 2000; Lee et al., 2003; Murray et al., 2009). Similarly, P23H mutants show impaired activation and delayed recovery after the photoresponse (Birch et al., 1995; Goto et al., 1995; Green et al., 2001; Lee et al., 2003). Since, Ca^{2+} ions regulate the processes associated with recovery phase of phototransduction, therefore, dysregulations of Ca^{2+} ions are expected in these mutants. High Ca^{2+} levels have often been implicated in neuronal and photoreceptor cell death (Fox et al., 1999; Kristian and Siesjo, 1998; Nicotera and Orrenius, 1992; Takano et al., 2005). Moreover, hyperactivation of Ca^{2+} sensitive calpain proteases has been reported during photoreceptor cell death in RP (Azarian and Williams, 1995; Azuma et al., 2004; Azuma and Shearer, 2008; Paquet-Durand et al., 2006). At normal physiological Ca^{2+} concentration, calpains regulate many calcium-regulated processes (Azuma and Shearer, 2008; Goll et al., 2003), however, high Ca^{2+} levels

trigger an overactivation of these proteases, which degrade a wide variety of proteins required for cell survival (Chan and Mattson, 1999; Croall and DeMartino, 1991).

In the present study a strong increase in calpain activity alongwith significant reduction in the expression level of its endogenous inhibitor calpastatin, was observed in both the mutants as explained earlier (Maki et al., 1989; Rao et al., 2008). These results are in agreement with earlier studies on *rd1* mouse model of RP, where calpain hyperactivity complemented with calpastatin down-regulation was reported during photoreceptor cell death (Paquet-Durand et al., 2006). Therefore, it can be inferred that calpain activation is a very important step in retinal degenerations induced by different mutation (Paquet-Durand et al., 2010; Sancho-Pelluz et al., 2008; Sharma and Rohrer, 2007).

Secondly, the present study revealed PARP enzyme to be over-activated along with PAR accumulation in degenerating S334ter and P23H rat retinas, in line with similar findings in *rd1* retina (Paquet-Durand et al., 2007). PARP-1 gets activated in response to DNA damage, which might occur due to pathological signals and/or oxidative stress (Jagtap and Szabo, 2005; Skaper, 2003), and helps to repair damaged DNA strands by generating poly-ADP-ribose (PAR) polymers from β-NAD+ (Herceg and Wang, 2001; Jagtap and Szabo, 2005). While, a basal level of PARP activity is necessary in conserving genomic integrity, however, its increased activity in response to excessive DNA damage could result in cell death (Boulu et al., 2001; Koh et al., 2005; Paquet-Durand et al., 2007). PARP over activation has been implicated in photoreceptor cell death in the past (Paquet-Durand et al., 2007; Sahaboglu et al., 2010). An excessive activation of PARP resulting in the production of high levels of neurotoxic PAR polymers (Andrabi et al., 2006; Vosler et al., 2009; Yu et al., 2006), has been linked to a novel form of cell death tentatively termed PARthanatos, which is characterized by caspase-independent pathway mediated by PARP (Wang et al., 2009).

Interestingly, in S334ter retina, PARP hyperactivity was matched with increased PARP-1 expression in retinal tissue, however, in P23H rats, activity did not complement the expression of PARP, as has been reported earlier in case of *rd1*. This intriguing discrepancy remains uncertain, as it does not seem to be

compensatory up regulation of PARP due to cleavage by the activated caspase-3, since S334ter retinal tissue did not show PARP cleavage product. In line with these findinds, a high oxidative stress, which could be the main reason of oxidative DNA damage, and hence of PARP activation, was observed in S334ter and P23H retinas. There may be different sources of oxidative stress in a cell, for example, excessive mitochondrial metabolism could produce reactive oxygen species (Halliwell, 2006) that creates characteristic oxidized compounds like 8-oxo-guanosine (observed in the studied models and earlier reported in *rd1* model (Oka et al., 2008; Paquet-Durand et al., 2007; Sanz et al., 2007). Clearly, in both mutant rats and *rd1* mice, oxidative DNA damage could be the key agent to induce PARP activation (Schreiber et al., 2006). Presently, it is not clear whether oxidative stress primarily causes the retinal degeneration (i.e. rod degeneration) or is it a secondary event causing mutation-independent death of cones and inner retinal neurons (Sancho-Pelluz et al., 2008).

Taken together, calpian and PARP activation are evidently important biochemical factors in photoreceptor degeneration independent of the nature of mutation. Therefore, targeting them by pharmacological approaches may address a wide spectrum of RP-causing mutations while working on a mutation independent neuroprotective therapeutic strategy.

1.3 Progression of cell death markers

S334ter and P23H transgenic rats are characterized by onset of cell death at early stages in the retina, when the developmental cell death is still taking place, therefore, mutation induced cell death showed a temporary overlap with developmental cell death (Young, 1984), However, the cell death markers examined in the the present study were strongly increased only in mutants, implying that developmental cell death probably had only a negligible influence on the overall degeneration of photoreceptors. Progression of photoreceptor cell death in case of P23H retina was slower than the S334ter retina, as reported by earlier studies (Liu et al., 1999; Machida et al., 2000). All studied cell death markers (i.e. TUNEL, caspase-3, calpain activity, PARP activity, PAR accumulation and oxidative DNA damage) followed a similar pattern of progression, coincided

temporally and showed maximum increase at the peak of degenerations in both the models (except caspase-3 in case of P23H). Further, S334ter retina showed both “caspase dependent” and “caspase independent” cell death, and interestingly, caspase-3 and calpain activation coincided with cell death during second PN week, suggesting that both proteolytic systems are activated and executed simultaneously. This co-activation suggests that probably some sort of cross-talk is involved between caspase and calpain proteases (Gomez-Vicente et al., 2005), and may explain at least partially, the excessively rapid degeneration of photoreceptors in this model. Alternatively, there is also a possibility of induction of separate cell death mechanisms to aid to the faster progression of S334ter degeneration possibly linked to a stronger genetic mutation at the C-terminus of opsin, which renders the cell more vulnerable to the biochemical irregularities, and ultimately switching on the multiple cell death pathways. On the other hand, in the *rd1* mouse, which also displays the same rate of cell death, is reported to undergo non-apoptotic mode of cell death (Chang et al., 1993), indicating that the speed of degeneration might not be the relevant factor to decide the mode of cell death. It may be postulated that various types of cell death signals from different cell organelles are often interconnected and they might act upstream or downstream to one another. Evident dominant cell death programme is decided by the relative speed of the available death programs. Generally, evident cell death program is the one, which essentially is the most effective and is executed at fastest speed (Bursch et al., 2000).

In P23H retina, caspase-3 activity seemingly plays only a minor, if any, role in cell death. However, calpain activity was clearly upregulated. These results are very important for therapeutic implications as targeting the events such as calpain or PARP activation may provide protection to the degenerating photoreceptors (Paquet-Durand et al., 2010; Sahaboglu et al., 2010). Undoubtedly, identification of all the associated and interacting proteins involved in photoreceptor cell death is fundamental for being able to develop novel therapies, because blocking of a single enzyme may not be enough, as the cell may still be able to die by other unidentified mechanisms or routes to die (Gomez-Vicente et al., 2005; Lockshin and Zakeri, 2004b). Additionally, as all of the cell death markers co-localized with

TUNEL staining (except caspase-3 in P23H), therefore, it may be inferred that that these events occurred relatively late during the degenerative process. Alternatively, this close correlation may also be explained by assuming that cell death, once triggered, is executed very rapidly.

Lastly, the present study showed AIF positive cells only in a subpopulation of S334ter retina, and not in P23H retina. PARP dependent cell death (PARthanatos) has often been reported to be mediated by the release of apoptosis inducing factor (AIF), a protein of mitochondrial inter membrane space during neuronal and photoreceptor cell death. As number of PARP positive cell is much higher than the AIF positive cells in ONL of S334ter retina, absence of AIF in P23H retina, which otherwise shows upregulation of PARP, the assumption that AIF release is triggered by PARP seems speculative, at least, in these models. Whether AIF release depends on excess PARP activity and consumption of NAD (Du et al., 2003; Sims et al., 1983) and/or by PAR polymers, needs further investigation in S334ter retina.

1.4 Conclusion

To conclude, the present study has unequivocally showed over activation PARP and calpain proteases along with other related processes such as oxidative stress in the transgenic rat models bearing S334ter and P23H rhodopsin mutations. All these markers have been linked to ‘caspase independent’ cell death programs. Further, S334ter retina additionally shows apoptotic mode of cell death. Probably cross-talk between these two passages of destruction causes a severe and faster loss of photoreceptors (Fig 5.1). Overall, S334ter and P23H rats show a great degree of overlap to the caspase independent cell death reported in *rd1* mouse, implying that different mutations induce similar death pathways and, and can be arrested by targeting common denominators of cell death.

2. Inhibitors of Calpain and PARP reduce photoreceptor cell death

Targeting key players of photoreceptor cell death could be a novel neuroprotective therapy in RP (Okoye et al., 2003). According to previously conceived view of cell

death in RP to be apoptotic (Chang et al., 1993; Delyfer et al., 2004; Portera-Cailliau et al., 1994), it was assumed that blocking this pathway would prevent the cell death and hence the disease progression. However, the attempts to rescue photoreceptors by caspase inhibition have not always brought the desired protective effect, indicating a gap in knowledge about the cell death pathways governing photoreceptor cell death. Accordingly, in recent times, alternate cell death players (*viz*., calpain and PARP), which don't fall under the category of 'classical apoptotic markers', have been identified during photoreceptor degeneration by several independent studies (Paquet-Durand et al., 2006; Paquet-Durand et al., 2010; Paquet-Durand et al., 2007; Sahaboglu et al., 2010). Importantly, in the first part of this study, it became clear that Rhodopsin mutant S334ter and P23H rat models also display the role of these newly identified non-apoptotic markers during degeneration of photoreceptors (Kaur et al., 2011). The fact that these key components of cell death are shared by different animal models regardless of mutations, targeting these key components as a neuroprotective strategy in retinal degenerations seems a relevant 'mutation independent' therapeutic approach that will benefit wide range of RP patients.

The present study showed that *in-vitro* treatment of S334ter-3 and P23H-1 transgenic rat retinas with selective inhibitors of calpains and PARP renders a protective effect to the dying photoreceptors under specifically defined regimens of treatment (viz., dose, single vs. combined treatment). The study also highlights that under certain paradigms, inhibitor treatment can result in increased cell death instead of imparting protection, and that, it is very important to use a suitable inhibitor in optimized paradigms of time and dose.

2.1 PARP inhibiton rescues photoreceptors in P23H rat

P23H explants treated with PJ34 showed significant rescue, however, S334ter retinal cultures showed enhanced cell death on application of PJ34. Since PARP-1 has been suggested to affect Ca^{2+} levels *via* PAR mediated pathway in the cells by by unknown pathway (Vosler et al., 2009), and calpain activitation is a downstream event after PARP activation (Moubarak et al., 2007), hence, targeting PARP may reduce cell death two fold ;i) inhibiting PARP activity, and ii) preventing the

propagation of death signal to calpains. However, S334ter retina PARP inhibition does not result in rescue, because even if it is presumed that PARP inhibition could influence calpain activation similar to P23H retina, the caspase mediated pathway still remains open, executing the death (Kaur et al., 2011; Liu et al., 1999).

2.2 Individual application of calpain inhibitors is not protective

Retinal cultures of mutants treated with calpain inhibitors at higher concentrations displayed a detrimental effect on photoreceptor cell survival and actually enhanced the cell death instead of reducing it. Furthermore, wild type retinal cultures treated in the same paradigm also displayed cell degeneration. These observations clearly marked the importance of calpain system for cell survival as described by a vast body of literature available (Carafoli and Molinari, 1998). Since, calpains regulate many calcium dependent processes in the cells, and not all the calpains are activated at the same times, a general inhibition therfore, may interfere with the functioning of some calpains resulting in imbalance in these processes causing adverse effects on the cells (Paquet-Durand, 2010).

2.3 Co-inhibition of Calapin and PARP rescues the Photoreceptors

Both calpain inhibitors when combined with PARP inhibitor at lower concentrations, provided rescue to P23H retinal cultures, nevertheless, calpain inhibitor VI (SJA6017) and PJ34 combination was more effective than Calpain inhibitor XI and PJ34 combination set. At the same time S334ter retinal cultures showed rescue in response to treatment by calpain inhibitor VI and PJ34 combination only, while no protective effect was seen for calpain inhibitor XI and PJ34 combined treatment in these mutants. The differential effects of calpain inhibition may be conceived with three considerations;

i) that photoreceptor cell survival depends on specially optimized regimens of inhibitor concentration and the treatment methods (separate or combined),

ii) that some calpain isoforms are required for cell survival more specifically and blocking them imparted no protective effect at all (Paquet-Durand, 2010),

iii) and that the specificities of various calpain inhibitors vary considerably (Azuma and Shearer, 2008), while trying to inhibit the calpains, there could be cross-inhibition of other cysteine-type proteases (Donkor, 2000; Gomez-Vicente et al., 2005; Ravid et al., 1994) that may be providing survival effects to the photoreceptors.

Taken together, these results advocate strongly the potential of inhibitors of calpains and PARP in reducing the photoreceptor cell death in RP due to different mutations. These results are also in line with previously reported neuroprotective effects of inhibitors used in this study. Calpain inhibitor VI (SJA6017), has been earlier shown to completely halt cell death in 661W murine photoreceptor-like cell line (Sharma and Rohrer, 2007), and calpain inhibitor XI has been reported to be protective in *rd1* photoreceptors under special paradigm of treatment (Paquet-Durand et al., 2010). Both SJA6017 and CX295 have been reported to reduce calpain activity in *rd1* retina *ex-vivo* with CX295 imparting more effect (Paquet-Durand et al., 2006).

Lastly, this study is the first to show the beneficial effect of co-inhibition of calpain and PARP in RP and hints strongly that exogenous regulation of the calpains and PARP could be the novel thrapeutic strategy in preventing or atleast slowing down the pace of photoreceptor cell death in retinal degenerations.

3. cGMP regulation in S334ter and P23H rats

cGMP plays a major role in visual transduction cascade as it regulates the cyclic nucleotide gated ion channels, and hence the intracellular Ca^{2+} levels. Importance of cGMP in photoreceptor survival is reflected by the fact that mutations in enzymes directly linked to cGMP metabolism cause retinal degeneration, e.g., PDE6, GC1 and GC-2 (Karan et al., 2008), or guanylyl cyclase activating protein (GCAP) (Nishiguchi et al., 2004; Olshevskaya et al., 2002). Additionally, mutations in cyclic-nucleotide-gated channel-a3 (CNGa3) (Michalakis et al., 2010) and aryl hydrocarbon receptor-interacting protein-like (AIPL1) (Ramamurthy et al., 2004) genes have also been reported to have abnormal cGMP levels, indicating that high cGMP is the common phenomenon in different models of retinal degenerations.

S334ter and P23H show functional abnormalities during recovery phase

(Birch et al., 1995; Goto et al., 1996), which possibly could have a bearing on Ca^{2+} and cGMP (Bush et al., 2000). In the present study, it was observed that there is an accumulation of cGMP in the photoreceptors of S334ter and P23H rat retinas possibly concomitant with other cell death markers. Further, mislocalization of RHO and arrestin was observed in both mutants. S334ter retina also showed less transducin and PDE6 expression corroborating earlier findings of impaired phototransduction in these mutants. This study is in agreement with another earlier published report of cGMP upregulation in P23H mice (Roof et al., 1994).

3.1 cGMP accumulation in S334ter and P23H rat retinas

High cGMP in photoreceptors may cause constitutive activation of cyclic nucleotide gated channels (CNGC) (Olshevskaya et al., 2002), which may further affect Ca^{2+}-dependent processes within the cells. Accordingly, an upregulation of calcium sensitive calpain proteases in the degenerating retinas of P23H and S334ter has been reported (Kaur et al., 2011). Apparently, presence of high cGMP level may explain high Ca^{2+} level related processes observed in these mutants, which could result from continuous influx of Ca^{2+} ions through open CNG channels. While, high intra-cellular Ca^{2+} may be responsible for calpain activation, on the other hand, high Ca^{2+} levels are responsible for GCAP inhibition (Olshevskaya et al., 2002), retGC inactivity and thus cGMP non-synthesis. Theoretically, therefore, this negative feedback mechanism should stop the GC activity and prevent the intracellular cGMP levels to rise, but, for reasons yet unknown, this feedback mechanism appear nonfunctional or ineffective in degenerating P23H and S334ter retina similar to *rd1* situation (Paquet-Durand, 2010), as an upregulation of GC level has been observed in both S334ter and P23H retina, which remains unexplainable as yet, however, raises the possibilty to relate the observed increased GC expression to a constitutive activity of GC.

3.2 Phototransduction proteins

Apart from cGMP and GC, status of other transduction protein components was also checked and it was found that in P23H retina there is less expression and a mislocalisation of RHO, while transducin, PDE6, and arrestin showed normal

expression and localisation. In S334ter also, rhodopsin was found to be mislocalised and less expressed as previously reported (Green et al., 2000; Kaur, 2010; Lee et al., 2003). PDE6 and transducin was less expressed, and arrestin was miscolalised despite normal expression. As the OS are not fully developed in S334ter retina, the observed abnormal profile of various phototransduction proteins may be explained as proposed earlier that upon degeneration of OS, the proteins normally meant to go to the OS may simply stop their movement, for lack of their final destination and may thus fill up the remaining photoreceptor cell bodies. Alternately, there could be passive concomitant misrouting of all the phototransduction proteins alongwith rhodopsin in this mutant, as these transduction proteins are suggested to be tightly associated with rhodopsin much before they reach the OS (Roof et al., 1994).

To conclude, it can be postulated from the available data that during photoreceptor cell death in S334ter and P23H transgenic rats, impairment in phototransduction machinery leads to accumulation of cGMP, possibly by constitutive activation of retGC. High cGMP cause the constitutive opening of CNG channels, which result in high intracellular calcium levels in the photoreceptors.

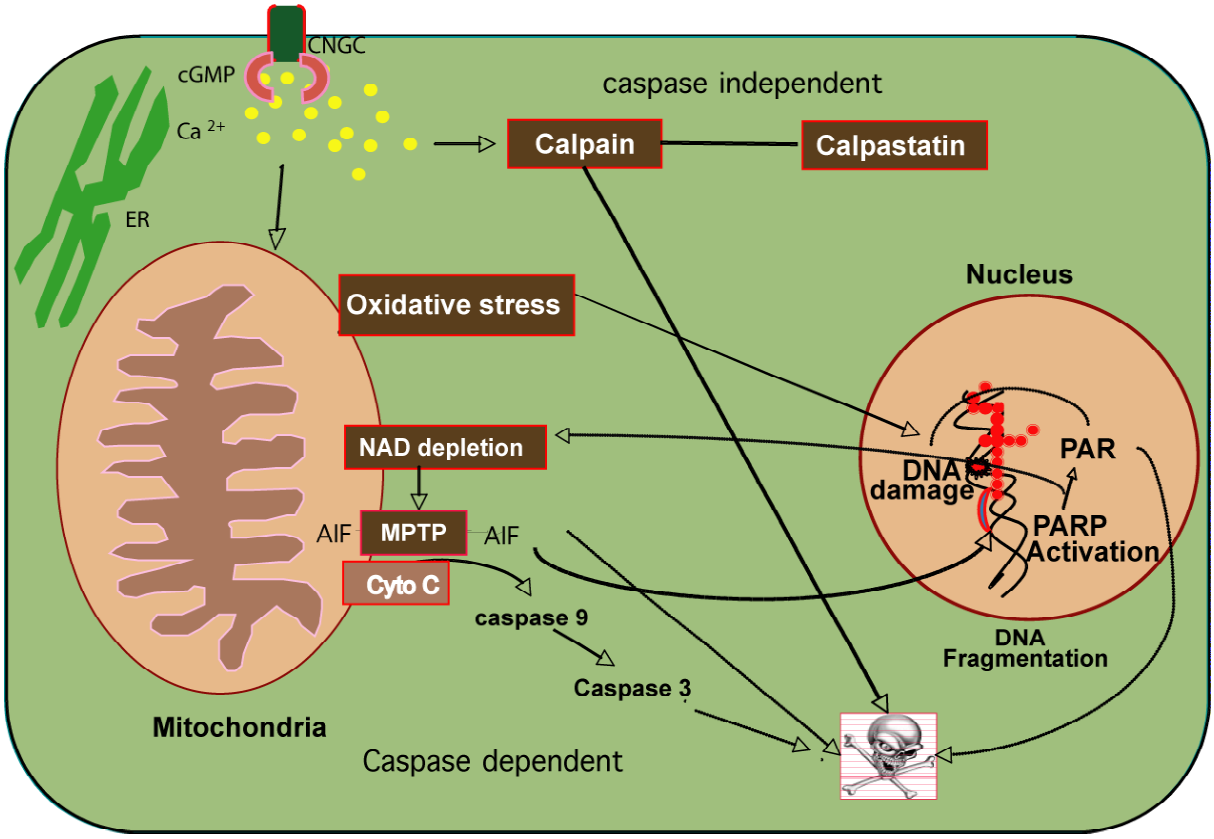

Figure 5.1 Caspase independent cell death pathway in S334ter and P23H models: Calcium dysregulation increases its intracellular concentration, which affects the Ca^{2+} related processes in the cell. On one hand, high level of Ca^{2+} triggers calpain activation. accompanied by Calpastain dissociation from calpains. On the other hand, elevated Ca^{2+}-levels may affect mitochondrial metabolism resulting in the production of oxidative stress This oxidative stress in turn may be the major factor to activate the DNA repair enzymes, specifically, PARP. Excessive activation of PARP causes NAD depletion and accumulation of PAR. Additionally, it may further increase the oxidative stress. All these processes eventually may lead to energy depletion, mitochondrial depolarization, opening of MPTP, and AIF translocation from mitochondria to nucleus. AIF translocation may also be mediated by calpain, independent of MPTP opening. The Ca^{2+} level increase could be due to constitutive cGMP activation may bet he reason of high Ca^{2+} levels. S334ter, additionally shows AIF translocation and caspase dependent apoptotic cell death. The molecular cell death pathway depicted in this diagram should be considered as hypothetical at this point in regard to chronology of events, as it has been constructed based on the available data only on S334ter and P23H models in comparison to PDE6 mutant *rd1* mouse model of RP. **Abbreviation**: AIF, apoptosis inducing factor; ER, endoplasmic reticulum; MPTP, mitochondrial permeabilty transition pores; PARP, poly(ADP)ribose polymerase; PAR; poly(ADP) ribose. ***Prepared with reference to*** **(Kaur et al., 2011; Paquet-Durand, 2010; Sancho-Pelluz et al., 2008)**.

High calcium levels may affect mitochondrial metabolism leading to oxidative stress, and also cause the activation of calpain type proteases. Oxidative stress leads to oxidized DNA damage, and hence, PARP activation, PAR synthesis and energy depletion. Calpain and PARP hyperactivation leads the cell towards its death (Fig. 5.1). S334ter retina shows presence of AIF and caspase activation in addition, showing that photoreceptor cell death in this mutant is executed by more than one pathway.

4. Perspective

The molecular pathways mediating the photoreceptor cell death during retinal degenerative diseases have not been completely understood till date. The present study discovered the role of calpain proteases and PARP alongwith oxidative stress during photoreceptor degeneration in two rat models of RP bearing separate RHO mutations (S334ter and P23H). Further, co-inhibition of these cell death players was found to impart beneficial effects to photoreceptors as survival incresed after their application. Further studies will shed more light on the potential effect of these specific therapeutic agents. Evaluation of calpain activity and expression together with calpastatin expression in the treated cultured tissues will provide additional insights about the proposed potential of calpain inhibitors used in this study. Also, examination of PARP levels and PARylation after inhibition of PARP would further reveal how potent PJ34 is, for its use as pharmacological therapeutic candidate in retinopathies. Preliminary studies already showed that long term cultures display increased thickness in the outer nuclear layer of the

retina after application of SJA60 and PJ34 inhibitors. Continuation of these studies would be necessary in order to ascertain overall survival and functional improvement, if any, rendered to photoreceptors by these calpain and PARP inhibitors. Finally, *in-vivo* studies using these inhibitors would be a necessary measures to compare the *in-vitro* effects.

Another important aspect obtained from the present study was the observation of high cGMP levels alongwith guanylyl cyclase upregulation in the photoreceptors of mutant rats. Further studies are required to establish a missing link between high cGMP, high calcium level dependent processes, and a possible constitutive activation of GC simultaneously, as in principle these processes should be counteractive. The findings in RHO mutants reflect the processes associated with cell death in PDE6 mutant *rd1* mouse, and it is noteworthy that all these events formulate a molecular pathway of degeneration, which deviates considerably from the generally assumed apoptotic cell death pathway. Since the retina perfectly depict central nervous system architecture, such findings could be of positive consequence for the treatment of neuronal cell death in general.

Chapter VI: Materials and methods

1. Materials

1.1 Chemicals and reagents

- Acetic acid — Merck, Damstadt, Germany
- (3-Aminopropyl) triethoxysilane — Sigma, St.Louis, MO, USA
- Ammonium chloride (NH_4Cl) — Sigma, St.Louis, MO, USA
- Ammonium persulfate (APS) — Merck, Damstadt, Germany
- Aquatex® — Merck, Damstadt, Germany
- Avidin biotin complex (ABC) kit — Vector, Burlingame, CA, USA
- Avidin Alexa conjugate — Molecular probes, Oregon, USA
- Biotinylated NAD — Trevigen, Gaithersburg, USA
- Bovine serum albumin (BSA) — Sigma, St.Louis, MO, USA
- Bradford reagent® — Sigma, St.Louis, MO, USA
- Bromophenol blue — GE Healthcare, Munich, Germany
- Calpain Inhibitor VI — Calbiochem, Darmstadt, Germany
- Calpain Inhibitor VI — Calbiochem, Darmstadt, Germany
- $CaCl_2$ — Sigma, St.Louis, MO, USA
- CMAC,t-BOC-Leu-Met — Molecular probes, Oregon, USA
- Coomassie blue — Merck, Damstadt, Germany
- DABCO (1,4-Diazabicyclo [2.2.2] octane) — Sigma, St.Louis, MO, USA
- Di-aminobenzidine (DAB) — Vector, Burlingame, CA, USA
- Dimethyl sulfoxide (DMSO) — Sigma, St.Louis, MO, USA
- Ethylenediaminetetraacetic acid (EDTA) — Roth, Karlsruhe, Germany
- Ethanol (EtOH) — Merck, Damstadt, Germany
- Fetal calf serum (FCS) — Gibco, Karlsruhe, Germany
- Fish gelatin — Aurion, Wageningen, Netherlands
- Glacial acetic acid — Merck,Damstadt, Germany
- Glucose — Merck, Damstadt, Germany
- Glucoseoxidase — Sigma, St.Louis, MO, USA
- Glycerol — Sigma, St.Louis, MO, USA
- Glycine — Sigma, St.Louis, MO, USA

- HEPES (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid) — Sigma, St.Louis, MO, USA
- Hydrogen chloride (HCl) — Merck, Damstadt, Germany
- Hydrogen peroxide ($H_2O_{2)}$) — Merck, Damstadt, Germany
- Isopropanol — Merck, Damstadt, Germany
- Jung freezing tissue medium — Leica, Nussloch, Germany
- Potassium chloride (KCl) — Merck, Damstadt, Germany
- Methanol (MeOH) — Merck, Damstadt, Germany
- Magnesium chloride ($MgCl_{2)}$) — Merck, Damstadt, Germany
- Moviol — Sigma, St.Louis, MO, USA
- Sodium chloride (NaCl) — Merck, Damstadt, Germany
- Sodium hydroxide (NaOH) — Merck, Damstadt, Germany
- Nicotinamide adenine dinucleotide (NAD^+) — Sigma, St.Louis, MO, USA
- Nickel ammonium sulphate — Sigma, St.Louis, MO, USA
- Normal goat and rabbit Serum — Sigma, St.Louis, MO, USA
- Paraformaldehide (PFA) — Sigma, St.Louis, MO, US
- PJ34 — Alexis Biochem., Switzerland
- PhenylMethylSulfonylFluoride (PMSF) — Sigma, St.Louis, MO, USA
- Pierce ECL WB substrate — Thermo Sci, Rockford, IL, USA
- Powder milk — Roth, Karlsruhe, Germany
- Proteases inhibitor cocktail — Calbiochem, Darmstadt, Germany
- Proteinase K — Sigma, St.Louis, MO, USA
- R16 medium — Invitrogen, Paisley, UK
- Rainbow marker — Amersham Bios., Freiburg, Germany
- Rotiblock® — Roth, Karlsruhe, Germany
- Rotiphorese® Gel 30 (Bis-methylene-Acrylamide+ 30% acrylamide) — Roth, Karlsruhe, Germany
- Di-SodiumHydrogenPhosphate (Na_2HPO_4 x $2H_2O$) — Merck, Damstadt, Germany
- SodiumDihydrogenPhospate-Monohydrate $NaH_2PO_{4)}$ — Merck, Damstadt, Germany
- Sodium dodecyl sulfate (SDS) — Sigma, St.Louis, MO, USA
- Tetramethyletilenedimine (TEMED) — Roth, Karlsruhe, Germany

- Triton X-100 — Sigma, St.Louis, MO, USA
- Tris (tris (hydroxymethyl) aminomethane) — Sigma, St.Louis, MO, USA
- Tris hydrochloride (Tris-HCl) — Sigma, St.Louis, MO, USA
- TUNEL® assay Kit — Roche, Mannheim, Germany
- Tween 20 — Sigma, St.Louis, MO, USA
- Vectashield® — Vector, Burlingame,
- Vectastatin ABC Elite® — Vector, Burlingame, CA, USA
- Trisma base — Sigma, St.Louis, MO, USA
- Dithiothreitol (DTT) — BioChemika, Germany

1.2 Buffers and Solutions

PB (Phosphate Buffer) Stock buffer (20X)

Solution A: Add 6,89 g of NaH_2PO_4 x H_2O in 250 ml ddH2O (0,2 M; pH4)

Solution B: Add 14,19 g of NaH_2PO_4 in 500 ml dd H_2O (0,2M; pH11)

- Add Solution A to Solution B
- Adjust pH to 7,4

PBS (Phosphate buffered saline)

Dilute PB stock (20X) solution in dd H_2O (1:20), to have PB (0,1 M)

- Add 9 g/L of NaCl to have PBS (0,1 M; pH7.4)

TBS (Tris Buffer Saline) 50mM

- Add 6,06g of Tris base in 900 ml dd H_2O
- Adjust pH to 7,6
- Addd more dd H_2O to make the final volume 1000 ml
- Add 9 g/L of NaCl

PFA (Paraformaldehyde)

4% PFA in 0,1 M PB

For 100 ml:

- Dissolve 4g of Paraformaldehyde in 50 ml PB (0,1M)

Heat on a hot plate at 60°C for 45 minutes (stir with magnet)

- Add 10 drops of NaOH (till the solution becomes clear)

- Again add 50ml of 0,1M PB (to make the final volume 100 ml)

- Adjust the pH to 7,4 (with NaOH/HCl)

1.3 Antibodies

Table 5.1: Primary Antibodies: mAb: monoclonal antibody; pAb: polyclonal antibody; Gt: goat; Ms: mouse; Rb: rabbit; Sh: sheep.

Antigen	Host	Source / Cat. Number	Dilution	
			IF/IHC	WB
Actin	Ms, mAb	Millipore/ MAB 1501	-	1:1000
Actin	Rb, pAb	Abcam/ ab1801	-	1:1000
Avidin-Alexa 488	--	Molecular Probes/ A-21370	1:80	1:1000
Avidin-texas red Biotin binding Protein	--	Molecular Probes/ A-820	1:200	-
Calpastatin	Ms, mAb	Novus Biologicals/NB120-5582	1:50	1:5000
Calpain LP85 and LP82 (Capn3)	Rb, pAb	Millipore Chemicon / AB81011	1:50	1:5000
m-Calpain, large (catalytic) subunit (Capn2)	Rb, pAb	Millipore Chemicon /AB81023	1:100	1:1000
M-Calpain large subunit, clone P-6 (Capn1)	Ms, mAb	Millipore Chemicon/ MAB3082	1:100	1:1000
Cleaved Caspase-3 (Asp175) (5A1E)	Rb, mAb	Cell Signalling / 9664	1:300	-
Cleaved Caspase-9 (Asp353)	Rb,pAb	Abcam/ab52298	1:100	-
Cytochrome-C (clone 7H8.2C12)	Ms, mAb	BD Pharmingen/556433	1:2000	
cGMP	Sh	Gift from Jan de Vente (Maastricht University, Netherlands)	1:500	-
GC	Rb	Gifted by Prof. Karl-Wilhelm Koch (University of Oldenburg, Germany)	-	1:1000
PDE6 ß	Rb, mAb	Dianova/ PA1-722	1:200	1:1000
PARP(cloneC2-10)	Ms, mAb	BD Pharmingen/ 556362	--	1:1000
PAR (10H)	Ms, mAb	Enzo / ALX-804-220	1:200	1:1000
Rho 1D4	Ms, mAb	Chemicon/ MAB5356	1:200	1:500
Rhodopsin	Ms, mAb	Chemicon/ MAB 5316	1:100	1:1000
Transducin Gαt1 (K-20)	Rb. pAb	Santa Cruz/ sc-389	1:100	1:1000
Vimentin	Gt, pAb	Millipore Chemicon/ AB1620	1:200	-
Visual Arrestin	Rb, pAb	Thermo scientific/ PA1-731	1:500	1:1000

Table 5.2: Secondary antibodies: gt: goat; ms: mouse; rb: rabbit; sh: sheep; dk: donkey; IgG: Immunoglobulin G.

Secondary antibody	Information	Source	Dilution	
			IF/IHC	WB
Alexa Fluor® 488	Gt anti Ms (IgG)	Molecular Probes, Oregon, USA	1:750	-
Alexa Fluor® 488	Gt anti Rb (IgG)	Molecular Probes, Oregon, USA	1:750	-
Alexa Fluor® 488	Dk anti Gt (IgG)	Molecular Probes, Oregon, USA	1:750	-
Alexa Fluor® 555	Gt anti Rb (IgG)	Molecular Probes, Oregon, USA	1:200	-
Alexa Fluor® 568	Gt anti Rb (IgG)	Molecular Probes, Oregon, USA	1:500	-
Biotinylated IgG (H+L)	Gt anti Ms	Vector Laboratories, CA, USA	1:150	-
Biotinylated IgG (H+L)	Gt anti Rb	Vector Laboratories, CA, USA	1:150	-
ECL®, HRP-linked	Dk anti Rb (IgG)	GE Healthcare, UK	-	1:2000
ECL®, HRP-Linked	Sh anti Ms (IgG)	GE Healthcare, UK	-	1:2000

2. Animals Procedures

Homozygous P23H and S334ter mutant RHO transgenic rats (produced by Chrysalis DNX Transgenic Sciences, Princeton, NJ) of the line Tg(P23H)1Lav and Tg(S334ter)3Lav (abbreviated P23H-1 and S334ter-3 respectively) were obtained from Dr. M. M. LaVail (University of California, San Francisco, CA; see http://ucsfeye.net/MLaVail%20RD%20Rat%20Model%20Resource.pdf) and bred in a colony at Institute of Experimental Ophthalmology, University Eye Hospital, Tuebingen. Only heterozygous animals were used for all experimental purposes. Homozygous S334ter-3 and P23H-1 breeding pairs were mated with normal albino Sprague-Dawley [CD: CD® IGS Rat, Crl:CD(SD); see http://www.criver.com/en-US/ProdServ/ByType/ResModOver/ResMod/Pages/CDRat.aspx] rats to produce heterozygous offspring for the S334ter and P23H transgene respectively. These heterozygous offsprings were subsequently used. Heterozygous animals were used in order to have the heterozygous genetic background of most human ADRP cases. Controls were age-matched Sprague Dawley (SD/CD) rats.

All procedures of animal use were approved by the Tuebingen University committee on animal protection and performed in compliance with the ARVO

statement for the use of animals in Ophthalmic and Visual Research. Protocols compliant with § 4 Abs. 3 TSchG of the German law on animal protection were reviewed and approved by Dr. Ulf Scheurlen and Dr. Susanne Gerold (Einrichtung für Tierschutz, Tierärztlichen Dienst und Labortierkunde directed by Dr. Franz Iglauer; Anzeige/Mitteilung nach § 4 vom 28.04.08 and 29.04.10). Animals were housed under standard cyclic 12/12-h light/dark conditions, had free access to food and water, and were selected and used irrespective of gender. All possible efforts were made to decrease the number of animals used and to minimize their suffering and discomfort.

Table 5.3: Animal models used in the study

Official Line Designation	Tg(P23H)1Lav	Genetic defect
Tg(S334ter)3Lav	S334ter Line 3 or S334ter-3	RHO S334ter mutation
Tg(P23H)1Lav	P23H Line 1 or P23H-1	RHO P23H mutation
Crl:CD(SD)	CD® IGS Rat or CD	Wild type

3. Tissue Preparation (Embedding and Section Preparation)

3.1 Method Overview

The technique of putting fixed or unfixed biological specimens/ samples (cells or tissue) in a medium in order to provide a firm support to them in order to obtain thin histological sections is called embedding. Commonly used medium for embedding are paraffin wax (paraffin embedding) or Cryomatrix (Cryoembedding). The sectioning of fixed tissue is done by means of a microtome (http://protocolsonline.com/category/histology).

3.2. Cryo-embedding

3.2.1 Requirement

4 % Paraformaldehyde (PFA)

PBS (0,1 M; pH7,4),

Sucrose (10%, 20%, 30%),

Liquid N_2

3.2.2 Procedure

P23H-1, S334ter-3 and CD rats were euthanised at different developmental ages (PN0 - PN30) by exposing them to an overdose of CO_2. The eyes were immediately enucleated and dissected to remove the anterior segments along with the lenses. The eyecups were fixed by immersing them in 4% PFA for 45 minutes at 4ºC, followed by washing in PBS (0,1 M; pH7,4) at room temperature. Afterwards, the eyecups were cryo-protected using PBS containing increasing concentrations of sucrose (10%, 20%, and 30%). During these steps, tissue was kept for 10 minutes in 10% sucrose, 20 minutes in 20% sucrose and overnight in 30% sucrose at 4°C. After cryo-protection, the tissue was embedded in cryomatrix (Tissuetek, Leica, Bensheim, Germany) and immediately frozen using or liquid N_2, and stored at -20°C. For unfixed tissues, eyes were immediately frozen in liquid N_2 followed by embedding in cryomatrix (Tissuetek, Leica, Bensheim, Germany). Later on, the fixed as well as unfixed retinal tissues were sectioned sagitally (12µm) using a cryostat (Jung CM 3000, Leica, Nussloch, Germany). These sections were collected on slides pre-coated with (3-Aminopropyl) triethoxysilane, and stored at -20ºC for further experiments.

3.3 Paraffin Embedding

3.3.1 Requirement

- 4 % Paraformaldehyde (PFA)
- PBS (0,1 M; pH7,4)
- Ethanol different grades: 100%, 96%, 70%
- Chloroform
- Paraffin

3.3.2 Procedure

As described in cryoembedding, the eyes were fixed in 4% PFA following enucleation from the eye sockets for 45 minutes at 4°C). Afterwards the eyes

were washed in PBS (0,1M; pH 7,4) followed by dehydrating the eyecups in different grades of ethanol at room temperature (three times for 20 minutes each in 70%, 96%, and 100%). Then the eyecups were infiltrated in chloroform and paraffin (1 hr each at RT). For embedding, the moulds made from aluminium foil were filled with hot paraffin wax and eyecups were placed inside them carefully. The moulds containing embedded eyes were subsequently placed on ice and stored at – 20°C. Later on, 12µm thin retinal tissue were sectioned sagitally using a microtome (Leica RM 2155, Nussloch, Germany). These sections were collected on slides pre-coated with 3-Aminopropyl-triethoxysilane, and stored at RT for further experiment.

4. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay

4.1 Method Overview

A widely used method for detecting *in-situ* DNA damage is TdT-mediated dUTP-biotin nick end labeling (TUNEL) labeling (Loo, 2002). Initially, TUNEL was described as a method to detect only apoptotic cells (Gavrieli et al., 1992), however, later it was shown to label cells undergoing non-apoptotic cell deaths as well (Grasl-Kraupp et al., 1995). Working principle of TUNEL staining is based on the ability of enzyme terminal deoxynucleotidyl transferase (TdT) to incorporate fluorescein labeled dUTP into free 3′-hydroxyl termini generated by DNA damage (Loo, 2002). Due to the incorporation of fluorescein, nucleotide polymers can be detected and quantified by fluorescence microscopy (Negoescu et al., 1998; Negoescu et al., 1996).

4.2 Solutions and buffers

1) Proteinase K

- 0,005 M TBS pH 7.6 100 ml
- Proteinase K 14,3 µl

2) Fixation buffer (Ethanol-acetic acid solution) (for 100 ml)

- 70% Ethanol (EtOH) 62 ml
- 30% Acetic acid 12 ml

- dH_2O 26 ml

3) Blocking solution

- 10% NGS
- 1% BSA
- 1% fish gelatin

0,003% PBST

4) TUNEL-kit (see table 3)

Table 5.4: TUNEL assay Reagents

Solutions	Components	for 100 µl
Enzyme solution (#1)	Terminal deoxynucleotidyl transferase (TdT) from calf thymus, recombinant in E. coli	5µl
Label solution (#2)	Nucleotide mixture in reaction buffer	45 µl
Blocking solution	See above	50µl

4.3 Procedure

TUNEL assay was performed using an in situ cell death detection kit (Fluorescein or TMR; Roche Diagnostics GmbH, Mannheim, Germany). Fixed cryo-sections from retinas or from retinal explant cultures were dried for 30 to 60 min at 37ºC, and washed in TBS. Subsequently, the sections were incubated in Proteinase K diluted in TBS (10 µg/ml in10 mM TRIS-HCL, pH 7,4–8,0) for 5 minutes at 37ºC. Proteinase K incubation is necessary to inactivate nucleases (DNAases and RNAases), which may otherwise degrade the nucleic acids. Then, the sections were washed thrice in TBS at room temperature for 5 minutes each, and re-fixed in a solution of EtOH and glacial acetic acid at -20ºC for 5 minutes. Subsequently, the slides were washed in TBS and incubated in Proteinase K (dissolved in TBS) for another 5 minutes at 37ºC. The sections were afterwards blocked for 1 hour in blocking solution at room temperature. After blocking, the sections were incubated with TUNEL

reaction mixture (see Table 5.4) using *in-situ* cell death detection kit at 37°C for 60 minutes or overnight at 4°C. Finally, the sections were washed in PBS (0,1 M) and covered by Vectashield mounting medium with DAPI (4'-6-Diamidino-2-phenylindole) (Vector, Burlingame, CA, USA). For negative controls, the enzyme TdT was omitted from the TUNEL reaction mixture. Negative controls gave no staining at all.

For co-localization with calpain or PARP activity, the activity-stained sections were fixed in 4% PFA and the TUNEL assay was performed afterwards. For co-localization with cleaved caspase-3 or avidin staining, TUNEL was performed prior to the theses two stainings respectively.

5. Immunohistochemistry

5.1 Method Overview

Immunohistochemistry is the method of detecting proteins or peptides in the tissue sections on the basis of antigen-antibody specific reactions. In this technique, a specialized antigen-recognizing antibody is applied to the tissue followed by the addition of a secondary antibody that binds specifically to the primary antibody. If the secondary antibody is linked to an enzyme, e.g., streptavidin-horseradish peroxidase, which reacts with DAB and produces a brown stain and the technique, is referred to as immunohistochemistry. If the secondary antibody is linked to a fluorescent dye, then the technique is known as immunofluorescence (http://www.piercenet.com/browse.cfm).

5.2 Solutions and buffers

- PBS (0,1M; pH 7,4)
- TBS (pH, M)
- PBST (with 0,1% or 0,3% Triton X-100)
- TBST (with 0,1% or 0,3% Triton X-100)
- Normal Goat/Rabbit serum
- BSA

- Ethanol
- Fetal Calf Serum (FCS)
- Aquatex mounting medium
- Vectashield mounting medium
- Vectastin ABC Elite
- Xylol

DAB solution:

o PB	40 ml
o 20% glucose	400 µl
o 0,4% NH_4Cl	400 µl
o 1% Nickel ammonium sulphate	3,2 ml
o DAB	40 mg
o Glucoseoxidase	40 µl

5.3 Procedure

5.3.1 Rehydration of Sections

1) Paraffin embedded retinal sections were dried at 60ºC for one hour followed by de-paraffinization with xylol (4 times for 5 minutes each). De-paraffinized sections were subsequently re-hydrated in ethanol alcohol of different grades (in decreasing concentration order; 100 % EtOH 2x2 min, 90 % EtOH, 2x2min, 80% EtOH for 2 min, 70 % EtOH for 5 min, 50% EtOH for 5 min). Then, the sections were washed in dH_2O followed by being rinsed in PBS.

2) Frozen sections from retinas or cultured retinas were dried at 37ºC for one hour. Subsequently, the tissue was re-hydrated in PBS.

5.3.2 Staining with antibodies

Rest of the procedure was same for the paraffin and cryosections. After washing step in PBS, the sections were incubated in 10% normal goat serum (NGS) and 1% BSA dissolved in Triton X-100 in PBS (0,1% or 0,3%, or 0.5% TBST) for 1 hour at room temperature in order to block all non-specific binding

sites. After blocking, immunohistochemistry/immunostaining was performed overnight at 4°C, using primary antibodies (see table 5.1). Each primary antibody was diluted in PBST containing 0,1% or 0,3% Triton X-100 along with 1%BSA and 10%NGS. After applying primary antibodies, sections were kept at 4°C overnight. The following day, sections were washed in PBS for 30 minutes (thrice for 10 minutes each) and afterwards incubated in secondary antibodies for 1 hour at room temperature in Alexa conjugated secondary antibody (Molecular Probes, Oregon, USA) (See Table5.2). Then, the sections were washed for 30 minutes with PBS (0,1M). Lastly, the sections were mounted by using the mounting medium (Vectashield with or without DAPI) (Vector, Burlingame, CA, USA) and coverslipped. Imaging was done with light or fluorescent microscopy. Controls consisted of slides for which the primary antibodies were omitted; nevertheless, the fluorescence detection system was applied.

Using a biotinylated secondary antibody, avidin-biotin complex (ABC) and Di-aminobenzidine (DAB), immunohistochemistry without fluorescence was performed. For this, after rinsing in PBS, the sections were incubated for 20 minute in quenching solution consisting of 100 µl of 30% H_2O_2, 400 µl MeOH, and 600 µl PBS-T (0, 1%) at room temperature for the purpose of re-fixing them. Immediately after this step, the sections were blocked in 10% normal goat serum (NGS) and 1% BSA dissolved in Triton X-100 in PBS (0.1% or 0.3%, or 0.5% TBST) for 1 hour at room temperature. Afterwards, the primary antibody was applied and sections were kept overnight at 4ºC. Subsequently, the sections were washed in PBS for 30 minutes and incubated for 1 hour with a biotinylated secondary antibody. This step was followed by washing in PBS and application of ABC dissolved in PBS (1µl component A, 1µl of component B dissolved in 148µl PBS per slide). The biotin group linked to secondary antibody was identified and localized by avidin (component of the ABC kit). After this, the slides are kept in freshly prepared DAB solution for 2-5 minutes in order to develop the signal. Coloration progress typical of DAB staining was tested under a simple light microscope (Olympus CH-20). Lastly, the sections are mounted with Aquatex® and coverslipped. Negative controls

lacked primary antibody incubation, however, the secondary antibodies were applied and they showed no staining at all.

6. Calpain activity Assay

6.1 Method Overview

Calpain activity at the cellular level can be measured by means of a cell permeable fluorescent calpain substrate called tert-butoxycarbonyl-L-leucyl-L-methionineamide-7-amino-4-chloromethylcoumarin (Boc-Leu-Met-CMAC). After entering cells, Boc-Leu-Met-CMAC gets conjugated to thiols, particularly glutathione, and becomes impermeable to the plasmamembrane. Cleavage of thiol-conjugated Boc-Leu-Met-CMAC by calpain proteases is known to release thiol-conjugated 7-amino-4-methylcoumarin (AMC) that generates constitutive fluorescence proportional to the activity of calpain. Fluorescence (blue) generated by calpain-dependent cleavage of t-Boc-Leu-Met-CMAC is observed at 430nm (Chen et al., 2001; Rosser et al., 1993).

In this study, calpain activity at the cellular level was investigated with an *in-situ* enzymatic assay, earlier used to assess the calpain activity in photoreceptors of *rd1* mouse model of retinal degeneration (Paquet-Durand et al., 2006).

6.2 Solutions and Buffers

Calpain Reaction Buffer (CRB) (10x)

- HEPES (25 mM) 5,96 g
- KCl (65mM) 4,85 g
- $MgCl_2$ (2 mM) 0,47 g
- $CaCl_2$ (1,5mM) 0,22 g

Dissolve all the above components in 60ml ddH_2O

Adjust pH to 7,2 and complete the volume to 100 ml with ddH_2O

Working solution of CRB (1x)

Dilute 10x CRB in dH_2O in1: 10 concentrations

Dithiothreitol (DTT) Stock solution, 1M

- 154, 25 mg dissolve in 1ml ddH_2O

Calpain Reaction mix

Working solution

- To 1ml CRB-working solution add
- µl DTT (from 1M Stock solution) to make the final concentration 2mM
- 5 µl 7-amino-4-chloromethylcoumarin, t-BOC-L-leucyl- L-methionine amide (CMAC, t-BOC-Lwu-Met) (2mM Stock solution) to make the final concentration 10mM

6.2 Procedure

Unfixed retinal sections were dried at RT for 10 minutes and subsequently washed in calpain reaction buffer (CRB; 25 mM HEPES, 65 mM KCl, 2 mM $MgCl_2$, 1,5 mM $CaCl_2$, 2 mM DTT) for 15 minutes. Afterwards, the sections were incubated in 2 µM fluorescent calpain substrate, CMAC, t-BOC-Leu-Met (Molecular Probes, Inc. Eugene, USA) dissolved in CRB at 35°C for 2 hours in the dark. Lastly, the sections were washed in CRB twice for 10 minutes each and cover slipped after adding moviol with DAPCO or Vectashild without DAPI.

7. Avidin Staining

7.1 Method Overview

Avidin is a natural component of egg white, and it has very high affinity for biotin (Vitamin B7). Structurally, avidin is very stable tetramer containing four biotin binding sites (Bayer and Wilchek, 1990). This affinity of avidin-biotin system is applied in experimental systems to detect the oxidative damage of DNA (Wilchek and Bayer, 1990). 8-Oxoguanine (8-hydroxyguanine (8-hOG) or 8-oxo-Gua) is one of the most common DNA lesions resulting from reactive oxygen species (Kanvah et al., 2010). Avidin labels 8-hOG, which is similar in its structure to biotin (Struthers et al., 1998) and that is why, it is a widely used method to detect oxidative DNA damage.

7.2 Solutions and Buffers

- Methanol (pre-cooled at -20°C)
- PBS (pH 7,4; 0,1M)
- NGS /PBS (10% and 0,2%)
- Avidin conjugated to Alexa 488 (dissolved in 0,1% PBST)
- Vectashield DAPI

7.3 Procedure

The retinal cryosections were dried for one hour at 37°C followed by incubation in methanol pre-cooled at -20°C in a choplin for 15 min. Washing the slides with PBS followed this step. Afterwards, the slides were clamped on cover plates tightly and blocked with 10% NGS/PBS for one hour. Again, slides were washed with 0,2% NGS/PBS three times and incubated with Avidin-Alexa 488 (1:80; Molecular Probes, Inc. Eugene, USA) or dissolved in 0,1%PBST for 90 minutes at room temperature. Negative controls were treated for 1 hour with avidin staining solution pre-adsorbed with 12 nM 8-Hydroxy-2′-deoxyguanosine (Calbiochem, Darmstadt, Germany) and 50nM biotin (Vector laboratories, Burlingame, CA). Lastly, the slides were washed with PBS and mounted with Vectashield medium containing DAPI.

8. PARP *in-situ* enzyme activity assay

8.1 Method Overview

PARP (Poly(ADP ribose) Polymerase is activated by DNA damage and help in the DNA repair process (Herceg and Wang, 2001). For this, PARP catalyze the synthesis of poly (ADP-ribose) from its substrate β-NAD+ at the expense of ATP. In situ activity of PARP enzyme can be measured by using a novel PARP substrate, 6-biotin-17-nicotinamide-adenine-dinucleotide (bio-NAD^+) (Bakondi et al., 2002; Zhang and Snyder, 1993).

In this study, PARP *in-situ* activity assay was used to assess the activity of PARP in degenerating retinas following the method earlier used to assess the

PARP activity in the photoreceptors of *rd1* retina (Paquet-Durand et al., 2007).

8.2 Solutions and Buffer

TB 100 mM, pH 8,0

(12, 14 g Trisma base dissolved in 600 ml ddH_2O (pH 8, 0 with 2N HCl)

Filled to 1000 ml with ddH_2O

PARP reaction buffer (PRB):

TB, 0,2% Triton, 10 mM $MgCl_2$

- 250 ml Trisma Base
- 500 µl Triton X100
- 0,508 g $MgCl_2$ dissolve

Dithiothreitol (DTT) Stock solution, 1 M

154, 25 mg dissolved in 1 ml ddH_2O

PARP reaction mixture: To 1 ml, PRB working solution added:

1 µl DTT Stock solution (1 M) to make the final concentration 1mM

20 µl biotinylated NAD (250 µM) to make the final concentration 5µM

Table 5.5 Components of PARP reaction mixture

Volume	Components	Stock Concentration	Working Concentration
980 µl	Reaction buffer (RB)	TB (100mM, pH8,0) 0,2% Triton, 10mM $MgCl_2$)	--
1 µl	DTT	1 M	1mM
20 µl	biotinylated NAD	250 µM	5 µM

8.3 Procedure

Unfixed retinal cryosections were dried for 10 minutes at room temperature followed by washing in TB (100mM; pH 8,0) in a choplin. Slides were clamped tightly in cover plates and washed in TB again. Afterwards the slides were

incubated in avidin/biotin blocking kit (Vector Laboratories, Burlingame, USA). For this, first avidin and then biotin were applied each for 15 minutes from the Biotin blocking kit. This step was followed washing in TB twice and then by incubation at 37°C for 2,5 h in PARP reaction mixture containing 10 mM $MgCl_2$, 1 mM DTT, 5 µM biotinylated NAD^+ (Trevigen, Gaithersburg, USA) in 100 mM Tris buffer with 0,2% Triton X-100 (pH 8,0). Thereafter, the slides were washed with PBS (pH7,4; 0,1M). For detection of incorporated biotin, Avidin, Alexa Fluor® 488 conjugate (1:80; Molecular Probes, Inc. Eugene, USA) was applied at room temperature for 1 hour followed by washing in PBS and finally the sections were mounted with vectashield containg DAPI (Vector, Burlingame, CA, USA). For negative controls, biotinylated NAD^+ was omitted from the reaction mixture and they showed no staining.

9. Western Blots (WB)

9.1 Method Overview

Western blot is the most widely used method to separate, locate and identify proteins from tissue homogenates based on their ability to bind to specific antibodies (http://www.molecularstation.com/protein/western-blot/).

In stepwise procedure, WB involves the proteins separation according to their sizes by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In the second step, the proteins are transferred from SDS-gel to a membrane (Nitrocellulose or PVDF- Polyvinylidene Fluoride). Third step consist of incubation of membrane with the primary antibody followed by addition of the secondary antibody (this antibody is an antibody-enzyme conjugate, e.g., horseradish peroxidase (HRP). Finally, with the detection solutions the proteins of interest are detected (Westermeier and Marouga, 2005).

9.2 Solutions and buffers

A. Homogenization buffer (to prepare 50 ml)

- 20 mM Tris-HCl 0,12g
- 0,25 M Sucrose 4,28g

1 mM EDTA	0,015g
- 0,05% BSA	0,025g
- 0,1M PMSF	500 µl
- Protease inhibitor cocktail Set III	500 µl

Bradford dye Reagent

B. Polyacrylamide Gel (SDS-PAGE)

Separating gel (10%):

- dd H_2O	9,9 ml
- 1.5 M Tris-HCl, pH: 8.8	6,3 ml
- 10 % SDS	250 µl
- 30 % Acrylamide Mix (Rotiphorese)	8,3ml
- 10 % APS	250 µl
- TEMED	10 µl

Stacking gel (4%):

- dd H_2O	6,8 ml
- 0.5 M Tris-HCl, pH: 6.8	1,25 ml
- 10 % SDS	100 µl
- 30 % Acrylamide Mix (Rotiphorese)	1,7 ml
- 10 % APS	100 µl
- TEMED	10 µl

C. Loading buffer (for 20 ml):

- 0.02% Bromophenol Blue	0,004 g
- 125 mM Tris, pH 6.8	2,5ml
- 20% Glycerol	4 ml
- 4% SDS	4 ml

- dd H_2O to 18,4 ml

(A mixture of loading buffer and ß-mercaptoethanol in 92:8 concentrations respectively was used)

D. Running Buffer (1 litre, 10x)

- 25 mM Tris 30,3g
- 190 mM Glycin 142,6g
- 0,1% SDS 50 ml from 20% SDS solution

To make 1 litre working solution (1x) of Running Buffer:

Added 900 ml of dd H_2O to 100ml of 10x Buffer

E. Transfer Buffer (1 litre, 10x)

- 25 mM Tris 30,3g
- 190 mM Glycin 142,6g

To make working solution (1x) of Running Buffer:

Added 15% Methanol to 1x transfer buffer (For one litre: 100ml from 10x Buffer + 150 ml Methanol + 750 ml of dd H_2O)

F. Triton-X-100 (10%)

To make 50ml: 5ml Triton-X-100 in 50 dd H_2O

G. TBS (Tris Buffer Saline) 1 litre 10x

- 150mM NaCl 87,66g
- 13mM Trisma Base 15,74g
- Adjust pH to 7,5

To make 1ml working solution of 1x TBST

- Added 900ml of dd H_2O to 100ml of 10x TBS (Either Tween 20 or Triton used as detergent)
- Added 0,02% Triton-X-100 (2ml from 10% Triton) or 0,05% Tween

H. Coomassie blue solution:

- Coomassie Blue 1,25 g
- MeOH 250 ml
- Glacial acetic acid 50 ml
- ddH_2O 200 ml

I. Tris-HCl (1,5M, pH 8,8)

For 100ml: Trisma base 18,165g

pH adjusted with HCl

J. Tris- HCl (1M, pH 6,8)

For 50 ml: Trisma base 6,05g

pH adjusted with HCl

K. SDS, 10%

For 50ml: SDS 5g in 50ml of distilled water

L. SDS, 20%

For 500ml: SDS 100g in 500 ml distilled water

M. APS, 10%

For 50ml: APS (Ammonium-per-sulfate) 5g in 50ml distilled water (after aliquoting kept at -20°C)

9.3 Procedure

9.3.1 Tissue Homogenization

A. The retinal tissue was collected from S334ter-3 PN12, P23H-1 PN15, CD PN 12 and CD PN15 animals and immediately frozen in liquid N_2. The retinal tissue was homogenized in freshly prepared homogenizing buffer using either a mechanical homogenizer (Diax 600; Heidolph, Schwabach, Germany) or a glass/glass (pestle and mortar system) homogenizer (3 ml tapered tissue grinder; Wheaton, Millville, New Jersey, USA). Eppendorf tubes containing tissue were kept on ice during entire homogenization process.

B. To perform WB for G-protein transducin and arrestin, a separate protocol was followed to solubilize the rod OS membrane protein complexes, which is explained as follows:

Buffers: 10mM Hepes pH 6.8
10 mM $MgCl_2$
20 mM KCl
10% Dodecyl-Maltoside (DM) in H2O

Solubilsation

-“freeze and thaw” (1mg) retinal tissue for three times
(and/or osmotic shock: Hepes (pH 7.4 , on ice)
-Centrifuge 5min, 5000rpm, 4°C
-Take supernatant and freeze (N_2 + store 80°C) = “soluble proteins”
-Wash the remaining pellet (=”membrane proteins”) 2 times with 500µl TMK or HMK-buffer
-Add 50-80µl TMK or HMK with 1% DM to the pellet
-Solubilize on ice (30min or up to 2h)
-Centrifuge 30min UZ, 100000g (50000rpm: Rotor TLA 110), 4°C
-Take supernatant to new tube (=”solubilised membrane proteins”)

9.3.2 Protein Estimation

To estimate the total proteins within each sample, a colorimetric reaction known as the Bradford assay was performed. For that purpose, the tissue was mixed with Bradford dye (Bradford reagent, Biorad 500-0006), which was diluted 1:5 in distilled H_2O (see table 5.7) in spectrophotometer cuvettes (Sarstedt, Nümbrecht, Germany). Absorption was recorded at 595 nm using a spectrophotometer (UltraSpec 2000; Pharmacia Biotech, Cambridge, UK). The data were calculated using an equation determined by the standard curve prepared by BSA (Roth, Karlsruhe, Germany) concentration row (1mg BSA/ml distilled H_2O., 500µg/ml, 250µg/ml, 100µg/ml, 50µg/ml). For each sample, the results were given in µg/µl.

Table 5.6: Protein Estimation (Bradford assay)

Components	BSA calibration cuvettes	Sample cuvettes	Blank cuvettes

Bradford solution	1ml	1ml	1ml
Homogenized tissue sample	-	1µl	-
dd H_2O	-	9µl	10µl
BSA (different concentrations)	10µl	-	-

Formula to determine the Protein content:

$$x = y+0,0218/0,0004$$

(x= Protein, y= average value of 2 different readings of Protein Sample)

9.3.3 Electrophoresis and Loading

For gel preparation, loading and electrophoresis, big gel western blot system was used (Amersham Bioscience, Freiburg, Germany). Freshly prepared separating gel (SDS-PAGE 10%) solution was poured between the two tightly fitted glass plates separated by spacers and standing vertically on a stand. The fresh gel solution was immediately covered with 1ml Iso-propanol and left to solidify for 30- 45 minutes at room temperature. After the solidification of gel, isopropanol was removed, subsequently rinsed with dH_2O and the fresh stacking gel was added over it. At this point, 1mm thick comb was inserted in the gel at the top in order to create the loading wells. The stacking gel was left to polymerize at room temperature for 30 minutes. Simultaneously, the loading buffer was prepared and mixed in vials with the sample tissue homogenates alongwith ddH_2O. In each well 20µl of sample was loaded which had 25µg protein in it (see Table 5.6 for various components of loading solution).

Table 5.6: components of loading solution

Sample (25µg/per well)	ddH_2O	Loading Buffer
(say) x µl	10-x µl	10µl

The samples were heated on a water bath at 90ºCfor 10 minutes for denaturation and immediately shifted to ice just before loading. When the gel polymerized, the comb was removed and the gel was shifted to an electrode assembly, which was placed in a tank filled with running buffer. The samples were loaded into the wells. A protein marker (rainbow marker) was loaded into

the first well. After sample loading, the system was covered with a lid connected to an external power supply (Biometra, Goettingen, Germany), and voltage was applied (50V). The electrophoresis was allowed to run overnight (about 16-18 h) at 4°C until the dye had reached to the bottom of the gel.

9.3.4 Protein Transfer

Protein transfers were performed using the protein transfer equipment for bigger gel (Hoefer Pharmacia Biotech, San Francisco CA, USA). After electrophoresis, the proteins were transferred from gel to a PVDF (Polyvinylidene Fluoride) membrane (GE Healthcare, UK). Before transferring proteins, the PVDF membrane was soaked in MeOH for 2 minutes, and subsequently washed in ddH_2O followed by rinsing in transfer buffer. Thereafter, the membrane and the gel were placed together tightly in a "sandwich" which consisted of whattman filter papers and sponges surrounding the central unit of gel and PVDF membrane. This "sandwich" was placed inside a gel holder cassette. All steps were performed keeping all components immersed in transfer buffer. The cassette was later placed into the buffer tank already filled with transfer buffer. The lid linked to the power supply covered the tank. The transfer was done for one hour at 400 mA at 4°C.

After the transfer step was over, the gel was exposed to coomasie blue to test the whether the transfer had taken place efficiently, as the coomasie blue is used to stain proteins in SDS gels.

9.3.5 Blocking the Membrane and Primary Antibody Incubation

The PVDF membrane containing the proteins was kept in Rotiblock® blocking solution (diluted in dH_2O 1:10), for 2 h on the shaker (Unimax 2010, Heidolph, Schwabach, Germany) at RT. Primary antibody (see table 1) was diluted in TBST (0.05%Tween 20 or 0,02% Triton-X-100) with 5% non-fat dry milk (Roth, Karlsruhe, Germany). The membrane was incubated in the Primary antibody solution overnight at 4°C with gentle shaking. However, some antibodies (PARP PAR, RHO-C, PDE6β) were diluted in PBST (0.05%Tween 20 or 0,02% Triton-X-100) with 5% non-fat dry milk (Roth, Karlsruhe, Germany).

9.3.6 Secondary antibody and development

The following day, the membrane was washed in TBST (0.05%Tween 20 or 0,02% Triton-X-100) or PBST (0.05%Tween 20 or 0,02% Triton-X-100) three times for 10 minutes on the shaker. Subsequently, a chemiluminescent secondary antibody, previously diluted in TBST or PBST with milk (5%), was added for 1 hour at room temperature. Afterwards, the membrane was washed in TBST or PBST for 1 hour (2x5 min, 2x10 min, 2x15 min). Lastly, the detection solutions (Pierce ECL WB substrates) were mixed (1:1) and applied to the membrane for 1 minute. Subsequently, the membrane was exposed to Amersham Hyperfilms™ (GE Healthcare, UK) which were further detected by chemiluminiscent detection method in a Curix 60® processor (Agfa, Mortsel, Belgium). Quantification of relative WB band was performed after scanning the films of resulting bands according to a method devised by Luke Miller (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots) by calculating the integrated intensity of each band and by comparison with loading control (Actin).

10. Organotypic Retinal Cultures/Retinal explants cultures

10.1 Method Overview

The term ‘organotypic cultures’ refers to the *ex-vivo* maintenance of an organ or tissue in the form of an *in-vitro* model system that imitates the structural and physiological aspects of a tissue *in-vivo*. Hence, this method has emerged as an easily accessible tool to observe and manipulate the biological processes of complete tissues in highly controlled *in-vitro* conditions (Bull et al., 2011). Mid-twentieth century witnessed the emergence of retinal organotypic culture with the works of Ames et al, which since then has proved to be extremely important in neurobiological research (Ames and Nesbett, 1981).

Organotypic retinal explant culture system has been employed for studying a wide spectrum of neurobiological processes including cell death and neuroprotection (Bull et al., 2011), making retinal explants a useful and applicable tool in vision research.

10.2 Solutions and buffers

- Proteinase K.
- R16 or basal medium (see Table 5.7)
- FCS (Fetal calf serum)
- Supplements for the preparation of complete medium

Table 5.7: Components of the R16 basal medium

Ingredient	Mg/L	Ingredient	mg/L
L-Alanine	2,01	Glucose	3443,0
L-Arginine HCl	104,12	D(+)-Galactose	15,0
L-Asparagine H_2O	3,38	D(+)-Mannose	10,0
L-Cystine Na_2	38,33	Choline chloride	6,07
L-Glycine	21,94	Pyridoxal HCl	2,72
L-Histidine HCl.H_2O	33,07	$CaCl_2.2H_2O$	188,74
L-Isoleucine	71,63	$Fe(NO_3)3.9H_2O$	0,068
L-Leucine	73,70	$FeSO_4.7H_2O$	0,19
L-Lysine HCl	106,90	KCl	320,34
L-Methionine	21,25	$MgSO_4.7H_2O$	168,27
L-Phenylalanine	45,67	$NaH_2PO_4.2H_2O$	95,38
L-Proline	7,78	Na_2HPO_4	31,95
L-Serine	30,72	$ZnSO_4.7H_2O$	0,20
L-Threonine	66,94	Folic acid	3,0
L-Tryptophan	11,26	i-Inositol	8,78
L-Tyrosine	49,82	NAM	2,71
L-Valine	65,82	Hypoxanthine	0,92
Putrescine	16,11	Riboflavine	0,28
L-Carnitine	2,0	Thymidine	0,162

Cytidine 5'-diphospho ethanolamine	1,28	NaCl	6030,0
Cytidine 5'-disphospho choline	2,56	D-calcium pantothenate	2,75
Sodium phenol red	5,0	-	-

Preparation of Basal Medium (see table 5.8)

· Dissolved 1 vial of R16 Medium (powder) in 500ml of ddH_2O

· Added following supplements:

2,73g $NaHCO_3$ (32,5mM in Complete Medium)

0,1ml $NaSeO_3/MnCl_2/CuSO_4$

1,0ml Biotin

1,0ml Ethanolamine (from Stock, see table above)

Filled up to 800ml with ddH_2O and filtered

Table 5.8: Preparation of the R16 basal medium

Substance	Amount	Dissolve in	Stock Conc.	Conc. In Complete Medium
$NaSeO_3$ x $5H_2O$ $MnCl_2$ x $4H_2O$ $CuSO_4$ x $5H_2O$	7,9 mg 1,0 mg 2,5 mg	·Dissolve together in 100ml ddH_2O	300µM 50 µM 100 µM	30/60nM 5nM 10/20nM
Biotin	10 mg	·Dissolve in 9,8ml ddH_2O ·Added 0,1ml 1N NaOH (pH 10-12) ·Heat to 35°C (~10min) ·Neutralised with 0,1ml 1N HCl ·Put there from 1ml in 9ml ddH_2O	0,1mg/ml	0,1µg/ml
Ethanolamine	10 µl	·Add to10ml ddH_2O	1mg/ml	1µg/ml

Complete medium: To fulfil optimum culturing conditions of the retina, following nutrients were added to the basal medium (Table 5.9).

Table 5.9: Supplements for the complete medium (Source: Sigma, St. Louis, MP, USA)

Supplement	Conc. Stock-solution	Conc. compl. Medium	Amount for 50ml

Basal R16 Medium	-	-	40 ml
BSA	4%	0,2%	2,5 ml
Transferrin	10mg/ml	10µg/ml	50 µl
Progesterone	6,3µg/ml	0,0063µg/ml	50 µl
Insulin	2mg/ml	2µg/ml	50 µl
T3 (Triiodo-L-thyronine sodium salt)	2µg/ml	0,002µg/ml	50 µl
Corticosterone	20µg/ml	0,020µg/ml	50 µl
Thiamine HCl	2,77mg/ml	2,77µg/ml	50 µl
Vitamin B12	0,31mg/ml	0,31µg/ml	50 µl
(+/-)-α-Lipoic Acid	45µg/ml	0,045µg/ml	100 µl
Retinol/Retinylacetate	0,05mg/ml	0,1 µg/ml	100 µl
DL-Tocopherol/acetate	0,5mg/ml	1µg/ml	100 µl
Linolic/Linolenic Acid	0,5mg/ml	1µg/ml	100 µl
L-Cysteine HCl	7,07mg/ml	7,09 µg/ml	50 µl
Glutathione	1mg/ml		
Na-pyruvate	50mg/ml	50 µg/ml	50 µl
Glutamine/Vitamine C	2,5mg/ml 10mg/ml	25 µg/ml 100 µg/ml	0,5 ml
ddH_2O			6,15 ml

10.3 Procedure

Organotypic Retinal cultures were prepared according to procedures devised in earlier publications (Caffe et al., 1989; Pinzon-Duarte et al., 2000). S334ter-3 and P23H-1 were sacrificed at PN5 and PN8 respectively. After enucleating the eyes under sterile conditions, they were incubated in R16 basal medium for 5 minutes followed by 15 minute incubation in 0,12% Proteinase K dissolved in R16 basal medium at 37°C. Proteinase K incubation is important to facilitate the explantation of the RPE together with the retina. Immediately after this step, the eyes were placed in R16 basal medium with 20% fetal calf serum (FCS) to deactivate the Proteinase K. The eyes were then dissected under a

laminar flow hood (Heraeus instruments, Hanau, Germany; danLAF®-o-matic Claus Damm, Humlebæk, Denmark) by using a binocular microscope (Zeiss, Oberkochen, Germany). The eyes were kept in basal medium during the whole procedure. The eyeballs were pierced at the ora serrata by means of a needle and then from the same point the cuts were given with dissecting scissors along the ora serrata. In this way, the anterior parts along with cornea and lens were removed. The optic nerve was cut and sclera was carefully removed to isolate the retina, together with the attached pigment epithelium. The retina so obtained was given four wedge shaped cuts to give it a cloverleaf like appearance. Afterwards, the retinas were transferred to a 0,4µm polycarbonate membrane (Costar, Corning, NY, USA), with the RPE facing the membrane. Subsequently, the membrane was placed in a six well culture plate (Falcon, BD Biosciences, San Jose, CA, USA) and incubated in 1,2 ml of complete R16 medium (see table 5.9) at 37°C in an incubator with humidity and 5% CO_2. The medium was replaced every two days.

10.4 *In-vitro* treatments with Calpain and PARP inhibitors

For *in-vitro* inhibitor treatment experiments, S334ter and P23H animals were sacrificed at PN5 and PN8 respectively. Before being treated with inhibitors, the explants were allowed to adjust to *in-vitro* culturing conditions for 2 days. The cultures received treatments for 5 days in case of S334ter cultures and 10 days in case of P23H cultures. The retinas remained in cultures for 7 and 12 days for S334ter and P23H retinas respectively.

Two calpain inhibitors were used: Calpain Inhibitor XI (CPI XI; Z-l-Abu-CONH $(CH2)_3$-morpholine; also referred to as CX295 and Calpain Inhibitor VI also referred as SJA-6017). Two concentrations of both calpain inhibitors were tested; 5µM and 100µM were used (Paquet-Durand et al., 2010).

Similarly, PARP inhibitor used in the study was PJ34 (N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide.HCl), which is highly potent PARP inhibitor. Concentration of PJ34 was 6µM (Paquet-Durand et al., 2007). Controls for treated cultured retinas received the regular complete medium.

Table 5.10. Inhibitors used in the study

Inhibitor	Source
Calpain Inhibitor VI (SJA-6017) N-(4-Fluorophenylsulfonyl)-L-valyl-L-leucinal	Calbiochem
Calpain Inhibitor XI(Z-L-Abu-ONH(CH2)3- morpholine)	Calbiochem
PARP inhibitor- PJ34 (N-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-N,Ndimethyl-acetamide.HCl)	Alexis Biochem

11. Microscopy, cell counting, and statistical analysis

Morphological observations and routine light microscopy were performed using a Zeiss Imager Z1 Apotome Microscope. Images were captured with a Zeiss Axiocam digital camera, using Zeiss Axiovision 4.7 software. Primary image processings were done by using Adobe Photoshop CS3 extended version 10.0, Adobe illustrator CS (Adobe Systems Incorporated, San Jose, CA) and Corel Draw X3 softwares. Representative pictures were taken from the central area of the retina. Labelled cells in radial sections were counted manually at 20X magnification in a blindfold fashion. The total number of cells was determined dividing the outer nuclear layer (ONL) area through the average cell size. The number of positive cells (for various cell death markers) was then divided by the total number of cells in the ONL giving the percentage of positive cells. All given data, represent the means and standard deviations from three sections of at least three different animals. Statistical comparisons between experimental groups were made using a One-way ANOVA test combined with Bonferroni's test using Prism 4 for Windows (GraphPad Software, La Jolla, CA). Values are given as mean ± standard deviation (SD) or standard error of means (SEM). Levels of significance were as follows: n.s., $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Annextures

I. Abbreviations

ADRP: Autosomal Dominant Retinitis Pigmentosa

AIF: Apoptosis Inductor Factor

Apaf-1: Apoptosis activating factor-1

ARRP: Autosomal Recessive Retinitis Pigmentosa

ARVO: Association for Research in Vision and Ophthalmology

ATP: Adenosine Triphosphate

cAMP: cyclic Adenosine Monophosphate

CaMKII: Calcium/calmodulin-dependent protein kinase II

COD: cone dystrophy

CORD: Cone rod dystrophy

CSNB: congenital stationary night blindness

cc-3: cleaved caspase-3

cc-9: cleaved caspase-9

CD: Charles river (sprague dawley) rats

cGMP: cyclic Guanosine Monophosphate

CI: Calpain Inhibitor

CIVI: Calpain Inhibitor VI

CIXI: Calpain Inhibitor XI

CNG: Cation Nucleotide-gated

CNS: Central Nervous System

Cyt C: Cytochrome C

DAB: Di-Amino Benzidine

DAPI: 4’,6-diamidino-2-phenylindole

dd: Double Distilled

DIV: Days *in-vitro*

DMSO: Dimethylsulfoxide

DNA: Deoxyribose nucleic acid

ESC: Embryonic Stem Cell

ER: Endoplasmic reticulum

GC: guanylate cyclase

GCAP: guanylate cyclase-activating protein

GDP: guanosine diphosphate

GCL: ganglion cell layer

GEF: Guanine effector protein

GFP: green fluorescent protein

GK: Guanylate kinase

GMP: guanosine monophosphate

GPCR: G-Protein Coupled Receptor

GTP: guanosine triphosphate

GTPase: guanosine triphosphatase

h: hour

INL: inner nuclear layer

IPL: inner plexiform layer

iPSC: induced pluripotent stem cell

IS: inner segment

KO: Knockout

LCA: Leber congenital amaurosis

MD: Macular Dystrophy

min: minute

MNU: N-methy-N-nitrosurea

MOMP: Mitochondrial Outer Membrane Pemeabilization

MPTP: Mitochondrial Permeability Transition Pore

mRNA: messenger RNA

NAD+: Nicotinamide Adenine Dinucleotide

NAM: Nicotinamide

NCKX: Sodium Calcium Potasium exchanger

NDPK: Nucleoside diphosphate kinase

NMDA: N-methyl-D-aspartate

OGG: 8-oxoG DNA Glycosylase

ONL: Outer Nuclear Layer

OPL: Outer Plexiform Layer

OS: Outer Segment

PAR: Poly ADP Ribose

PARP: Poly ADP ribode polymerase

PBS: Phosphate Buffered Saline

PDE6: Phosphodiesterase 6

PN: Post-natal day

P23H Proline 23 histidine

rd1: retinal degeneration 1

rds: retinal degeneration slow

retGC: Retinal Guanylyl Cyclase

RHO: Rhodopsin

RK: Rhodopsin kinase

RP: Retinitis Pigmentosa

RPE: retinal pigment epithelium

RT: room temperature

S334ter Serine 334 termination

SD: Sprague Dawley

TBS: tris buffered saline

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling

UPR: Unfolded protein response

wt: wild-type

XLRP: X-linked retinitis pigmentosa

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IV. Curriculum Vitae

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Academic Qualification and Experience

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Theme: Mechanism of Photoreceptor cell death in Rhodopsin mutant rat models (S334ter and P23H) of Autosomal Dominant Retinitis Pigmentosa

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M.Sc. (Zoology) 1997-1999 (First Division):

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1999 – 2001: Lecturer, Dept of Zoology

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Additional Qualification

1. Qualified the combined CSIR-UGC National Eligibility Test (NET) for research and Lecturereship conducted by Council of Scientific and Industrial Research (CSIR), Govt. of India, New Delhi (June, 2000).

2. Diploma thesis in Biology, Eberhard Karls University, Tübingen (Germany). March, 2011

Meetings/Conferences

1. Association for Research in Vision and Ophthalmology (ARVO) Fort Lauderdale USA 2009 (Poster presented)

2. European Retina Meeting (ERM-2009) Oldenburg, Germany (Poster presented)

3. Pro Retina Meeting 2009, Potsdam, Berlin, Germany (Poster presented)

4. Pro Retina Meeting 2010, Potsdam, Berlin, Germany (Poster presented)

5. Association for Research in Vision and Ophthalmology (ARVO) Fort Lauderdale- FL USA 2010 (Poster presented)

6. Tübingen Outer Retina Conference 2010, Tübingen, Germany 2010 (Poster presented)

V. Publications

1. Calpain and PARP Activation during Photoreceptor Cell Death in P23H and S334ter Rhodopsin Mutant Rats

Jasvir Kaur, Stine Mencl, Ayse Sahaboglu, Pietro Farinelli, Theo van Veen, Eberhart Zrenner, Per Ekström, François Paquet-Durand, and Blanca Arango-Gonzalez (**PLoS One** July, 2011, Chapter I of the thesis)

2. PARP1 gene knock-out increases resistance to retinal degeneration without affecting retinal function

Ayse Sahaboglu, Naoyuki Tanimoto, **Jasvir Kaur**, Javier Sancho-Pelluz, Gesine Huber, Edda Fahl, Blanca Arango-Gonzalez, Eberhart Zrenner, Per Ekström, Hubert Loewenheim, Mathias Seeliger, Francois Paquet-Durand (**PLoS ONE** November 24, 2010)

3. Neuroprotective strategies for the treatment of inherited photoreceptor degeneration

Dragana Trifunovic, Ayse Sahaboglu, **Jasvir Kaur**, Stine Mencl, Eberhard Zrenner, Marius Ueffing, Blanca Arango-Gonzalez François Paquet-Durand (accepted, December, 2011: **Current molecular medicine**)

4. Photoreceptor neuroprotection by using inhibitors of calpains and PARP

Jasvir Kaur & Blanca Arango Gonzalez *(Manuscript under preparation) Chapter III of the thesis*

5. cGMP signalling in S334ter and P23H rat models of Retinitis Pigmentosa

Jasvir Kaur, Ayse Sahaboglu, Andreas Vogt, Blanca Arango Gonzalez *(Manuscript under preparation) Chapter IV of the thesis*

6. Synaptic organization of synapses during retinal degeneration in S334ter-3 and P23H-1 rat models of Retinitis Pigmentosa

Jasvir Kaur, Sylvia Bolz, Blanca Arango Gonzalez *(Manuscript under preparation)*

VI. Contributions

All experiments designed, executed and analysed together with **Dr. Blanca Arango Gonzalez** at the **Institute for Experimental Ophthalmology, University Eye Hospital, Tübingen, Germany.**

Ayse Sahaboglu: Helped in quantification of co-labeling of cell death markers with TUNEL, and in completing WB for chapter IV (transducin, arrestin, PDE6)

Andreas Vogt: Helped in homogenisation of retinal tissue for cytoplasmic and outer segment proteins

Francois Paquet Durand: Helped in manuscript editing, and inputs in scientific ideas (published part of the study, chapter II)

Per Ekström (University of Lund, Sweden): Helped in manuscript editing (published part of the study: chapter II)

Pietro Farinelli (University of Lund, Sweden): Cytochrome-c immunostaining pictures

Stine Mencl: Calpastatin WB

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Jasvir

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