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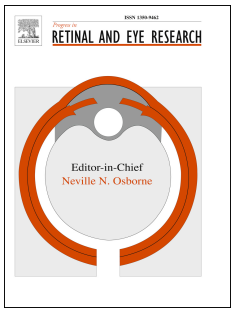

Cellular mechanisms of hereditary photoreceptor degeneration – Focus on cGMP

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– Title page –

Title: **Cellular mechanisms of hereditary photoreceptor degeneration – Focus on cGMP**

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Declaration of interest

VM, PE, and FPD have filed for three patents on the synthesis and use of cGMP analogues (PCTWO2016/146669A1, PCT/EP2017/066113, PCT/EP2017/071859) and have obtained a European Medicine Agency orphan drug designation for the use of a cGMP analogue for the treatment of Retinitis Pigmentosa (EU/3/15/1462). VM, PE, and FPD are shareholders of, or have other financial interest in, the company Mireca Medicines, which intends to forward clinical testing of cGMP analogues. KS is owner and CEO of CellTool GmbH, which commercializes Raman microscopes.

Title

Cellular mechanisms of hereditary photoreceptor degeneration – Focus on cGMP

Abstract

The cellular mechanisms underlying hereditary photoreceptor degeneration are still poorly understood, a problem that is exacerbated by the enormous genetic heterogeneity of this disease group. However, the last decade has yielded a wealth of new knowledge on degenerative pathways and their diversity. Notably, a central role of cGMP-signalling has surfaced for photoreceptor cell death triggered by a subset of disease-causing mutations.

In this review, we examine key aspects relevant for photoreceptor degeneration of hereditary origin. The topics covered include energy metabolism, epigenetics, protein quality control, as well as cGMP- and Ca^{2+}-signalling, and how the related molecular and metabolic processes may trigger photoreceptor demise. We compare and integrate evidence on different cell death mechanisms that have been associated with photoreceptor degeneration, including apoptosis, necrosis, necroptosis, and PARthanatos. A special focus is then put on the mechanisms of cGMP-dependent cell death and how exceedingly high photoreceptor cGMP levels may cause activation of Ca^{2+}-dependent calpain-type proteases, histone deacetylases and poly-ADP-ribose polymerase. An evaluation of the available literature reveals that a large group of patients suffering from hereditary photoreceptor degeneration carry mutations that are likely to trigger cGMP-dependent cell death, making this pathway a prime target for future therapy development.

Finally, an outlook is given into technological and methodological developments that will with time likely contribute to a comprehensive overview over the entire metabolic complexity of photoreceptor cell death. Building on such developments, new imaging technology and novel biomarkers may be used to develop clinical test strategies, that fully consider the genetic heterogeneity of hereditary retinal degenerations, in order to facilitate clinical testing of novel treatment approaches.

Keywords

cGMP, Apoptosis, PARthanatos, Necroptosis, PKG, Raman microscopy

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1. The retina and hereditary photoreceptor degeneration

The retina is the neuronal tissue located at the back of the eye and its primary function is the perception of light, the processing of light induced stimuli, and the transmission of light-dependent information to various parts of the central nervous system (Hoon et al., 2014). While the human retina and its constituent cells can in principle remain viable and functional life-long (*i.e.* for 120 years or even more), it is affected by a large number of hereditary, typically monogenic, diseases that will result in severe vision impairment or blindness (Verbakel et al., 2018). These genetic diseases can be grouped under the name of hereditary retinal degeneration (RD) and usually result in the degeneration and loss of the light-sensitive photoreceptors in the retina (Hamel, 2007; Kennan et al., 2005).

The degeneration and loss of photoreceptors in RD-type diseases is a major unmet medical problem, with most of these diseases still untreatable today (Trifunovic et al., 2012; Verbakel et al., 2018). This review focuses on the cellular pathways underlying the diseases and aims at promoting the further elucidation and understanding of their mechanisms. A particular emphasis is put on the hypothesis that photoreceptor degeneration is often triggered by high intracellular levels of cGMP.

1. 1. The retina and hereditary retinal degeneration

The neuroretina is arranged in three layers of cells, namely the outer nuclear layer (ONL), the inner nuclear layer (INL), and the ganglion cell layer (GCL), separated by two synaptic, or plexiform, layers, called the outer and inner plexiform layers, respectively (Figure 1) (Hoon et al., 2014). The photoreceptors (rods and cones), whose nuclei are located in the ONL, are highly specialized types of neurons capable of transforming photons of light into electrochemical messages. Rod photoreceptors (rods) respond to dim light and enable vision at night, whereas cone photoreceptors (cones) respond to bright daylight. In humans, cones are essential for high-resolution colour vison (Kolb, 2003). The visual stimuli perceived by photoreceptors are transmitted to bipolar cells and then on to ganglion cells. Both bipolar and ganglion cells integrate and process visual input (Franke et al., 2017; Schubert and Euler, 2010), before relaying it further to higher parts of the central nervous system, such as the lateral geniculate nucleus (Roska, 2019). Two other cell types, amacrine cells and horizontal cells, are important for additional integration, modulation, and interpretation of visual stimuli (Chapot et al., 2017; Franke et al., 2017).

All the main components of the phototransduction cascade, which represents the highly specific cellular processes responsible for translation of light to electrical information, are located in the so-called outer segment, the part of the photoreceptor that is farthest away from the incoming light (Figure 1). The outer segments are constantly growing and constantly engulfed by the processes of the retinal pigment epithelial (RPE) cells. RPE cells engage in the phagocytosis and renewal of the outer segments and moreover recycle the visual pigment retinal (Bertolotti et al., 2014; Ward et al., 2018), which is an integral part of the rhodopsin molecule (see 2. d. below).

RPE cells are linked to each other by tight junctions, resulting in the formation of a “shield” that limits access to the neuroretina. This so called outer blood-retinal barrier delimits the neuroretina towards the endothelial cells in the blood vessel rich choroidea and protects against pathogens or toxins that might otherwise enter the neuroretina via the blood

stream (Campbell and Humphries, 2012). An additional barrier is the outer limiting membrane, which is formed by the apical ends of the Müller glial cells (Hauck et al., 2010; Reichenbach and Bringmann, 2013), and which in turn are linked by both adherent and tight junctions (Omri et al., 2010), further limiting access to the neuroretina (West et al., 2008). The endothelial cells of the vasculature in the inner retina are connected by tight junctions as well (Figure 1), and in concert with the processes of pericytes and Müller cells they create the inner blood-retinal barrier. Finally, the neuroretina is shielded towards the vitreous by the inner limiting membrane, which consists of the end-feet (basal ends) of Müller cells (Peynshaert et al., 2019). Importantly, the outer and inner blood-retinal barriers constitute important obstacles for therapeutic interventions aimed at the neuroretina (Koo et al., 2012).

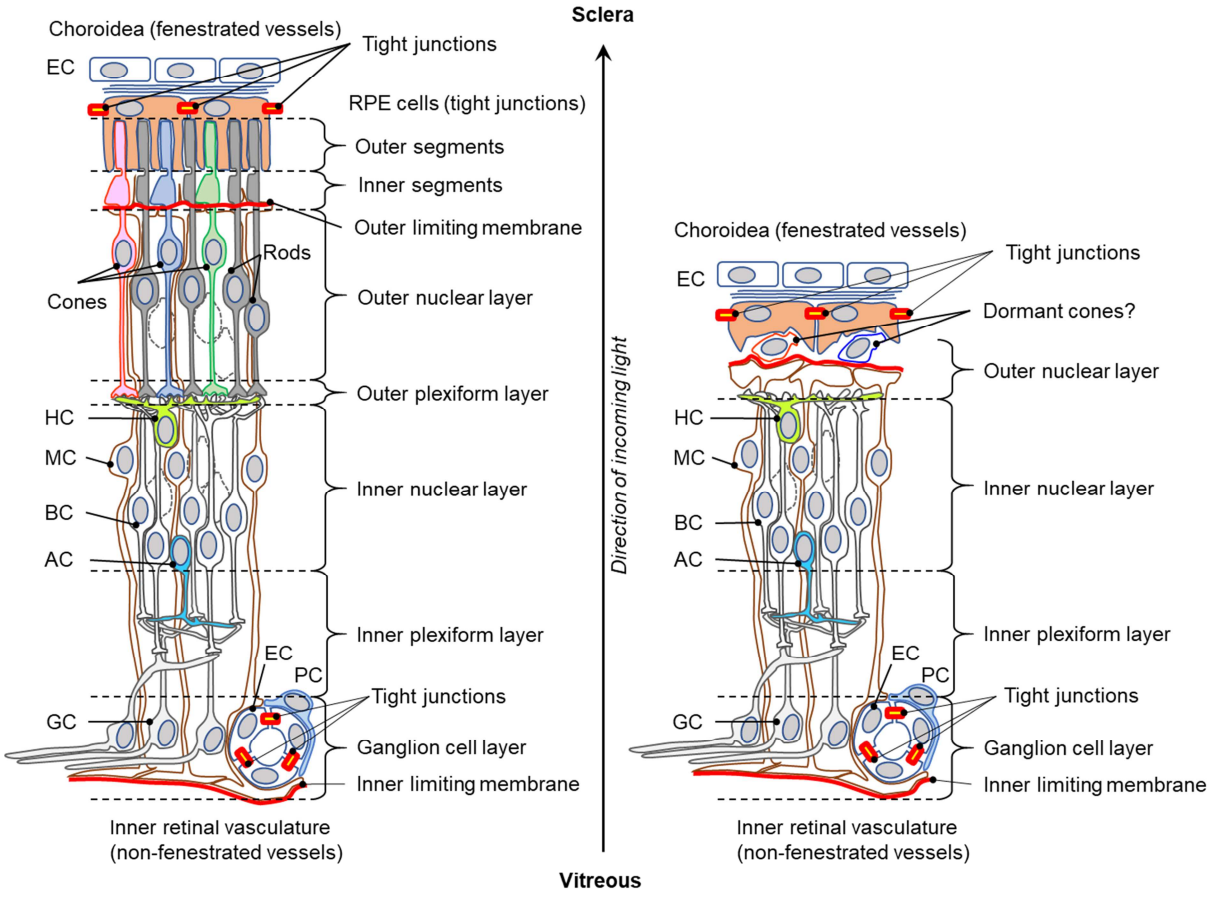

Figure 1: Idealized cross-sections through healthy and degenerated retina. Left: Cartoon displaying the various layers of an intact, healthy retina, from the choroidea to the ganglion cell layer. Rod photoreceptors in the outer nuclear layer are shown in grey, while cones are indicated by red, green, and blue outlines. Also shown are components of the outer and inner blood-retinal barrier, as well as outer and inner limiting membranes (red).

Right: Retina in the final stages of retinal degeneration. Note that the outer nuclear layer is almost completely lost, and that the outer plexiform layer has nearly vanished. Remarkably, even in late degeneration stages, when the retina has lost all functionality, some cone photoreceptors may remain; these have sometimes been addressed as “dormant cones”.

AC=amacrine cells; BC=bipolar cells; EC=endothelial cell; GC=ganglion cell; HC=horizontal cells; MC=Müller glial cell; PC=Pericyte; RPE=retinal pigment epithelium. Note that the retinal structure has been simplified for clarity and that not all retinal cell types are shown.

Hereditary retinal degeneration (RD) is a group of rare retinopathies that cause progressive loss of vision. Within the group of RD-type diseases, adult-onset Retinitis Pigmentosa (RP) is the most common, with a prevalence of approximately 1:3.500 (Bertelsen et al., 2014). A genetically related disease is Leber congenital amaurosis (LCA), with a prevalence of circa 1:8.000 and a disease onset already in early childhood or even in infancy (den Hollander et al., 2008). These most common forms of RD are caused by a genetic defect in a single gene that compromises the viability of photoreceptors (Hartong et al., 2006; Verbakel et al., 2018). In RP, an initial, primary degeneration of rods is usually followed by a secondary degeneration of cones, eventually leading to complete blindness (Kennan et al., 2005). The result is a complete, or near complete, loss of the ONL, while the inner retina remains mostly intact, even though the dendrites of bipolar and horizontal cells eventually retract (Gargini et al., 2007). In LCA, the disease-causing mutations may affect both rods and cones simultaneously, sometimes leading to a very severe disease phenotype with no discernible retinal function as measured in electroretinography (Jacobson et al., 2017; Preising et al., 2012).

It is important to note that the clinical terms RP and LCA are somewhat ambiguous when it comes to disease onset and progression, as well as their clinical characterization, which may sometimes overlap. There is also some correspondence between these two disease groups from the point of view of affected genes and biochemical pathways, especially as different mutations in the same genes may cause either RP or LCA (Goldberg et al., 2016; Sharon et al., 2018). In addition to the initial and primary rod degeneration in RP and LCA, there is usually a secondary cone degeneration, even when cones are not directly affected by the mutation. Such secondary cone loss, which so far has not been explained in detail (but see chapter 2.1.2. below), can be a remarkably protracted process (Carter-Dawson et al., 1978). In fact, this phenomenon has sometimes been referred to as “cone dormancy”, raising the possibility of a re-activation of dormant cones for therapeutic purposes (Busskamp et al., 2010) (Figure 1).

Cones may also be subject to primary degeneration in RD-type diseases, for instance in achromatopsia (ACHM). In ACHM the primary genetic defect of genes specifically expressed in cones leads to cone dysfunction and degeneration (Hamel, 2007). ACHM has a prevalence of approx. 1:30.000 and is usually characterized by severe visual impairments (photophobia, low visual resolution) from birth. However, as opposed to RP and LCA, ACHM does not usually entail secondary loss of (in this case) rods and complete blindness (Kohl and Hamel, 2013).

1. 2. Genetic heterogeneity of hereditary retinal degeneration

The RD group of diseases is characterized by a vast genetic heterogeneity, with disease causing mutations known in over 270 genes (https://sph.uth.edu/retnet; information retrieved May 2019). This fact severely hinders both the understanding of degenerative mechanisms and the development of treatments. To complicate matters further, each of these RD-linked genes may carry many different types of recessive, dominant, or X-linked mutations, ranging from complete loss-of-function to gain-of-function (Chizzolini et al., 2011).

An example for such a situation are mutations in the *GUCY2D* gene encoding for retinal guanylyl cyclase (retGC). retGC produces the second messenger molecule cyclic guanosine- 3’-5’-mono-phosphate (cGMP) in photoreceptor outer segments, and over 100

different mutations have been described in the *GUCY2D* gene (Sharon et al., 2018). Some of these mutations will result in a loss-of-function and lack of cGMP synthesis in photoreceptors, causing photoreceptors to die from low cGMP levels (Williams et al., 2006). However, it appears that many of the *GUCY2D* mutations – rather than abolishing enzymatic function – will prevent a proper regulation of the enzyme, resulting in retGC gain-of-function and constitutive activation. The net result of such mutations is thus an excessive production of cGMP in photoreceptors (Dizhoor et al., 2016; Wimberg et al., 2018).

Similarly, the genes *GUCA1A* and *GUCA1B* can be affected by a large variety of different mutations (Manes et al., 2017; Sato et al., 2005). In rods and cones these encode for the guanylyl cyclase activating protein (GCAP), the protein that regulates retGC activity in a Ca^{2+}-dependent manner (Vinberg et al., 2018b). In the Ca^{2+} bound-state GCAP inhibits retGC. Loss-of-function mutations frequently reduce the binding of Ca^{2+} to GCAP, leading to a lack of inhibition on retGC and an over-production of cGMP (Nishiguchi et al., 2004; Peshenko et al., 2019). Hence, loss-of-function mutations in GCAP genes produce the same net effect as gain-of-function mutations in retGC, *i.e.* high levels of cGMP in photoreceptors.

Further examples for the variability of mutation effects in RD genes are the genes encoding for photoreceptor phosphodiesterase-6 (PDE6) (Cote, 2004). Rod PDE6 is a heterotetramer composed of the catalytic α and β subunits, encoded by the *PDE6A* and *PDE6B* genes, respectively, and two inhibitory γ subunits, encoded for by the *PDE6G* gene. Cone PDE6 consists of two α subunits encoded for by the *PDE6C* gene and two inhibitory γ subunits encoded for by the *PDE6H* gene. Numerous mutations have been found in all PDE6 genes, causing RP (*PDE6A*, *PDE6B, PDE6G*) (Corton et al., 2010; Dvir et al., 2010; Muradov et al., 2012) or ACHM (*PDE6C, PDE6H*) (Gopalakrishna et al., 2017; Kohl et al., 2012; Thiadens et al., 2009). Typically, a mutation causing PDE6 loss-of-function leads to extremely high cGMP levels and the death of the affected photoreceptor cell type (Farber and Lolley, 1974; Paquet-Durand et al., 2009; Sothilingam et al., 2015; Trifunovic et al., 2010). On the other hand, some *PDE6A* mutations are known to produce “only” a loss of rod functionality, without cell death, resulting in a clinical phenotype referred to as congenital stationary night blindness (CSNB) (Zeitz et al., 2015).

These are just a few examples for the complexity of RD gene mutations, taken from only one photoreceptor aspect, namely its handling of the cGMP metabolism, but which nevertheless highlight the need for a careful analysis of each individual gene mutation, so as to guide further research and therapy development.

1. 3. Animal models for hereditary retinal degeneration

Studies into the mechanisms of photoreceptor degeneration require the use of suitable model systems, that will reproduce degenerative processes as faithfully as possible. Since the physiological processes related to vision and light perception are evolutionarily conserved over a wide range of organisms (Nilsson, 2009), gene mutations causing RD in humans will often also cause RD in animals. Accordingly, a large number of animal models is available for RD research, in species/orders as diverse as *Drosophila* (Griciuc et al., 2010), zebrafish (Ward et al., 2018), and mammals (Shaw et al., 2001). For the latter, the typically used species are mice and rats (Dalke and Graw, 2005; Won et al., 2011) though larger mammalian models, including cats (Menotti-Raymond et al., 2010), dogs (Beltran, 2009), and pigs are also available (Petters et al., 1997).

Among the mouse models for RD, the "retinal degeneration-1", *rd1*, mouse stands out as probably the earliest and likely the most studied animal model for RD. The first report about the *rd1* mouse dates back to 1924, where it is described as having a "rodless retina" (Keeler, 1924) and in older literature it is usually referred to as "rd" or "rd/rd" mouse. The *rd1* mouse is characterized by a mutation in the gene encoding for the β subunit of rod PDE6 (Bowes et al., 1990), a lack of PDE6 protein (Yan et al., 1998) and exceedingly high levels of cGMP in rods (Farber and Lolley, 1974; Paquet-Durand et al., 2009). This leads to rapid loss of most of the rods within the first two weeks after birth (Sahaboglu et al., 2013), followed by a mutation-independent, secondary death of cones (LaVail et al., 1997). Another mouse model with a mutation (albeit different from that of *rd1*) in the *Pde6b* gene is the *rd10* mouse (Chang et al., 2002). Compared to *rd1*, the loss of rods in the *rd10* retina starts later, at around post-natal day 18 (P18), from when on it takes about 10 days until most rod photoreceptors are lost (Arango-Gonzalez et al., 2014; Gargini et al., 2007).

More recently several new mouse models for mutations in the *Pde6a* gene became available (Sakamoto et al., 2009; Sothilingam et al., 2015), which carry point mutations leading to single amino acid changes in the PDE6A protein and are named accordingly, *i.e.* R562W, D670G, or V685M. All these mutations impair, to varying extents, PDE6A protein expression and activity, and correspondingly display an accumulation of cGMP in rods prior to rod loss (Jiao et al., 2016; Sothilingam et al., 2015). Since homologous *PDE6A* point mutations have been found in RD patients, these animals allow to precisely match patient genotypes and model both homozygous and compound heterozygous disease conditions (Sothilingam et al., 2015).

Primary cone degeneration may also be studied in mouse models, prominent examples of which are the cone-photoreceptor-function-loss *cpfl1* mouse (Chang et al., 2009) and the *Cnga3* knock-out (KO) mouse (Biel and Michalakis, 2007). While the latter suffers from a lack of expression of a subunit of the cyclic nucleotide gated channel (CNGC), leading to slow cone degeneration over the course of about four months, the *cpfl1* mouse carries a mutation in the cone-specific α subunit of PDE6, leading to a loss of cones approx. two months after birth (Trifunovic et al., 2010). Incidentally, both the *Cnga3* KO and the *cpfl1* mouse show strong accumulation of cGMP in degenerating cones (Arango-Gonzalez et al., 2014).

Many more RD animal models exist, for many of the known RD-genes (Chang et al., 2002; Won et al., 2011), enabling comparative studies into disease mechanisms (Arango-Gonzalez et al., 2014), but also a validation of novel therapeutic approaches across various models (Vighi et al., 2018b). Interestingly, in a number of different RD models photoreceptor cell death appears to be caused by high levels cGMP (Arango-Gonzalez et al., 2014; Iribarne and Masai, 2017; Paquet-Durand et al., 2009; Wang et al., 2017); further details on this finding and its significance will be presented in chapter 3. Taken together, animal models for RD, carrying similar or even homologous mutations to those identified in human patients, and with similar disease phenotype, offer an enormous potential to disentangle the underlying disease mechanisms.

2. Photoreceptors: Highly specialized neurons with special needs

Photoreceptors are neurons that have taken cellular specialization to extremes. Unlike any other mammalian cell, they are highly adapted for photon capture and the transformation of this information into electrical signals, with subsequent transmission to 2nd order neurons of the retinal networks (Kolb, 2003). In order to do so, photoreceptors are equipped with distinctive features, including the complex signalling cascades involved in phototransduction, as well as the architecture of the transduction compartments and synapses, which indeed are illustrative of how far structural and molecular adjustments of specialized neurons may go (Goldberg et al., 2016; Molday and Moritz, 2015; Wensel et al., 2016).

Insights into the molecular function of photoreceptors may enlighten the operative mechanisms in neurons in general. However, as a consequence of all their unique specializations, photoreceptors may display distinctive features when it comes to their degeneration, *i.e*. the mechanisms of cell death that photoreceptors may resort to could be different from what other neuronal cell types might use, and thus need to be identified. In this context, we will here discuss four topics that appear to be particularly relevant for photoreceptor degeneration: 1) Energy metabolism, 2) Epigenetic processes, 3) Protein quality control and transport, 4) Phototransduction.

2. 1. Photoreceptor energy metabolism

While energy metabolism is fundamental for the survival of any cell, surprisingly little is known about how photoreceptors satisfy their energetic demands. The retina is known to be one of the most metabolically active tissues in the body (Trick and Berkowitz, 2005) and this high energy demand is likely caused to a major extent by photoreceptors. Curiously, the retina as a whole appears to use mostly 'aerobic glycolysis' (Ames et al., 1992), *i.e*. the conversion of glucose to pyruvate and then to lactate, under aerobic conditions, instead of using the much more energy efficient direct mitochondrial oxidation. This phenomenon was discovered already in the early 1920s by Otto Warburg and is referred to as the 'Warburg effect' (Leveillard and Sahel, 2016; Warburg, 1925). Yet, the details on how photoreceptors acquire their cellular fuels (glucose, lactate, *etc*.) and generate from these the necessary energy containing substrates (ATP, NADH, *etc*.) are still unknown today, although two alternative concepts have been forwarded, that may be applicable for neuronal metabolism and/or the retina.

2. 1. 1. The astrocyte-neuron-lactate-shuttle (ANLS)

The ANLS hypothesis proposes that glia cells convert glucose into lactate, which then serves as fuel for neurons (Brooks, 2018; Pellerin and Magistretti, 1994). Among other advantages, this separation of glycolysis from energy consumption may allow for faster adaptation to rapid changes in neuronal energy demand and may furthermore reduce neuronal oxidative stress (Kane, 2014). For the retina, the ANLS hypothesis would mean that the Müller glial cells (alternatively the RPE cells) would perform glycolysis and then pass lactate on to photoreceptors. Indeed, the expression of the lactate transporter monocarboxylate transporter 1 (MCT1) by photoreceptors and MCT2 by Müller glia cells may support the ANLS hypothesis (Gerhart et al., 1999). However, the lactate shuttle

hypothesis is not universally accepted and in practice its usage may depend on specific cell and tissue properties, as well as metabolic status.

2. 1. 2. The photoreceptor-RPE-lactate-shuttle

For the retina an inverted lactate shuttle has recently been proposed, in which photoreceptors would consume glucose and generate lactate, which would then be released to serve as fuel for Müller glial cells and RPE cells (Kanow et al., 2017). The study also showed that high levels of lactate can suppress glycolysis in RPE cells, which is interpreted as a means to increase the passage of glucose to photoreceptors. While this alternative shuttle hypothesis elegantly resolves some of the contradictions of retinal metabolism, it does not explain the enormous density of mitochondria in the photoreceptor inner segments. Moreover, the hypothesis proposes the expression of the glucose transporter-1 (GLUT1) in rod and cone photoreceptors, something that is still controversial. Older studies using electron microscopy confirmed GLUT1 expression in RPE, but could not detect it in photoreceptors (Bergersen et al., 1999; Gerhart et al., 1999). Further electron microscopic studies showed that the expression of GLUT1 previously proposed for photoreceptor outer segments (Mantych et al., 1993) was in fact localized to RPE cell microvilli (Gospe et al., 2010). More recently GLUT1 expression was suggested to be present on cones only (Ait-Ali et al., 2015).

To comprehend precisely how photoreceptors are nourished and how they generate ATP and NADH is key to understanding photoreceptor demise, including in hereditary retinal diseases (Joyal et al., 2018). For instance, in RP and LCA, the secondary loss of cones may be associated with alterations in retinal vasculature and loss of trophic support (Ambati and Fowler, 2012), both of which may influence the metabolic environment of the cones. Here, cones may be dependent on trophic factors from rods, such as the rod-derived cone-viability factor (RdCVF) (Leveillard et al., 2004), which has been hypothesized to regulate glucose uptake in cones (Ait-Ali et al., 2015). Others suggest that, based for instance on the temporal aspects of the secondary cone death (which is very protracted (Carter-Dawson et al., 1978)), the loss of rods produce a strongly oxidative environment, which will push the cones towards their death (Campochiaro and Mir, 2018). Furthermore, because of the dark current (Hagins et al., 1970) (chapter 2.4), there may be significant differences in energy consumption between light and dark, raising the possibility that energy supply and shuttling of metabolites may be switched according to lighting conditions.

Addressing these questions has important ramifications for understanding the pathophysiology of RD, not the least for secondary cone degeneration, but is highly relevant also for diabetic retinopathy and possibly even age-related macular degeneration (AMD). Hence, the identification of early pathogenic events related to energy metabolism may provide new insights into degenerative mechanisms that could facilitate the development of novel diagnostic and therapeutic approaches (Gross and Glassman, 2016).

2. 2. Epigenetics in retinal degeneration

Apart from the heritable mutations in distinct genes, many retinal degenerations connect with several functional alterations of the chromatin. Such epigenetic changes include DNA methylation and different types of histone modifications, including histone

acetylation/deacetylation, methylation, and poly-ADP-ribosylation (PARylation). Figure 2 shows some examples of typical staining patterns when markers for these aspects are used on an RP-model retina.

With respect to other retinal degenerations, epigenetic changes may potentially affect the disease course also in glaucoma, AMD, and diabetic retinopathies (Corso-Diaz et al., 2018; Gemenetzi and Lotery, 2014; Pennington and DeAngelis, 2016; Wei et al., 2012a). A similar situation may apply to yet other ocular diseases (He et al., 2013; Liu et al., 2013).

2. 2. 1. DNA methylation

At the DNA level, hypermethylation of cytosine bases frequently targets upstream promoter regions, usually with a gene repressing function with methylation carried out via DNA methyl transferases (DNMTs) (Smith and Meissner, 2013). While hypermethylation appears to be the most common epigenetic change, hypomethylation also occurs. Increased methylation in photoreceptor genomic DNA has been detected by immunostaining in several models of RP (Figure 2), indicating an involvement of DNA methylation in the degeneration process (Farinelli et al., 2014; Wahlin et al., 2013). The methylation of photoreceptor DNA can further be modified to hydroxymethylation (Wahlin et al., 2013). Moreover, the use of a pharmacological inhibitor of DNMTs on *rd1*-model based retinal explants reduced the extent of TUNEL-labelled, dying cells (Farinelli et al., 2014). Even if this was not clearly translated to a photoreceptor survival it caused a delay in the degeneration. Since DNA methylation labelling of the degenerating photoreceptors did not appear until wide-spread DNA fragmentation set in (Farinelli et al., 2014; Wahlin et al., 2013), this suggests that DNA hypermethylation is a consequence rather than a cause of the disease, although it may be a consequence that aggravates the situation and accelerates the progression. Therefore, the growing insights into the retinal genes that may be under control of DNA methylation during conditions of hereditary degeneration (Farinelli et al., 2014) might very well serve as a starting point for future, ameliorating treatment options in RD.

2. 2. 2. Histone acetylation and deacetylation

Acetylation and deacetylation of histones is carried out by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, and relate to the addition or removal of acetyl groups to/from histone lysine residues (Haberland et al., 2009). The HDACs form a large family of at least 18 enzymes, classified according to sequence similarities to their counterparts in yeast (Delcuve et al., 2012; Seto and Yoshida, 2014). There is considerable variation in expression patterns among the HDAC classes and the HDAC species, as well as in cellular functions, although transcriptional control via chromatin organisation appears as a theme for many of them, usually with deacetylation being correlated with gene repression (Delcuve et al., 2012; Seto and Yoshida, 2014).

In the context of retinal degeneration, it has been known for a number of years that HDACs play a role for photoreceptor survival, including in models of RP (Figure 2). Interestingly, though, the exact role of these enzymes may be dependent on the type of degeneration and/or the type of intervention used to investigate the function of a given HDAC, or class of HDACs. A positive regulation of photoreceptor survival by HDAC4 was suggested, since experimentally reduced expression of this HDAC variant in normal retinas led to photoreceptor cell death, while overexpression of HDAC4 in the *rd1* model conversely

prolonged the life of the diseased photoreceptors (Chen and Cepko, 2009). However, the latter effect may be unrelated to actual deacetylase activity (Guo et al., 2015). In contrast, a detrimental HDAC effect on survival was indicated by pharmacological experiments, in which the relatively broad HDAC inhibitors trichostatin A (TSA) and scriptaid were able to protect rod photoreceptors of the *rd1* model from cell death in retinal explants (Sancho-Pelluz et al., 2010). TSA was similarly able to reduce cone cell death in the cone degeneration model *cpfl1* in explant culturing, and, more importantly, *in vivo* through intravitreal injection (Trifunovic et al., 2016). On the other hand, yet another broad HDAC inhibitor, valproic acid (VPA), exhibited either protective or detrimental effects in *Xenopus laevis* models of rhodopsin mutation-based RP, depending on the exact type of genetic defect (Vent-Schmidt et al., 2017). Likewise, VPA had opposing effects on the degeneration of rod photoreceptors in two *Pde6b* mutation models, the *rd1* and the *rd10* mouse, in which *rd1* photoreceptors were protected by VPA treatment, whereas it, in contrast, accelerated the *rd10* photoreceptor degeneration (Mitton et al., 2014). Similar discrepancies have been seen in other studies (Berner and Kleinman, 2016), and the use of VPA in people with RP has not resulted in a consensus on whether or not this is a valuable treatment option, or if it may actually be negative (Dias et al., 2018; Vent-Schmidt et al., 2017).

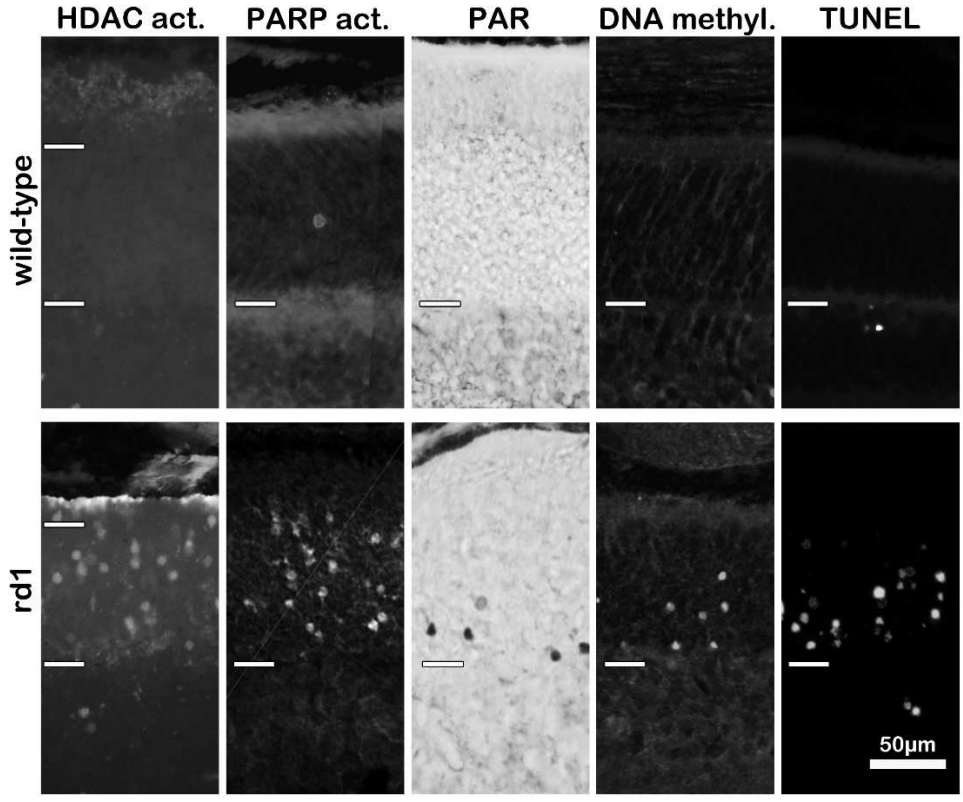

Figure 2: Differential regulation of epigenetic markers early in *rd1* retinal degeneration. At post-natal day 11, wild-type photoreceptors (top panel) show no signs of increased activity of epigenetic processes, while *rd1* photoreceptors (bottom panel) show activation of HDAC and PARP, increased PARylation and DNA methylation, as well as a DNA fragmentation as detected by the TUNEL assay. ONL, INL = outer, inner nuclear layer, respectively. White horizontal bars in leftmost figures indicate the outer (top) and inner (bottom) limits of the ONL, while in the other figures the bars indicate the inner limit of this layer.

The situation with HDAC involvement in RD, which as discussed above are indeed heterogenous, is thus extremely complex. In turn, this may be related to the nature of the HDAC family (Haberland et al., 2009; Seto and Yoshida, 2014), with its varied distribution and functional patterns – even outside the nucleus – in combination with the use of often not-so-very specific inhibitors as explorative agents (see above). Adding to the complexity, there also exist non-histone substrates for acetylation, which could have clinical implications in neurologic diseases (Schneider et al., 2013).

Interestingly, a recent study on the role of so called bromodomain and extra-terminal domain (BET) family proteins, which are involved in the actual reading of the acetylation marks and translating them into gene expression, suggests that acetylation marks are critical for the microglial response of the *rd10* model of RP. Inhibition of BET by a specific blocker preserved photoreceptor structure and function, likely via the suppression of microglial activation (Zhao et al., 2017). If various models and/or test systems differ in their susceptibility to, for instance, microglial responses, this could help explain why an interference with acetylation via HDAC inhibition is not a straightforward route to protection. The identification of the bromodomain proteins binding to acetylated residues during photoreceptor degeneration may be an important finding, which could advance the understanding of the role of histone acetylation for the progression of the disease.

2. 2. 3. Histone methylation

In addition to acetylation, histones may also be methylated, usually at defined lysine residues, with such reactions carried out by histone methyl transferases (HMTs). In the same manner as for the acetylation, the methylation of histones in mouse rod photoreceptor nuclei is distributed in particular patterns, including concentric ones, depending on which exact histone and which exact amino acid residue is modified (Eberhart et al., 2013; Kizilyaprak et al., 2010). It is possible that this is somehow related to the inverted organization of rod nuclei (of nocturnal mammals), in which the euchromatin is placed to the outside of the heterochromatin (Eberhart et al., 2013; Solovei et al., 2009). In comparison to histone acetylation though, the possible participation of histone methylation in retinal degenerations is not as well studied. Still, a very recent report showed histone hypermethylation in the *rd1* retina, although the cellular origin of this alteration was not determined. The report furthermore demonstrated that methyltransferase inhibition via subretinal injections provided both structural and functional protection for the degenerating rods (Zheng et al., 2018). It will be interesting to follow future developments in the area of histone methylation and RD, and the exact cell type(s) in which the hypermethylation takes place.

2. 2. 4. PARP activity and PARylation

Yet another epigenetic modification is represented by a process coined PARylation, which involves the attachment of multiple poly-ADP-ribose molecules to specific acceptor or target proteins, by means of the activity of poly-(ADP-ribose) polymerase, PARP. As described below in chapter 3, PARP activity is related to the PARthanatos type of caspase-independent cell death. An involvement and role for PARP and PARylation in inherited retinal degeneration is well documented by different laboratories and in several models for RP (Arango-Gonzalez et al., 2014; Camara et al., 2015; Jiao et al., 2016; Paquet-Durand et

al., 2007; Sahaboglu et al., 2016). The epigenetic importance of PARP and PARylation is still being investigated, but it is intriguing to see that it seems to connect with DNA methylation, to the point that it may exert some sort of control over the DNA methylation processes (Ciccarone et al., 2017). Recent data suggest that PARylation may also occur on the DNA molecule itself (Talhaoui et al., 2016). In a broad sense, PARP is coming up as a significant player in chromatin regulation and has been shown to also have links to histone acetylation and methylation, with PARP targets including both HDAC and histone proteins (Ciccarone et al., 2017). This could indicate that most, if not all, of the epigenetic changes seen in inherited retinal degenerations may indeed be coupled (for a discussion of such links in general, see *e.g*. (Jin et al., 2011)). In fact, it was noted that PARylation of the degenerating photoreceptors in the *rd1* model overlapped very well with de-acetylated photoreceptor nuclei, *i.e.* where HDAC activity was high (Sancho-Pelluz et al., 2010). When HDAC activity was blocked by TSA, the PARylation disappeared, indicating that PARP activation occurred downstream of HDAC activity (Sancho-Pelluz et al., 2010). This and other observations in the same study point to a rather late position of these events in the degeneration process of an individual affected photoreceptor, just as was observed for the DNA methylation (Figure 2).

As a final remark, it is interesting to note that many different compounds directed at these epigenetic processes are either already out on the market as registered drugs or in clinical trials (Da Costa et al., 2017; Mirza et al., 2018; Yan et al., 2016). Although these drugs are foremost aimed at non-retinal diseases, often in the area of oncology, their status holds promise for a quick transfer or repurposing for a use in the field of RD whenever deemed relevant.

2. 3. Protein synthesis, quality control, and transport

Phototransduction relies on isomerization of 11-*cis* retinal by photons, but light can also oxidize and damage proteins and lipids. Photoreceptors are constantly exposed to light and they metabolize and function under high oxygen conditions, making them vulnerable to oxidative stress (Campochiaro et al., 2015; Stefansson et al., 2019; Usui et al., 2009). Moreover, the polyunsaturated fatty acids present at the photoreceptor disc membrane are highly susceptible to oxidative damage (Beatty et al., 2000). Together, this imposes significant stress on the cellular machinery of photoreceptors, which they cope with by daily regeneration of the outer segments (Athanasiou et al., 2013; Molday and Moritz, 2015). This requires a high rate protein synthesis and efficient quality control systems for correct folding and transport to the outer segment (Leveillard and Sahel, 2016).

2.3.1. Protein quality control and endoplasmic reticulum (ER) stress

Protein quality control resides at the endoplasmic reticulum (ER) and is mediated by sensors located at the ER membrane. In case of defects, these sensors activate the unfolded protein response (UPR) to decrease protein synthesis (Gorbatyuk and Gorbatyuk, 2013), enhance the protein folding mechanism, and remove misfolded proteins (Chan et al., 2016). ER stress and UPR are intricately connected (Hetz, 2012) and are transduced by three ER resident proteins: 1) the inositol-requiring enzyme 1 (IRE1), 2) the activating transcription factor-6 (ATF6), and 3) the protein kinase R-like ER protein kinase (PERK). The three ER sensors can regulate expression of chaperones, such as binding-immunoglobulin-

protein (BIP), reduce protein synthesis through phosphorylation of eukaryotic initiation factor-2 α (eIF2α) or activate apoptotic responses by expression of several genes such as *CHOP/GADD153* encoding for a transcription factor that negatively regulates, among others, the anti-apoptotic factor BCL2 (Hetz, 2012). During retinal degeneration these pathways can be activated (Chan et al., 2016), as shown by the progressive increase of *CHOP* and decrease of BIP expression in photoreceptors expressing a misfolding mutation in rhodopsin (Lin et al., 2007).

ER stress arises after the accumulation of misfolded proteins in the ER and can reduce the production of functional proteins and even lead to cell death in case the UPR cannot relieve the cell from the stress. Moreover, ER stress can be activated also by oxidative stress and reactive oxygen species (ROS) (Zhang et al., 2014). Recent studies support the view that ER protein folding highly correlates with ROS production, because redox homeostasis is crucial for the protein folding process and disulphide bond formation (Plaisance et al., 2016). Furthermore, the ER plays an essential role in regulation of Ca^{2+} homeostasis. The chaperone BIP contributes to Ca^{2+} buffering in the ER lumen and is involved in sensing misfolded proteins and the activation of ER stress. BIP contributes to the prevention of ER Ca^{2+} leakage and helps to maintain ER homeostasis (Krebs et al., 2015). Mutations in different genes can lead to differential dysfunctions in photoreceptor cells, such as protein misfolding, oxidative stress, and Ca^{2+} dysregulation in the ER, triggering ER stress, that has been linked to photoreceptor cell death in different models of RD (Chan et al., 2016). Thus, photoreceptors have properties that could make them particularly vulnerable to ER stress related processes, opening also the possibility to target such processes for therapeutic purposes.

2.3.2. Protein misfolding in RD

Rhodopsin is the most abundant protein in rods and the majority of mutations in rhodopsin cause autosomal dominant RP due to failure of rhodopsin to fold correctly or defects in the transport to the outer segment (Mendes et al., 2005). Several studies characterized the molecular responses to misfolded mutant rhodopsin in different animal models and led to the proteostatic stress hypothesis for this type of mutations (Athanasiou et al., 2013). The best studied dominant mutation in rhodopsin is the P23H mutation, a misfolding mutation that has been linked to ER-stress, UPR, and impaired proteasome activity (Athanasiou et al., 2014; Chiang et al., 2012; Chiang et al., 2015; Comitato et al., 2016; Gorbatyuk et al., 2010). Recent studies on the P23H rhodopsin mutation demonstrated that this mutation does not cause ER stress, but rather UPR. To this end, in P23H mutant photoreceptors activation of the ER sensors, such as IRE1, is possibly a compensatory response to help the degradation of the misfolded protein and strengthening of the proteasome, which then has protective effects (Chiang et al., 2015). Further studies showed limited or even negative neuroprotection by interfering with ER stress mechanisms (Athanasiou et al., 2017; Comitato et al., 2016). To explain these results, we need to keep in mind that different molecular effects can be triggered by different mutations in rhodopsin. In fact, a recent molecular study characterized the effects on the protein structure of 33 rhodopsin mutations and showed that different mutations have distinctive effects on the protein. This study also identified a group of mutations for which misfolding is relieved upon interaction with retinal, which acts as a chaperone for rhodopsin (Behnen et al., 2018).

Based on the differential mechanism and the numerous mutations causing protein misfolding, different therapeutic strategies have been proposed. In this context, alleviation of misfolding has been addressed in preclinical studies by treatment with molecular chaperones, which showed positive results *in vitro* and *in vivo* (Behnen et al., 2018; Chen et al., 2018; Mendes et al., 2005). A second approach would be activation of the proteasome activity (Lobanova et al., 2018). Finally, targeting the dominant allele either by ribozyme or CRISPR/Cas9 or expression downregulation may eliminate the toxic effect of the mutated protein (Latella et al., 2016; Millington-Ward et al., 2011; Mussolino et al., 2011).

2.3.3. Protein mistrafficking

The delivery of proteins to the outer segment is a tightly regulated mechanism (Kandachar et al., 2018; Wang and Deretic, 2014). Mutations causing defects in the transport of rhodopsin to the rod outer segment, or defects in the connecting cilium that affect protein translocation to the disks, are linked to retinal degeneration. In fact, mutations in intraflagellar transport proteins, such as ITF172, a component of the connecting cilium, can lead to RP and rhodopsin mislocalization (Gupta et al., 2018). Cell death is likely triggered by protein mislocalization because the severity of photoreceptor degeneration directly correlates with the rate of missorting (Green et al., 2000).

The mechanisms of photoreceptor demise caused by protein mistrafficking are still not well characterized. Different explanations have been forwarded as to how mislocalized proteins, especially rhodopsin, may cause photoreceptor cell death in RD: When in the inner segment rhodopsin may activate G-α transducin, which in turn cannot activate PDE6 (see also chapter 2.4.) outside the outer segment, and may instead act on adenylate cyclase to increase cAMP, with subsequent activation of caspases (Nakao et al., 2012; Wang et al., 2012). Interestingly, genetic deletion of transducin does not completely prevent photoreceptor loss caused by mislocalized rhodopsin, arguing for transducin-independent cell death promoting processes (Concepcion and Chen, 2010). These processes could potentially be due to constitutive binding of mislocalized rhodopsin to arrestin (Chen et al., 2006; Chuang et al., 2004).

2. 4. The phototransduction cascade and the regulation of cGMP and Ca^{2+} levels

The physiology of photoreceptors and the phototransduction cascade critically depends on the regulation and interplay of the second messenger signalling molecules cGMP and Ca^{2+} (Pugh and Lamb, 1990; Vinberg et al., 2018b). Mutations affecting genes related to the phototransduction cascade often cause a dysregulation of cGMP and/or Ca^{2+}, triggering a series of down-stream processes, which eventually kill photoreceptors (Kulkarni et al., 2016).

The phototransduction cascade employs high levels of cGMP in photoreceptor outer segments to fully sensitize photoreceptor cells in the dark (Figure 3). cGMP is synthesized by retGC in a Ca^{2+} -dependent way. When Ca^{2+} levels are low, GCAP stimulates retGC to produce cGMP. Conversely, under high Ca^{2+} concentrations GCAP inhibits retGC (Tucker et al., 1999) providing for a negative feedback loop that limits photoreceptor cGMP to physiological levels of 1-5 µM (Burns et al., 2009; Dell'Orco et al., 2009; Pugh and Lamb, 1990). Independent of Ca^{2+}, retGC activity is additionally controlled by the RD3 protein (Peshenko et al., 2016).

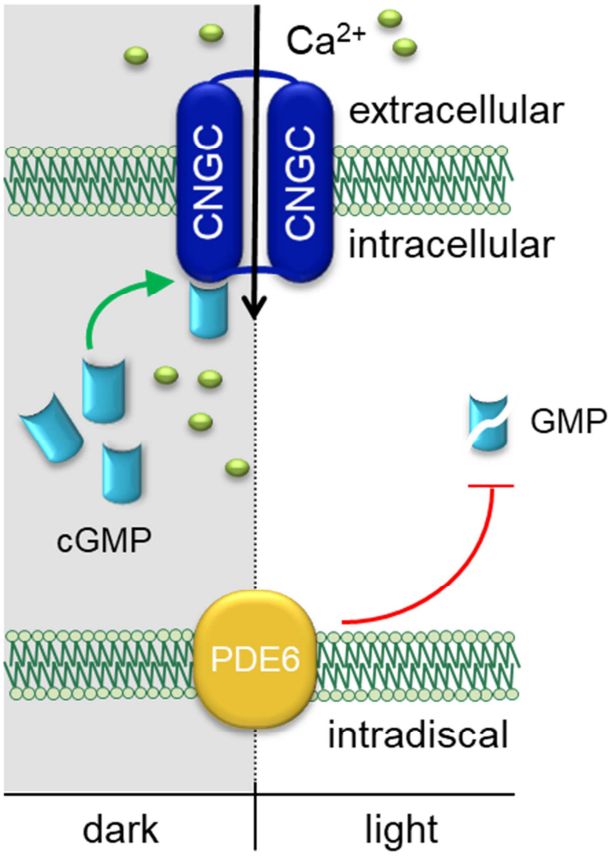

Figure 3: Regulation of cGMP in dark and light. Schematic drawing representing the location and interaction between phosphodiesterase-6 (PDE6), cGMP, and the cyclic nucleotide gated channel (CNGC). In photoreceptor outer segments, in the dark (left side), high levels of cGMP open CNGC and allow for influx of Ca^{2+} ions. In light (right), PDE6 in photoreceptor disc membranes is activated and hydrolyses cGMP to GMP. This in turns leads to the closure of CNGC and a decrease of intracellular Ca^{2+} levels.

In the dark high levels of cGMP bind to and open the prototypic phototransduction target, the CNGC, located in the outer membrane of the photoreceptor outer segments. CNGC opening allows for an influx of Na^+ and Ca^{2+} into the outer segment, yet, at the same time Ca^{2+} ions are constantly extruded via the $Na^+/Ca^{2+}/K^+$ exchanger (NCKX). This continuous influx and outflow of ions in the absence of light is referred to as the dark current (Hagins et al., 1970; Vinberg et al., 2018a). When a photon of light hits rhodopsin it sets in motion the sequential activation of the G-protein transducin and the enzyme PDE6. PDE6, which is located to the membranous disks within photoreceptor outer segments (Figure 3), hydrolyses cGMP, leading to the closure of CNGC and the subsequent hyperpolarization of the cell, which, in turn, leads to the cessation of glutamate release at the photoreceptor synapse (Kolb, 2003). Closing of CNGC also lowers outer segment Ca^{2+}-levels, stimulating cGMP production via retGC and GCAP (Figure 4) (Burns et al., 2002; Olshevskaya et al., 2002). In each step of the phototransduction cascade the original light signal is massively amplified, resulting in the remarkable single-photon sensitivity of rods (Hagins et al., 1970).

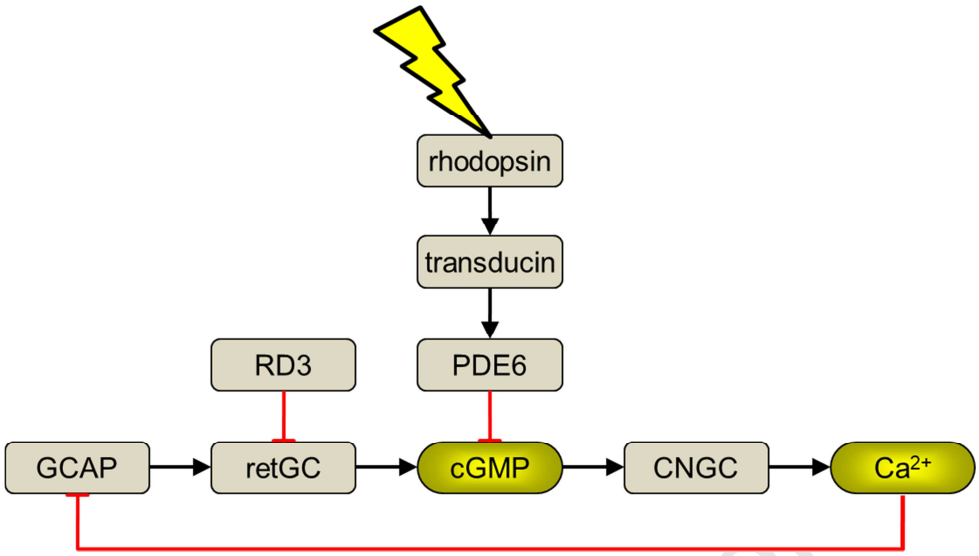

Figure 4: Feedback regulation of photoreceptor cGMP and Ca^{2+} levels. The conformational change caused when a photon of light strikes a rhodopsin molecule sequentially activates transducin and phosphodiesterase-6 (PDE6), which in turn hydrolyses cGMP. Guanylyl cyclase activating protein (GCAP) is regulated by Ca^{2+} in its capacity to activate retinal guanylyl cyclase (retGC). retGC produces cGMP which opens the cyclic nucleotide gated channel (CNGC), allowing for influx of Ca^{2+}. retGC is additionally regulated by the RD3 protein. Importantly, mutations in any of the genes encoding for these proteins can cause RD.

Seminal research performed already in the 1970s, established that high levels of cGMP were associated with and likely causal for photoreceptor degeneration (Farber and Lolley, 1974; Lolley et al., 1977). How exactly RD mutations led to the rise of photoreceptor cGMP levels became clearer when some of the first disease-causing mutations were discovered in the PDE6 α and β genes ((Huang et al., 1995; McLaughlin et al., 1993). Since then it has become evident that many other gene mutations can affect cGMP and Ca^{2+} signalling in very similar ways.

For instance, the G86R point-mutation in the *GCAP1* gene alters the Ca^{2+}-dependent regulation of retGC in a way that leads to increased cGMP production, causing a dominant cone-rod degeneration (Peshenko et al., 2019). Moreover, a disruption of the cGMP – Ca^{2+} negative feedback loop (Figure 4) (Burns et al., 2002; Olshevskaya et al., 2002), as triggered for example by mutations in CNGC genes (Biel and Michalakis, 2009; Paquet-Durand et al., 2011; Reuter et al., 2008), will permit cGMP levels to rise to extremely high and apparently photoreceptor toxic concentrations. A similar effect is produced by loss-of-function mutations in the *RD3* gene also causing high cGMP levels and photoreceptor death (Peshenko et al., 2016).

2. 5. Ca^{2+} and CNGC in photoreceptor degeneration

The precise regulation of intracellular Ca^{2+} levels is critical for neuronal survival in general (Yamashima, 2004) and, correspondingly, for almost 20 years Ca^{2+} channels have been studied as potential targets for RD therapy. The general hypothesis is that an excessive activation of Ca^{2+} channels causes Ca^{2+} overload inside the cell and triggers photoreceptor

cell death (Fox et al., 1999; Orrenius et al., 2003). According to this hypothesis, Ca^{2+} channel blockers should in principle be able to prevent or delay photoreceptor death.

In photoreceptors there are two major sources for Ca^{2+} influx: 1) CNGC in the outer segment, and 2) voltage gated Ca^{2+} channels (VGCC) located in the photoreceptor's synapse (Van Hook et al., 2019). A seminal study by Frasson and colleagues (Frasson et al., 1999) suggested the use of D-*cis*-diltiazem – a registered drug used to treat hypertension – to prevent *rd1* mutant rod degeneration and to preserve cone visual function. Since D-*cis*-diltiazem was known to target the voltage-gated Ca^{2+}-channels (VGCC) in the photoreceptor synapse, the authors assumed a deleterious Ca^{2+}-influx to cause photoreceptor death and that this influx occurred mainly via synaptic VGCC. However, a number of follow-up studies were unable to reproduce the proposed protective effects of D-*cis*-diltiazem (Bush et al., 2000; Pawlyk et al., 2002; Read et al., 2002) reviewed in (Barabas et al., 2010). Diltiazem exists in two enantiomers and commercially available preparations usually contain mixtures of D-*cis*- and L-*cis*-diltiazem. Importantly, while D-*cis*-diltiazem targets VGCCs in the photoreceptor synapse (Hart et al., 2003), L-*cis*-diltiazem targets CNGCs in the photoreceptor outer segment (Haynes, 1992; Stern et al., 1986). In light of these facts, it is plausible to think that the protective effects observed by Frasson and colleagues were in fact not due to D-*cis*-diltiazem acting on VGCC, but instead due to L-*cis*-diltiazem acting on CNGCs. This idea would go well with observations from two later studies performed with genetic knock-out models: After the genetic deletion of rod VGCCs in *rd1* mice, there was essentially no rescue of photoreceptor viability or function (Schon et al., 2016). By contrast, when CNGCs were genetically inactivated in *rd1* mice, there was a marked improvement of rod viability and cone function (Paquet-Durand et al., 2011). These results strongly suggest the inhibition of CNGC as a viable therapeutic approach, at least in those patients where the causal mutation does not affect CNGC genes.

An important problem in targeting CNGC, however, is the isoform specificity. In recessive forms of RP, the disease-causing mutations typically abolish rod function. Hence, an inhibition of rod CNGC could likely be pursued with impunity, without further reduction in retinal function. However, the cones in RP are genetically functional and the inhibition of cone CNGC would decrease the remaining cone vision, likely aggravating a patient's visual impairment. Thus, a therapeutic approach aimed at inhibiting CNGC must have a strong isoform specificity, inhibiting rod CNGC while leaving cone CNGC functional. Pharmacological approaches thus far have not yielded such a strong isoform specificity, even though cGMP analogues – especially dimers or tetramers (Kramer and Karpen, 1998; Vighi et al., 2018b) – could in principle be developed to show such a strong specificity.

An alternative approach to pharmacological CNGC inhibition could be its genetic downregulation. CNGC is a heterotetramer comprised of two different subunits, an α and a β subunit. Although the function of the channels is similar in both rods and cones, they are encoded by different sets of genes. In rods, CNGC is encoded for by *CNGA1* and *CNGB1* genes, with the respective proteins assembled in the ratio of 3:1, while in cones it is encoded by *CNGA3* and *CNGB3*, and was for some time thought to be assembled in the ratio of 2:2 (Biel and Michalakis, 2009). However, more recent studies suggest the CNGA3 to CNGB3 ratio to be the same as in rods, *i.e.* 3:1 (Ding et al., 2012).

Since loss-of *Cngb1* expression in the mouse (Huttl et al., 2005; Paquet-Durand et al., 2011) causes rod CNGC function loss and protects *rd1* photoreceptors, it is possible that even a relatively incomplete knock-down of *CNGB1* will significantly reduce rod CNGC

function, Ca^{2+} influx, and photoreceptor cell death. This raises the possibility to develop knock-down approaches targeting the single CNGB1 subunit in rods. However, the structural organisation of *CNGB1* gene is somewhat complex and displays a unique bipartite structure in which 33 exons also encode glutamic acid rich protein (GARP) as a result of alternative splicing (Ardell et al., 2000; Korschen et al., 1995; Sugimoto et al., 1991). GARP interacts with the structural protein peripherin-2 to connect photoreceptor outer segment disks to the plasma membrane, a function that is also critical for photoreceptor function and survival (Goldberg et al., 2016). Therefore, a molecular approach targeting *CNGB1* must leave the GARP portion of the gene intact.

Paradoxically, both RP and ACHM can be caused by mutations in rod or cone CNGC subunits (Bareil et al., 2001; Johnson et al., 2004; Michalakis et al., 2014; Muhlfriedel et al., 2017). The likely explanation is that in the absence of functional CNGC the negative Ca^{2+}-mediated feedback on retGC is missing, allowing for an excessive production of cGMP up to toxic levels (see chapter 2. d.). Any therapeutic strategy focusing on inhibition or down-regulation of CNGC will thus have to carefully titrate the positive effects of reducing Ca^{2+} influx against the negative effects of low Ca^{2+} and unbalanced cGMP production.

Yet another alternative therapeutic strategy to pharmacological Ca^{2+} channel inhibition may be to increase Ca^{2+} extrusion. Photoreceptors express plasma membrane Ca^{2+} ATPase (PMCA) pumps to extrude Ca^{2+} from photoreceptor cells (Johnson et al., 2007). PMCA is activated by the neurotrophic factor pigment epithelium-derived factor (PEDF), and a recent study showed that PEDF could reduce intracellular levels of Ca^{2+} and protect photoreceptors from cell death (Comitato et al., 2018).

Regardless of what strategy is pursued to lower photoreceptor Ca^{2+}-levels, it is important to consider the down-stream effectors of Ca^{2+}-signalling. Apart from GCAP (see chapter 2.4) these may include a number of different kinases, including calmodulin-kinases (Hauck et al., 2006) and kinases belonging to the protein kinase C (PKC) family (Azadi et al., 2006). However, as detailed in the next chapter, Ca^{2+}-activated calpain type proteases may be even more critical for photoreceptor degeneration.

2. 6. Downstream of Ca^{2+}: calpain-type proteases

Calpains are a family of cytosolic cysteine proteases whose enzymatic activities are dependent on Ca^{2+}, in the sense that while they are expressed in every cell, the calpains are not constitutively active but rather are activated in a number of steps by Ca^{2+} (Suzuki et al., 2004). To date, 15 calpain isoforms have been discovered in mammals and the calpain family can be subdivided into typical (calpain 1, 2, 3, 8, 9, 11, and 12) and atypical calpains (calpain 5, 6, 7, 8b, 10a, and 15) (Huang and Wang, 2001; Suzuki et al., 2004). Calpain isoforms have been implicated in cellular functions such as signal transduction, cell cycle, proliferation, differentiation, migration, apoptosis, membrane function, formation of muscle fibres, dendritic spine formation and pruning, and many others (Goll et al., 2003; Kanamori et al., 2013; Smalheiser and Lugli, 2009; Suzuki et al., 2004).

Calpains could be involved in a variety of the physiological changes seen during necrosis and necrosis-like forms of cell death (see chapter 3. b. below). This includes calpain-mediated alterations in cellular membrane permeability which are thought to occur in three stages, with each stage showing increasing permeability, from permeability to propidium iodide in stage one, to allowing the release of the 130kDa lactate dehydrogenase (LDH)

enzyme in stage three (Chen et al., 2001). Increases in calpain proteolytic activity have been linked to the increased plasma membrane permeability, such that hydrolysis of paxillin, a cytoskeletal protein, occurs at the same time as propidium iodide entry, while hydrolysis of the cytoskeletal proteins talin and vinculin occurs concomitantly with LDH release (Liu et al., 2004).

Furthermore, calpain promotes disruption and hyperpermeability in the blood-brain barrier (BBB) through disruption of the tight junctions *in vitro* (Alluri et al., 2016). Calpain mediated dysfunction of the BBB was induced by interleukin-1β (IL-1β) and this was abolished with calpain inhibition (Alluri et al., 2016). IL-1β is a marker for inflammation in mammalian tissues and the involvement of calpain in an inflammatory response (also associated with oncosis) suggests yet more links between calpain and necrotic cell death.

Apoptosis is a programmed form of regulated cell death carried out by the caspase family of proteins (discussed below). The role of calpains in apoptosis is difficult to fully elucidate due to a combination of improper nomenclature and the use of calpain inhibitors which also inhibit other molecules governing the apoptosis pathway. Especially the interactions between caspases and calpains are complex to understand. Caspases are proteolytic enzymes with a particular role in programmed cell death (see chapter 3. b. below). Calpains cleave caspase-7, -8 and -9, and by doing so inactivate capase-7 and -8 (Chua et al., 2000), which may then be seen as an anti-apoptotic action. Indeed, given that a regulatory step in the formation of the necrosome is the inhibition of caspase-8 (Geng et al., 2017; Oberst et al., 2011) the cleavage of caspase-8 by calpains may be seen as a pro-necrotic action by the protease. By contrast, calpain-2 has been suggested to be a promotor of apoptosis by cleaving and activating pro-caspase-12, and by cleaving the loop region of the large BCL isoform, BCL-XL, and changing it from an anti-apoptotic molecule into a pro-apoptotic molecule (Nakagawa and Yuan, 2000).

Activation of calpains, specifically of calpain-1 and -2, was linked to increased intracellular Ca^{2+} in several models of RD caused by increased cGMP or protein misfolding (Arango-Gonzalez et al., 2014; Comitato et al., 2016; Comitato et al., 2014). With respect to such activation, a reduction of the expression levels of calpastatin, the endogenous inhibitor of calpains, was observed in the *rd1* mouse model (Paquet-Durand et al., 2006). neuroprotection of the retina of *rd1*, *Rho*$^{-/-}$ mice as well as mice expressing the P23H mutant rhodopsin has been reported with several calpain inhibitors (Comitato et al., 2016; Comitato et al., 2014; Paquet-Durand et al., 2010). Treatments with the calpastatin peptide, a peptide derived from the endogenous inhibitor calpastatin, showed the best protection results on *rd1* mutant retinas (Paquet-Durand et al., 2010) when it comes to calpain inhibition so far.

2. 7. cGMP and the activity of protein kinase G (PKG)

Controlled elevation of cGMP has been discussed extensively as a strategy for neuroprotection, notably via its activation of cGMP-dependent protein kinase (PKG) and its effect on regulating gene expression (Pilz and Broderick, 2005). Protection could, for instance, be achieved by using PDE inhibitors to prevent cGMP hydrolysis and thus to keep its level high (Heckman et al., 2018). An equivalent protective effect might be obtained by a nitric oxide (NO) donor (Mejia-Garcia and Paes-de-Carvalho, 2007), since it is well established that NO activates a soluble guanylyl cyclase (sGC), increasing cGMP levels and activating PKG (Hofmann et al., 2006).

Yet, at the same time we know that cGMP can be detrimental to neurons, as it may cause neuronal cell death when raised (Canals et al., 2003; Canzoniero et al., 2006; Gonzalez-Forero et al., 2007). Perhaps the retinal photoreceptors represent the most well described case of such a cGMP-dependent neuronal calamity, since high levels of cGMP have for a long time been related to the degeneration of these cells (Farber and Lolley, 1974; Lolley et al., 1977). We may therefore ask whether the photoreceptors have some particular features that put them in this situation.

An aspect that distinguishes cGMP-signalling in photoreceptors from that of (most) other neurons is the presence of the CNGCs and the Ca^{2+} influx they mediate, with the possibility that the elevated cGMP leads to activation of for example calpains (as detailed above). Another photoreceptor-specific feature is the very rapid turn-over rates of cGMP, which are at least 10-fold higher here than in any other cell type (Granovsky and Artemyev, 2001; Pugh and Lamb, 1990, 1993). This high turn-over is most likely mandated by the necessity for extreme sensitivity in photon capture. In fact, in photoreceptor outer segments retGC was found to have a synthesis rate allowing for a cGMP concentration change of 600 µM/s (Peshenko et al., 2011), while a single PDE6 enzyme, at its Vmax rate, can hydrolyse approx. 5000 cGMP molecules/s (Leskov et al., 2000). The high cGMP turn-over may therefore help explaining why even seemingly minor alterations in cell physiology can cause photoreceptor degeneration, while leaving most other cells of the body unharmed. Along these lines, even a small alteration of the hydrolytic capacity of the photoreceptor's PDE6 actions, *e.g.* by any perturbation of the phototransduction cascade, would most likely produce a large deviation of the cellular cGMP level. The same would hold true for changes in retGC activity, and in both cases this could be expected to add stress to the photoreceptor when it tries to regain homeostasis or when it responds to the new cGMP levels by altering the activity of the downstream components of the cGMP signalling.

A critical question then is how the high cGMP would be able to exert any negative effects. *i.e.* what kind of downstream effector(s) can we surmise? Even though the regulation of CNGCs by cGMP is a key process in phototransduction, and as such could affect cellular Ca^{2+} balance, the prototypic target for cGMP is PKG. Excessive activation of PKG is known to cause cell death in different cancer cell lines (Deguchi et al., 2004; Hoffmann et al., 2017; Vighi et al., 2018a). On the other hand, PKG inhibition can have cytoprotective effects (Brunetti et al., 2002). Indeed, the effects of PKG activation in different types of cancer are complex: Activation of PKG1α and PKG1β appears to favour cancer progression (Arozarena et al., 2011; Dhayade et al., 2016), while the activation of the PKG2 isoform had anti-tumour effects (Hoffmann et al., 2017; Vighi et al., 2018a). The situation with respect to how PKG activity affects cancer cell death is thus not a straightforward decision between death or protection.

The PKG type kinases are also the dedicated effectors of the NO – sGC – cGMP – PKG signalling pathway (Hofmann et al., 2006). In the nervous system, an overactivation of this pathway has been associated with neuronal cell death (Canals et al., 2003; Canzoniero et al., 2006; Gonzalez-Forero et al., 2007), suggesting that PKG activity is a candidate for being key to at least some neurodegenerative events. Yet, it is not clear which PKG isoform mediates these responses. Furthermore, while in the inner retina neurons express sGC and respond to NO stimulation with a rise in intracellular cGMP levels, the photoreceptors in stark contrast to this appear to respond with a NO dependent decrease in cGMP (Gotzes et al., 1998; Wei et al., 2012b), which in some yet unknown way probably relates to their lack of

sGC (Wei et al., 2012b). Together this makes it unlikely that NO signalling contributes to photoreceptor PKG activity, which also re-emphasizes the peculiarity of the photoreceptors among neuronal cells in general.

Even though NO-signalling is unlikely to be responsible for high cGMP in photoreceptors, there are good reasons to believe that PKG is taking a central role in photoreceptor degeneration. For instance, the treatment of wild-type retina with cGMP analogues that will selectively activate PKG was found to cause strong photoreceptor degeneration (Paquet-Durand et al., 2009). On the other hand, cGMP analogues with PKG inhibitory actions were very efficient in protecting the degenerating photoreceptors of several RP models (*i.e.* in *rd1*, *rd2*, and *rd10* mice) (Paquet-Durand et al., 2009; Vighi et al., 2018b). Even so, the degenerative process is not completely stopped when such analogues are used. This could be due to insufficient inhibition of PKG, or the differential contribution of specific PKG isoforms to photoreceptor degeneration. Moreover, we cannot exclude the execution of additional PKG-independent cell death mechanisms. The latter would correspond to earlier findings on multiple cell death mechanisms being triggered concurrently during retinal degeneration and which could also include CNGC activation (Arango-Gonzalez et al., 2014; Gomez-Vicente et al., 2005; Sancho-Pelluz et al., 2008). Nevertheless, the connection between cGMP and RD (see Table 1) and of the clear protective effect of PKG inhibiting analogues (Paquet-Durand et al., 2009; Vighi et al., 2018b), point to a major importance of PKG-dependent cell death mechanisms in photoreceptor degeneration.

3. The diversity of neuronal cell death mechanisms and their relevance for photoreceptor degeneration

Neuronal cell death can be triggered by a variety of stimuli, resulting in the execution of different cell death pathways that are highly adapted to the type of stimulus, developmental stage, energetic status, pathogen load, *etc*. (Leist and Jaattela, 2001). This leads to a relatively large number of different routes for cell death, with clear ramifications for anti-cell death therapy developments (Kepp et al., 2011). These different cell death mechanisms may in fact not be clearly delineated pathways but a continuum of processes and metabolic subroutines, the boundaries between which may be hard to define (Galluzzi et al., 2018). The information on such pathways and processes has been obtained from several biological systems, and there seems to be a remarkable consistency throughout the various organisms. Yet it appears reasonable that different systems can have quite different prerequisites, and certain findings may therefore not always be applicable to every cell and situation. Moreover, in pre-clinical RD research, a further confounding factor arises in which early mutation-induced degeneration often coincides with developmental processes as well as with secondary and tertiary degenerative processes in certain animal models (Sancho-Pelluz et al., 2008). What we see in animal models therefore has the potential to be somehow different from the situation in patients.

In this chapter, we give an overview of the topic, from the evolution of cell death, to details and differences between mechanisms, to finally address the question of timing of the degenerative processes in individual neurons. Nevertheless, the reader should bear in mind that our knowledge on photoreceptor death specifically is still limited and that the information given in this review can thus not be considered exhaustive.

3. 1. On the evolution of cell death mechanisms

Until the 1950s, cell death was thought to be a biological accident that occurred when a cell was physically destroyed (trauma, intoxication, disease, *etc*.) and the term “necrosis” was introduced for this (Glucksmann, 1951). In the following decades other forms of cell death unequivocally different from necrosis, driven by intrinsic programs, were increasingly recognized as fundamental biological processes. This view was formalized in the early 1970s by the works of Kerr, Wyllie, and Currie who coined the term “apoptosis” for a specific type of programmed cell death (Kerr et al., 1972).

Because of the importance of apoptosis for ontogenesis in multicellular organisms, programmed cell death was initially thought to have evolved when life on earth became multicellular, *i.e.* about 1 billion years ago (Vaux et al., 1994). However, programmed cell death evolved much earlier, since even single celled eubacteria – the oldest and still living life form known – have the ability to undergo programmed cell death (Ameisen, 2002). But to what benefit would single-cell organisms kill themselves? One possible explanation is that when colonies get too big, bacteria use the so-called quorum sensing, to trigger cell death in the colony centre, while cells at the rim will survive (Kaiser, 1996). Without access to orderly cell death, resource depletion would cause the demise of the entire colony and possibly the extinction of the species (Fiegna and Velicer, 2003). Correspondingly, in multicellular organisms, the death of individual cells can promote the survival of the organism.

In the context of this review it is interesting to note that cyclic-diguanylate (c-di-GMP), a signalling molecule used already by bacteria (Jenal et al., 2017), serves as a trigger of cell death in single-celled eukaryotes (Luciani et al., 2017). We may speculate that in higher eukaryotes some of the signalling functions of c-di-GMP, including the induction of cell death, may have been taken over by cGMP-signalling (chapter 3.4).

As the mechanisms of cell death likely evolved over billions of years, they are hard-wired into the genomes, possibly with manifold "back-up systems", and hence difficult to overcome. The multitude of new, non-apoptotic cell death pathways discovered in the last decade attests to the diversity of these mechanisms (Galluzzi et al., 2018). Together, this may explain why in neurodegenerative diseases programmed cell death has proven hard to defeat by modern therapeutics and highlights the need for in depth studies to resolve the underlying mechanisms.

3. 2. The classical mechanisms: Necrosis and Apoptosis

Since the 1950s the definition of necrosis has been updated numerous times and the original description (Glucksmann, 1951) would today be related to as "accidental cell death". For necrosis the general understanding now appears to be that it is a death process that is mostly passive, involves cell and/or tissue swelling, and is typically associated with a subsequent inflammatory response at the site of the event (Edinger and Thompson, 2004). In line with this view, a more recent definition labels necrosis as "cell death caused by loss of membrane integrity, intracellular organelle swelling and adenosine-triphosphate (ATP) depletion leading to an influx of Ca^{2+} (Cullen, 2010). This influx of Ca^{2+} has been associated with the activation of Ca^{2+} -dependent calpain-type proteases and the disruption of the cellular cytoskeleton (Liu et al., 2004).

The conceptual counterpart to necrosis is apoptosis, which is a genetically regulated, ATP-dependent, and finely tuned process of cell elimination essential for tissue maintenance, embryogenesis, and development (Kerr et al., 1972), and may thus be seen as a type of physiological cell death. As such, apoptosis plays an essential part in normal development and is a mechanism that is highly conserved between organisms as diverse as nematodes, insects, and humans (Twomey and McCarthy, 2005). Hence, our understanding of the mechanisms involved in apoptosis in mammalian cells comes largely from the investigation of programmed cell death in the nematode *Caenorhabditis elegans* (Ellis and Horvitz, 1986). Cell death by apoptosis is characterised by a series of events including cell collapse, formation of membrane blebs, chromatin condensation, and DNA fragmentation (Kerr et al., 1972). The fragmentation of DNA is a feature that is often used for the visualization of dying cells using the terminal-deoxynucleotidyl-transferase dUTP-nick-end-labelling (TUNEL) technique. This technique was originally highlighted as specific for the detection of apoptosis (Gavrieli et al., 1992), but it soon became evident that the TUNEL technique detects a variety of other forms of cell death, including necrosis, with similar efficiency (Grasl-Kraupp et al., 1995).

Due to the shrinkage of the cells during apoptosis, the cytoplasm appears denser and the organelles more tightly packed than in unaffected cells. The apoptotic process occurs without inflammation or tissue swelling for three reasons: 1) apoptotic cells do not release their cellular contents into the surrounding tissue, 2) they are quickly phagocytosed by surrounding cells, and 3) the engulfing cells appear to produce neither pro- nor anti-

inflammatory cytokines (Haslett et al., 1994; Kurosaka et al., 2003). Apoptosis as such is usually set in motion by an intrinsic signal (*e.g.* lack of trophic factor support) which leads to the expression of pro-apoptotic genes and proteins, including of caspase-type proteases (Kroemer et al., 2005). The caspase family has at least 14 members, that when expressed are found in cells as pro-enzymes before activation (Chan and Mattson, 1999). Other proteins act on the mitochondrial integrity, such as proteins of the so called BCL2 family, which in a process coined mitochondrial outer membrane permeabilization (MOMP) form a pore across the outer membrane of the cell's mitochondria. MOMP is a critical event during apoptosis as it allows the release of mitochondrial proteins into the cytoplasm. Among such released proteins is cytochrome c, which, when in the cytoplasm, can aggregate with apoptotic protease activating factor 1 (APAF1) and caspase-9, that acts as an initiator caspase, to activate down-stream executioner caspases, such as caspase-3 (Figure 5). This proteolytic cascade then allows for a rapid degradation and clearance of the dying cell (Galluzzi et al., 2018). While caspase activity is considered necessary for the completion of apoptosis, some works suggests that the formation of the MOMP is indeed the critical step in the process of apoptotic cell death (Tait and Green, 2010).

The ATP-dependency of apoptotic processes – notably the activation of caspase-type proteases (Liu et al., 1996) – provides for a possible switch between apoptotic and necrotic forms of cell death, *i.e.* in the absence of ATP, necrosis or necrosis-like forms of cell death might be favoured (Leist et al., 1997). Remarkably, caspases are also targets for proteolytic cleavage by calpains. Such calpain cleavage, however, causes caspase inactivation and may provide for another molecular switch between necrotic and apoptotic forms of cell death (Chua et al., 2000; Lankiewicz et al., 2000).

In consideration of the photoreceptor cell death in RD, this was for a long time thought to be governed by apoptosis based mostly on the observation of DNA fragmentation (Chang et al., 1993), the absence of inflammation and clumping of dying cells (Sancho-Pelluz et al., 2008), and the fact that photoreceptors are lost by an intrinsic, cell autonomous process (Clarke et al., 2000a). However, the views on this have changed over the past decade (Arango-Gonzalez et al., 2014), not the least since there has at the same time been a rising awareness of alternative cell death mechanisms, decidedly different from both necrosis and apoptosis (Galluzzi et al., 2018). Interestingly, the discovery of caspase-independent forms of regulated, program-driven cell death, including in photoreceptors, was one of the first clues as to the existence of further non-apoptotic and non-necrotic degenerative mechanisms (Donovan and Cotter, 2002; Kroemer and Martin, 2005)

3. 3. Brief overview of alternative cell death mechanisms

The use of the terms necrosis and apoptosis to describe cell death rather than specific forms of cell death led to considerable confusion in the scientific literature. In the late 90s/early 2000s several groups tried to define each form of cell death more clearly to build a proper nomenclature and these efforts eventually led to the formation of the Nomenclature Committee on Cell Death in 2005 (Kroemer et al., 2005). Here, we will briefly discuss two of the forms of cell death defined by this committee, necroptosis and PARthanatos, whereas the next section, 3.d, concentrates on cGMP-dependent photoreceptor degeneration.

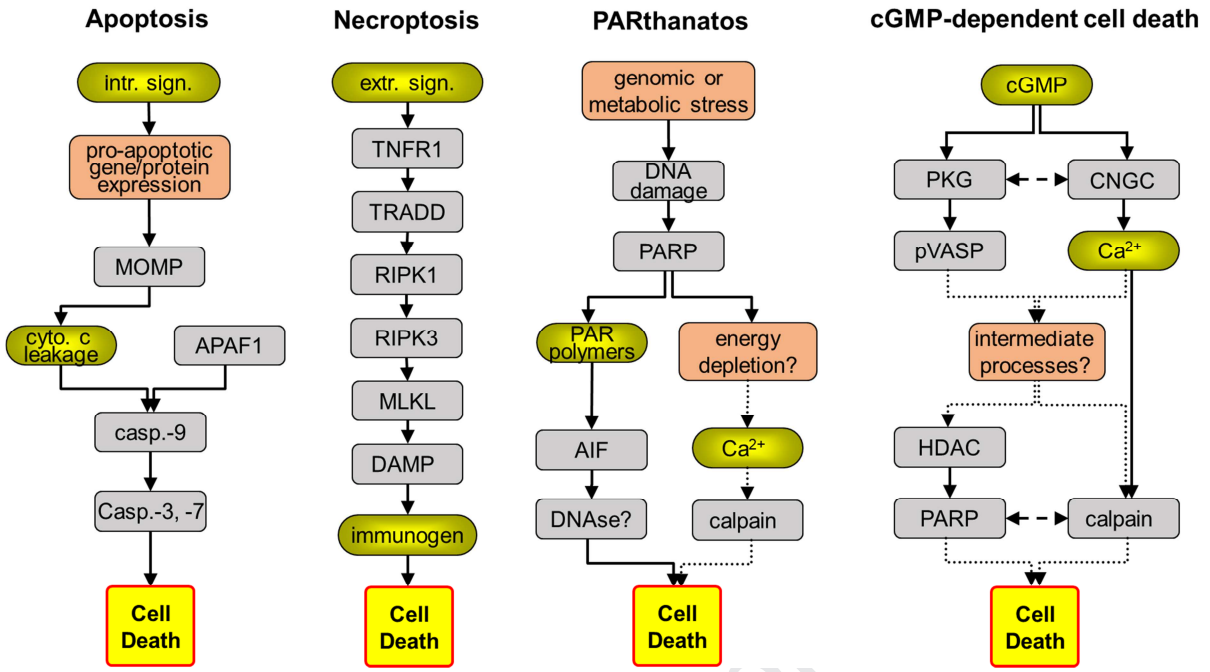

Figure 5: Comparison of different cell death mechanisms. Shown are mechanistic diagrams illustrating the cellular processes executed during apoptosis, necroptosis, PARthanatos, and cGMP-dependent cell death in the retina. See text for more details.

Classical apoptosis involves an intracellular signal (intr. sign.) generated expression of pro-apoptotic genes and proteins and the translocation of BCL2 family proteins to produce mitochondrial outer membrane permeabilization (MOMP). The resulting leakage of cytochrome c (cyto. c) from the mitochondria to the cytoplasm leads to its combination with apoptotic protease activating factor-1 (APAF1) and caspase-9 to activate executioner caspases, such as caspase-3 and -7.

Necroptosis is triggered by extracellular signals (extr. sign.) leading to activation of tumour necrosis factor receptor-1 (TNFR1), which when associated with its adaptor protein TRADD drives the activation of receptor interacting protein kinase-1 (RIPK1). RIPK1 activates RIPK3 and then mixed-lineage-kinase-domain-like pseudokinase (MLKL) resulting in the extracellular release of highly immunogenic damage-associated molecular patterns (DAMPs) and the production of a strong inflammatory response.

In PARthanatos genomic or metabolic stress and resultant DNA damage causes over-activation of poly ADP-ribose polymerase (PARP). PARP will produce poly-ADP-ribose (PAR) polymers and deplete cellular energy resources in the process. PAR polymers can apoptosis inducing factor (AIF) leading to DNA degradation, while energy depletion will induce increased levels of intracellular Ca^{2+} and activation of calpain-type proteases.

In cGMP-dependent photoreceptor cell death a mutation-induced up-regulation of cGMP on the one hand causes activation of cyclic-nucleotide-gated-channel (CNGC), leading to Ca^{2+} influx and calpain activation. On the other hand, cGMP-dependent activation of protein kinase G (PKG) is somehow (perhaps involving the phosphorylation of the PKG substrate VASP) associated with histone deacetylase (HDAC) and PARP activation. Importantly, cGMP-dependent photoreceptor cell death offers new targets for photoreceptor neuroprotection.

Yellow highlight indicates signalling molecules/processes; orange highlight indicates complex processes likely involving multiple proteins and molecules.

3. 3. 1. Necroptosis

Necroptosis, in a typical setting, is initiated by signalling of tumour necrosis factor (TNF) to tumour necrosis factor receptor-1 (TNFR1) (Conrad et al., 2016). TNFR1 when associated with the adaptor protein tumour necrosis factor receptor-1 death domain (TRADD) drives the activation of receptor interacting protein kinase-1 (RIPK1) (Hsu et al., 1996). This in turn sets in motion a cascade of events that activates RIPK3 and mixed-lineage-kinase-domain-like pseudokinase (MLKL) (Kaiser et al., 2013). MLKL forms oligomers, usually tetramers or trimers, which translocate to the plasma membrane, where they bind specific phosphatidylinositol phosphate species, triggering plasma membrane permeabilization resulting in the extracellular release of so-called damage-associated molecular patterns (DAMPs) (Trichonas et al., 2010). These DAMPs are strongly immunogenic and will produce a marked inflammation in the affected tissue (Figure 5). The latter is thought to be beneficial under conditions of a pathogen infection (*e.g*. viruses), but could be highly detrimental if executed in healthy tissue (Kaczmarek et al., 2013). Even though the activation of RIPK3 is considered a key element of the necroptotic pathway (Galluzzi et al., 2018), caution is necessary when trying to qualify a certain observation of cell death, since to date necroptosis can be confirmed only indirectly by plasma membrane rupture concomitant with an absence of caspase activation. In the retina necroptosis was reported to occur as a response to injuries related to the activation of microglia (Huang et al., 2018).

3. 3. 2. PARthanatos

PARthanatos is a form of cell death resulting from the hyperactivation of PARP1 (Galluzzi et al., 2018). PARthanatos may be triggered not only by excessive DNA damage but also by stressors such as, oxidative stress, hypoxia, hypoglycaemia or inflammatory cues (David et al., 2009; Virag and Szabo, 2002). PARP1 overactivation is thought to mediate cytotoxic effects through the depletion of NAD^+ and ATP, resulting in bio-energetic and redox collapse (Andrabi et al., 2008; Ha and Snyder, 1999; Sims et al., 1983). The ATP-depletion caused by excessive PARP activity will prevent Ca^{2+}-ATPases from further extruding Ca^{2+} (Guerini et al., 2005) and likely lead to rising intracellular Ca^{2+} levels. Similar to what was reported from necrosis-like forms of cell death, high Ca^{2+} will activate calpain type proteases, which indeed is a phenomenon that has been associated with PARthanatos in retinal photoreceptors (Prado Spalm et al., 2018).

Another consequence of PARP over-activation is an accumulation of PAR polymers (Fatokun et al., 2014), which can bind to the mitochondrial protein apoptosis inducing factor (AIF) (Moubarak et al., 2007). Upon its release from the mitochondria AIF can translocate to the nucleus where it in turn can activate DNAses, to further precipitate cell death (Wang et al., 2009). Interestingly, mitochondrial AIF release may be dependent on calpain activity (Polster et al., 2005). The execution of cell death in PARthanatos is thus driven by two PARP-dependent processes, energy depletion, as well as PAR accumulation and AIF release (Figure 5).

3. 4. cGMP-dependent photoreceptor cell death

All the cell death mechanisms mentioned above have been associated with photoreceptor degeneration in the past, in a variety of different RD disease models (Allocca et al., 2019; Chang et al., 1993; Liu et al., 1999; Rohrer et al., 2004). However, many genetically distinct forms of RD share a strong increase in the photoreceptor intracellular concentration of cGMP (Arango-Gonzalez et al., 2014; Farber and Lolley, 1974; Paquet-Durand et al., 2009). Together with a number of other findings, this has prompted us to propose a cGMP-dependent pathway for photoreceptor degeneration, in which high cGMP concomitantly activates CNGC and PKG, producing excessive Ca^{2+}-influx and protein phosphorylation, respectively (Arango-Gonzalez et al., 2014). As a possible consequence of the latter, PKG dependent phosphorylation could trigger HDAC activation (Hao et al., 2011), which appears to be upstream of PARP activation (Sancho-Pelluz et al., 2010). On the other hand, and likely in parallel, CNGC-mediated Ca^{2+}-influx can activate calpains (Kulkarni et al., 2016; Paquet-Durand et al., 2011; Wei et al., 2012b). Both of these two cGMP-dependent pathways, alone or in concert, may drive photoreceptor cell death (Figure 5). This cGMP-induced alternative form of cell death appears to be significantly slower than other forms of cell death (Sahaboglu et al., 2013) (see also 3.f below). Importantly, this pathway offers a number of new targets for therapeutic intervention, some of which appear early during the process, as with cGMP-signalling, while others act further down-stream, as with calpains, HDAC, and PARP.

In this context, it is worth noting that the pathways of cGMP-dependent photoreceptor cell death display some overlap with what is seen during PARthanatos. Both cell death mechanisms share an over-activation of PARP and accumulation of PAR (Paquet-Durand et al., 2007), likely associated with mitochondrial release of AIF (Sanges et al., 2006), as well as with excessive Ca^{2+} influx and calpain protease activation (Kulkarni et al., 2016; Vighi et al., 2018b). On the other hand, the upstream events of cGMP-dependent cell death appear to be different from PARthanatos and one question for future studies may be whether PARthanatos could be a “subroutine” of cGMP-dependent cell death.

3. 5. RD genes related with high photoreceptor cGMP

Because of this pathologic aspect of cGMP in photoreceptors, we hypothesized several years ago that interventions in cGMP-signalling might constitute a viable therapeutic avenue applicable to many different RD-causing mutations. While we were able to show that such interventions are indeed feasible from a pharmacological and drug delivery standpoint (Mencl et al., 2018; Paquet-Durand et al., 2009; Vighi et al., 2018b), a question that remained is how many RD causing mutations, in how many genes, and affecting how many patients, would actually be amenable to such a treatment. Here, an initial graphical overview of the relationship between certain RD genes and photoreceptor cGMP levels is given in Figure 6.

As detailed above (see chapter 2.d.), RD mutations in the genes encoding for PDE6 subunits (*PDE6A*, *PDE6B*, *PDE6C*, *PDE6G*, *PDE6H*) (Brennenstuhl et al., 2015; Dryja et al., 1995; Dvir et al., 2010; McLaughlin et al., 1993; Thiadens et al., 2009; Trifunovic et al., 2010) prevent cGMP hydrolysis and therefore lead to excessive accumulation in photoreceptors. Similarly, mutations in the aryl hydrocarbon receptor-interacting protein-like gene (*AIPL1*) (den Hollander et al., 2008) thwart the functional assembly of PDE6 enzyme dimers

(Ramamurthy et al., 2004), also causing cGMP accumulation. During phototransduction PDE6 is activated by transducin, a protein encoded by the *GNAT1* gene in rods and *GNAT2* in cones, explaining why certain mutations in these genes lead to insufficient cGMP hydrolysis (Kohl et al., 2002; Mejecase et al., 2016).

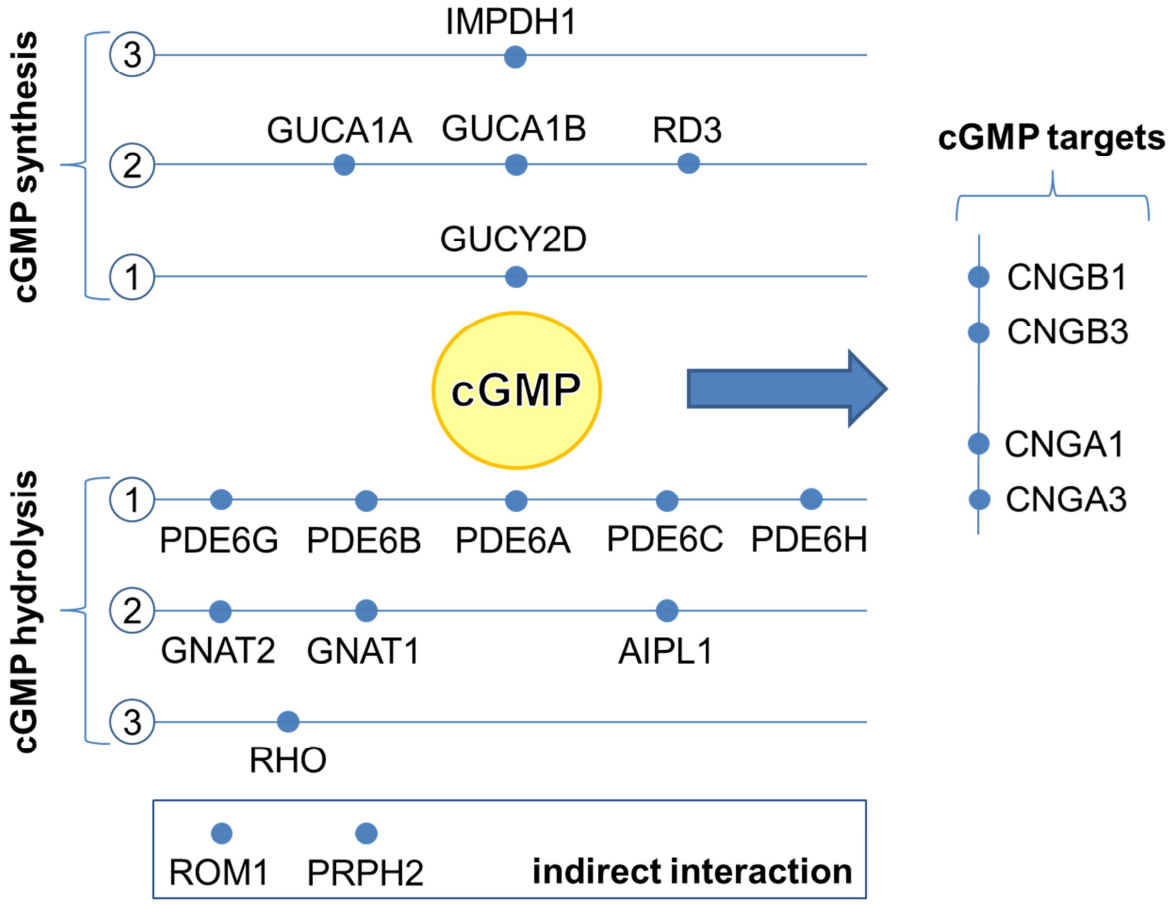

Figure 6: Relationship of RD genes to photoreceptor cGMP. The upper part of the diagram shows genes involved in cGMP synthesis, while hydrolysis genes are below. cGMP targets are shown on the right. The different lines indicate levels of interaction of the gene products with cGMP. Level 1 indicates direct binding, level 2 and 3 indicate second and third order interaction, respectively.

While impaired cGMP hydrolysis certainly leads to high cGMP levels in photoreceptors, excessive production may produce the same effect. Hence, gain-of-function mutations in retGC, a protein encoded by the *GUCY2D* gene (Sato et al., 2018), as well as in GCAP, encoded for by the *GUCA1A* and *GUCA1B* genes in rods and cones (Peshenko et al., 2019; Sato et al., 2005), respectively, may result in excessive production of cGMP. retGC is additionally inhibited by the RD3 protein, so that loss-of-function mutations in the *RD3* gene will also cause excessive cGMP production and photoreceptor death (Peshenko et al., 2016).

An accumulation of cGMP in photoreceptors is also observed in CNGC mutations in the genes *CNGA1*, *CNGA3*, *CNGB1*, and *CNGB3*, (Arango-Gonzalez et al., 2014; Huttl et al., 2005; Ma et al., 2013; Paquet-Durand et al., 2011; Reuter et al., 2008), presumably because of the lack of negative feedback on retGC and cGMP synthesis in the absence of Ca^{2+} influx in the outer segment (see chapter 2.d.) (Olshevskaya et al., 2002).

High cGMP is interestingly also observed in animal models suffering from mutations in several other genes not directly related to cGMP synthesis or hydrolysis. For instance, several different mutations in the gene encoding for rhodopsin (*RHO*), ranging from a full knock-out, to the P23H point-mutation, to the S334ter truncation, were all displaying high cGMP levels in photoreceptors (Arango-Gonzalez et al., 2014). While this does not allow to conclude that all of the currently known 150 *RHO* mutations (Athanasiou et al., 2018) cause cGMP accumulation in photoreceptors, it seems likely that it will be the case in a large number of these.

Photoreceptor cGMP accumulation was also found in mutations in the gene encoding for the outer segment structural protein peripherin (*PRPH2*) (Arango-Gonzalez et al., 2014; Paquet-Durand et al., 2009). Knowledge on the indirect effects of such gene mutations on photoreceptor cGMP may allow to infer the situation in yet other gene mutations. For example, mutations in *PRPH2* lead to an absence of outer segments, which likely leads to an ectopic and dysregulated expression of outer segment enzymes. Peripherin assembles with its ortholog retinal outer segment protein-1 (*ROM1*) to anchor photoreceptor disks to the outer membrane (Goldberg et al., 2016), and just as *PRPH2* loss-of-function, *Rom1* knock-out in the mouse causes outer segment disorganisation and shortening, and photoreceptor death (Clarke et al., 2000b). Because of its parallel functions with *PRPH2* it appears likely that also *ROM1* mutations will be associated with high cGMP, although this remains to be studied. Mutations in the inosine mono phosphate dehydrogenase-1 (*IMPDH1*) gene may likewise cause elevated cGMP levels, since the IMPDH1 enzyme catalyses the rate-limiting step of GTP production, the substrate employed by retGC for cGMP synthesis. RD-causing mutations in *IMPDH1* do not reduce enzyme activity (Aherne et al., 2004) but may affect negative regulation of the enzyme (Xu et al., 2008). This would lead to an increased production of GTP, which, given the comparatively high Michaelis constant of GC (Aparicio and Applebury, 1995), could result in higher than normal synthesis of cGMP.

Table 1: Disease genes likely associated with high photoreceptor cGMP-levels

No.	Gene	Full name	ACHM	LCA	RP	Reference
1	AIPL1	Aryl-hydrocarbon-interacting protein-like 1	X	5.3%	X	(den Hollander et al., 2008)
2	CNGA1	Cyclic-nucleotide-gated-channel A1	X	X	1%	(Dryja et al., 1995)
3	CNGB1	Cyclic-nucleotide-gated-channel B1	X	X	2%	(Hartong et al., 2006)
4	CNGA3	Cyclic-nucleotide-gated-channel A3	30-40%	X	X	(Johnson et al., 2004)
5	CNGB3	Cyclic-nucleotide-gated-channel B3	40-50%	X	X	(Kohl et al., 2005)
6	GNAT1	Guanine nucleotide binding protein, rod-specific transducin α subunit	X	X	0.26%	(Mejecase et al., 2016)

7	GNAT2	Guanine nucleotide binding protein, cone-specific transducin α subunit	1.8%	X	X	(Rosenberg et al., 2004)
8	GUCA1A	GCAP1; Guanylate cyclase activating protein	1.6%	X	X	(Gill et al., 2019) Dell'Orco et al., 2018
9	GUCA1B	GCAP2; Guanylate cyclase activating protein	X	X	2.3%	(Sato et al., 2005)
10	GUCY2D	Retinal-specific guanylate cyclase	X	11.7%	X	(den Hollander et al., 2008)
11	IMPDH1	Inosine mono phosphate dehydrogenase-1	X	8.3%	2%	(den Hollander et al., 2008; Kennan et al., 2002)
12	PDE6A	Phosphodiesterase-6 α	X	X	2-3%	(Dryja et al., 1999)
13	PDE6B	Phosphodiesterase-6 β	X	X	3%	(McLaughlin et al., 1993)
14	PDE6C	Phosphodiesterase-6 α (cone)	2%	X	X	(Grau et al., 2011)
15	PDE6G	Phosphodiesterase-6 gamma (rod)	X	X	1%	(Tsang et al., 1996)
16	PDE6H	Phosphodiesterase-6 gamma (cone)	0.3%	X	X	(Kohl et al., 2012)
17	PRPH2	Peripherin	X	2.2%	3.5-4.1%	(Dryja et al., 1997; Manes et al., 2015; Wang et al., 2013)
18	RHO	Rhodopsin	X	X	7.5-10%	(Hartong et al., 2006)
19	RD3	RD3	X	1%	X	(Li et al., 2009)
20	ROM1	Retinal outer segment membrane protein 1	X	X	1%	(Bascom et al., 1995)

	ACHM	**LCA**	**RP**
Total patient prevalence	**75-95%**	**28.5%**	**25-30%**

Apart from the RD-genes mentioned above, where a connection to high photoreceptor cGMP has either been demonstrated already or appears as very likely, it is reasonable to think that this will also be the case for other photoreceptor-specific genes. Candidates for such a relationship may be the transcription factors *CRX*, *NRL*, or *NR2E3*, which regulate the expression of genes linked to phototransduction (Pittler et al., 2004; Xu et al., 2013), and, when mutated, may eventually lead to lack of phototransduction activity and consequently increased cGMP. Likewise, mutations in genes involved in the trafficking of phototransduction proteins could result in aberrant cGMP production. For instance, the REEP6 protein may mediate the trafficking of retGC to the photoreceptor outer segment (Agrawal et al., 2017). Still, to date it is not known whether *REEP6* mutations cause RD via aberrant cGMP ectopic production in the photoreceptor cytoplasm. Additionally, high cGMP may be connected to the pathogenesis of Stargardt disease, an RD-type disease which is caused predominantly by mutations in the *ABCA4* gene (Gill et al., 2019). ABCA4 activity is essential for the shuttling of all-trans-retinal out of the photoreceptor disks so that it may be recycled by RPE cells (Lenis et al., 2018; Quazi et al., 2012). Accordingly, *ABCA4* mutations are associated with early impairments in the electroretinographic responses of the retina (Abed et al., 2018; Fujinami et al., 2013), indicating that insufficient recycling of retinal decreases phototransduction activity. It is tempting to speculate that this may then also entail a decrease in cGMP hydrolysis and an accumulation of cGMP over time.

Taken together, many RD genes are related, or likely related, to high levels of cGMP in photoreceptors. Most of these RD genes are connected to cGMP synthesis, hydrolysis, or are coding for CNGCs (Figure 6), while in some other cases the relation to cGMP appears to be more indirect. For an overview of RD genes connected to photoreceptor cGMP see Table 1, which for the ACHM, LCA, and RP disease groups also details the approximate percentages of patients suffering from mutations that are likely causing excess photoreceptor cGMP concentrations. While at the moment there may still be considerable uncertainty about the disease gene distribution in different patient cohorts, ethnicities, and geographical locations, it is remarkable that, in the numerically most important disease group – RP – up to 30% of the patient population appears to be related to high cGMP.

3. 6. On the temporal progression of cell death

The above chapters focused on the question as to ***what*** happens during neuronal cell death, yet, a key question that is frequently overlooked is ***how long*** the cell death process takes in an individual cell. Although this is a rather simple question, to address it experimentally has proven to be a difficult task (Henson and Hume, 2006; Skommer et al., 2010), especially when it comes to investigations in complex neuronal tissues such as the retina. Since the onset of neuronal cell death in a diseased tissue in most cases follows a stochastic, non-synchronised process over the entire cell population (Clarke et al., 2000a), there will be a widespread distribution of different death stages that may be hard to disentangle. During many of our studies on the mechanisms behind RD, we have therefore tried to analyse co-appearances (or lack thereof) of various markers, in order to focus on and understand the degeneration sequence for individual photoreceptors (Ekstrom et al., 2014; Farinelli et al., 2014; Paquet-Durand et al., 2006; Paquet-Durand et al., 2007; Sancho-Pelluz et al., 2010), rather than for the retina as a whole. In turn this has enabled us to suggest a chronology, or "order of appearance", for the degeneration components in a given photoreceptor (Figure 7). While this alone does not reveal the time needed for the

degeneration (but see below) it can provide important information on causalities.

The question on the duration of cell death is obviously connected to the underlying mechanisms, especially as different cell death pathways run on different timescales. For instance, necrosis is seen as a fairly rapid destruction of the cell, taking between a few minutes and 1-2h to complete (Zong and Thompson, 2006), whereas apoptosis as a program driven and orderly disintegration of the cell, is much slower, taking 6-18h to complete (Oppenheim, 1991; Wong and Hughes, 1987). Importantly, information on the time-course and sequence of degenerative events will define the temporal window-of-opportunity, with strong implications for future therapeutic strategies.

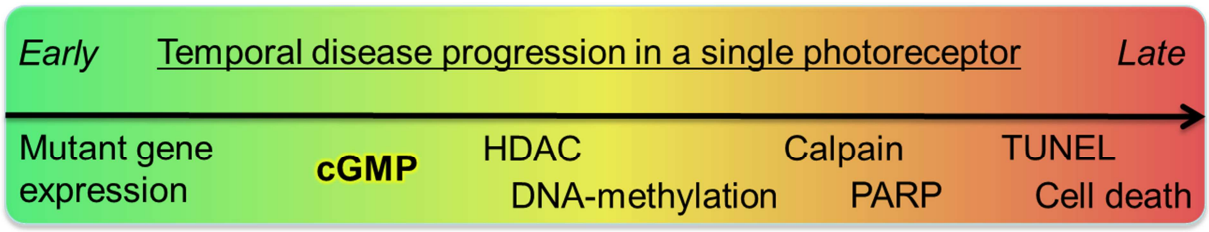

Figure 7: Players and supposed temporal order in *rd1* degeneration.

Overactivation of the cGMP system triggered by mutations in different genes promotes the disease. High cGMP triggers the execution of cell death, likely via concurrent activation of histone deacetylase (HDAC), calpain, DNA-methylation, and poly-ADP-ribose polymerase (PARP). Since the rise in cGMP occurs early in the degenerative process, targeting of the cGMP system gives a chance for protection in time. TUNEL refers to a technique detecting dying cells based on chromatin fragmentation.

In a previous study, we used the PDE6 inhibitor zaprinast (Zhang et al., 2005) in wild-type retina explant cultures to induce cGMP-dependent photoreceptor cell death in a relatively synchronized fashion (Sahaboglu et al., 2013). Curiously, even though zaprinast started to exert its inhibitory effect almost immediately after drug application (Wei et al., 2012b), it took about 36-48h before a clear rise in photoreceptor cGMP levels could be observed (Sahaboglu et al., 2013). This suggests that a photoreceptor cell can sustain PDE6 inactivation for 1-2 days, keeping cGMP levels within physiological limits, possibly via the cGMP – Ca^{2+} feedback regulation detailed in Figure 4 (Burns et al., 2002; Olshevskaya et al., 2002). Prolonged PDE6 inactivity may then alter photoreceptor metabolism in a way that leads to a catastrophic rise of cGMP, eventually causing the cell to die. Even so, after the strong rise in cGMP levels it took another 40h for a photoreceptor to activate DNA fragmentation (visualized by the TUNEL assay) and eventually disappear. With a period of about 80h – from initiation, to cGMP accumulation, to TUNEL positive reaction, to clearance – the time an individual cell needs to die is remarkably long in comparison with the times indicated above (a few minutes to 1-2h for necrosis, 6-18h for apoptosis), which therefore points towards execution of non-necrotic, non-apoptotic, and also relatively slow, cell death mechanisms in RD.

4. Targeting cGMP-signalling for therapy development

The finding that cGMP-dependent cell death may be the prevalent pathogenic mechanism in a large subset of RD patients raises the possibility to target this pathway for gene- and mutation-independent therapeutic purposes. Generally speaking, drugs that target cGMP-signalling have been marketed for decades (*e.g.* nitro-glycerine, Viagra) illustrating the feasibility of the concept. However, currently available drugs usually work by *raising* intracellular cGMP-levels, while the treatment of RD would in most cases require *inhibiting* cGMP-signalling. Prospective RD drugs could either inhibit cGMP synthesis directly or target cGMP-signalling indirectly by inhibiting its effectors. Furthermore, such drugs would have to do this in a highly photoreceptor specific manner, ideally discriminating between rods and cones.

4.1. Inhibiting cGMP synthesis

In a situation were too much cGMP causes cell death, an ideal treatment approach might be to reduce the synthesis of cGMP. However, because of the general importance of cGMP signalling in almost all cells of the body (Pilz and Broderick, 2005), a prospective drug will need to be highly specific for retGC in photoreceptors, without affecting other GCs elsewhere. In RP and other rod-cone dystrophies, this would mean that only rod GC should be inhibited, while cone GC should be unaffected. The key substrate that retGC requires for cGMP synthesis is GTP and in principle inhibitory GTP analogues such as *Rp*-GTPαS exhibit a very high specificity for retGC on *in vitro* enzyme preparations (Garger et al., 2001; Gorczyca et al., 1994). However, such trisphosphates, carrying three negative charges, are highly membrane impermeable. To drive such compounds into a photoreceptor cell, *in vivo*, will require a very specific and highly efficient drug delivery system (DDS), something that may still not be available for trisphosphates (but see 4.2 below for monophosphate cGMP analogues). Thus, currently, the direct targeting of retGC for therapeutic purposes does not appear to be feasible as long as no suitable DDS has been generated.

An alternative approach to reducing cGMP synthesis could be to inhibit upstream enzymes in the GTP synthesis pathway. One such target is IMPDH1, which catalyses the rate-limiting step of GTP production and likely produces most of the photoreceptor GTP (Aherne et al., 2004). IMPDH1 can be inhibited selectively by the registered immunosuppressive drug mycophenolate (Allison and Eugui, 2000). Accordingly, mycophenolic acid was suggested to reduce photoreceptor cGMP and to have protective effects in the *rd1* and *rd10* mouse models (ARVO2018 abstract (Yang et al., 2018)). If confirmed further, mycophenolate, or similar IMPDH1 targeting drugs, could potentially be developed into effective RD treatments, with the wealth of already available clinical data likely facilitating such repurposing.

4.2. Blocking the effects of cGMP-signalling

Other than inhibiting cGMP synthesis, a therapeutic agent may instead block the targets of cGMP-signalling, without reducing cGMP-levels. As stated above (chapters 2.5 and 2.7), in the photoreceptor these targets include the diseases drivers PKG and CNGC (Paquet-Durand et al., 2011; Paquet-Durand et al., 2009). For the inhibition of CNGC the same conditions as for targeting retGC would apply, namely a drug should be able to discriminate

between rod and cone CNGC, so as to preserve the functionality of cone phototransduction (chapter 2.5). PKG, on the other hand, is not known to exist in specific rod or cone variants (Ekstrom et al., 2014). In a situation where cGMP levels are very high in rods, but normal in cones, dampening PKG activity, with its signal amplified by downstream kinases and transcriptional activity (Pilz and Broderick, 2005), may provide for stronger leverage compared to drugs targeting retGC or CNGC. Inhibition of PKG additionally has the advantage that it is unlikely to interfere with phototransduction (Vighi et al., 2018b).

Intriguingly, the photoreceptor cGMP targets CNGC and PKG can be inhibited with very high specificity and selectivity by analogues of cGMP (Butt et al., 1990; Vighi et al., 2018b; Wei et al., 1998). Compared to trisphosphate GTP analogues, cGMP analogues carry only one negative charge and, by adding electronegative or lipophilic substituents, can be designed to have a high membrane permeability, enabling *in vivo* applications even without the use of dedicated DDS (Rapoport et al., 1982; Werner et al., 2011; Zhuo et al., 1994).

However, for a long time an important obstacle for the clinical development of cyclic nucleotide analogues was their rapid clearance via the kidney and their correspondingly very low bioavailability (Coulson et al., 1983; Schwede et al., 2000). Thus, for drugs aimed at photoreceptor proteins it is essential to use a suitable DDS that can deliver such compounds across the different retinal barriers to the photoreceptors, for prolonged periods of time (Himawan et al., 2019). With such DDS technology now becoming increasingly available, clinical development of cGMP analogues also becomes feasible. For instance, the European DRUGSFORD project (*i.e.* “drugs for RD”; www.drugsford.eu) generated over 80 novel inhibitory cGMP analogues and tested these together with a liposomal DDS to enable efficient and sustained delivery to the neuroretina. Notably, this combination resulted in the morphological and functional preservation in different pre-clinical RD models (Vighi et al., 2018b).

While these results highlight the potential of inhibitory cGMP analogues in forms of RD connected to high photoreceptor cGMP levels, a clinical proof-of-concept may be several years away still. Importantly, for all patients in such future clinical trials, the causative mutations and their connection to high photoreceptor cGMP must be clearly established prior to their enrolment (see chapter 5.2.1). To turn around the perspective, cGMP analogues activating PKG were shown to kill healthy, wild-type photoreceptors (Paquet-Durand et al., 2009), and have furthermore displayed significant anti-proliferative capacities in certain cancer cell lines (Hoffmann et al., 2017; Vighi et al., 2018a). It thus remains to be seen whether such activatory analogues could instead have protective capacity in situations where photoreceptor cGMP is too low, as may be the case in *GUCY2D* or *IMPDH1* loss-of-function mutations (Aherne et al., 2004; Williams et al., 2006).

5. The future of retinal degeneration research

In the final chapter of this review, we give an overview of the technological and methodological developments that we feel will be needed to forward a deeper understanding of the complexity of cell death and hereditary retinal degeneration. Another point will be to advance clinical testing, notably to develop clinical test strategies that fully consider the genetic heterogeneity of RD-type diseases and exploit it to enable faster and more meaningful clinical test results.

5. 1. What kind of methods do we need in the future for the study of photoreceptor cell death mechanisms?

A key problem of all current technical approaches is that these do not allow for a temporo-spatial resolution of the multitude of metabolic processes happening during cell death at the level of an individual dying cell. While single-cell RNA sequencing allows to study gene expression profiles in different cell types within a tissue (Peng et al., 2019), similar insight into cellular biochemistry is far more difficult to obtain.

To capture the processes governing photoreceptor cell death in their entirety, we will need to be able to analyse single cell metabolism, ideally following the fate of a single cell over prolonged periods of time. Single cell analysis at this level will likely require the development and use of novel technology, collectively referred to as hyperspectral imaging. Among the techniques that may be useful here in the near future are near-infrared (NIR) spectroscopy, fluorescence life-time-imaging (FLIM) (Dysli et al., 2018) and the combination of Raman spectroscopy with laser-confocal microscopy or Raman imaging (Gaifulina et al., 2016; Manley, 2014). Another technique that may resolve individual metabolites on retinal preparations is matrix-associated-laser-desorption/ionization (MALDI) mass-spectrometry imaging (MSI) (Bowrey et al., 2016; Ly et al., 2015). However, MALDI-MSI is destructive, and its spatial resolution is currently still limited to approx. 30-50 µm, *i.e.* too large to resolve individual cells.

Raman microscopy is an attractive analytical choice since it is a non-destructive technique in which single cells or entire tissues may be sampled many times over (Karuna et al., 2019). It is a label-free technique that provides chemical information about the metabolic status of biological samples, detecting structural changes within the major macromolecules such as proteins, lipids, carbohydrate and nucleic acids. Each molecule contributes to a spectral pattern that is considered as a fingerprint of the analysed cell. Raman spectra enable discrimination, for example, between healthy and diseased or between living and dead cells (Brauchle et al., 2014). Furthermore, Raman microscopy can reach a high lateral and spatial resolution of about 1 μm^3 measurement volume using a confocal setup. Raman data can be transformed to the corresponding heat-maps and pseudo color-coded images using multivariate analysis methods. In these constructed images, clusters with similar spectra are grouped and coded with the same colour (Miljkovic et al., 2010), making it possible to depict cell types and different cell states within the Raman spectral image.

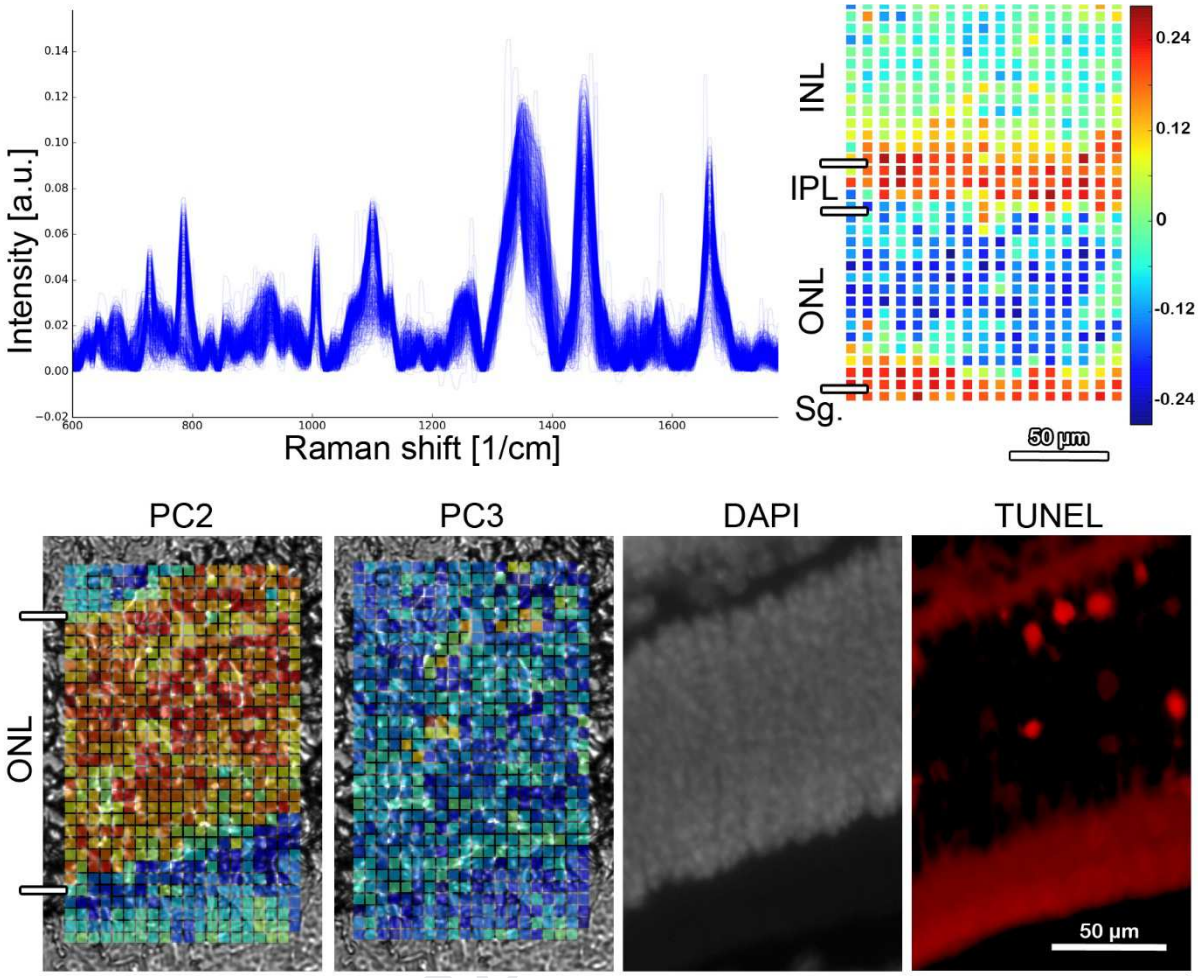

Figure 8: Raman imaging detects dying photoreceptors: Top left: Raman spectra collected on cross-sections of *rd1* retina at post-natal day 11, a time of active degeneration in this animal model. Top right: Principal component analysis (PCA) of Raman spectra obtained by scanning with a confocal Raman microscope. PC2 identifies outer nuclear layer (ONL) photoreceptor nuclei (negative values, dark blue). Membranous structures in photoreceptor segments (Sg.) and inner plexiform layer (IPL) show positive PC2 values (red). PC2 also distinguished neurons in inner nuclear layer (INL, green-yellow). Bottom panel: PCA analysis and subsequent DAPI and TUNEL staining. PC2 labels photoreceptor nuclei (orange-red) and conforms to a DAPI staining performed on the same specimen. PC3 colocalizes with TUNEL assay identifying dying cells. Note: retinal specimens may have been distorted by staining procedures.

In a pilot study, Raman imaging was performed using the Raman microscope system BioRam® (CellTool GmbH Tutzing, Germany). We found that the diversity of neuronal cells in the retina can easily by depicted based on their Raman spectroscopic fingerprints. Excitingly, a comparatively simple principal component analysis (PCA) readily identified dying photoreceptors in RD mutant retina (Figure 8). While still very early, these results highlight the promise of label-free and non-destructive hyperspectral imaging for future studies of neuronal cell death mechanisms within the retina.

Further development of this technology may allow the identification of individual metabolites based on the Raman spectra obtained from pure reference compounds and the informatic mixing of a large number of reference spectra (Scheier et al., 2014). Eventually,

this may enable us to temporally and spatially resolve key metabolites (Gaifulina et al., 2016) relevant for cellular metabolism. Comparing the metabolism of healthy and dying cells will lead to the construction of the "activitome" (also referred to as "reactome") of cell death, promising to deliver a wealth of new data on cell death mechanisms.

5. 2. From bench to bedside: Why is clinical translation so difficult?

The lack of treatments for RD is in part due to an incomplete understanding of the underlying photoreceptor cell death mechanisms and a lack of targets that could be exploited for therapy development, especially for pharmacology-based therapies. The missing mechanistic knowledge furthermore makes it difficult to develop biomarkers for early disease diagnosis and an assessment of treatment efficacy. Nevertheless, the wealth of new information on degenerative mechanisms that has become available within the last decade promises to change this situation (Scholl et al., 2016).

5. 2. 1. Genetic heterogeneity combined with rarity: A unique problem with unique opportunities

RD-type diseases show an enormous genetic heterogeneity with disease-causing mutations in more than 270 genes (see chapter 1). Since each of these disease genes can carry from several dozens to several hundred or more individual mutations (Athanasiou et al., 2018; Messchaert et al., 2018), we may, at present, estimate the total number of disease mutations to amount to several tens of thousands. This situation severely hinders the design of clinical trials as the numbers of patients carrying a specific disease-causing mutation will be small, even in a best-case scenario. However, a careful choice of the patients to be included in a clinical trial, with precisely known genotypes, is critical for success during clinical testing. The typically very slow progression of RD-type diseases over the course of many decades makes it difficult to identify patients that will be in a suitable stage of the disease (Iftikhar et al., 2019). Moreover, in preventive treatment trials the slow disease progression and the lack of biomarkers, that can identify treatment effects early on, will likely require very long clinical trial timelines, multiplying the associated clinical development costs.

Interestingly, RD-type diseases may allow to solve this problem in a way that is not available in other, common neurodegenerative diseases of the retina such as diabetic retinopathy or age-related-macular degeneration, and even less so for diseases of the brain such as Alzheimer's, or Parkinson's. RD-type diseases of the RP and LCA group are not only closely connected genetically but the causative mutations typically affect the same biochemical pathways. An example for this closeness may be mutations in the *AIPL1* gene, which cause fast progressing LCA (den Hollander et al., 2008; Ramamurthy et al., 2004), and mutations in *PDE6* genes, which produce the relatively slow progression RP phenotype (Gopalakrishna et al., 2017). Since AIPL1 is needed to functionally assemble the PDE6 α-β dimer, mutations in both genes cause an excessive accumulation of cGMP and photoreceptor degeneration. In some cases, mutations in the very same gene may produce a very rapid LCA-type degeneration, while a different mutation results in the less aggressive RP-type disease progression. An example for this situation are mutations in the *PRPH2* gene, which can give rise to both LCA and RP depending on where in the gene exactly the mutation resides (den Hollander et al., 2008; Gill et al., 2019).

This interconnection between LCA and RP enables a clinical trial strategy which has the potential to significantly shorten clinical test-timeframes. Clinical testing must typically first establish safety and tolerability in adult subjects in a phase 1 or phase 1/2a trial. Once this has been demonstrated, a phase 2 (2b) trial can move to children suffering from rapid progression LCA, to establish clinical efficacy in the relatively short timeframe of 6 to 12 months. If efficacy in LCA can indeed be demonstrated, then there will be sufficient rationale to perform (and fund) a similar long-term clinical trial also in slow progressing adult RP patients. Besides, the numbers of patients required for such clinical trials, and even as far as market registration, can be rather small thanks to special rare disease regulations and legislation. Thus, as opposed to the situation in most other neurodegenerative diseases, the interrelation of RD-type diseases provides a unique opportunity to accelerate clinical testing, at comparatively moderate development costs.

5. 2. 2. Biomarkers for retinal degeneration

Another important problem for clinical translation is a lack of *in vivo* biomarkers that could be used for the rapid assessment of treatment efficacy. Ideally biomarkers should allow for a live, non-invasive visualization of cell death in the retina, using techniques such as scanning laser ophthalmoscopy (SLO) (Beck et al., 2010; Paquet-Durand et al., 2019) or adaptive optics SLO (AO-SLO) (Walters et al., 2019). For instance, the binding of the protein annexin-5 to phosphatidylserine, may be utilized for *in vivo* detection of apoptotic cells (Kurosaka et al., 2003). Such methodology has been developed for studies on glaucoma and retinal ganglion cell death, initially in a mouse model (Cordeiro et al., 2010) and eventually in a clinical trial (Cordeiro et al., 2017), using intravitreal injection of a fluorescently labelled derivative of annexin-5. Similar methodology may be applicable also to the *in vivo* cell death detection in RD-type diseases. A future combination of non-invasive retinal imaging, such as SLO, with label-free cell death detection techniques, such as Raman spectroscopy (see chapter 5. a.), could advance pre-clinical and clinical examinations even further.

Recent developments in the field of magnetic resonance imaging (MRI) suggest that it may be possible to non-invasively observe oxidative stress, or production of free radicals, in for instance the retina (Berkowitz, 2018), at least in experimental animals. While this approach, coined QUEST-MRI, would perhaps not detect ongoing photoreceptor degeneration as such, it may, once transferred to the clinic, still be useful in providing a snapshot on the oxidative stress load, and could serve as an index, or surrogate marker, on the photoreceptor status.

As an example of potential blood-based parameters, it is interesting to note that there are reports on increased cGMP in blood from RP patients compared to healthy counterparts (Camara et al., 2013; Kjellstrom et al., 2016). This may be related to the exaggerated cGMP levels in the photoreceptors of many RP types, *i.e.* the phenomenon discussed in several of the sections above, especially since at least some of the patients had mutations in the *PDE6A* gene (Kjellstrom et al., 2016). When connected with other measurements and parameters obtained from at least certain cohorts of RP patients, a future blood-test may allow to assess the retinal status and disease progression in genetically defined RP patient cohorts (Lains et al., 2019).

5. 2. 3. Ocular barriers and retinal drug delivery

The translation of research findings from the lab to the clinic faces another important hurdle, namely the blood-retinal barrier (BRB) and other ocular barriers that prevent therapeutic agents from reaching the photoreceptor cells in the retina. As laid out in chapter 1 the retina is shielded against external agents (*e.g.* toxins, pathogens) by the inner and outer blood-retinal barrier. To overcome this barrier, a variety of different technical approaches have been pursued, using different routes of administration, suprachoroidal injection (Yeh et al., 2018), subretinal injection (Ochakovski et al., 2017), injection into the capsule of Tenon (Ohira et al., 2015), and intravitreal injection (Meyer et al., 2016). Each of these administration routes have specific advantages and disadvantages, but whichever administration route is chosen, the drug formulation and the delivery system used will be critical for successful treatment development.

Different drug delivery systems (DDS) have been developed to allow therapeutic agents to reach the retinal photoreceptors. This includes, light-responsive polymers for non-invasive triggering of intraocular drug release (Huu et al., 2015), polymeric nanoparticles (Koo et al., 2012), or glutathione-conjugated liposomes originally intended for drug delivery to the brain (Birngruber et al., 2014; Vighi et al., 2018b). Indeed liposomes – decorated or not with polyethylenglycol (PEG) chains – may be used for direct compound administration to the vitreous (Bochot and Fattal, 2012) where, for instance, non-PEGylated liposomes have shown improved cargo delivery to photoreceptors (Asteriti et al., 2015).

Targeted downregulation of crucial BRB components has also been suggested for drug delivery to the retina. A proof-of-concept for this approach has been delivered with an siRNA-mediated transient knock-down of a claudin-5, a protein needed to form tight junctions in the BRB (Campbell et al., 2013). With this approach, a compound may be applied systemically via the blood stream, and could, within a specific time-frame penetrate the retina, as long as BRB component remains open (Campbell et al., 2018).

In terms of retinal drug delivery, the last ten years have seen an important development and the appearance of many innovative materials, designs, and technologies. Still, efficient and sustained drug delivery to the photoreceptors remains a major challenge. Importantly, each compound or therapeutic agent may require highly adapted DDS, which additionally must comply with regulatory requirements (Himawan et al., 2019). Therefore, future research into new treatments for RD should take the retinal delivery problem into consideration as early as possible and synchronize compound and delivery development.

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Title

Cellular mechanisms of hereditary photoreceptor degeneration

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Author statement

The concept for this article was developed by VM, PE, and FPD. The figures were prepared by FPD and SD. Table 1 was compiled by FPD. The manuscript was written jointly by all authors.

Declaration of interest

VM, PE, and FPD have filed for three patents on the synthesis and use of cGMP analogues (PCTWO2016/146669A1, PCT/EP2017/066113, PCT/EP2017/071859) and have obtained a European Medicine Agency orphan drug designation for the use of a cGMP analogue for the treatment of Retinitis Pigmentosa (EU/3/15/1462). VM, PE, and FPD are shareholders of, or have other financial interest in, the company Mireca Medicines, which intends to forward clinical testing of cGMP analogues. KS is owner and CEO of CellTool GmbH, which commercializes Raman microscopes.