New aspects of retinal photoreceptor morphology and development in normal and pathological conditions

PhD thesis outline

Dr. Ágnes Ida Berta

Doctoral School of Molecular Medicine
Semmelweis University

PhD Supervisor: Dr. Ágoston Szél, D.Sc.

Official reviewers: Dr. Róbert Gábriel, D.Sc.
Dr. Gábor Somfai, Ph.D.

Head of the examination board: Dr. András Csillag, D.Sc.

Members of the examination board: Dr. Gábor Gerber, Ph.D.
Dr. Zsuzsanna Szepessy, Ph.D.

Budapest, 2013

Introduction

The major cause of blindness in industrialized countries are diseases caused by the progressive dysfunction and the loss of retinal photoreceptors. It is one of the most genetically heterogeneous disorders in man. (Hartong et al., 2006). The inherited forms of photoreceptor degeneration are a common cause of visual impairment and the majority of them is monogenic, with a prevalence of ~1 in 3,000 (Pacione et al., 2003; Rattner et al., 1999). The most common subtype is retinitis pigmentosa, which is one of the two main causes of blindness in the adult population (20–64 year olds, (Buch et al., 2004). Usually retinitis pigmentosa results from a primary defect in rods, but this also leads to secondary cone loss at later stages and this is why it is classified as a rod–cone degeneration. Other inherited photoreceptor degenerations include macular, cone and cone-rod degenerations, which are clinically distinguishable from retinitis pigmentosa. (Wright et al., 2010).

The vertebrate retina is made of six major cell types: rod and cone photoreceptors, and horizontal, bipolar, amacrine and ganglion cells. The visual process begins at the photoreceptors, which are sensory neurons specialized to capture light quanta. The chemical output of photoreceptors is integrated and processed by interneurons (bipolar, horizontal and amacrine cells) and transmitted to visual centers in the brain by ganglion cells. Cone photoreceptors respond to bright light, mediate colour vision and foveal cones make high resolution of visual images ("sharp vision") possible. Rod photoreceptors function only under dim light conditions. They are so sensible that they can respond to single light quanta (Masland, 2001; Swaroop et al., 2010). Across the retina, rods and various cone subtypes always have a well-defined arrangement, occurring with different patterns in different species. In mice and humans, photoreceptors represent over 70% of retinal cells, but rods outnumber cones by 30:1 in mice and 18–20:1 in humans (Carter-Dawson and LaVail, 1979; Roorda and Williams, 1999). The mammalian retina has only one type of rod visual pigment called rhodopsin, which has a peak spectral sensitivity at ~500 nm. Most mammals, including the mouse, have two types of cone opsins defining dichromatic colour vision: S opsin (also known as blue sensitive opsin), which has peak sensitivity in the short wavelength (ultraviolet or blue) region of the spectrum; and M opsin (also known as green-sensitive opsin),

which has peak sensitivity in the medium–long wavelength (green) region of the spectrum (Swaroop et al., 2010). In humans and diurnal primates an additional opsin, L opsin (also known as red-sensitive opsin) is also present, which is sensitive to longer wavelength (red) light. (Deeb, 2006; Nathans et al., 1986). In mice M opsins and S opsins are expressed in opposing gradients across the retina (Szel et al., 1993)

The outer segment, where the primary photoreceptive processes take place, is a specially transformed primary cilium (Davenport and Yoder, 2005). It is made of a dense stack of flattened membrane discs covered with the plasma membrane and contains more than 500 proteins species. Important steps along the visual transduction are closely associated with the disc and plasma membranes (Khorana, 1992) of the outer segment. The unique structural and functional organization of the vertebrate retina is finely adapted to the initial capture and processing of visual signals, but this organization also makes it unusually vulnerable to dysfunction (Masland, 2001). Mutations affecting functions that are photoreceptor specific, such as phototransduction or the visual cycle, are only marginally more numerous than mutations affecting more general cell functions, such as protein folding, lipid metabolism or the extracellular matrix. Most of the mutations show widespread rather than photoreceptor specific expression patterns. There is no explanation why mutations in so many different genes cause photoreceptor degeneration (see review at (Wright et al., 2010). The dog as a model for hereditary diseases has attracted the attention of basic research in the last 15 years, as numerous inherited canine retinal diseases show close homology to human diseases, however the retinal structure in the two species is in many ways different. Early retinal degeneration (*erd*) is an autosomal recessive, early onset form of canine retinal degeneration characterized by aberrant functional and structural development of rod inner and outer segments, and rod and cone synapses. Abnormal development is followed by rapid degeneration of rods and cones. Affected dogs are initially night blind, and become totally blind between 48 and 72 weeks of age. (Acland and Aguirre, 1987).

In mammals, the genesis of photoreceptors takes relatively long, weeks to months depending on the species, occurring pre- and postnatally (Carter-Dawson and LaVail, 1979; Morrow et al., 1998; Rapaport et al., 2004; Young, 1985). In humans, all photoreceptors are formed prenatally (Cornish et al., 2004; Hendrickson et al., 2008). In

the mouse, photoreceptor development is less advanced than in humans at birth. The eyes of the newborn animals remain closed for almost 2 weeks. Cone genesis starts at embryonic day 11 and is essentially complete at birth, while peak of rod genesis occurs in the first few postnatal days. S opsin is already expressed at late embryonic stages, but rhodopsin only subsequently after birth. Expression of M opsin begins later around postnatal day 6 (Carter-Dawson and LaVail, 1979; Young, 1985).

Lately it was demonstrated that a group of integral membrane proteins, the caveolins play a critical role in early vertebrate development. They are especially important in notochord and neuromast formation (Fang et al., 2006; Nixon et al., 2007). Caveolins are integral membrane proteins that are principal components of the special, Ω-shaped plasma membrane invaginations called caveolae. For formation of caveolae cholesterol is essential, as cholesterol binds caveolins and also influences caveolin transcription (Fielding et al., 1997; Murata et al., 1995). Caveolae were first identified in endothelial cells about 50 years ago and they were only later recognized, as a type of lipid rafts on the basis of their lipid composition. Although lipid rafts and caveolae are similar biochemically, they are morphologically different. Because of their unique, Ω-shaped appearance caveolae can be detected by electron microscopy, whereas lipid rafts are not visible. The different structure also suggest different functions (Head and Insel, 2007). It is now known, that the lateral organization of the lipids in photoreceptor outer segment membranes is not uniform, since the membrane contains microdomains, that are resistant to nonionic detergents, also known as lipid rafts (Martin et al., 2005). A general feature in various cell types is the presence of caveolin-1 and c-src in detergent-resistant membranes (Pike, 2003). Similarly to detergent-resistant membranes in other cell types, photoreceptor DRMs also contain caveolin-1 and c-src as shown by biochemical studies (Ghalayini et al., 2002; Martin et al., 2005). Until now immunocytochemical studies showed no or minimal amount of caveolin-1 at the rod outer segment level (Kim et al., 2006). Caveolin was originally identified in transformed chick fibroblasts as a tyrosine-phosphorylated substrate of Src (Glenney, 1989; Murata et al., 1995). Multiple isoforms of caveolin have been identified: caveolin-1-α, caveolin-1-β, caveolin-2, and caveolin-3. It has been proposed that members of the caveolin family members function as scaffolding proteins to organize and concentrate specific lipids (cholesterol and glycosphingolipids), lipid-modified

signaling molecules and G proteins within caveolae, as binding may suppress or inhibit enzyme activity through the caveolin scaffolding domain, which is a common caveolin domain (Schlegel et al., 1998; Swaney et al., 2006).

There are only a few reports available about the presence and distribution of caveolins in the retina. In the mouse retina, caveolin-1 was found to be present in the outer plexiform layer in the synaptic ribbon of photoreceptor terminals (Kachi et al., 2001). In the rat retina caveolin-1 was detected in various retinal layers, between the inner plexiform layer and the outer limiting membrane, suggesting that caveolin-1 is expressed in Müller cells, this was also confirmed using Müller cell specific markers (Ueda, 2002). It was also shown with laser scanning confocal microscope that caveolin-1 is located in apical and basal surfaces of pigment epithelial cells. (Bridges et al., 2001). Few years later a more detailed histological study was published showing caveolin-1 to be present in the majority of retinal layers of the rat. These first studies did not offer a comprehensive and accurate picture about the expression of caveolin isoforms in the mammalian retina.

Objectives

1. **Localization and expression pattern of caveolin-1 and c-src during retinal development** in order to understand their potential function in photoreceptor development. We wanted to compare the expression of these molecules to two highly abundant, well characterized proteins, rhodopsin and rhodopsin kinase. In addition, we also aimed at investigating the distribution profile of a kinase responsible for caveolin-1 phosphorylation, c-src, as well as the phosphorylated product, phospho-caveolin-1. To study potential colocalization and coexpression, double immunolabeling and co-immunoprecititation were used.
2. **Expression and distribution of caveolin isoforms in the retina of different species and in different retinal diseases, especially focusing on the human retina,** where relevant data on the topic were until now completely missing. We also wanted to compare degenerating and normal retina of the dog to reveal a possible role of caveolin and caveolin-related proteins in these retinal disorders. The dog retina and inherited canine retinal degenerations are especially interesting, because they show close homology to similar diseases in human.
3. **Detailed morphological and biochemical description of early retinal degeneration (*erd*), a canine retinal degeneration** characterized by abnormal photoreceptor development, followed by rapid photoreceptor degeneration. Although many other canine degenerations are now well characterized, in this disease the mechanism of photoreceptor degeneration was until now not described. Since in *erd* a special, elongated cell death period was observed, which is not followed by an appropriate change in the number of photoreceptors, our aim was to investigate the possibility if a cell proliferation compensates for the loss of photoreceptors. Various cell proliferation and cell-specific markers were planned to assess cell proliferation and to identify which cell types are proliferating.
4. **Comparison of *erd* with other canine retinal degenerations.** As in *erd* proliferating cells were found it the photoreceptor layer, we also wanted to investigate if the cell proliferation period is a unique characteristic of early retinal degeneration or not. We aimed at evaluating the cell proliferation rate in other canine retinal degenerations and comparing it to that of *erd*.

Material and methods

Animals and human samples

- Syrian (golden) hamsters were used in different stages of postnatal development. Retinal samples were collected on postnatal days 1, 5, 10, 15 (P1, P5, P10 and P15) and in adulthood. More than 18-days-old hamsters were considered as adults.
- Retinal samples from one *Xenopus laevis* were also used in this study.
- One black-and-white ruffed lemur (*Varecia variegata*), which lived in captivity and died naturally at the age of 11. The retinas were postmortem collected.
- Three patients were involved in the study. Two males (ages: 50, 57) and one female (age: 50). They were operated for posterior uveal melanoma (melanoma malignum choroideae) in 2005 at the Department of Ophthalmology, University of Debrecen. For our study we used retinal samples of the enucleated eyes.
- *Erd* dog retinas. Dogs were maintained at the Retinal Disease Studies (RDS) facility in Kennett Square, PA. The dogs represent an outbred population with a common genetic background segregating *erd* and other retinal disease alleles. 70 dogs were used in the study, including 44 crossbred *erd* affected dogs (age range, 4.3-165 weeks), and 26 non-affected dogs that were used as normal control subjects (age range, 4.7–25.7 weeks).

Laboratory methods

- Toluidine blue staining and electron microscopy
- Immunocytochemistry using specific antibodies
- Double immunolabeling and confocal microscopy
- Immunoblot analysis of retinal lysates and isolation of ROS
- Immunoprecipitation and Western Blot analysis
- Immunoblot and densitometric analysis
- Rod outer segment renewal and opsin biosynthesis
- Gene expression studies with quantitative real time-PCR (qRT-PCR)

Results

1. Expression of raft-associated proteins during photoreceptor development

In order to study the caveolin-1 localization during photoreceptor development, we compared it to two highly abundant, well characterized proteins, rhodopsin and rhodopsin kinase. In addition, we also investigated the distribution profile of a kinase responsible for caveolin-1 phosphorylation, c-src, as well as the phosphorylated product, phospho-caveolin-1. Interestingly, the localization and distribution of rhodopsin, rhodopsin kinase, caveolin-1 and c-src is very similar in the ONL during retinal development. The signals are located throughout the ONL and have a granular, punctuate appearance. The extent of protein expression during development was followed by Western blot analysis. Rhodopsin, rhodopsin kinase, caveolin-1 and phospho-caveolin-1 were not detected at P1, while their expression gradually increased through P5, P10 and P15 until adulthood. In comparison, there is no marked change in the expression of c-src during development. Since the distribution of rhodopsin, rhodopsin kinase, caveolin-1 and c-src was similar at P10 and P15, the question arose whether these proteins are located in a common compartment or not. In order to test if these proteins are co-distributed, double-labeling immunocytochemistry was used. As the similarity of the distribution was most obvious at postnatal day 10, we chose this age to compare the distribution of rhodopsin and caveolin-1 as well as the distribution of rhodopsin with c-src. Individual punctuate structures between the outer limiting membrane and outer plexiform layer were seen to be labeled by both rhodopsin and caveolin-1, indicated by yellow color in the merged images. The heavy staining of the OS/IS layer by the rhodopsin antibody made observation of an eventual colocalization difficult. A similar overall observation could be made on specimens double-labeled with rhodopsin and c-src. In order to determine if rhodopsin formed a complex with other raft-associated OS proteins, as identified by immuncytochemical colocalization, we used immuoprecipitation. We also investigated the co-immunoprecipitation with two other proteins of the OS: ROM-1 and RDS. Previously ROM-1 was reported to be a part of the OS raft membrane domains, but RDS was not found in this same fraction(Boesze-Battaglia et al., 2002). Whole retinal lysates from P1, P5, P10, P15 and adult were immunoprecipitated with the anti-opsin antibody. Immunoprecipitated complexes were

isolated and their protein content determined by Western blots. Rhodopsin was found to co-immunoprecipitate with caveolin-1, c-src and ROM-1, but not with RDS

2. Localization of raft-associated proteins in the mature retina

To characterize the rod photoreceptors with immunocytochemistry we used specific antibodies against rhodopsin, as well as rhodopsin kinase. The labeling of rhodopsin is restricted to the OS, while rhodopsin kinase is present in OS/IS and in the outer nuclear layer. We determined the caveolin-1 distribution in the photoreceptor layer of the retina and also studied the localization of c-src (an enzyme that phosphorylates caveolin-1, (Schlegel et al., 1998), as well as the phosphorylated form of caveolin-1. The presence of caveolin-1 is clearly seen in the IS, however, it can only be detected in traces in the OS. The ONL, which is generally made of the cell bodies of photoreceptors and Müller cell processes, also contains caveolin-1. In contrast to caveolin-1, phospho-caveolin-1 is restricted to the OS while the IS and the ONL lacking phospho-caveolin-1 labeling. The distribution of c-src in the photoreceptor layer is analogous to that of caveolin-1: c-src is more dominant in IS, weakly stains OS and shows a scattered punctuate labeling in the ONL. The pigment epithelium also contains caveolin-1, phospho-caveolin-1 and c-src, but it is not labeled with rhodopsin or rhodopsin kinase antibodies. The phospho-caveolin-1 content was determined in whole retinal lysates and isolated ROS fractions. As expected, the ROS lysate is enriched in rhodopsin and rhodopsin kinase when compared to the whole retinal lysate. Caveolin-1, phospho-caveolin-1 and c-src are all detectable in both ROS and whole retinal lysates. As the hamster retina, similarly to other rodents, has very thin photoreceptors and therefore a finer localization in ROS/RIS is not possible, we made parallel immunolabeling of caveolin-1 and phospho-caveolin-1 in the large rod photoreceptors of the toad *Xenopus laevis*. The caveolin-1 antibody did not give specific labeling in this species. Phosho-caveolin-1 could be localized to the thin rim of OS and to structures likely to be cilia.

3. Localization of caveolins in dog retina and in inherited canine retinal degenerations

First we wanted to describe the expression of the different caveolin isoforms in the normal dog retina. Our results show that caveolin-1, caveolin-2 and c-src give a diffuse labeling throughout the ONL, indicating that the cell bodies of photoreceptors and possibly Müller cells are labeled. Horseshoe-like structures and layers of dotted signals can be observed on the border of the ONL and the OPL labeled with these antibodies. This appearance at this location is typical for rod and cone presynaptic terminals. The IS are visibly labeled, but the OS are very weakly or not labeled at all. Caveolin-3 does not label photoreceptors in this species. Phospho-caveolin-1 is restricted to the OS, while phospho-caveolin-2 gives a labeling very similar to that of caveolin-2. The pigment epithelium contains caveolin-1, caveolin-2, phospho-caveolin-1, phospho-caveolin-2 and c-src, but no caveolin-3. In the inner retinal layers caveolin-1, caveolin-2, phospho-caveolin-2 and c-src gave a very strong labeling around the blood vessels and a weaker labeling in the INL and GCL. Interestingly caveolin-3 labeled only structures that, according to their location, can be assumed to be horizontal cells. Immunocytochemistry with the same antibodies were done also in different retinal degenerations of the dog: *erd, rcd1, crd2* and *XLPRA2*. Meaningful differences could be observed, the degeneration of photoreceptors also affected the expression of the investigated raft-associated proteins. The most striking observation of all was that phospho-caveolin-1 labeling was missing in all diseases, but caveolin-1 labeling was intact, indicating the possibility that the diseased photoreceptors are not capable of phosphorylating caveolin-1. Interestingly *XLPRA2* and *crd2* retinas were not labeled with the caveolin-2 antibody, while the labeling was intact in *erd* and *rcd1*.

4. Distribution of caveolin isoforms in the lemur retina

Caveolin-1: In the macular region, caveolin-1 was detected in every layer. Among these layers, the density of immunolabeling showed only slight differences. Both the outer and inner segments harbored caveolin-1. In the outer and inner nuclear layers, caveolin-1 was localized along the cell membranes. The ganglion layer and the outer

and inner plexiform layers diffusely expressed caveolin-1. In the periphery, caveolin-1 was localized in the same layers, but at lower densities. The INL and ONL both contained caveolin-1, but the immunoreactivity was weaker in the inner layer. In the ciliary body, both layers were labeled with anti-caveolin-1.

Caveolin-2: The immunostaining of caveolin-2 differed from that of caveolin-1. The lemur retina barely evidenced any caveolin-2 signals. Weak immunoreactivity was detected within the ganglion layer. No signal was detected in the ciliary body samples.

Caveolin-3: In the macular region and the periphery, the immunostaining pattern of caveolin-3 was similar to that of caveolin-1, but the density was significantly lower. Labeling was detected only between the ganglion cell layer and the inner limiting membrane. Immunostaining densities ranged from low to moderate. Caveolin-3 was also detected within the ciliary body.

5. Caveolins in the (melanoma malignum affected) human retina

Caveolin-1: The bound antibody was present in all layers of the retina. The weakest staining could be found in the pigment epithelium. In other layers the density was estimated to vary from low to very high. The distribution of immunolabel exhibited a characteristic center-to-peripheral gradient; increasing from very low, low, moderate and high density, reaching the maximum level, then decreasing towards the extreme peripheral part of the retina.

Caveolin-2: The caveolin-2 immunoreactivities were found to be present from the outer nuclear layer to the inner limiting membrane, showing very low, low and moderate densities, however at the peripheral part in the inner plexiform layer, the layer of the optic nerve fibers and the inner limiting membrane no immunoreactivities could be detected. There was no immunolabeling in the epithelium of the ciliary body.

Caveolin-3: In the macular region, the caveolin-3 immunoreactivity was present from the outer nuclear layer to the inner limiting membrane with very low, low and moderate densities. At the peripheral part IRs also occurred in the layer of the outer segments. As to the ciliary body, caveolin-3 signals could be seen in both epithelial layers, with low and moderate densities.

6. Early retinal degeneration (*erd*) in dogs

First we described the development and degeneration of the *erd* affected dog retina using 7 μm H&E sections using light microscopy. The retinal development appears to be normal until 7.7 wks of age. From 8.3 wks abnormalities begin to be visible. At 12.3 wks loss of OS, shortening and loss of IS are obvious. These details become even more pronounced at 14.1 wks. Until this time point the ONL and INL thickness do not seem to change. By 48.1 wks OS and IS become extremely short, ONL thickness decreases dramatically, INL thickness decreases moderately. Thereafter at 62 wks degeneration becomes severe, ONL has only one layer. The retina seems to be completely degenerated at 101 wks, severe gliosis is dominant. The retina is entirely lacking both rods and cones. At 165 wks the disease progresses even more, the retina is extremely thin.

Visualized with immunocytochemistry as early as 4.3 wks a partial mislocalization of rhodopsin is visible in the ONL. The OS appear to be of variable length and already have irregular contours. Between 7.7-14.1 wks progressive rod disease develops: OS become more disorganized, more variable in shape and length, they also shorten, and some disappear. Rod opsin mislocalization becomes severe. Both red/green (COS-1) and blue opsin (OS-2) were expressed in the hCAR-labeled cones. Both the number and distribution pattern of blue- and red/green cones were similar to normal in all ages examined (4.3-14.1 wks). As for their morphology, cone outer segments progressively started to have irregular contours, similarly to rods. Surprisingly in rods mAb OS-2 not only localized to the blue cones, but also to the rod outer segments in the age range of 7.7-14.1 wks.

TUNEL labeling was used to examine the kinetics of photoreceptor apoptosis/cell death in the disease during (4.3 wks), or after (7.7-14.1 wks) the completion of postnatal retinal differentiation. The period of cell death is prolonged and has two peaks: 7.7 and 11.6 wks in the superior retina, 8.3 and 12.3 wks in the inferior retina. To quantify the changes in the ONL the thickness was measured in μm. After a small decrease between 4.3 and 7.7 wks the ONL thickness stays constant until 14.1 wks of age. Thereafter the ONL decreases dramatically by 48.1 wks and gets even thinner at 62 wks. In spite of the high rate of cell death between 4.7 and 14.1 wks, ONL thickness did not decrease in this period, which suggested the possibility of cell

proliferation in the ONL. PCNA labeling was used to determine cell proliferation in the ONL of the *erd* retina. Between 7.7 and 14.1 wks high amount of PCNA positive cells were observed in the *erd* retina. This is the same period, when high cell death kinetics were observed. Interestingly, the course of cell proliferation also has two peaks, occurring at the same time points as cell death peaks

We used qRT-PCR to characterize retinal expression of the mutated gene, STK38L, and another member of the NDR family, LATS1, at different ages during normal development and disease. STK38L expression in control retinas was unchanged during development. Although mutant mRNA lacked exon 4, the altered transcript showed a slight increase in expression at the 2 older disease time periods (8.3/9.9wks, 11.9/14.1wks) examined when using an exon 6 probe ($0.05 \leq p \leq 0.1$). LATS1 expression was increased at the 3 wk time point in normals, and at the 2 older disease time points (8.3/9.9wks, 11.9/14.1wks).

Immunoblotting demonstrated increased expression of three of four photoreceptor-specific proteins evaluated (S opsin, L/M opsin, RDS peripherin) in lysates from 6.4 and 9.9 wk *erd*-retinas compared to 8wk normal control.

Rod OS renewal in control and mutant retinas was examined by autoradiography at different time points (1, 2, 3 and 4 days) following the intravitreal injection of ^{3}H-leucine or ^{3}H-fucose. As expected, normal rod renewal was by band displacement. In mutant rods, however, a distinct band of radioactivity was not present, and a renewal rate could not be established. Diffuse label was present at all levels of the OS layer, and the labeling pattern was similar in rods and cones. To complement the autoradiographic renewal studies, a series of biochemical experiments were carried out to examine rod opsin biosynthesis and disposal kinetics in control and mutant retinas at 1, 2 and 4 days following the intravitreal injection of ^{3}H-fucose/^{14}C-leucine. Relative incorporation of ^{3}H-fucose/^{14}C-leucine label into control and mutant rod opsin differed between control and mutant animals. In normals, opsin labeling with both precursors increased at post injection days 2 and 4 as the labeled precursor pool in the vitreous was incorporated into newly synthesized protein. In contrast, after the 1 day time point, the mutant retinas demonstrated abnormal kinetics of label incorporation into opsin, and the intensity decreased at 2 and 4 days.

Conclusions

Initially it was believed that caveolin expression in the central nervous system is limited to glial cells. However, more recent studies showed that all three caveolin isoforms are expressed in neurons of the central nervous system (Head and Insel, 2007). Interestingly their expression in neuronal cells has been detected independently without any conclusive evidence for caveolae, even though detergent-resistant, low-density membranes (lipid rafts) can be isolated from these cells. It is now widely accepted that caveolins do exist and function also independent from caveolae. (Head and Insel, 2007). Generally caveolins have two significant functions in neuronal cells. First, they play a role in synaptic maintenance and stabilization. Second, they have a crucial function in regulating intracellular signaling in neurons. (Stern and Mermelstein, 2010).

Despite their inter-species differences, based on recent publications and our studies, caveolin isoforms seem to be inherent components of the vertebrate retinas. Our research group was first to investigate the expression and localization of caveolin isoforms in the human and the primate retina – previously only common laboratory animals (mouse and rat) were used in these studies (Bridges et al., 2001; Kachi et al., 2001; Ueda, 2002). Studies on mammalian retinas suggest, that the different caveolin types occur less frequently than the ones found in our sections from melanoma malignum-affected human eyes, as caveolin-1 was only observed in a few cell types in rodents (Bridges et al., 2001; Kim et al., 2006; Ueda, 2002). Our observations show, that caveolin-1 is evenly distributed in the different layers of not just the lemur, but also of the human retina, but with an obviously higher density in the human as estimated in our semiquantitative analysis (Berta et al., 2007a; Berta et al., 2007b). Regarding caveolin-2, previous studies described this protein to be present only around blood vessels of the retina (Ueda, 2002). Our observations about caveolin-2 distribution in the lemur retina confirm these facts: caveolin-2 was localized mainly around blood vessels. In the human retina caveolin-2 was also localized in several layers: between and including the ONL and the ILM. The estimated densities were markedly higher in the human (Berta et al., 2007a; Berta et al., 2007b). Until now no studies were previously performed on the presence of caveolin-3 in the retina in any species. Traditionally, caveolin-3 was thought to be muscle-specific, however recently it was also detected in

astroglial cells and vegetative ganglions (Kiss et al., 2002). It is also known that the expression of caveolin-3 is intense in the central nervous system in early embryonic stages in the chicken (Shin et al., 2003). Comparing the human and the lemur retina the estimated expression density of caveolin-3 did not show such obvious differences, as seen at caveolin-1 and -2. Still, caveolin-3 was expressed in more layers of the human retina compared to those of the lemur. The distribution of caveolin-3 in the human retina was similar to caveolin-2: immunoreactive labeling was present between and including the ONL and the ILM. In comparison, the lemur retina contained caveolin-3 specific labeling only in the ganglion cell layer and the ILM (Berta et al., 2007a; Berta et al., 2007b). Summarizing these results, the distribution and the quantity of the caveolin isoforms were different in the two species. Although caveolin is a conservative protein, the amount and distribution of caveolin in different tissues may alter across species. We also cannot rule out, that other factors, i.e. during sample preparation or pathological conditions provoked by the choroideal tumor also influence the results.

Parallel to the first morphological findings other study groups also investigated the retinal caveolin content with biochemical methods and confirmed the presence of caveolin-1 in the retina of various species, namely in rod outer segment (ROS) -derived detergent-resistant membranes (DRMs) (Boesze-Battaglia et al., 2002). Later rhodopsin (Seno et al., 2001), rhodopsin kinase (Senin et al., 2004) and c-src (Martin et al., 2005) were also described in ROS DRMs. Since rhodopsin and rhodopsin kinase are important participants in the phototransduction process, while caveolin-1 and c-src are characteristic markers of lipid rafts (Pike, 2003), their presence in the same membrane domains (DRMs) initiated speculations about functional connections between the two systems. Although previous biochemical data suggest the enrichment of caveolin-1 and c-src in the DRM fraction of ROS (Boesze-Battaglia et al., 2002; Martin et al., 2005), their localization using immunocytochemistry is more pronounced in the IS and in the ONL than in the OS (Berta et al., 2007a; Berta et al., 2007b; Elliott et al., 2008; Kachi et al., 2001; Kim et al., 2006). We described a similar distribution of these proteins in photoreceptors of the Syrian hamster with immunocytochemistry. (Berta et al., 2011b).

Caveolin-1 is phosphorylated on tyrosine 14 and it has been proposed that the phosphorylated form of caveolin-1 may play a role in membrane domain internalization processes similar to those observed during OS disc formation (del Pozo et al., 2005).

Yet until now, there have been no publications devoted to phosphorylation of caveolins in retinal photoreceptors. In our studies we described that interestingly phospho-caveolin-1 was almost exclusively localized to photoreceptor outer segments of the hamster, but precise localization was not possible due to the thinness of the photoreceptor OS (Berta et al., 2011b). The large photoreceptors of the *Xenopus laevis* made it possible to more precisely localize this protein. In the *Xenopus laevis* phospho-caveolin-1 is located in the photoreceptor OS rim and the cilia. We also confirmed the presence of phospho-caveolin-1 biochemically in the isolated OS membranes. Based on these findings the idea that caveolin-1 entering the OS is phosphorylated seems reasonable. This phosphorylation event may take place either in the OS or at the IS/OS junction. This latter option seems a little more plausible, since c-src, the kinase that phosphorylates caveolin-1 (Li et al., 1996) is preferentially present in the inner segment (Berta et al., 2011b). In vitro experiments also support this idea (Elliott and Ghalayini, 2008).

The idea of caveolin-1 as a passive or active participant of rhodopsin transport originated from the fact that the localization pattern of rhodopsin, caveolin-1, rhodopsin kinase and c-src during hamster retinal development was very similar. We showed their morphological colocalization using double immunolabeling and confocal microscopy This was also supported by immunoprecipitation: rhodopsin was shown to co-immunoprecipitate with caveolin-1 and c-src not only at different developmental stages, but also in the adult. Our findings were similar using co-immunoprecipitation (Berta et al., 2011b).

The rhodopsin content and distribution of these punctuate structures in the photoreceptor cytoplasm led us to the concept that they represent rhodopsin transport carriers (Deretic, 2006), delivering rhodopsin, a characteristic transmembrane protein, to the outer segment. If so, rhodopsin transport carriers should contain such lipid rafts that scaffold caveolin-1, c-src, ROM-1 and other molecules on the way to the base of the connecting cilium. This is in good agreement with recent finding (Baker et al., 2008), that integral membrane proteins or proteins anchored to the membrane by geranyl-geranylation or palmitoylation move collectively along a default pathway to the outer segment and raises the possibility of caveolin-1 mediating and/or regulating rhodopsin transport mechanism in photoreceptor cells.

The exact function of lipid rafts or caveolin-1 in photoreceptors is still not clear and probably longer discussion has to be expected in the future. Function of lipid rafts in trafficking of membrane proteins or in the regulation of phototransduction are possible options, as lipid rafts have been implicated in many important cellular processes, such as polarized sorting of apical membrane proteins in epithelial cells and signal transduction (Kurzchalia and Parton, 1999). As for the caveolins, caveolin-1 and phospho-caveolin-1 can suppress or inhibit enzyme activity through bounding and releasing enzymes in their scaffolding domain (Schlegel et al., 1998; Swaney et al., 2006). There are several enzymes in phototransduction that are possible candidates to have interactions with caveolin-1 or phospho-caveolin-1, but this issue remains unresolved. Further studies are also needed to specify the exact role of caveolin-1 and its phosphorylated form in the primary photoreceptor processes in the OS. Especially intriguing is the question, why and by what mechanism is caveolin phosphorylated on its way at the IS/OS border. Comparative morphological studies are essential in order to understand the distribution and function of raft-associated proteins in the retina. In our studies, we also examined the expression of caveolins in the normal dog retina, but great differences in comparison to the Syrian hamster were not seen.

In the dog the mutation in STK38L results in the *erd* phenotype in abnormal photoreceptor development, particularly and primarily affecting rods. (Acland and Aguirre, 1987). The early retinal degeneration is unique among other retinal degenerations. The extensive variation in length between rod IS and OS, and the lack of a synchronized developmental plan between adjacent groups of photoreceptors are such characteristics, that until now were not seen in other retinal disorders.

Opsin mislocalization is characteristic of photoreceptor disease, and cell-class specific effects are presumed to reflect the expression of the mutated gene in rods or cones (Beltran et al., 2006; Rohrer et al., 2005). That opsin mislocalization and neurite sprouting is a feature of rods, but not cones would suggest that STK38L is a rod-specific gene, and that the apparent cone abnormalities, e.g. failure of pedicles to fully mature, and cone cell death, are secondary to the rod disease and degeneration.

In *erd* apoptosis detected by TUNEL labeling occurs in terminally differentiated photoreceptors, after retinal development is completed (Acland and Aguirre, 1987). Concurrently with cell death, cell proliferation in the ONL is occurring in mutants. The

existence of this proliferation event was confirmed by labeling with two different cell proliferation markers, PCNA and KI-67. It was ruled out with immunochemistry that these labeled cells would be Müller cells, microglia, retinal progenitor cells. Based on our results, we conclude that the cells labeled with proliferation markers are decisively photoreceptors (Berta et al., 2011a).

Other than the present study, generation of new photoreceptors in a naturally occurring retinal degeneration has not been demonstrated. What is not clear at this time are the signals that commit terminally differentiated photoreceptors to die or divide.

Coincident with the photoreceptor cell death and proliferation phase is a change in the visual cell population. Although both cone types remain, most rod cells (presumably generated after the proliferation event in the ONL) have features similar to cones. Our biochemical analysis and rod OS renewal/ opsin synthesis studies also support this idea (Berta et al., 2011a). Afterwards just like in other retinal degenerations the loss of photoreceptors becomes dominant and leads to the unstoppable damage of the whole retinal structure. Still the proliferation event is exceptionally interesting and may represent a hidden capability of the retina of self renewal or regeneration possibly controlled by the STK38L gene. This might have consequences not only on further basic research in this topic, but also on clinical studies developing new strategies to heal or prevent retinal degenerations.

Retinal degenerations are presently thought to be incurable. Studies about photoreceptor development and degeneration are essential to find such photoreceptor differentiation mechanisms that might be manipulated experimentally in the future to reconstitute and preserve a diseased photoreceptor layer. In our work we described that a new protein, caveolin-1 may have a crucial function in photoreceptor development and that a new gene, STK38L may the control of cell division and morphogenesis in photoreceptors and possibly other retinal neurons.

List of publications

Publications related to the theme of the PhD Thesis:

1. Berta, A.I., Kiss, A.L., Kemeny-Beke, A., Lukats, A., Szabo, A., and Szel, A. (2007a). Different caveolin isoforms in the retina of melanoma malignum affected human eye. Mol Vis *13*, 881-886. IF:2,329
2. Berta, A.I., Kiss, A.L., Lukats, A., Szabo, A., and Szel, A. (2007b). Distribution of caveolin isoforms in the lemur retina. J Vet Sci *8*, 295-297.
3. Berta, A.I., Boesze-Battaglia, K., Genini, S., Goldstein, O., O'Brien, P.J., Szel, A., Acland, G.M., Beltran, W.A., and Aguirre, G.D. (2011a). Photoreceptor Cell Death, Proliferation and Formation of Hybrid Rod/S-Cone Photoreceptors in the Degenerating STK38L Mutant Retina. PLoS One *6*, e24074. IF:4,092
4. Berta, A.I., Boesze-Battaglia, K., Magyar, A., Szel, A., and Kiss, A.L. (2011b). Localization of caveolin-1 and c-src in mature and differentiating photoreceptors: raft proteins co-distribute with rhodopsin during development. J Mol Histol *2011*, 22. IF: 1,484

Publications not related to the theme of the PhD Thesis:

None.

Acknowledgements

I would like thank all my colleagues and friends in the Institute of Human Morphology, especially the members of the Laboratory of Cell- and Molecular Biology; and the co-workers of the Eye Research Lab, School of Veterinary Medicine at the University of Pennsylvania in Philadelphia.

In both labs I received a lot of technical support from Katalin Lőcsey, Margit Kutasi, Svetlana Savina, Jessica Rowling and Lydia Melnyk.

Special thanks to my teachers: Ágoston Szél, Anna L. Kiss, Gustavo Aguirre, Kathleen Boesze-Battaglia, Magyar Attila and Pál Röhlich.