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Autophagy and ER-stress contribute to photoreceptor degeneration in cultured adult porcine retina

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Abbreviations**:** AMD**,** age-related macular degeneration; BSA, bovine serum albumin; CHOP, C/EBP homology protein; CtBP2, C-terminal binding protein 2; ER, endoplasmic reticulum; GRP78/BiP, glucose-regulated protein 78kDa/Binding immunoglobulin protein, INL, inner nuclear layer; IPL, inner plexiform layer; LC3B, microtubule-associated protein light chain 3B; mTOR, mammalian target of rapamycin; ONL, outer nuclear layer; OPL, outer nuclear layer; PBS, phosphate buffered saline; PNA, peanut agglutinin; p62/SQSTM1; nucleoporin p62/sequestosom 1; PSD-95, postsynaptic density protein 95**;** rd1, retinal degeneration 1; RPE, retinal pigment epithelium; UPS, ubiquitin-proteasome system;

Abstract

The aim of this study was to investigate rod and cone photoreceptor degeneration in organotypic cultures of adult porcine retina. Our hypothesis was that the photoreceptors accumulate opsins, which, together with exposure to cyclic dim light illumination, induce autophagy and endoplasmic reticulum stress (ER-stress) to overcome damaging protein overload. For this purpose, retinas were cultured for 48 h and 72 h during which they were illuminated with dim light for 8 h/day; specimens were analyzed by means of immunohistochemistry, western blot and transmission electron microscopy. ER-stress and photoreceptor degeneration was observed in conventionally cultured retinas. The additional stress in the form of dim light illumination for 8 h/day resulted in increased levels of the ER-stress markers GRP78/BiP and CHOP, as well as increased level of active caspase-12. Increased autophagic processes in cone and rod photoreceptors were detected by LC3B-II increases and occurrence of autophagosomes at the ultrastructural level. Illumination also resulted in altered protein expression for autophagy inducers such as p62 and Beclin-1. Moreover, there was a decrease in phosphorylated mammalian target of rapamycin (mTOR), which further indicate an increase of autophagy. Rod and cone photoreceptors in retinas from a diurnal animal that were exposed to dim light illumination *in vitro* displayed autophagy and ER-stress processes. In particular, these processes resulted in decreased protein levels for rhodopsin.

1. Introduction

Rhodopsin and the different cone opsins are light-sensitive proteins in photoreceptor cells that initiate the phototransduction process in rod and cone photoreceptors, respectively. Photoreceptors are neural cells that are vulnerable to degenerative apoptotic processes following environmental and genetic defects, by mechanisms that are similar to other neurodegenerative diseases (Sivak, 2013). A common retinal degeneration, retinitis pigmentosa, is linked to over 40 genes of which most generate mutated rhodopsin proteins (http://www.sph.uth.tmc.edu/retNet). Mutations in the rhodopsin molecule result in the formation and accumulation of misfolded rhodopsin proteins in the endoplasmic reticulum (ER) and recognition by molecular chaperones (Chapple et al., 2003; Sung et al., 2003). When challenged to overcome this overload of misfolded proteins, ER-chaperones and protein folding enzymes activate and subject the abnormal proteins to degradation via the ubiquitin-proteasome system (UPS) reviewed by Shore and colleagues (2011). Initially the cell implements an adaptive response(s) but chronic ER dysfunction and protein accumulation can dramatically alter photoreceptor cell homeostasis and thereby induce ER-stress genes for the chaperone glucose regulated protein78 binding immunoglobulin protein (GRP78/BiP) as well as apoptosis-inducing transcription factor C/EBP-homology protein (CHOP) (Kroeger et al., 2012). Prolonged ER-stress eventually results in apoptotic rod photoreceptor death through caspase-12 activation (Griciuc el al., 2011; Lindholm et al., 2006; Mohlin and Johansson, 2011; Shinde et al., 2012; Yang et al., 2007). However, the death mechanism(s) associated with the degeneration of otherwise healthy cones following the degeneration of rods in retinitis pigmentosa is still yet to be fully understood.

Age-related macular degeneration (AMD) results in visual impairment due to cone photoreceptor degeneration. The etiology involves dysfunctional retinal pigment epithelium (RPE), accumulation of drusen and occasionally choroidal neovascularization, which eventually leads to a physical separation of the retina and the RPE from the underlying choroid. Irrespective of vessel ingrowth, the separation from the RPE/choroid results in complicated retinal degenerative cascades involving photoreceptor apoptosis, aberrant localization and accumulation of rhodopsin, loss of cone photoreceptor opsin, and disassembly of photoreceptor synapses as well as retraction of photoreceptor axon

terminals (Fariss et al., 2000; Johnson et al., 2003; Li et al., 1995; Parmeggiani et al., 2013). Importantly, rod photoreceptors overlaying drusen detach and show aberrant accumulation of rhodopsin (Johnson et al., 2003).

Autophagy has been shown to be an important housekeeping activity in the aging retina (Mitter et al., 2012; Rodriguez-Muela et al., 2013) and increases of lipofuscin granules (Iwasaki et al., 1988).   Recent data implies that autophagy may be a complementary pathway for degradation of misfolded proteins in photoreceptor cells (Kunchithapautham, and Rohrer, 2007a). Autophagy has been implicated to light-induced cellular remodeling/apoptosis in photoreceptor cells (Kunchithapautham and Rohrer, 2007b; Remé et al., 1999) and in retinal ganglion cells in experimental glaucoma models (Park et al., 2012; Rodriguez-Muela et al., 2012). The autophagy housekeeping-activity is involved in the disposal of cellular organelles and proteins through induction of autophagosomes and transport to lysosomes (Levine and Kroemer, 2008). The natural occurrence of autophagy in photoreceptors might prevent degeneration, but during starvation, oxidative stress and protein accumulation autophagy increases and cells start to degenerate. The mammalian target of rapamycin (mTOR) controls a balance between protein synthesis and degradation, and the dysregulation of mTOR has been linked to neurodegenerative disorders (Reiling and Sabatini, 2006). Regulation of mTOR (Sarkar, 2013) and the autophagy marker microtubule-associated protein light chain 3B (LC3B)(Tanida et al., 2005) has been shown to be important during starvation. Autophagy related genes convert LC3B into LC3B-I directly after synthesis. During autophagic conditions LC3B-I is lipidated to LC3B-II, which is incorporated into the membranes of autophagosomes. LC3B-II is consequently closely associated with the quantity of autophagosomes (Kabeya et al., 2000). Other regulators of autophagy involve the ubiquitin- and LC3B-binding protein p62 (Moscat et al., 2007) and Beclin-1, which is a regulator for initial autophagosome formation (Pickford et al., 2008).

Other proteins that are afflicted during photoreceptor degeneration are localized to the synaptic terminals in the outer plexiform layer, the site where synapses with retinal interneurons are established. In particular, the ribeye or C-terminal binding protein 2 (CtBP2) in the synaptic ribbon seems to be vulnerable during a variety of injuries (Specht

et al., 2007; tom Dieck et al., 2005). Under normal conditions ribeye gives the ribbon an arciformed shape that is lost during degeneration and electron microscopy shows that the ribbon may detach and float in the synaptic terminal (tom Dieck et al., 2005). Another synapse-related protein that appears to be afflicted the postsynaptic protein 95 (PSD-95), a scaffold protein in rod and cone synaptic terminals (Blackmon et al., 2000; Takada et al., 2008). Once the photoreceptors start to follow a degenerative pathway, synaptic disassembly is initiated and appropriate communication with interneurons is impaired.

This current study was undertaken to explore whether autophagy and ER-stress participates in the degenerative process of photoreceptors. In particular, we have chosen to investigate the autophagy and ER-stress that arises following accumulation of naive rhodopsin and so-called macroautophagy by studying illuminated explants of porcine retinas. As pigs are diurnal animals with a central cone-enriched area similar to the macula in the human retina (Hendrickson and Hicks, 2002), examinations of retinal explants makes is possible to study subsets of photoreceptor populations in tissue segments with all subsets of retinal cells and their synaptic connectivity patterns maintained.

2. Results

2.1. Morphological observations

All cultured specimens showed preserved retinal architecture with separated nuclear and synaptic (plexiform) layers. Preserved tissue lamination was observed during the entire culture period, although longer culture periods resulted in tissue vacuoles in the inner- and outer nuclear layer (INL and ONL), which are separated by the outer plexiform layer (OPL) (Fig. S1 A-C). In cultured specimens the ONL appeared less compact and tissue vacuole development may depend on Müller cells sprouting into places of the dying cells (Garcia and Vecino, 2003), Müller cell hypertrophy and/or phagocytosis of apoptotic cell debris by activated microglial cells (Engelsberg et al., 2004; Harada et al., 2002).

Photoreceptor cell degeneration in general was monitored during the first 72 h *in vitro* by using TUNEL as well as immunolabeling for caspase-12 and cleaved caspase-3. Essentially, similar labeling pattern of degenerating photoreceptors was observed in conventional and illuminated cultures, therefore images of the latter are shown. After 48 h, photoreceptor somata exhibiting TUNEL were mostly found in parts of ONL that houses rod photoreceptors (Fig. 1 B and Fig. S2 B); in case of longer time-points (Fig. 1 C) TUNEL was also evident in the outermost part of the ONL where cone photoreceptor nuclei reside.

Caspase-12 immunoreactivity was observed in not only in cultured retinas but also in the normal controls. Caspase-12 immunoreactivity was found in the outer part of the ONL (Fig. 1 A-C and Fig. S2 A-B) and single cells displayed both TUNEL and caspase-12 immunoreactivity (Fig. S2 B). Cleaved caspase-3 was only observed in single cells in the INL and ONL of cultured retinas (Fig. 1 F and Fig. S2 F and G), and no cells displayed cleaved caspase-3 immunoreactivity in normal controls (Fig. 1 F and Fig S2 F, G).

2.2. Quantification of photoreceptor degeneration in illuminated cultures

Photoreceptor degeneration occurred both in illuminated (Fig. 1 B-C) and conventional cultured retinas (Fig. S2 B). Significant cellular alterations were only detected in illuminated cultures and are the focus of the current study; hence experimentation and

quantifications of degenerative events carried out on conventional cultures are mostly given as supplementary (S) data.

There were a significantly higher number of TUNEL-positive cells in illuminated cultures after 72 h (1627 ± 337 cells/mm^2; n = 3) compared to the normal controls (Fig. 1 A) (6.70 ± 2.36 cells/mm^2; n = 3). In addition to TUNEL, caspase-12 immunoreactivity was observed in somata close to the outer aspect of the ONL indicating expression in cone photoreceptors (Fig. 1 A-C). Unexpectedly, the number of caspase-12 immunoreactive photoreceptors in the ONL declined with time *in vitro*. Compared with normal controls (670 ± 148 caspase-12 labeled cells/mm^2; n = 3), there was a reduction of the caspase-12 labeled cells after 48 h (203 ± 18.7 cells/mm^2; n = 3) and a further reduction of caspase-12 labeled cells (Fig. 1 D) after 72 h (133 ± 23.0 cells/mm^2; n = 3) in illuminated retinas.

The antibody against caspase-12 is supposed to detect both the pro- and the active cleaved form of caspase-12 (Fig. 1 E), and western blot analysis showed pro-caspase-12 expression in illuminated cultures. However, the cleaved (active) form of caspase-12 increased significantly (41.0 ± 5.42 o.d. units; n = 3) after 72 h illuminated cultures compared with normal controls (14.6 ± 2.08 o.d. units; n = 3) (Fig. 1 E).

Immunoreactivity for cleaved caspase-3, an executioner of apoptosis, was evident in single cells after 72 h in illuminated cultures (Fig. 1 F). Labeled cells included photoreceptors, horizontal cells and ganglion cells; however, only single cells were caspase-3 active and it was not possible to gain any statistically reliable cell counts. Western blot showed a small but insignificant increase of cleaved caspase-3 expression (Fig. 1 G).

Positive controls of experimentally induced activation of caspase-3 and caspase-12 were achieved by staurosporine and tunicamycin treatment, respectively, and appropriate protein expressions were observed with western blots (Fig. S2 D and Fig. S2 H).

2.3. ER-stress in illuminated cultures

Retinal culture usually leads to a rapid destruction of the photoreceptor outer segments (OS). The disrupted transport of rhodopsin to this OS-compartment results in rhodopsin accumulation in the ER and plasma membranes; such outcomes lead to ER-stress and

degeneration of photoreceptors (Mohlin and Johansson, 2011). Rhodopsin immunoreactivity was not easily observed in the ONL of illuminated cultures, but was mainly restricted to the outer and inner segments. Rhodopsin immunoreactivity appeared to decrease with time in illuminated cultures (Fig. 2 B-C), an observation that was confirmed by western blot (Fig. 2 D). After 48 h there was a decrease (52.7 ± 12.1 o.d. units; $n = 3$) of the rhodopsin expression compared with the normal control (141 ± 13.9 o.d. units; $n = 3$). The 72 h illuminated cultures showed even more reduced rhodopsin expression (31.8 ± 20.2 o.d. units; $n = 3$) compared with normal controls. Four different rhodopsin isomers can normally be observed with this antibody (Fig. S3 C), but a temporal down-regulation of the different rhodopsin isomers was noted in illuminated cultures (Fig. 2 D). During western blot investigations it was difficult to separate the trimer and the tetramer rhodopsin subunits (see Fig. S3) and/or due to the loss of minor rhodopsin subunits (Fig. 2 D), western blot measurements were only made on the major rhodopsin monomer. Conventional culture did not induce this temporal downregulation of rhodopsin in the ONL (Fig. S3 B and Fig. S3 C).

Expression pattern of red/green opsin-immunoreactivity was altered in cone photoreceptors of cultured specimens. In normal controls, red/green opsin immunoreactivity was distributed to the outer segments (Fig. 3 A). With increasing time *in vitro,* individual cones of illuminated cultures showed an erroneous localization of red/green opsin immunoreactivity from the outer segments to the axon terminals (arrowheads in Fig. 3 B-C). Similar erroneous localization of red/green opsin immunoreactivity photoreceptor red/green opsin was also observed in cones of conventional cultures (arrowheads in Fig. S4 B-C).

The fidelity of rhodopsin folding is monitored by chaperones and sustained ER-stress results in activation of different ER-stress genes, of which GRP78/BiP and CHOP are associated with retinal degenerations *in vitro* (Mohlin and Johansson, 2011) and *in vivo* (Kroeger et al., 2012). Significant temporal increases of GRP78/BiP (Fig. 4 A) and CHOP (Fig. 4 B) proteins were only observed in illuminated cultures suggesting that the retina is under ER-stress. The expression of the chaperon GRP78/BiP in illuminated

cultures increased after 48 h (29.3 ± 2.4 o.d. units; n = 3) compared with the normal controls (15.6 ± 2.4 o.d. units; n = 3), and after 72 h the increase was still significantly more (26.5 ± 2.6 o.d. units; n = 3) than the control. In illuminated cultures for 72 h resulted in increased expression of CHOP (39.2 ± 4.7 o.d. units; n = 3) compared to the normal control (18.9 ± 3.7 o.d. units; n = 3). Conventional culture in dark was not correlated with changed ER-stress markers (i.e. CHOP or GRP78/BiP protein expressions; Fig. S5), implying that illumination induced significant ER-stress in the porcine retinal tissue.

2.4. Retinal culture affects synaptic integrity

Structural changes of synapses were investigated at time points as above with PSD-95 (Fig. 3) and CtBP 2/ribeye (Fig. 5) immunoreactivity as well as by transmission electron microscopy (TEM) (Figs. 7 and S6). Both rod spherules and cone pedicles are known to express PSD-95 (Blackmon et al., 2000) and CtBP 2/ribeye (Sterling and Matthews, 2005). PSD-95 immunolabeling was apparent in the OPL of normal controls where it occasionally (Fig. 3 A) coincided with the opsin labeling in cone pedicles. In illuminated cultures, there was a loss of PSD-95 immunoreactive pedicles (arrows in Fig. 3 B-C) and a concomitant decline of PSD-95 protein levels (Fig. 3 D) showed by western blot. In addition, the PSD-95 expression in illuminated cultures appeared to change localization, and was found scattered in the ONL (asterisks in Fig. 3 B-C). In the 48 h and 72 h illuminated cultures (Fig. 3 D) there was a significant reduction of the PSD-95 expression (84.5 ± 6.19 and 167 ± 28.0, respectively, o.d. units; n = 3) compared to the normal controls (167 ± 28.0 o.d. units; n = 3). Similar alterations of PSD-95 immunoreactivity pattern were also observed in conventional cultures (Fig. S4 A-C). Western blot also showed a decreased PSD-95 protein expression after 48 h and 72 h of conventional cultures (Fig. S4 D).

Diminished immunoreactivity (Fig. 5 B-C) and significantly reduced protein levels (Fig. 5 E) were also found for the synaptic protein CtBP 2/ribeye in illuminated cultures; however, no such changes were observed in the conventional cultures (data not shown). CtBP 2/ribeye labeling was seen to be in a horseshoe shaped manner (Fig. 5A and insert)

close to and between the PNA labeling in normal controls. The horseshoe shaped CtBP 2/ribeye labeling (insert Fig. 5 A) disappeared in illuminated cultures after 48 h (Fig. 5 B and C). In addition, there were obvious regions devoid of ribeye (Fig. 5 B and C) in the OPL of in illuminated cultures. CtBP 2/ribeye protein expression showed a concomitant decline in the illuminated cultures (Fig. 5 E). After 48 h there was a decrease in the CtBP 2/ribeye expression (16.8 ± 1.16 o.d. units; n = 3) compared to normal controls (30.9 ± 1.41 o.d. units; n =3). After 72 h the CtBP 2/ribeye protein level was reduced even more (11.6 ± 3.16 o.d. units; n = 3). Both PSD-95 (Fig. 3 A) and CtBP 2/ribeye (Fig. 5 A) were expressed in the cone pedicles, here indicated by red/green opsin and peanut agglutinin (PNA) labeling, respectively.

The loss of the synaptic proteins was paralleled with a declined density of PNA-labeled cone pedicles in illuminated cultures (Fig. 5 B-D). After 48 h there was a loss of the PNA labeled pedicles (9.85 ± 3.49 labeled PNA pedicles/200 μm; n = 2) and after 72 h there was an increased reduction of the labeled cone pedicles (6.07 ± 0.93 labeled PNA pedicles/200 μm; n =3) compared to normal control retinas (20.3 ± 0.93 labeled PNA pedicles/200 μm; n = 3) (Fig. 5 D). Remaining cone pedicles retained their position in the OPL, suggesting minimal sprouting of cone terminals during injury.

2.5. *Cyclic illumination during culture results in autophagy and altered protein expression*

Pigs are diurnal and in an attempt to mimic this situation were retinas cultured for 48 h and 72 h exposed to dim illumination for about eight hours during daytime. Photoreceptors are sensitive to high oxygen tension and in combination with light-induced oxidative stress, autophagy is supposed to increase (Wenzel et al., 2005). To assess the effect of dim light on cultured retinas, LC3B, p62, Beclin-1 together with mTOR and p-mTOR were used to monitor autophagic activity (Fig. 6). Autophagy was further examined using electron microscopy by observing ultrastructural changes (Fig. 7).

Immunolabeling and western blotting indicated a ubiquitous expression of LC3B-I in normal controls, particularly in horizontal cells (arrows in Fig. 6 A) and slightly in the

10

photoreceptor layer, ONL. In illuminated cultures, western blot revealed significant increases of LC3B-II (Fig. 6 D) relative to LC3B-I. LC3B-II is phosphatidylethanolamine-conjugated LC3B-I and closely correlated with autophagosome formation, and commonly used as a marker to detect autophagic activity (Kabeya et al., 2000; Komatsu and Ichimura, 2010). The 48 h (10.5 ± 1.1 o.d. units; n = 3) and the 72 h (8.9 ± 2.1 o.d. units; n = 3) illuminated cultures were accompanied with an increase in the LC3B-II expression compared to the normal control retinas (2.5 ± 0.75 o.d. units; n = 3), providing biochemical verification supporting the occurrence of autophagosomes in the retina. The LC3B immunolabeling increased after illumination and was confined to both cone (see insert in Fig. 6 B, double labeled with PNA (red)) and rod photoreceptor somata and to the inner segments (arrows in Fig. 6 B-C). Conventional culture of retinas in dark did not induce conversion of LC3B-I into LC3B-II (Fig. S5).

To further assess if the autophagic process was potentiated by illumination, we examined the temporal expression of the ubiquitin binding protein p62 (Fig. 6 E), the autophagosome initiator Beclin 1 (Fig. 6 F) together with the regulator of protein synthesis and degeneration mTOR and p-mTOR (Fig. 6 G-H). The expression of p62 increased (Fig. 6 E) after 48 h (68.2 ± 7.1 o.d. units; n = 3) and after 72 h (77.7 ± 9.5 o.d. units; n = 3) in illuminated cultures compared with normal control retinas (34.1 ± 1.9 o.d. units; n = 3). Beclin-1 expression decreased significantly (Fig. 6 F) only after 48 h of cyclic illumination (29.8 ± 4.22 o.d. units; n = 3) compared to the normal control (47.9 ± 0.62 o.d. units; n = 3). Western blot indicated a ubiquitous expression of mTOR in the illuminated cultures (Fig. 6 G, n = 3), while p-mTOR (Fig. 6 H) decreased in the 48 h illuminated cultures (27.5 ± 3.81 o.d. units; n = 3) compared to normal controls (46.4 ± 3.93 o.d. units; n = 3). In the 72 h illuminated cultures, p-mTOR decreased even further (23.7 ± 5.41 o.d. units; n = 3) compared with the normal control.

To test if autophagy and ER-stress had a functional consequence on protein expression, rhodopsin and ribeye protein levels were measured illuminated cultures. Concomitant to increased autophagy and ER-stress described above, western blot revealed significant decrease of both rhodopsin (Fig. 2 D) and ribeye (Fig. 5 A-E)

protein. Our hypothesis predicts that rhodopsin accumulation results in autophagy and decreased levels of phosphorylated mTOR, which is known to regulate protein synthesis (Reiling and Sabatini, 2006) and inhibit autophagy (Ravikumar et al., 2004). Cyclic illumination to retinal cultures was associated with a significant decline of phosphorylated mTOR (Fig. 6 G, H), a feature that occurred simultaneously with the increase of autophagic activity.

Finally, electron microscopy revealed the presence of smooth endoplasmic reticulum (sER) cisternae in the synaptic terminals of rods and cones. Transmission electron microscopy of all cultured specimens showed that rod spherules and cone pedicles remained in the OPL and formed synaptic complexes *per se* (Fig. 7 see also Fig. S6). Compared to normal controls (Fig. S6 A-C), there was an abundance of sER in cone pedicles (p) (Fig. 7 B, C, E) and rod spherules (s) (Fig. 7 D, F) of both illuminated and conventional cultures (Fig. S6 D-F). Double-membranous autophagosomes (arrows in Fig. 7 C, F) as well as electron-dense lysosomes (arrow in Fig. 7 E) in the synaptic terminals were only detected in illuminated specimens and not in retina cultured during normal conventional conditions in darkness. In addition, TEM also revealed the presence of numerous electron-dense cellular profiles, probably horizontal cells (h) as judged by their post-synaptic position (Fig. 7 D), supporting the observation of LC3B-II expression in horizontal cells. Some spherules were located in close vicinity to the cell soma, indicating retraction of the axon terminal (Fig. 7 F). The typical rod triad with a rod spherule and two post-synaptic horizontal cell processes and one bipolar cell process is shown in figure S6 B.

3. Discussion

The current study describes developmental retinopathy in adult porcine retinas shortly after explantation. During the experimental process the retina is detached from the pigmented retinal epithelium, which results in accelerated photoreceptor death that may likely occur in rods and cones. TUNEL was mostly observed in the rod subpopulation, but a temporal increase of TUNEL was noted in the cone subpopulation. Cones exhibited caspase-12 immunoreactivity and later increases of TUNEL may suggest that the cones are more resistant to stress. Surprisingly, the density of caspase-12 immunoreactive photoreceptors was lower in cultured specimens compared to normal controls. One explanation may be that the shift from pro-active to active form of caspase-12 is only distinguished on western blot and not by immunohistochemistry.

The initial mechanism for retinopathy may be an accumulation of rhodopsin and opsin in the ER of rods and cones, respectively, and the photoreceptor cells respond to protein overload with ER-stress and autophagy. Dim light illumination for 8h/day was enough to elicit the significant regulations of autophagy markers, down-regulations of rhodopsin and ribeye protein expression levels. Dim light illumination was also required to induce morphological signs of autophagy at the TEM level.

3.1. Opsin accumulation and cyclic illumination promote ER-stress

Conventional retinal culture with accompanied rhodopsin and opsin accumulations in rods and cones, respectively, led to induction of ER-stress and caspase-12 activation. Increases of the chaperone were only observed upon exposing the cultured specimens to cyclic illumination of dim light during the culture period. Sustained ER-stress resulted in a significant upregulation of CHOP, a transcription factor that inhibits anti-apoptotic BCL-2 (McCullough et al., 2001), after three days of culture and cyclic dim light exposure. Induction of GRP78/BiP and CHOP is known to occur as a consequence of misfolded rhodopsin in the rat P23H rhodopsin mutation (Gorbatyuk et al., 2010) as well erroneous localized rhodopsin in rods in cultured rat retinas (Mohlin and Johansson, 2011). ER-stress seems mainly to be associated with erroneous localized rhodopsin in

rods, but various degeneration models suggest ER-stress via misfolded cone opsins as well (Nakanishi et al., 2013; Zhang et al., 2011).

ER-stress has also been reported from studies on the *rd1* mouse, in which photoreceptor degeneration is caused by a mutation in a phototransduction enzyme (Yang et al., 2007). In the current study, the erroneous localization of red/green opsin immunolabeling and caspase-12 immunolabeling were pronounced in some cones, suggesting that ER-stress can be induced in these cells as well. Because of the separation from the RPE in the culture paradigm, it is hypothesized that opsin shuttling to cone photoreceptor outer segments is impaired and starts to accumulate and induce ER-stress. If this means that cones separated from the underlying choroid by drusen in AMD retinas also degenerate by ER-stress remains to be established.

In a series of studies by Johnson and colleagues (Johnsson et al., 2003; 2005) on human AMD retinas, it has been shown that photoreceptors directly overlying drusen depict cellular and molecular changes. In particular, rhodopsin becomes mislocalized and cone opsin is absent although their terminals appear normal. The synaptic proteins and synapse ultrastructure overlying drusen is altered, and synaptic protein disorganization is also found lateral to the drusen border. The loss of cone-specific proteins in human retinas with rhodopsin-mutant *retinitis pigmentosa* is also known John et al., 2000). Expression of stress-response protein apolipoprotein E increases in cones and rods overlying and flanking drusen. The current study described loss of PNA-labeled cone terminals, decreased protein levels of PSD-95 and CtBP 2/ribeye, but TEM data indicated that cone terminals retained their position in the OPL. If these findings correspond to a decreased expression of specific synaptic proteins (John et al., 2000) and not retraction of synaptic terminals still remains to be established. In comparison, the AMD data and the current study most likely represent a degenerative process at different time scales: our data may represent the initial events whereas stress-response in diverse proteins may be a later event. Synaptic disassembly appears to arise early and is sustained for a long period (Johnson et al., 2005).

Caspase-3-mediated photoreceptor degenerations have been observed previously in cultured rodent retinas (Mohlin and Johansson, 2011; Englund-Johansson et al., 2010),

which possess a rod-dominated retina with a small subpopulation of cones. Although insignificant, we also observed increased protein levels of cleaved caspase-3 in homogenates of illuminated cultured specimens. Our positive control showed fidelity of the cleaved capsase-3 antibody, but only single cleaved caspase-3 immunolabeled cells were observed in the different retinal layers in each section and statistical analysis was not possible. Conventional cultured retinas exhibited similar expression pattern of cleaved caspase-3, indicating that this apoptotic pathway does not contribute to photoreceptor apoptosis in the porcine retina following culture and low-intensity illumination. Even though only periodic low-intensity illumination was tested in the current study, it is known that different apoptotic pathways can be induced depending on light intensity and duration in rodent models (Contin et al., 2013; Hao et al., 2002; Perche et al., 2007).

3.2.   Cyclic illumination causes additional stress and induces autophagy

In addition to ER-stress as discussed above, autophagic processes also had an impact on the photoreceptor degeneration after cyclic light exposure during culturing of retinal explants. Autophagy is a naturally occurring process but dysregulation may contribute to not only neurodegeneration but also to photoreceptor degeneration. As shown in the current study, phosphorylated mTOR was down regulated probably to increase autophagy, allowing the photoreceptors to cope with increasing protein accumulation. Alterations of mTOR signaling in the retina have been shown in mouse models of *retinitis pigmentosa* (Punzo et al., 2009), most likely stemming from the starvation of cones. Increased autophagy as a consequence of down-regulated mTOR activity has been suggested in models of Huntington disease (Ravikumar et al., 2004) and taupathies (Caccamo et al., 2013). The etiology of Huntington and taupathies involves protein accumulation in the nervous system. However, TOR-independent routes of autophagy exist (Lipinski et al., 2010).

Autophagy involves autophagosome formation and the enclosure of injured cell organelles and proteins, which are ultimately transported and destroyed in lysosomes (Komatsu and Ichimura, 2010). Our TEM data showed an abundance of ER in the

synaptic terminals as well as single autophagosome enclosed structures after light exposure. Furthermore, it was also possible to observe lysosomes in the synaptic and photoreceptor terminals. This was paralleled by increases of LC3B-II and p62 protein level, both closely associated with autophagy. Increase of p62 protein levels has been found in the aging retina (Rodrigues-Muela et al., 2013) and is known to interact with LC3B-I and ubiquitinated proteins, perhaps acting as an autophagy receptor (Moscat et al., 2007).

Beclin-1 is a known regulator of initial autophagosome formation. Alteration of Beclin 1 protein levels appears to be associated with altered LC3B-II levels under retinal ischemia and starvation (Russo et al., 2011). In the current study, Beclin 1 protein levels declined after 48 h in illuminated cultures whereas LC3B-II increased. Other studies on photoreceptor cells show that deletion/inhibition of the Beclin 1 gene is associated with increases of light-induced retinal damages (Chen et al., 2013) and oxidative stress (Kunchithapautham and Rohrer, 2007b). Reduced Beclin-1 expression is also correlated with age-related autophagosome formation in the aging mouse retina (Rodrigues-Muela et al., 2013) and amyloid accumulation early in Alzheimer's disease Pickford et al., 2008). However, in experimental glaucoma, retinal ganglion cells show increases of Beclin 1 and LC3B-II protein levels with increased autophagy (Park et al., 2012). The reason for reduced Beclin-1 and decreased autophagosome formation in the cultured retinas seems as a contradiction to other indications of increased autophagy. If this means that two separate autophagic pathways might be active, as suggested for the aging retina (Rodrigues-Muela et al., 2013), has to be determined.

3.3.   *ER-stress and autophagy collectively reduce protein expressions*

Autophagy and ER-stress are two routes by which photoreceptor cells can degrade misfolded proteins from the ER and overcome protein accumulation. Low intensity light-induced stress only engages autophagy and ER-stress, which results in a down-regulation of rhodopsin protein levels. Autophagic degradation of rhodopsin in altered light environment has been described in rod inner and outer segments (Remé et al., 1999). In the current study, lysosomes were evident in cone and rod terminals as well. The

induction of autophagy in the porcine retina following illumination is further substantiated by the increased expression of LC3B-II in cones and perhaps also rods.

The ER-stress induced transcription factor CHOP is known to regulate rhodopsin protein expression via an ER-stress inducible microRNA (Behrman et al., 2011), and may be accounted for the down-regulation of rhodopsin described in the current study. Rhodopsin is here considered to be the main trigger for ER-stress, which in turn acts as a feedback process to decrease overload of this protein. Due to the lack of appropriate and commercial antibodies it could not be established if red/green opsin protein level is affected as well.

Another affected protein by autophagy and ER-stress is the synaptic protein ribeye, which is a structural protein in the synaptic ribbons of cone and rod synaptic terminals (tom Dieck et al., 2005). In the current study, the synaptic protein ribeye appeared altered at the ultrastructural level and subsequently, concomitant and temporal decreases of the ribeye protein level was evident. Other models of retinal degeneration show that ribeye alteration may most likely be associated to impaired synaptic transmission. In addition to its suggested role in initial autophagosome formation, Beclin 1 seems to be important for synaptic integrity in Alzheimer's (Pickford et al., 2008). The rapid and sustained disassembly of synaptic proteins described in this and other studies may be to uncouple dying photoreceptors from interneurons and thereby stop dysfunctional signals, however, with impaired vision.

3.4.   Conclusion

Under the stress situations *in vitro* together with protein accumulation and cyclic illumination, two degenerative pathways were found to be activated: ER-stress and autophagy. Judging from the current data both rods and cones showed rhodopsin and opsin accumumlation, respectively, and were affected with an apoptotic end-stage. As the photoreceptors degenerated there was a parallel disassembly of synaptic ribbons in pedicles and spherules, particularly after activation of autophagy through exposure to dim light. Rhodopsin accumulation in the ER was hypothesized as a trigger of ER-stress, and

by using dim light as an additional stressor, the amounts of rhodopsin was significantly reduced.

It is suggested that cultured porcine retina may be an attractive model to study rod and, in paticular, cone photoreceptor degenerations in intact tissue. Eventhough porcine retinas have similarities to the human retina, some limitiations have to be adressed. First, porcine tissue requires antigen retrivial and occasionally longer incubation times for succesfull antibody recognition in the fixed tissue; hence antibody specificity has to be carefully checked. In this study, we checked fidelities of caspase-12 and cleaved caspase-3 immoreactivities in in homogenates of stimulated cells of other origin. Second, western blots were done on tissue homogenate and as immunolabeling failed for some antibodies, it was not possible to identify the cellular origen for some proteins.

4. Experimental procedures

4.1. Tissue preparation and culture of porcine retinas

The local Ethics Committee of Human Experimentation approved all experimental procedures. Adult porcine eyes were collected from a local abattoir and transported to the laboratory in cold CO_2-independent media (Gibco, Life Technologies, Carlsbad, CA). After a rapid spraying with ethanol the cornea, lens and vitreous body were removed. The neural retina was gently detached from the pigmented epithelium and sections of about 10 mm^2 from cone-enriched central areas (Hendrickson and Hicks, 2002) were punched out. Retinal specimens were divided into four equal sections and explanted onto a Millicell®-PCF 0.4 μm culture plate insert (Millipore, Bedford, MA, USA) with the vitreal side oriented upwards. One or two retinal sections were explanted onto the same insert and placed into multiwell culture dish to which 1.0-1.2 ml of medium was added, covering the explants with a moist film. Culture medium was composed of Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; Gibco) supplemented with 5 % B27-supplement (Gibco), 1 % penicillin/streptomycin and 2 mM glutamine (Sigma-Aldrich, St. Louis, MO).

In cultured control groups, retinas were conventionally cultured (CC) in the dark for 12 h, 24 h, 48 h and 72 h at 37°C with 95 % humidity and 5 % CO_2. For experiments, retinas were cultured as above but at light/dark cycle for 48 h and 72 h (illuminated cultures). The light illumination consisted of 8 h illumination of warm white light with an illuminance of 80 lux *i.e.* dim light (Hagner Screenmaster digital luxmeter, Solna, Sweden).

For comparison, normal and non-cultured control retinas were dissected as above, placed onto inserts, but not cultured, before fixation. All specimens were immersion fixed in 4 % paraformaldehyde (Apoteket, Gothenburg, Sweden) in phosphate buffered saline (PBS, Medicago, Uppsala, Sweden) over night at 4°C. After several rinses with PBS, the specimens were cryoprotected (25 % sucrose, Sigma) in PBS. The tissue samples were embedded in cryomount[TM] (HistoLab, Gothenburg, Sweden) and cryosectioned at 12 μm and stored at -20°C.

4.2. Immunohistochemistry

For immunolabeling, sections were rinsed in PBS and incubated for 15 min in 1 % bovine serum albumin (BSA; Sigma) in PBS with or without 0.25 % Triton X-100 (Sigma). For antigen retrieval, some sections were microwave irradiated at 370 W for approximately four minutes in 0.01 M citrate buffer (pH 6) (Sigma), modified from (Negoescu et al., 1997). After irradiation, slides were quickly immersed in cold PBS following blocking with 20 % goat serum dissolved in PBS with 1 % BSA for 30 min at room temperature, or directly labeled by diverse primary antibodies.

Apoptosis was estimated by using rabbit polyclonal antibodies against cleaved caspase-3 (Asp175) (1:500; Cell Signaling, Beverly, MA) and caspase-12 (1:2000; Abcam, Cambridge, UK). Autophagy was assessed in retinal explants by using an antibody against (LC3B, 1:1000; Cell Signaling). The antibody against LC3B identifies LC3B-I and the autophagy vesicle-specific LC3B-II proteins. Photoreceptors were identified using a rabbit polyclonal antibody specific for red/green opsin in cone photoreceptors (1:1000; Millipore). A mouse monoclonal antibody was used to identify rod rhodopsin in rod photoreceptors (1:2000; Millipore). According to the manufacturer's information, the rhodopsin antibody detects the four different subunits of the rhodopsin protein. A mouse monoclonal antibody against PSD-95 (1:1000; Millipore) was used to identify presynaptic photoreceptor terminals. Synaptic ribbon proteins were identified by using an antibody against CtBP 2/ribeye (1:1000; Synaptic Systems, Göttingen, Germany). Rhodamine-conjugated peanut agglutinin (PNA) (1:1000; Vector Laboratories, Burlingame, USA) was used for labeling cone photoreceptor synaptic terminals and cone outer segments.

For the immunohistochemical staining, the primary antibodies were either applied overnight at 4°C or, for double-labeling experiments, applied for three hours at room temperature. The primary antibodies were detected by using appropriate FITC-, Texas Red-conjugated donkey/goat anti-mouse IgG Fab/ or donkey/goat anti-rabbit IgG Fab (1:500; Jackson Laboratories, West Grove, PA). All secondary antibodies were applied for 45 min at room temperature. The immunolabeled sections were mounted using Vectashield mounting medium (Vector Laboratories) with and without 4′,6-diamidine-2-phenylindole-dihydrochloride (DAPI). Both the primary and the secondary antibodies

were diluted in PBS containing 1 % BSA. Omission of the primary antibody served as negative control in all experiments.

4.3. Western blot

Retinal protein extraction was performed in buffer containing 100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 0.1 % sodium deoxycholate, 2 mM Na_3VO_4 and 100 mM NaF (all purchased from Sigma). One protease inhibitor cocktail mini tablet (Roche Diagnostic, Mannheim, Germany) per 10 mL of extraction buffer was used. The lysed retinas were centrifuged at 12000 x g, 11300 rpm at 4°C for 20 min. The supernatant was recovered and the pellet was resuspended in buffer, recentrifuged and the two supernatants were combined. Protein concentration was determined by using Pierce® BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

Equal amounts of proteins were separated onto 10 %, 18 % Novex Tris-glycine or onto 4-12 % Novex Bis-Tris pre-cast gels (Life Technologies, Paisley, UK) and transferred onto a Hybond^{TM} P+ membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated with a rabbit polyclonal antibody against the apoptosis executioner cleaved caspase-3 (1:500) and caspase-12 (1:2000). Rod rhodopsin was investigated using anti-rhodopsin (1:100000). Synapse protein expression was assessed using anti-PSD-95 (1:1000) and anti-CtBP 2/ribeye (1:1000). ER-stress markers used were a mouse monoclonal anti-CHOP (1:1000; Cell Signaling) and a rabbit polyclonal anti-GRP78/BiP (1:1000; Abcam). Autophagy was estimated by using antibodies (all purchased from Cell Signaling) against Beclin-1 (1:1000), LC3B (1:1000), anti-mTOR (1:1000), anti-p-mTOR (Ser2448) (1:1000) and anti-sequestosome 1, (SQSTM1/p62) (1:1000). Antibodies were visualized with horseradish peroxidase conjugated secondary goat anti-rabbit or a rabbit anti-mouse antibody (1:5000-1:20000; Dako, Glostrup, Denmark). All membranes were washed, stripped and reincubated with a loading control primary antibody against beta-actin (1:5000-1:20000; Abcam). Detection was performed with ECL Plus Western Blotting Detection System (Amersham).

4.4. Transmission electron microscopy

Specimens were fixed with 2 % paraformaldehyde (Taab Laboratory, Berkshire, UK) and 2 % glutaraldehyde (Agar Scientific, Stansted, Essex, UK) in 0.1 M PBS, pH 7.4, for 2 h and post-fixed in 1 % osmium tetroxide (Agar Scientific) for 1 h. The specimens were embedded in Epon resin (Sigma), and semi-thin sections were obtained and examined. Ultrathin sections were taken from selected areas, which were counterstained with uranyl acetate (Ted Pella, Inc, Reading, CA) and lead nitrate (BDH, Dubai, UAE) and examined using a JEOL 1230 transmission electron microscope (Jeol, Tokyo, Japan).

4.5. TUNEL assay

A commercial terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche) system with fluorescein-conjugated dUTP was used on fixed crysectioned retinas according to the manufacturer's instructions. To improve the sensitivity of the TUNEL labeling, sections were microwave irradiated in citrate buffer (pH 6) for approximately four minutes at 370 W, followed by rapid cooling in cold PBS at room temperature, modified from[58]. Blocking with 20 % goat serum diluted in PBS containing 1 % BSA for thirty minutes at room temperature inhibited unspecific binding. TUNEL labeled sections were thereafter immunolabeled for caspase-12. These sections were immersed in PBS where after the coverslips were gently removed and immune stained for caspase-12.

4.6. Microscopy and data analysis

Sections were examined using an epifluorescence microscope (Nikon, Tokyo, Japan) equipped with appropriate filters, and images were captured with digital acquisition systems (Nikon DS-U1, Optronics). Image brightness/contrast adjustments were made using Adobe Photoshop (Adobe, San José, CA). Degenerations of photoreceptors were assessed and light microscopy observations were made during the first 72 h of culture. Caspase-12, cleaved caspase-3 and TUNEL labeled cells were quantified by counting the number of positive stained cells in three-four separate areas in the outer nuclear layer, respectively, and in 2-3 non-consecutive sections. Cone photoreceptor synapses were counted in three separate 200 μm OPL-lengths, in PNA stained sections. To avoid

eccentricity-dependent effects, no measurements/counts were made on the very peripheral parts of the sectioned explants. Image J software was used for cell counting and for immunoblotting image analysis; results were obtained in uncalibrated optical density (o.d.) units. In the final analysis all signals were normalized to the loading control β-actin. Data in this study were assumed to be normally distributed and ANOVA followed by the Dunnett's post hoc test was used for statistical comparison using Graph Pad Prism software. All data are presented as mean values ± standard error of the mean (SEM) and $p < 0.05$ was considered as statistically significant. Independent experiments are indicated by n. For the Western blot analysis of each separate experiment (n), tissue homogenates from two separate and merged cultures, which in turn consisted of 2-3 retinas each, were used.

Appendix A. Supporting information

Figure S1. Fluorescence images of DAPI labeled retinas. Normal control (**A**) and illuminated 48 h (**B**) and 72 h (**C**) cultures. The retinal architecture with separate nuclear layers: ganglion cell layer, inner nuclear layer, outer nuclear layer (GCL, INL and ONL, respectively) and the synaptic outer plexiform layer (OPL) are retained during the culture period. After 72 h, tissue vacuoles are evident (asterisks in **C**) in the nuclear layers and the ONL appears loose. Fluorescence images are representative for all cultured specimens. Scale bars = 10 μm, (n = 3).

Figure S2. Immunofluorescence images and analysis of photoreceptor cell death depicted by TUNEL, caspase-12 and cleaved caspase-3 in conventionally cultured retina. Fluorescence images of normal control (**A**) and conventional culture for 48 h (**B**), stained by caspase-12 (red) and TUNEL (green). Insert in **B** shows colocalized TUNEL and caspase-12. (**C**) Quantitative analysis of TUNEL and caspase-12 immunoreactive cells in ONL; there was a significant temporal increase of TUNEL-labeled cells in the ONL but no changes in caspase-12 immunoreactivity. (**D**) Quantitative analysis of caspase-12 optical density (o.d.) showed no significant changes in the protein expression for pro- or cleaved-caspase-12 compared to the control. ER-stress induced increase of caspase-12 was confirmed in cultured retinas by using tunicamycin (Tu). (**E-G**) Fluorescence images and analysis of cleaved caspase-3 (red) in normal control (**E**), conventional culture for 48 h (**F**) and for 72h (**G**). No cleaved caspase-3 immunoreactive cells in normal control (**F**), and only single cleaved caspase-3 immunoreactive cells in the inner nuclear layer (INL) and ONL of cultured specimens (**F** and **G**). Quantitative analysis of caspase-3 optical

density (o.d.) confirmed the slight increase of cleaved caspase-3 expression (**H**) after in culture and apoptosis was confirmed in cultured retinas by using the apoptosis inducer, staurosporine (St). The molecular masses of the immunolabeled fragments are indicated in the right margin of the respective western blot images. Data are expressed as mean ± SEM and *, $p < 0.05$, **, $p < 0.01$. Scale bars = 10 µm, (n = 3 and n = 5).

Figure S3. Fluorescence images of rhodopsin labeled normal control (**A**) and conventional culture for 72 h (**B**). Rhodopsin immunoreactivity (red) is normally localized to the outer segments (OS) but shows an erronoeous localiztion in the ONL of cultured specimens (**B**). (**C**) Western blot analysis (n=3) showed the presence of several rhodopsin isomers during the entire culture period and quantitative measurements of rhodopsin optical density (o.d.) did not show any significant changes of the rhodopsin expression in conventional cultures. The different rhodopisn isomers are shown in the left margin and molecular masses of the immunolabeled fragments are indicated in the right margin. Scale bars = 10 µm (n = 3).

Figure S4. Immunofluorescence images and analysis of PSD-95 and red/green opsin immunoreactivity in normal control and conventionally cultured retina. Fluorescence microscopy of normal control (**A**), conventional culture for 12 h (**B**) and 48 h (**C**) showing PSD-95 (red) and red/green opsin (green) immunoreactivity. In normal control PSD-95 immunoreactivity (arrows in **A**) is confined to the OPL. After 12 h PSD-95 expression in the OPL seems to decrease (arrows in **B**), which is confirmed after 48 h in conventional cultures (**C**). (**A-C**) Red/green opsin immunoreactivity detected in the cone inner/outer segments of the normal control (**A**). In cultured retinas, an erroneous localization of red/green opsin protein becomes evident in the entire cone photoreceptor (arrowheads in **B** and **C**). (**D**) Quantitative analysis of PSD-95 in conventional cultures (n = 5) show that PSD-95 expression decreased significantley over time. Scale bars = 10 µm.

Figure S5. Western blot analysis (n=3) showed the presence of GRP78 BiP, CHOP, LC3B in normal control and conventional cultures. GRP78 BiP, CHOP and LC3B-I did

not show any changes in their protein expression in conventional cultures (n =3). The molecular masses are indicated in the right margin of the respective western blot and an representative image of β-actin is shown.

Figure S6. Electron micrographic analyses of photoreceptor synaptic terminals in normal control and conventional culture for 48 h. (**A-C**) Electron micrographs of normal non-cultured controls (**A**) showing cone pedicles (p) and rod spherule (s) aligned in the outer plexiform layer and a rod nucleus (n). (**B**) Higher magnification of rod spherule (s) with synaptic ribbon (arrow) and postsynaptic horizontal cells (h). (**C**) Cone pedicle (p) with synaptic ribbons (arrows). Note the rather even cytoplasmic appearance of both the rod spherule and the cone pedicle. (**D-F**) Electron micrographs of cone pedicles in 48 h conventional cultures. (**D**) Cone pedicle (p) with even cytoplasm (uppermost) and pedicles with an abundance of smaller vesicles/sER cisternae were found. Ribbons (arrows) are aligned to the active zone. (**E**) Cone pedicles (p) with abundance of smaller vesicles/sER cisternae and floating ribbons (arrow). (**F**) Cone pedicle (p) with single small vesicles/sER cisternae and mitochondria (arrow). Scale bars A = 2 μm, B-F = 0.5 μm.

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Figure legends

Figure 1. Immunofluorescence images and analysis of photoreceptor cell death depicted by TUNEL, caspase-12 and cleaved caspase-3 in cultured retina exposed to cyclic illumination. Fluorescence microscopy of non-cultured control retina (**A:** Ctrl), cultured for 48 h (**B**) and cultured for 72 h (**C**), stained by caspase-12 (red) (arrows in **A**: Ctrl, **B** and **C**) immunoreactivity and TUNEL (green) (arrows in **B** and **C**) in the ONL. (**D**) TUNEL labeled cells (white bars) in the ONL increase with time and caspase-12 immunoreactive cells (checkered bars) decrease significantly with time *in vitro.* (**E**) Caspase-12 optical density (o.d.) shows no changes for the pro-caspase-12 isoform (white bars), whereas cleaved caspase-12 (black bars) increase significantley after 72 h of culture. (**F**) Fluorescence microscopy showing cleaved caspase-3 (red) immunoreactivity. Single cells (photoreceptor in ONL, horizontal cells and amacrine cells in the INL) were cleaved caspase-3 immunoreactive (arrows) (**G**) Quantitative analysis of cleaved caspase-3 optical density (o.d.) showed a slight increase in cultured specimens. The molecular masses are indicated in the right margin of the respective western blot images. Data are expressed as mean ± SEM and *, $p < 0.05$, **, $p < 0.01$. Scale bars = 10 μm.

Figure 2. Immunofluorescence images and analysis of rhodopsin expression in cultured retina exposed to cyclic illumination. Fluorescence microscopy of non-cultured control retina marked for rhodopsin immunoreactivity (red) in outer segments (OS) (**A**). Rhodopsin immunoreactivity is mostly localized to the OS after 48 h (**B**) and 72 h (**C**). (**D**) Quantitative analysis of rhodopsin optical density (o.d.) revealed a significantly decreased rhodopsin expression after 48 h and 72 h. The densitometric meassurements of rhodopsin was made on the rhodopsin monomer and not on the smaller subunits, due to lack of those after culturing. The molecular masses of the isomers are indicated in the right margin. Data are expressed as mean ± SEM and **, $p < 0.01$. Scale bars = 10 μm.

Figure 3. Immunofluorescence images and analysis of PSD-95 and red/green opsin immunoreactivity in cultured retina exposed to cyclic illumination. Fluorescence microscopy of normal control (**A**), cultured for 48 h (**B**) and 72 h (**C**) showing PSD-95

(red) and red/green opsin (green) immunoreactivity. PSD-95 immunoreactivity in the OPL decreases over time and shows an uneven distribution in illuminated cultures during (arrows in **B** and **C**) compared to the control (arrows in **A**). PSD-95 immunoreactiviy shows abberant localizion within the ONL (asterisks in **B** and **C**) after cyclic illumination. In controls (**A**), red/green opsin (green) is expressed in cone inner and outer segments. After culture for 48 h (**B**) and 72 h (**C**) opsin shows erroneous localization to the cone cell somata in ONL (arrowheads **B** and **C**) and to the synapse terminal in OPL (OPL in **B** and **C**). (**D**) Quantitative analysis of PSD-95 optical density (o.d.) showed a significant decrease of PSD-95 after culture for 48 and 72 h. The molecular masses of the immunolabeled fragments are indicated in the right margin. Data are expressed as mean ± SEM and *, $p < 0.05$, **, $p < 0.01$. Scale bars = 10 μm.

Figure 4. Analysis of GRP78/BiP and CHOP expression in cultured retina exposed to cyclic illumination. (**A**) Quantitative analysis of GRP78/BiP optical density (o.d.) revealed significantly increased expression after 48 h and 72 h. (**B**) Quantitative analysis of CHOP optical density (o.d.) revealed significantly increased expression after 72 h. Data are expressed as mean ± SEM and *, $p < 0.05$, **, $p < 0.01$.

Figure 5. Fluorescence images of PNA labeled cone pendicles, immunofluorescence images of CtBP 2/ribeye expression and quantitative analysis of cultured retina exposed to cyclic illumination. Fluorescence microscopy of normal control (**A**), 48 h (**B**) and 72 h (**C**) illuminated cultures labeled with PNA (red) and CtBP2/ribeye (green). CtBP 2/ribeye staining shows a (**A**) punctate apperance in the OPL with immunorective (boxed area; arrowheads) horseshoe shaped structures underneath. Both PNA (arrows in **A-C**) and CtBP 2/ribeye (arrowheads in boxed area **A**) immunoreactivity decreased over time; the CtBP 2/ribeye staining in the OPL became uneven and non-stained gaps were evident (asterisks in **B** and **C**). (**D**) Quantitative analysis showing decreased density of PNA labeled cone pendicles after cyclic illumination. (**E**) Quantitative analysis of CTBP 2/ribeye optical density (o.d.) revealed a significant decline of CTBP 2/ribeye expression after 48 h and 72 h of cyclic illumination. The molecular masses are indicated in the right

margin of the respective western blot images. Data are expressed as mean ± SEM and *, p < 0.05. **, p < 0.01. Scale bars = 10 μm.

Figure 6. Immunofluorescence images and analysis of LC3B-I and LC3B-II, SQSTM1/p62, Beclin-1, mTOR and p-mTOR expression in retina cultured for 48 and 72 h and exposed to cyclic illumination. Fluorescence microscopy of normal control (**A**), and retinas cultured for 48 h (**B**) and 72 h (**C**) labeled by LC3B (green), and 48 h insert (**B**) labeled for LC3B (green) and PNA labeled cone photorecptors (red). (**A**) LC3B immunoreactivity localizes to horizontal cells (arrows) near the OPL and sparsley in the ONL in the non-cultured control. With time (**B** and **C**), the LC3B immunoreactivity appears to increase in the ONL (arrows in **B**, arrowheads in 48 h insert in **B** and arrows in **C**) in the ONL. (**D**) Quantitative analysis of LC3B optical density (o.d.) abundantly expressed LC3B-I expression and significantly increased LC3B-II expression after illumination. (**E**) Quantitative analysis of SQSTM1/p62 optical density (o.d.) revealed significantly increased SQSTM1/p62 expression. (**F**) Quantitative analysis of Beclin-1 optical density (o.d.) revealed significantly decreased Beclin 1 expression after 48 h but not after 72 h. (**G**) Quantitative analysis of mTOR optical density (o.d.). mTOR protein expression is not affected during the current culture conditions. (**H**) Quantitative analysis of p-mTOR optical density (o.d.) revealed significantly decreased p-mTOR. The molecular masses of the immunolabeled fragments are indicated in the right margin of the respective western blot images. Data are expressed as mean ± SEM and *, p < 0.05. **, p < 0.01. Scale bars = 10 μm.

Figure 7. Electron micrographic analyses of photoreceptor synaptic terminals in cultured retina exposed to cyclic illumination. (**A**) Low magnification of the OPL (72 h) showing cone pedicles (p) and rod spherule (s). Synaptic ribbons (arrows) and nucleus (n) are indicated. (**B**) High magnification of a cone pedicle (48 h) with numerous vesicles and/or dilated sER as well as several ribbons (arrow). Note also darkly stained structures of horizontal cells (asterisks) in the OPL. (**C**) High magnification of a presumptive (72 h) cone pedicle (p) with an autophagosome (arrow) and presumptive dilated sER cisternae (asterisk). (**D**) High magnification of a presumptive rod spherule (s) (48 h) probably

retracted close to the soma (judged from the vicinity of the nucleus (n)). Presumptive dilated sER cisternae (asterisks) and synaptic ribbons (arrows) are visible. Postsynaptic horizontal cells (h) seen as darkly stained structures appear to be aligned close to the spherule synaptic structures. (**E**) Cone pedicle (p) with vesicles and presumptive dilated sER cisternae (asterisks) and a lysosome (arrow). (**F**) Presumptive rod soma (72 h) with several sER cisternae (asterisk) near the nucleus (n). A visible autophagosome (arrow) wrapped around unidentified cell organelle/s. Scale bars A = 2 μm, B-E = 0.5 μm, F = 1 μm.