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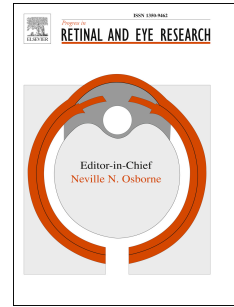
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Michael Power, Soumyaparna Das, Karin Schütze, Valeria Marigo, Per Ekström, François Paquet-Durand



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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.

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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 Ca²⁺-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 Ca²⁺-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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79 1. The retina and hereditary photoreceptor degeneration

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

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

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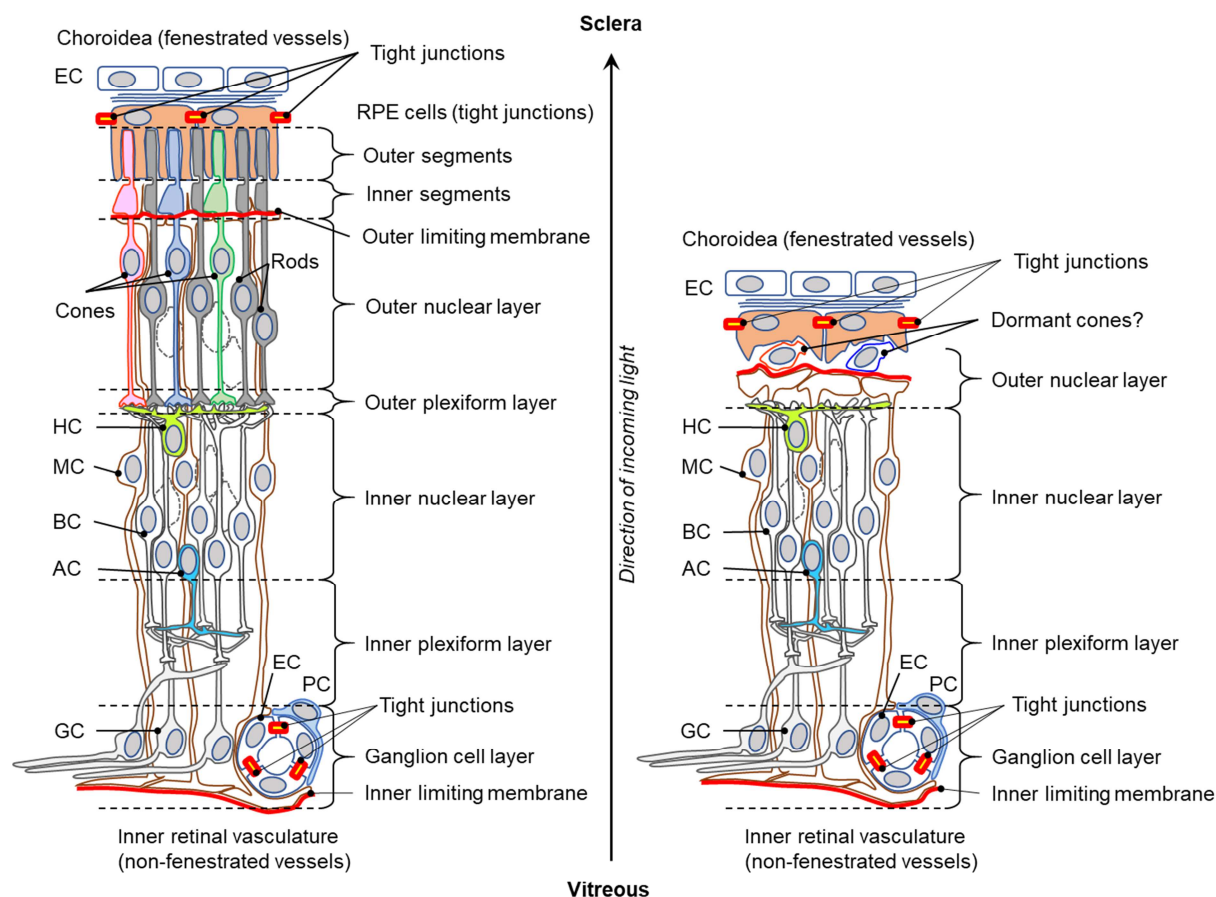
96 1. 1. The retina and hereditary retinal degeneration

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

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

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

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



133

134 **Figure 1: Idealized cross-sections through healthy and degenerated retina.**

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

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

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

146 Hereditary retinal degeneration (RD) is a group of rare retinopathies that cause
147 progressive loss of vision. Within the group of RD-type diseases, adult-onset Retinitis
148 Pigmentosa (RP) is the most common, with a prevalence of approximately 1:3.500
149 (Bertelsen et al., 2014). A genetically related disease is Leber congenital amaurosis (LCA),
150 with a prevalence of circa 1:8.000 and a disease onset already in early childhood or even in
151 infancy (den Hollander et al., 2008). These most common forms of RD are caused by a
152 genetic defect in a single gene that compromises the viability of photoreceptors (Hartong et
153 al., 2006; Verbakel et al., 2018). In RP, an initial, primary degeneration of rods is usually
154 followed by a secondary degeneration of cones, eventually leading to complete blindness
155 (Kennan et al., 2005). The result is a complete, or near complete, loss of the ONL, while the
156 inner retina remains mostly intact, even though the dendrites of bipolar and horizontal cells
157 eventually retract (Gargini et al., 2007). In LCA, the disease-causing mutations may affect
158 both rods and cones simultaneously, sometimes leading to a very severe disease phenotype
159 with no discernible retinal function as measured in electroretinography (Jacobson et al.,
160 2017; Preising et al., 2012).

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

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

180

181 1. 2. Genetic heterogeneity of hereditary retinal degeneration

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

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

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

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

206 Further examples for the variability of mutation effects in RD genes are the genes
207 encoding for photoreceptor phosphodiesterase-6 (PDE6) (Cote, 2004). Rod PDE6 is a
208 heterotetramer composed of the catalytic α and β subunits, encoded by the *PDE6A* and
209 *PDE6B* genes, respectively, and two inhibitory γ subunits, encoded for by the *PDE6G* gene.
210 Cone PDE6 consists of two α subunits encoded for by the *PDE6C* gene and two inhibitory γ
211 subunits encoded for by the *PDE6H* gene. Numerous mutations have been found in all
212 PDE6 genes, causing RP (*PDE6A*, *PDE6B*, *PDE6G*) (Corton et al., 2010; Dvir et al., 2010;
213 Muradov et al., 2012) or ACHM (*PDE6C*, *PDE6H*) (Gopalakrishna et al., 2017; Kohl et al.,
214 2012; Thiadens et al., 2009). Typically, a mutation causing PDE6 loss-of-function leads to
215 extremely high cGMP levels and the death of the affected photoreceptor cell type (Farber
216 and Lolley, 1974; Paquet-Durand et al., 2009; Sothilingam et al., 2015; Trifunovic et al.,
217 2010). On the other hand, some *PDE6A* mutations are known to produce “only” a loss of rod
218 functionality, without cell death, resulting in a clinical phenotype referred to as congenital
219 stationary night blindness (CSNB) (Zeitze et al., 2015).

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

224

225 1. 3. Animal models for hereditary retinal degeneration

226 Studies into the mechanisms of photoreceptor degeneration require the use of suitable
227 model systems, that will reproduce degenerative processes as faithfully as possible. Since
228 the physiological processes related to vision and light perception are evolutionarily
229 conserved over a wide range of organisms (Nilsson, 2009), gene mutations causing RD in
230 humans will often also cause RD in animals. Accordingly, a large number of animal models
231 is available for RD research, in species/orders as diverse as *Drosophila* (Griciuc et al.,
232 2010), zebrafish (Ward et al., 2018), and mammals (Shaw et al., 2001). For the latter, the
233 typically used species are mice and rats (Dalke and Graw, 2005; Won et al., 2011) though
234 larger mammalian models, including cats (Menotti-Raymond et al., 2010), dogs (Beltran,
235 2009), and pigs are also available (Petters et al., 1997).

236 Among the mouse models for RD, the “retinal degeneration-1”, *rd1*, mouse stands out as
237 probably the earliest and likely the most studied animal model for RD. The first report about
238 the *rd1* mouse dates back to 1924, where it is described as having a “rodless retina” (Keeler,
239 1924) and in older literature it is usually referred to as “rd” or “rd/rd” mouse. The *rd1* mouse
240 is characterized by a mutation in the gene encoding for the β subunit of rod PDE6 (Bowes et
241 al., 1990), a lack of PDE6 protein (Yan et al., 1998) and exceedingly high levels of cGMP in
242 rods (Farber and Lolley, 1974; Paquet-Durand et al., 2009). This leads to rapid loss of most
243 of the rods within the first two weeks after birth (Sahaboglu et al., 2013), followed by a
244 mutation-independent, secondary death of cones (LaVail et al., 1997). Another mouse model
245 with a mutation (albeit different from that of *rd1*) in the *Pde6b* gene is the *rd10* mouse
246 (Chang et al., 2002). Compared to *rd1*, the loss of rods in the *rd10* retina starts later, at
247 around post-natal day 18 (P18), from when on it takes about 10 days until most rod
248 photoreceptors are lost (Arango-Gonzalez et al., 2014; Gargini et al., 2007).

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

258 Primary cone degeneration may also be studied in mouse models, prominent examples of
259 which are the cone-photoreceptor-function-loss *cpfl1* mouse (Chang et al., 2009) and the
260 *Cnga3* knock-out (KO) mouse (Biel and Michalakis, 2007). While the latter suffers from a
261 lack of expression of a subunit of the cyclic nucleotide gated channel (CNGC), leading to
262 slow cone degeneration over the course of about four months, the *cpfl1* mouse carries a
263 mutation in the cone-specific α subunit of PDE6, leading to a loss of cones approx. two
264 months after birth (Trifunovic et al., 2010). Incidentally, both the *Cnga3* KO and the *cpfl1*
265 mouse show strong accumulation of cGMP in degenerating cones (Arango-Gonzalez et al.,
266 2014).

267 Many more RD animal models exist, for many of the known RD-genes (Chang et al.,
268 2002; Won et al., 2011), enabling comparative studies into disease mechanisms (Arango-
269 Gonzalez et al., 2014), but also a validation of novel therapeutic approaches across various
270 models (Vighi et al., 2018b). Interestingly, in a number of different RD models photoreceptor
271 cell death appears to be caused by high levels cGMP (Arango-Gonzalez et al., 2014;
272 Iribarne and Masai, 2017; Paquet-Durand et al., 2009; Wang et al., 2017); further details on
273 this finding and its significance will be presented in chapter 3. Taken together, animal
274 models for RD, carrying similar or even homologous mutations to those identified in human
275 patients, and with similar disease phenotype, offer an enormous potential to disentangle the
276 underlying disease mechanisms.

277

278 2. Photoreceptors: Highly specialized neurons with special 279 needs

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

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

296

297 2. 1. Photoreceptor energy metabolism

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

311

312 2. 1. 1. *The astrocyte-neuron-lactate-shuttle (ANLS)*

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

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

324

325 2. 1. 2. *The photoreceptor-RPE-lactate-shuttle*

326 For the retina an inverted lactate shuttle has recently been proposed, in which
327 photoreceptors would consume glucose and generate lactate, which would then be released
328 to serve as fuel for Müller glial cells and RPE cells (Kanow et al., 2017). The study also
329 showed that high levels of lactate can suppress glycolysis in RPE cells, which is interpreted
330 as a means to increase the passage of glucose to photoreceptors. While this alternative
331 shuttle hypothesis elegantly resolves some of the contradictions of retinal metabolism, it
332 does not explain the enormous density of mitochondria in the photoreceptor inner segments.
333 Moreover, the hypothesis proposes the expression of the glucose transporter-1 (GLUT1) in
334 rod and cone photoreceptors, something that is still controversial. Older studies using
335 electron microscopy confirmed GLUT1 expression in RPE, but could not detect it in
336 photoreceptors (Bergersen et al., 1999; Gerhart et al., 1999). Further electron microscopic
337 studies showed that the expression of GLUT1 previously proposed for photoreceptor outer
338 segments (Mantych et al., 1993) was in fact localized to RPE cell microvilli (Gospe et al.,
339 2010). More recently GLUT1 expression was suggested to be present on cones only (Ait-Ali
340 et al., 2015).

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

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

361

362 2. 2. Epigenetics in retinal degeneration

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

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

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

373

374 2. 2. 1. DNA methylation

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

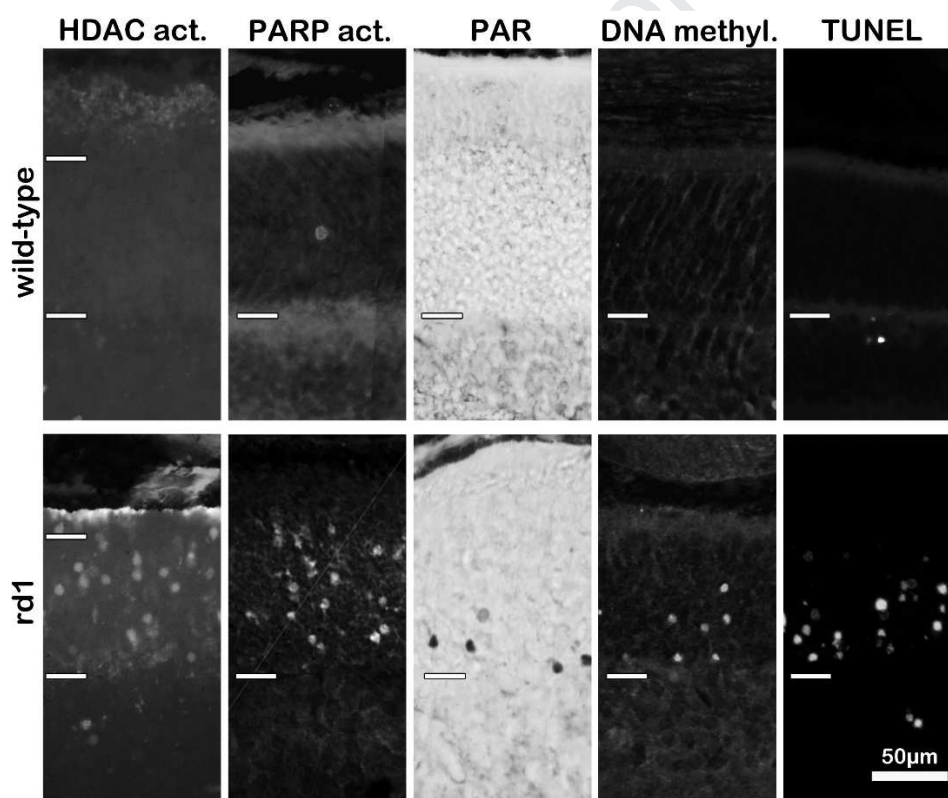
393

394 2. 2. 2. Histone acetylation and deacetylation

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

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

411 prolonged the life of the diseased photoreceptors (Chen and Cepko, 2009). However, the
 412 latter effect may be unrelated to actual deacetylase activity (Guo et al., 2015). In contrast, a
 413 detrimental HDAC effect on survival was indicated by pharmacological experiments, in which
 414 the relatively broad HDAC inhibitors trichostatin A (TSA) and scriptaid were able to protect
 415 rod photoreceptors of the *rd1* model from cell death in retinal explants (Sancho-Pelluz et al.,
 416 2010). TSA was similarly able to reduce cone cell death in the cone degeneration model
 417 *cpfl1* in explant culturing, and, more importantly, *in vivo* through intravitreal injection
 418 (Trifunovic et al., 2016). On the other hand, yet another broad HDAC inhibitor, valproic acid
 419 (VPA), exhibited either protective or detrimental effects in *Xenopus laevis* models of
 420 rhodopsin mutation-based RP, depending on the exact type of genetic defect (Vent-Schmidt
 421 et al., 2017). Likewise, VPA had opposing effects on the degeneration of rod photoreceptors
 422 in two *Pde6b* mutation models, the *rd1* and the *rd10* mouse, in which *rd1* photoreceptors
 423 were protected by VPA treatment, whereas it, in contrast, accelerated the *rd10*
 424 photoreceptor degeneration (Mitton et al., 2014). Similar discrepancies have been seen in
 425 other studies (Berner and Kleinman, 2016), and the use of VPA in people with RP has not
 426 resulted in a consensus on whether or not this is a valuable treatment option, or if it may
 427 actually be negative (Dias et al., 2018; Vent-Schmidt et al., 2017).



428

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

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

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

455

456 2. 2. 3. *Histone methylation*

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

473

474 2. 2. 4. *PARP activity and PARylation*

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

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

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

505

506 2. 3. Protein synthesis, quality control, and transport

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

517 2.3.1. Protein quality control and endoplasmic reticulum (ER) stress

518 Protein quality control resides at the endoplasmic reticulum (ER) and is mediated by
519 sensors located at the ER membrane. In case of defects, these sensors activate the
520 unfolded protein response (UPR) to decrease protein synthesis (Gorbatyuk and Gorbatyuk,
521 2013), enhance the protein folding mechanism, and remove misfolded proteins (Chan et al.,
522 2016). ER stress and UPR are intricately connected (Hetz, 2012) and are transduced by
523 three ER resident proteins: 1) the inositol-requiring enzyme 1 (IRE1), 2) the activating
524 transcription factor-6 (ATF6), and 3) the protein kinase R-like ER protein kinase (PERK). The
525 three ER sensors can regulate expression of chaperones, such as binding-immunoglobulin-

526 protein (BIP), reduce protein synthesis through phosphorylation of eukaryotic initiation factor-
527 2 α (eIF2 α) or activate apoptotic responses by expression of several genes such as
528 *CHOP/GADD153* encoding for a transcription factor that negatively regulates, among others,
529 the anti-apoptotic factor BCL2 (Hetz, 2012). During retinal degeneration these pathways can
530 be activated (Chan et al., 2016), as shown by the progressive increase of *CHOP* and
531 decrease of BIP expression in photoreceptors expressing a misfolding mutation in rhodopsin
532 (Lin et al., 2007).

533 ER stress arises after the accumulation of misfolded proteins in the ER and can reduce
534 the production of functional proteins and even lead to cell death in case the UPR cannot
535 relieve the cell from the stress. Moreover, ER stress can be activated also by oxidative
536 stress and reactive oxygen species (ROS) (Zhang et al., 2014). Recent studies support the
537 view that ER protein folding highly correlates with ROS production, because redox
538 homeostasis is crucial for the protein folding process and disulphide bond formation
539 (Plaisance et al., 2016). Furthermore, the ER plays an essential role in regulation of Ca²⁺
540 homeostasis. The chaperone BIP contributes to Ca²⁺ buffering in the ER lumen and is
541 involved in sensing misfolded proteins and the activation of ER stress. BIP contributes to the
542 prevention of ER Ca²⁺ leakage and helps to maintain ER homeostasis (Krebs et al., 2015).
543 Mutations in different genes can lead to differential dysfunctions in photoreceptor cells, such
544 as protein misfolding, oxidative stress, and Ca²⁺ dysregulation in the ER, triggering ER
545 stress, that has been linked to photoreceptor cell death in different models of RD (Chan et
546 al., 2016). Thus, photoreceptors have properties that could make them particularly
547 vulnerable to ER stress related processes, opening also the possibility to target such
548 processes for therapeutic purposes.

549 2.3.2. Protein misfolding in RD

550 Rhodopsin is the most abundant protein in rods and the majority of mutations in
551 rhodopsin cause autosomal dominant RP due to failure of rhodopsin to fold correctly or
552 defects in the transport to the outer segment (Mendes et al., 2005). Several studies
553 characterized the molecular responses to misfolded mutant rhodopsin in different animal
554 models and led to the proteostatic stress hypothesis for this type of mutations (Athanasίου et
555 al., 2013). The best studied dominant mutation in rhodopsin is the P23H mutation, a
556 misfolding mutation that has been linked to ER-stress, UPR, and impaired proteasome
557 activity (Athanasίου et al., 2014; Chiang et al., 2012; Chiang et al., 2015; Comitato et al.,
558 2016; Gorbatyuk et al., 2010). Recent studies on the P23H rhodopsin mutation
559 demonstrated that this mutation does not cause ER stress, but rather UPR. To this end, in
560 P23H mutant photoreceptors activation of the ER sensors, such as IRE1, is possibly a
561 compensatory response to help the degradation of the misfolded protein and strengthening
562 of the proteasome, which then has protective effects (Chiang et al., 2015). Further studies
563 showed limited or even negative neuroprotection by interfering with ER stress mechanisms
564 (Athanasίου et al., 2017; Comitato et al., 2016). To explain these results, we need to keep in
565 mind that different molecular effects can be triggered by different mutations in rhodopsin. In
566 fact, a recent molecular study characterized the effects on the protein structure of 33
567 rhodopsin mutations and showed that different mutations have distinctive effects on the
568 protein. This study also identified a group of mutations for which misfolding is relieved upon
569 interaction with retinal, which acts as a chaperone for rhodopsin (Behnen et al., 2018).

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

578 2.3.3. Protein mistrafficking

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

587 The mechanisms of photoreceptor demise caused by protein mistrafficking are still not
588 well characterized. Different explanations have been forwarded as to how mislocalized
589 proteins, especially rhodopsin, may cause photoreceptor cell death in RD: When in the inner
590 segment rhodopsin may activate G- α transducin, which in turn cannot activate PDE6 (see
591 also chapter 2.4.) outside the outer segment, and may instead act on adenylate cyclase to
592 increase cAMP, with subsequent activation of caspases (Nakao et al., 2012; Wang et al.,
593 2012). Interestingly, genetic deletion of transducin does not completely prevent
594 photoreceptor loss caused by mislocalized rhodopsin, arguing for transducin-independent
595 cell death promoting processes (Concepcion and Chen, 2010). These processes could
596 potentially be due to constitutive binding of mislocalized rhodopsin to arrestin (Chen et al.,
597 2006; Chuang et al., 2004).

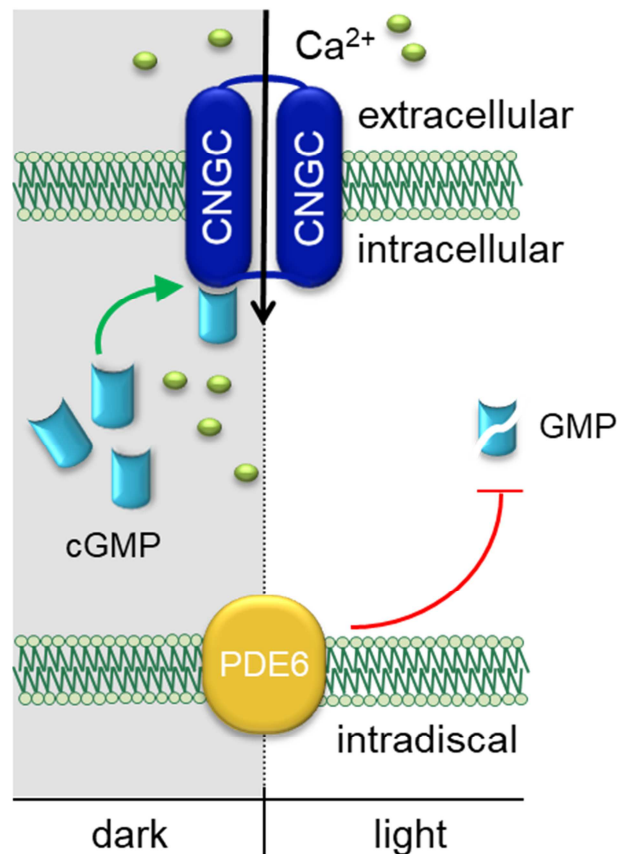
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599 2. 4. The phototransduction cascade and the regulation of cGMP and Ca²⁺ 600 levels

601 The physiology of photoreceptors and the phototransduction cascade critically depends
602 on the regulation and interplay of the second messenger signalling molecules cGMP and
603 Ca²⁺ (Pugh and Lamb, 1990; Vinberg et al., 2018b). Mutations affecting genes related to the
604 phototransduction cascade often cause a dysregulation of cGMP and/or Ca²⁺, triggering a
605 series of down-stream processes, which eventually kill photoreceptors (Kulkarni et al., 2016).

606 The phototransduction cascade employs high levels of cGMP in photoreceptor outer
607 segments to fully sensitize photoreceptor cells in the dark (Figure 3). cGMP is synthesized
608 by retGC in a Ca²⁺-dependent way. When Ca²⁺ levels are low, GCAP stimulates retGC to
609 produce cGMP. Conversely, under high Ca²⁺ concentrations GCAP inhibits retGC (Tucker et
610 al., 1999) providing for a negative feedback loop that limits photoreceptor cGMP to
611 physiological levels of 1-5 μ M (Burns et al., 2009; Dell'Orco et al., 2009; Pugh and Lamb,
612 1990). Independent of Ca²⁺, retGC activity is additionally controlled by the RD3 protein
613 (Peshenko et al., 2016).

614

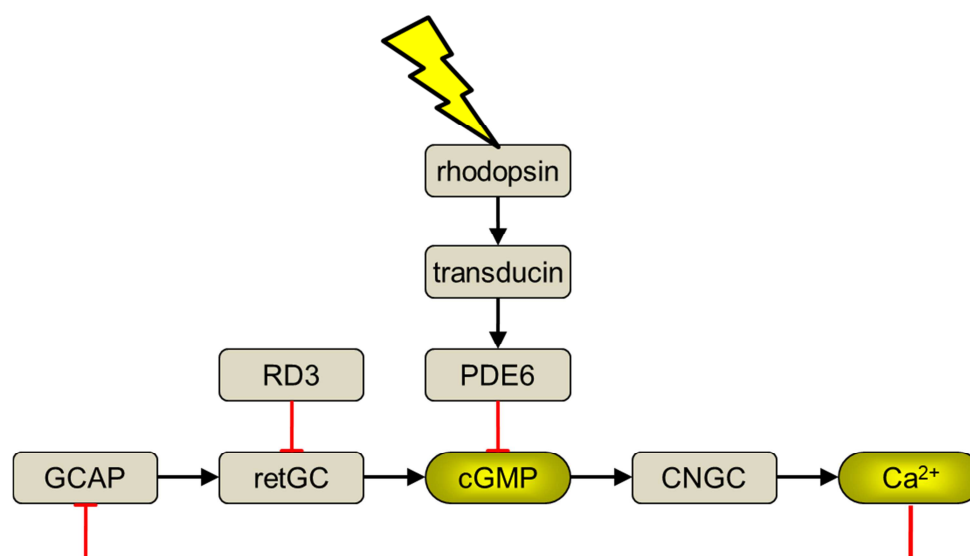


615

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.

622

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 $\text{Na}^+/\text{Ca}^{2+}/\text{K}^+$ 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).



637

638 **Figure 4: Feedback regulation of photoreceptor cGMP and Ca²⁺ levels.** The
 639 conformational change caused when a photon of light strikes a rhodopsin molecule
 640 sequentially activates transducin and phosphodiesterase-6 (PDE6), which in turn hydrolyses
 641 cGMP. Guanylyl cyclase activating protein (GCAP) is regulated by Ca²⁺ in its capacity to
 642 activate retinal guanylyl cyclase (retGC). retGC produces cGMP which opens the cyclic
 643 nucleotide gated channel (CNGC), allowing for influx of Ca²⁺. retGC is additionally regulated
 644 by the RD3 protein. Importantly, mutations in any of the genes encoding for these proteins
 645 can cause RD.

646

647 Seminal research performed already in the 1970s, established that high levels of cGMP
 648 were associated with and likely causal for photoreceptor degeneration (Farber and Lolley,
 649 1974; Lolley et al., 1977). How exactly RD mutations led to the rise of photoreceptor cGMP
 650 levels became clearer when some of the first disease-causing mutations were discovered in
 651 the PDE6 α and β genes ((Huang et al., 1995; McLaughlin et al., 1993). Since then it has
 652 become evident that many other gene mutations can affect cGMP and Ca²⁺ signalling in very
 653 similar ways.

654 For instance, the G86R point-mutation in the *GCAP1* gene alters the Ca²⁺-dependent
 655 regulation of retGC in a way that leads to increased cGMP production, causing a dominant
 656 cone-rod degeneration (Peshenko et al., 2019). Moreover, a disruption of the cGMP – Ca²⁺
 657 negative feedback loop (Figure 4) (Burns et al., 2002; Olshevskaya et al., 2002), as triggered
 658 for example by mutations in CNGC genes (Biel and Michalakakis, 2009; Paquet-Durand et al.,
 659 2011; Reuter et al., 2008), will permit cGMP levels to rise to extremely high and apparently
 660 photoreceptor toxic concentrations. A similar effect is produced by loss-of-function mutations
 661 in the *RD3* gene also causing high cGMP levels and photoreceptor death (Peshenko et al.,
 662 2016).

663

664 2. 5. Ca²⁺ and CNGC in photoreceptor degeneration

665 The precise regulation of intracellular Ca²⁺ levels is critical for neuronal survival in general
 666 (Yamashima, 2004) and, correspondingly, for almost 20 years Ca²⁺ channels have been
 667 studied as potential targets for RD therapy. The general hypothesis is that an excessive
 668 activation of Ca²⁺ channels causes Ca²⁺ overload inside the cell and triggers photoreceptor

669 cell death (Fox et al., 1999; Orrenius et al., 2003). According to this hypothesis, Ca²⁺
670 channel blockers should in principle be able to prevent or delay photoreceptor death.

671 In photoreceptors there are two major sources for Ca²⁺ influx: 1) CNGC in the outer
672 segment, and 2) voltage gated Ca²⁺ channels (VGCC) located in the photoreceptor's
673 synapse (Van Hook et al., 2019). A seminal study by Frasson and colleagues (Frasson et
674 al., 1999) suggested the use of D-*cis*-diltiazem – a registered drug used to treat
675 hypertension – to prevent *rd1* mutant rod degeneration and to preserve cone visual function.
676 Since D-*cis*-diltiazem was known to target the voltage-gated Ca²⁺-channels (VGCC) in the
677 photoreceptor synapse, the authors assumed a deleterious Ca²⁺-influx to cause
678 photoreceptor death and that this influx occurred mainly via synaptic VGCC. However, a
679 number of follow-up studies were unable to reproduce the proposed protective effects of D-
680 *cis*-diltiazem (Bush et al., 2000; Pawlyk et al., 2002; Read et al., 2002) reviewed in (Barabas
681 et al., 2010). Diltiazem exists in two enantiomers and commercially available preparations
682 usually contain mixtures of D-*cis*- and L-*cis*-diltiazem. Importantly, while D-*cis*-diltiazem
683 targets VGCCs in the photoreceptor synapse (Hart et al., 2003), L-*cis*-diltiazem targets
684 CNGCs in the photoreceptor outer segment (Haynes, 1992; Stern et al., 1986). In light of
685 these facts, it is plausible to think that the protective effects observed by Frasson and
686 colleagues were in fact not due to D-*cis*-diltiazem acting on VGCC, but instead due to L-*cis*-
687 diltiazem acting on CNGCs. This idea would go well with observations from two later studies
688 performed with genetic knock-out models: After the genetic deletion of rod VGCCs in *rd1*
689 mice, there was essentially no rescue of photoreceptor viability or function (Schon et al.,
690 2016). By contrast, when CNGCs were genetically inactivated in *rd1* mice, there was a
691 marked improvement of rod viability and cone function (Paquet-Durand et al., 2011). These
692 results strongly suggest the inhibition of CNGC as a viable therapeutic approach, at least in
693 those patients where the causal mutation does not affect CNGC genes.

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

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

712 Since loss-of *Cngeb1* expression in the mouse (Huttl et al., 2005; Paquet-Durand et al.,
713 2011) causes rod CNGC function loss and protects *rd1* photoreceptors, it is possible that
714 even a relatively incomplete knock-down of *CNGB1* will significantly reduce rod CNGC

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

724 Paradoxically, both RP and ACHM can be caused by mutations in rod or cone CNGC
725 subunits (Bareil et al., 2001; Johnson et al., 2004; Michalakis et al., 2014; Muhlfridel et al.,
726 2017). The likely explanation is that in the absence of functional CNGC the negative Ca^{2+} -
727 mediated feedback on retGC is missing, allowing for an excessive production of cGMP up to
728 toxic levels (see chapter 2. d.). Any therapeutic strategy focusing on inhibition or down-
729 regulation of CNGC will thus have to carefully titrate the positive effects of reducing Ca^{2+}
730 influx against the negative effects of low Ca^{2+} and unbalanced cGMP production.

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

737 Regardless of what strategy is pursued to lower photoreceptor Ca^{2+} -levels, it is important
738 to consider the down-stream effectors of Ca^{2+} -signalling. Apart from GCAP (see chapter 2.4)
739 these may include a number of different kinases, including calmodulin-kinases (Hauck et al.,
740 2006) and kinases belonging to the protein kinase C (PKC) family (Azadi et al., 2006).
741 However, as detailed in the next chapter, Ca^{2+} -activated calpain type proteases may be even
742 more critical for photoreceptor degeneration.

743

744 2. 6. Downstream of Ca^{2+} : calpain-type proteases

745 Calpains are a family of cytosolic cysteine proteases whose enzymatic activities are
746 dependent on Ca^{2+} , in the sense that while they are expressed in every cell, the calpains are
747 not constitutively active but rather are activated in a number of steps by Ca^{2+} (Suzuki et al.,
748 2004). To date, 15 calpain isoforms have been discovered in mammals and the calpain
749 family can be subdivided into typical (calpain 1, 2, 3, 8, 9, 11, and 12) and atypical calpains
750 (calpain 5, 6, 7, 8b, 10a, and 15) (Huang and Wang, 2001; Suzuki et al., 2004). Calpain
751 isoforms have been implicated in cellular functions such as signal transduction, cell cycle,
752 proliferation, differentiation, migration, apoptosis, membrane function, formation of muscle
753 fibres, dendritic spine formation and pruning, and many others (Goll et al., 2003; Kanamori et
754 al., 2013; Smalheiser and Lugli, 2009; Suzuki et al., 2004).

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

760 enzyme in stage three (Chen et al., 2001). Increases in calpain proteolytic activity have been
761 linked to the increased plasma membrane permeability, such that hydrolysis of paxillin, a
762 cytoskeletal protein, occurs at the same time as propidium iodide entry, while hydrolysis of
763 the cytoskeletal proteins talin and vinculin occurs concomitantly with LDH release (Liu et al.,
764 2004).

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

771 Apoptosis is a programmed form of regulated cell death carried out by the caspase family
772 of proteins (discussed below). The role of calpains in apoptosis is difficult to fully elucidate
773 due to a combination of improper nomenclature and the use of calpain inhibitors which also
774 inhibit other molecules governing the apoptosis pathway. Especially the interactions between
775 caspases and calpains are complex to understand. Caspases are proteolytic enzymes with a
776 particular role in programmed cell death (see chapter 3. b. below). Calpains cleave caspase-
777 7, -8 and -9, and by doing so inactivate caspase-7 and -8 (Chua et al., 2000), which may then
778 be seen as an anti-apoptotic action. Indeed, given that a regulatory step in the formation of
779 the necrosome is the inhibition of caspase-8 (Geng et al., 2017; Oberst et al., 2011) the
780 cleavage of caspase-8 by calpains may be seen as a pro-necrotic action by the protease.
781 By contrast, calpain-2 has been suggested to be a promotor of apoptosis by cleaving and
782 activating pro-caspase-12, and by cleaving the loop region of the large BCL isoform, BCL-
783 XL, and changing it from an anti-apoptotic molecule into a pro-apoptotic molecule
784 (Nakagawa and Yuan, 2000).

785 Activation of calpains, specifically of calpain-1 and -2, was linked to increased intracellular
786 Ca²⁺ in several models of RD caused by increased cGMP or protein misfolding (Arango-
787 Gonzalez et al., 2014; Comitato et al., 2016; Comitato et al., 2014). With respect to such
788 activation, a reduction of the expression levels of calpastatin, the endogenous inhibitor of
789 calpains, was observed in the *rd1* mouse model (Paquet-Durand et al., 2006).
790 neuroprotection of the retina of *rd1*, *Rho*^{-/-} mice as well as mice expressing the P23H mutant
791 rhodopsin has been reported with several calpain inhibitors (Comitato et al., 2016; Comitato
792 et al., 2014; Paquet-Durand et al., 2010). Treatments with the calpastatin peptide, a peptide
793 derived from the endogenous inhibitor calpastatin, showed the best protection results on *rd1*
794 mutant retinas (Paquet-Durand et al., 2010) when it comes to calpain inhibition so far.

795

796 2. 7. cGMP and the activity of protein kinase G (PKG)

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

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

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

830 A critical question then is how the high cGMP would be able to exert any negative effects.
831 *i.e.* what kind of downstream effector(s) can we surmise? Even though the regulation of
832 CNGCs by cGMP is a key process in phototransduction, and as such could affect cellular
833 Ca^{2+} balance, the prototypic target for cGMP is PKG. Excessive activation of PKG is known
834 to cause cell death in different cancer cell lines (Deguchi et al., 2004; Hoffmann et al., 2017;
835 Vighi et al., 2018a). On the other hand, PKG inhibition can have cytoprotective effects
836 (Brunetti et al., 2002). Indeed, the effects of PKG activation in different types of cancer are
837 complex: Activation of PKG1 α and PKG1 β appears to favour cancer progression (Arozarena
838 et al., 2011; Dhayade et al., 2016), while the activation of the PKG2 isoform had anti-tumour
839 effects (Hoffmann et al., 2017; Vighi et al., 2018a). The situation with respect to how PKG
840 activity affects cancer cell death is thus not a straightforward decision between death or
841 protection.

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

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

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

871

872

873 3. The diversity of neuronal cell death mechanisms and their 874 relevance for photoreceptor degeneration

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

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

896

897 3. 1. On the evolution of cell death mechanisms

898 Until the 1950s, cell death was thought to be a biological accident that occurred when a
899 cell was physically destroyed (trauma, intoxication, disease, *etc.*) and the term “necrosis”
900 was introduced for this (Glucksmann, 1951). In the following decades other forms of cell
901 death unequivocally different from necrosis, driven by intrinsic programs, were increasingly
902 recognized as fundamental biological processes. This view was formalized in the early
903 1970s by the works of Kerr, Wyllie, and Currie who coined the term “apoptosis” for a specific
904 type of programmed cell death (Kerr et al., 1972).

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

916

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

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

929

930 3. 2. The classical mechanisms: Necrosis and Apoptosis

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

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

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

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

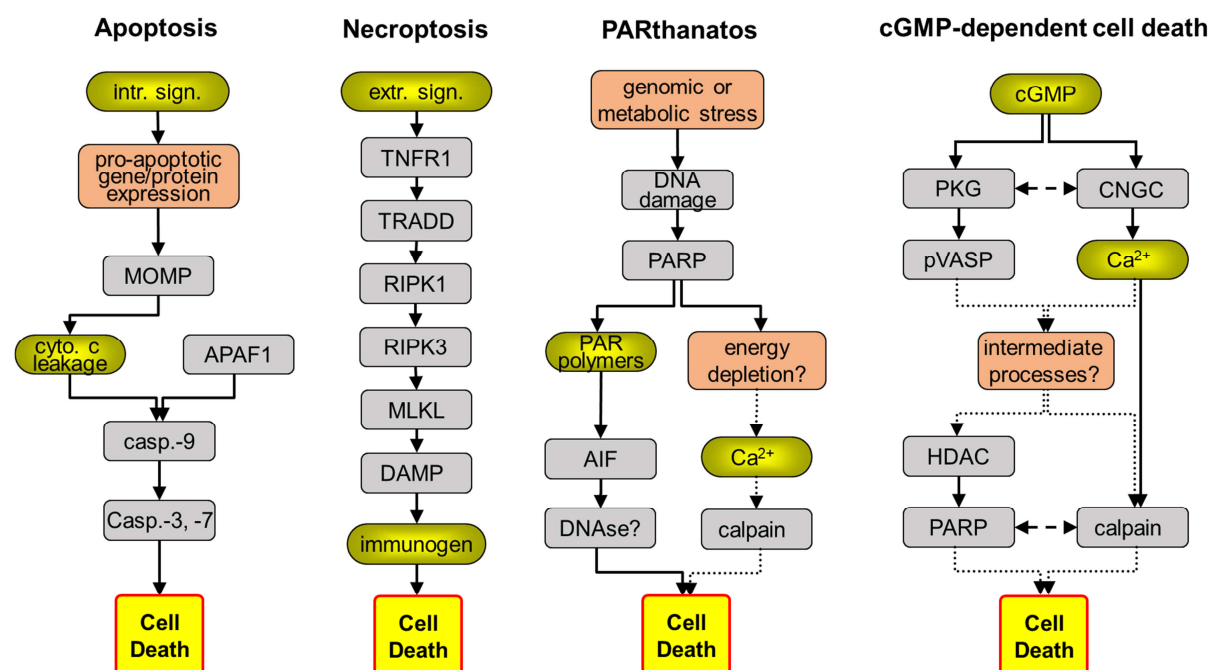
978 The ATP-dependency of apoptotic processes – notably the activation of caspase-type
979 proteases (Liu et al., 1996) – provides for a possible switch between apoptotic and necrotic
980 forms of cell death, *i.e.* in the absence of ATP, necrosis or necrosis-like forms of cell death
981 might be favoured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

985 In consideration of the photoreceptor cell death in RD, this was for a long time thought to
986 be governed by apoptosis based mostly on the observation of DNA fragmentation (Chang et
987 al., 1993), the absence of inflammation and clumping of dying cells (Sancho-Pelluz et al.,
988 2008), and the fact that photoreceptors are lost by an intrinsic, cell autonomous process
989 (Clarke et al., 2000a). However, the views on this have changed over the past decade
990 (Arango-Gonzalez et al., 2014), not the least since there has at the same time been a rising
991 awareness of alternative cell death mechanisms, decidedly different from both necrosis and
992 apoptosis (Galluzzi et al., 2018). Interestingly, the discovery of caspase-independent forms
993 of regulated, program-driven cell death, including in photoreceptors, was one of the first
994 clues as to the existence of further non-apoptotic and non-necrotic degenerative
995 mechanisms (Donovan and Cotter, 2002; Kroemer and Martin, 2005)

996

997 3. 3. Brief overview of alternative cell death mechanisms

998 The use of the terms necrosis and apoptosis to describe cell death rather than specific
999 forms of cell death led to considerable confusion in the scientific literature. In the late
1000 90s/early 2000s several groups tried to define each form of cell death more clearly to build a
1001 proper nomenclature and these efforts eventually led to the formation of the Nomenclature
1002 Committee on Cell Death in 2005 (Kroemer et al., 2005). Here, we will briefly discuss two
1003 of the forms of cell death defined by this committee, necroptosis and PARthanatos,
1004 whereas the next section, 3.d, concentrates on cGMP-dependent photoreceptor
1005 degeneration.



1006

1007 **Figure 5: Comparison of different cell death mechanisms.** Shown are mechanistic
 1008 diagrams illustrating the cellular processes executed during apoptosis, necroptosis,
 1009 PARthanatos, and cGMP-dependent cell death in the retina. See text for more details.

1010 Classical apoptosis involves an intracellular signal (intr. sign.) generated expression of pro-
 1011 apoptotic genes and proteins and the translocation of BCL2 family proteins to produce
 1012 mitochondrial outer membrane permeabilization (MOMP). The resulting leakage of
 1013 cytochrome c (cyto. c) from the mitochondria to the cytoplasm leads to its combination with
 1014 apoptotic protease activating factor-1 (APAF1) and caspase-9 to activate executioner
 1015 caspases, such as caspase-3 and -7.

1016 Necroptosis is triggered by extracellular signals (extr. sign.) leading to activation of
 1017 tumour necrosis factor receptor-1 (TNFR1), which when associated with its adaptor
 1018 protein TRADD drives the activation of receptor interacting protein kinase-1 (RIPK1).
 1019 RIPK1 activates RIPK3 and then mixed-lineage-kinase-domain-like pseudokinase (MLKL)
 1020 resulting in the extracellular release of highly immunogenic damage-associated molecular
 1021 patterns (DAMPs) and the production of a strong inflammatory response.

1022 In PARthanatos genomic or metabolic stress and resultant DNA damage causes over-
 1023 activation of poly ADP-ribose polymerase (PARP). PARP will produce poly-ADP-ribose
 1024 (PAR) polymers and deplete cellular energy resources in the process. PAR polymers can
 1025 apoptosis inducing factor (AIF) leading to DNA degradation, while energy depletion will
 1026 induce increased levels of intracellular Ca^{2+} and activation of calpain-type proteases.

1027 In cGMP-dependent photoreceptor cell death a mutation-induced up-regulation of cGMP on
 1028 the one hand causes activation of cyclic-nucleotide-gated-channel (CNGC), leading to Ca^{2+}
 1029 influx and calpain activation. On the other hand, cGMP-dependent activation of protein
 1030 kinase G (PKG) is somehow (perhaps involving the phosphorylation of the PKG substrate
 1031 VASP) associated with histone deacetylase (HDAC) and PARP activation. Importantly,
 1032 cGMP-dependent photoreceptor cell death offers new targets for photoreceptor
 1033 neuroprotection.

1034 Yellow highlight indicates signalling molecules/processes; orange highlight indicates
 1035 complex processes likely involving multiple proteins and molecules.

1036 3. 3. 1. *Necroptosis*

1037 Necroptosis, in a typical setting, is initiated by signalling of tumour necrosis factor
1038 (TNF) to tumour necrosis factor receptor-1 (TNFR1) (Conrad et al., 2016). TNFR1 when
1039 associated with the adaptor protein tumour necrosis factor receptor-1 death domain
1040 (TRADD) drives the activation of receptor interacting protein kinase-1 (RIPK1) (Hsu et al.,
1041 1996). This in turn sets in motion a cascade of events that activates RIPK3 and mixed-
1042 lineage-kinase-domain-like pseudokinase (MLKL) (Kaiser et al., 2013). MLKL forms
1043 oligomers, usually tetramers or trimers, which translocate to the plasma membrane,
1044 where they bind specific phosphatidylinositol phosphate species, triggering plasma
1045 membrane permeabilization resulting in the extracellular release of so-called damage-
1046 associated molecular patterns (DAMPs) (Trichonas et al., 2010). These DAMPs are
1047 strongly immunogenic and will produce a marked inflammation in the affected tissue
1048 (Figure 5). The latter is thought to be beneficial under conditions of a pathogen infection
1049 (e.g. viruses), but could be highly detrimental if executed in healthy tissue (Kaczmarek et
1050 al., 2013). Even though the activation of RIPK3 is considered a key element of the
1051 necroptotic pathway (Galluzzi et al., 2018), caution is necessary when trying to qualify a
1052 certain observation of cell death, since to date necroptosis can be confirmed only
1053 indirectly by plasma membrane rupture concomitant with an absence of caspase
1054 activation. In the retina necroptosis was reported to occur as a response to injuries
1055 related to the activation of microglia (Huang et al., 2018).

1056

1057 3. 3. 2. *PARthanatos*

1058 PARthanatos is a form of cell death resulting from the hyperactivation of PARP1
1059 (Galluzzi et al., 2018). PARthanatos may be triggered not only by excessive DNA damage
1060 but also by stressors such as, oxidative stress, hypoxia, hypoglycaemia or inflammatory
1061 cues (David et al., 2009; Virag and Szabo, 2002). PARP1 overactivation is thought to
1062 mediate cytotoxic effects through the depletion of NAD⁺ and ATP, resulting in bio-
1063 energetic and redox collapse (Andrabi et al., 2008; Ha and Snyder, 1999; Sims et al.,
1064 1983). The ATP-depletion caused by excessive PARP activity will prevent Ca²⁺-ATPases
1065 from further extruding Ca²⁺ (Guerini et al., 2005) and likely lead to rising intracellular Ca²⁺
1066 levels. Similar to what was reported from necrosis-like forms of cell death, high Ca²⁺ will
1067 activate calpain type proteases, which indeed is a phenomenon that has been associated
1068 with PARthanatos in retinal photoreceptors (Prado Spalm et al., 2018).

1069 Another consequence of PARP over-activation is an accumulation of PAR polymers
1070 (Fatokun et al., 2014), which can bind to the mitochondrial protein apoptosis inducing
1071 factor (AIF) (Moubarak et al., 2007). Upon its release from the mitochondria AIF can
1072 translocate to the nucleus where it in turn can activate DNases, to further precipitate cell
1073 death (Wang et al., 2009). Interestingly, mitochondrial AIF release may be dependent on
1074 calpain activity (Polster et al., 2005). The execution of cell death in PARthanatos is thus
1075 driven by two PARP-dependent processes, energy depletion, as well as PAR
1076 accumulation and AIF release (Figure 5).

1077

1078

1079 3. 4. cGMP-dependent photoreceptor cell death

1080 All the cell death mechanisms mentioned above have been associated with photoreceptor
1081 degeneration in the past, in a variety of different RD disease models (Allocca et al., 2019;
1082 Chang et al., 1993; Liu et al., 1999; Rohrer et al., 2004). However, many genetically distinct
1083 forms of RD share a strong increase in the photoreceptor intracellular concentration of
1084 cGMP (Arango-Gonzalez et al., 2014; Farber and Lolley, 1974; Paquet-Durand et al., 2009).
1085 Together with a number of other findings, this has prompted us to propose a cGMP-
1086 dependent pathway for photoreceptor degeneration, in which high cGMP concomitantly
1087 activates CNGC and PKG, producing excessive Ca^{2+} -influx and protein phosphorylation,
1088 respectively (Arango-Gonzalez et al., 2014). As a possible consequence of the latter, PKG
1089 dependent phosphorylation could trigger HDAC activation (Hao et al., 2011), which appears
1090 to be upstream of PARP activation (Sancho-Pelluz et al., 2010). On the other hand, and
1091 likely in parallel, CNGC-mediated Ca^{2+} -influx can activate calpains (Kulkarni et al., 2016;
1092 Paquet-Durand et al., 2011; Wei et al., 2012b). Both of these two cGMP-dependent
1093 pathways, alone or in concert, may drive photoreceptor cell death (Figure 5). This cGMP-
1094 induced alternative form of cell death appears to be significantly slower than other forms of
1095 cell death (Sahaboglu et al., 2013) (see also 3.f below). Importantly, this pathway offers a
1096 number of new targets for therapeutic intervention, some of which appear early during the
1097 process, as with cGMP-signalling, while others act further down-stream, as with calpains,
1098 HDAC, and PARP.

1099 In this context, it is worth noting that the pathways of cGMP-dependent photoreceptor cell
1100 death display some overlap with what is seen during PARthanatos. Both cell death
1101 mechanisms share an over-activation of PARP and accumulation of PAR (Paquet-Durand et
1102 al., 2007), likely associated with mitochondrial release of AIF (Sanges et al., 2006), as well
1103 as with excessive Ca^{2+} influx and calpain protease activation (Kulkarni et al., 2016; Vighi et
1104 al., 2018b). On the other hand, the upstream events of cGMP-dependent cell death appear
1105 to be different from PARthanatos and one question for future studies may be whether
1106 PARthanatos could be a “subroutine” of cGMP-dependent cell death.

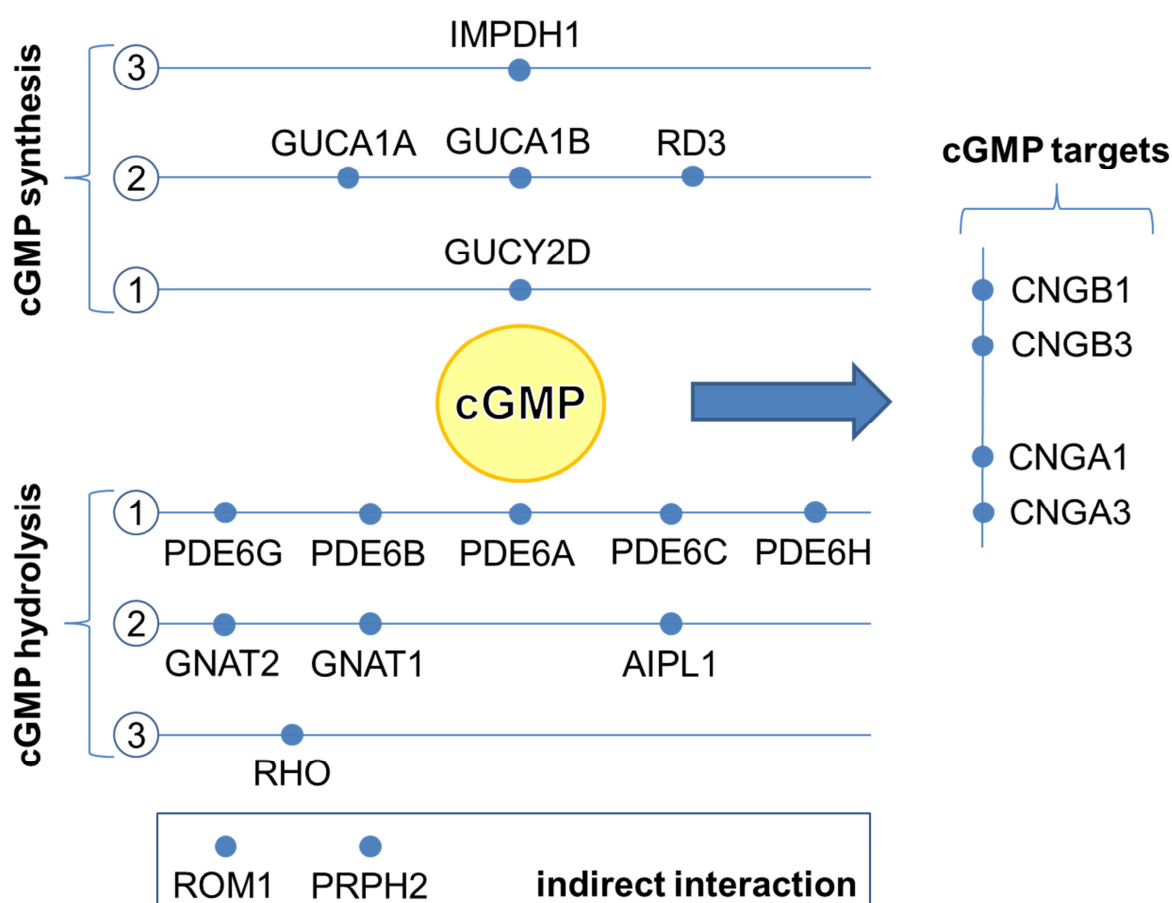
1107

1108 3. 5. RD genes related with high photoreceptor cGMP

1109 Because of this pathologic aspect of cGMP in photoreceptors, we hypothesized several
1110 years ago that interventions in cGMP-signalling might constitute a viable therapeutic avenue
1111 applicable to many different RD-causing mutations. While we were able to show that such
1112 interventions are indeed feasible from a pharmacological and drug delivery standpoint
1113 (Mencil et al., 2018; Paquet-Durand et al., 2009; Vighi et al., 2018b), a question that
1114 remained is how many RD causing mutations, in how many genes, and affecting how many
1115 patients, would actually be amenable to such a treatment. Here, an initial graphical overview
1116 of the relationship between certain RD genes and photoreceptor cGMP levels is given in
1117 Figure 6.

1118 As detailed above (see chapter 2.d.), RD mutations in the genes encoding for PDE6
1119 subunits (*PDE6A*, *PDE6B*, *PDE6C*, *PDE6G*, *PDE6H*) (Brennenstuhl et al., 2015; Dryja et al.,
1120 1995; Dvir et al., 2010; McLaughlin et al., 1993; Thiadens et al., 2009; Trifunovic et al., 2010)
1121 prevent cGMP hydrolysis and therefore lead to excessive accumulation in photoreceptors.
1122 Similarly, mutations in the aryl hydrocarbon receptor-interacting protein-like gene (*AIPL1*)
1123 (den Hollander et al., 2008) thwart the functional assembly of PDE6 enzyme dimers

1124 (Ramamurthy et al., 2004), also causing cGMP accumulation. During phototransduction
 1125 PDE6 is activated by transducin, a protein encoded by the *GNAT1* gene in rods and *GNAT2*
 1126 in cones, explaining why certain mutations in these genes lead to insufficient cGMP
 1127 hydrolysis (Kohl et al., 2002; Mejecase et al., 2016).



1128
 1129 **Figure 6: Relationship of RD genes to photoreceptor cGMP.** The upper part of the
 1130 diagram shows genes involved in cGMP synthesis, while hydrolysis genes are below. cGMP
 1131 targets are shown on the right. The different lines indicate levels of interaction of the gene
 1132 products with cGMP. Level 1 indicates direct binding, level 2 and 3 indicate second and third
 1133 order interaction, respectively.

1134
 1135 While impaired cGMP hydrolysis certainly leads to high cGMP levels in photoreceptors,
 1136 excessive production may produce the same effect. Hence, gain-of-function mutations in
 1137 retGC, a protein encoded by the *GUCY2D* gene (Sato et al., 2018), as well as in GCAP,
 1138 encoded for by the *GUCA1A* and *GUCA1B* genes in rods and cones (Peshenko et al., 2019;
 1139 Sato et al., 2005), respectively, may result in excessive production of cGMP. retGC is
 1140 additionally inhibited by the RD3 protein, so that loss-of-function mutations in the *RD3* gene
 1141 will also cause excessive cGMP production and photoreceptor death (Peshenko et al.,
 1142 2016).

1143 An accumulation of cGMP in photoreceptors is also observed in CNGC mutations in the
 1144 genes *CNGA1*, *CNGA3*, *CNGB1*, and *CNGB3*, (Arango-Gonzalez et al., 2014; Huttli et al.,
 1145 2005; Ma et al., 2013; Paquet-Durand et al., 2011; Reuter et al., 2008), presumably because
 1146 of the lack of negative feedback on retGC and cGMP synthesis in the absence of Ca^{2+} influx
 1147 in the outer segment (see chapter 2.d.) (Olshevskaya et al., 2002).

1148 High cGMP is interestingly also observed in animal models suffering from mutations in
 1149 several other genes not directly related to cGMP synthesis or hydrolysis. For instance,
 1150 several different mutations in the gene encoding for rhodopsin (*RHO*), ranging from a full
 1151 knock-out, to the P23H point-mutation, to the S334ter truncation, were all displaying high
 1152 cGMP levels in photoreceptors (Arango-Gonzalez et al., 2014). While this does not allow to
 1153 conclude that all of the currently known 150 *RHO* mutations (Athanasίου et al., 2018) cause
 1154 cGMP accumulation in photoreceptors, it seems likely that it will be the case in a large
 1155 number of these.

1156 Photoreceptor cGMP accumulation was also found in mutations in the gene encoding for
 1157 the outer segment structural protein peripherin (*PRPH2*) (Arango-Gonzalez et al., 2014;
 1158 Paquet-Durand et al., 2009). Knowledge on the indirect effects of such gene mutations on
 1159 photoreceptor cGMP may allow to infer the situation in yet other gene mutations. For
 1160 example, mutations in *PRPH2* lead to an absence of outer segments, which likely leads to
 1161 an ectopic and dysregulated expression of outer segment enzymes. Peripherin assembles
 1162 with its ortholog retinal outer segment protein-1 (*ROM1*) to anchor photoreceptor disks to the
 1163 outer membrane (Goldberg et al., 2016), and just as *PRPH2* loss-of-function, *Rom1* knock-
 1164 out in the mouse causes outer segment disorganisation and shortening, and photoreceptor
 1165 death (Clarke et al., 2000b). Because of its parallel functions with *PRPH2* it appears likely
 1166 that also *ROM1* mutations will be associated with high cGMP, although this remains to be
 1167 studied. Mutations in the inosine mono phosphate dehydrogenase-1 (*IMPDH1*) gene may
 1168 likewise cause elevated cGMP levels, since the IMPDH1 enzyme catalyses the rate-limiting
 1169 step of GTP production, the substrate employed by retGC for cGMP synthesis. RD-causing
 1170 mutations in *IMPDH1* do not reduce enzyme activity (Aherne et al., 2004) but may affect
 1171 negative regulation of the enzyme (Xu et al., 2008). This would lead to an increased
 1172 production of GTP, which, given the comparatively high Michaelis constant of GC (Aparicio
 1173 and Applebury, 1995), could result in higher than normal synthesis of cGMP.

1174

1175 **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%	(Mejcase et al., 2016)

1178 Apart from the RD-genes mentioned above, where a connection to high photoreceptor
1179 cGMP has either been demonstrated already or appears as very likely, it is reasonable to
1180 think that this will also be the case for other photoreceptor-specific genes. Candidates for
1181 such a relationship may be the transcription factors *CRX*, *NRL*, or *NR2E3*, which regulate
1182 the expression of genes linked to phototransduction (Pittler et al., 2004; Xu et al., 2013),
1183 and, when mutated, may eventually lead to lack of phototransduction activity and
1184 consequently increased cGMP. Likewise, mutations in genes involved in the trafficking of
1185 phototransduction proteins could result in aberrant cGMP production. For instance, the
1186 *REEP6* protein may mediate the trafficking of retGC to the photoreceptor outer segment
1187 (Agrawal et al., 2017). Still, to date it is not known whether *REEP6* mutations cause RD via
1188 aberrant cGMP ectopic production in the photoreceptor cytoplasm. Additionally, high cGMP
1189 may be connected to the pathogenesis of Stargardt disease, an RD-type disease which is
1190 caused predominantly by mutations in the *ABCA4* gene (Gill et al., 2019). *ABCA4* activity is
1191 essential for the shuttling of all-trans-retinal out of the photoreceptor disks so that it may be
1192 recycled by RPE cells (Lenis et al., 2018; Quazi et al., 2012). Accordingly, *ABCA4* mutations
1193 are associated with early impairments in the electroretinographic responses of the retina
1194 (Abed et al., 2018; Fujinami et al., 2013), indicating that insufficient recycling of retinal
1195 decreases phototransduction activity. It is tempting to speculate that this may then also entail
1196 a decrease in cGMP hydrolysis and an accumulation of cGMP over time.

1197 Taken together, many RD genes are related, or likely related, to high levels of cGMP in
1198 photoreceptors. Most of these RD genes are connected to cGMP synthesis, hydrolysis, or
1199 are coding for CNGCs (Figure 6), while in some other cases the relation to cGMP appears to
1200 be more indirect. For an overview of RD genes connected to photoreceptor cGMP see Table
1201 1, which for the ACHM, LCA, and RP disease groups also details the approximate
1202 percentages of patients suffering from mutations that are likely causing excess
1203 photoreceptor cGMP concentrations. While at the moment there may still be considerable
1204 uncertainty about the disease gene distribution in different patient cohorts, ethnicities, and
1205 geographical locations, it is remarkable that, in the numerically most important disease group
1206 – RP – up to 30% of the patient population appears to be related to high cGMP.

1207

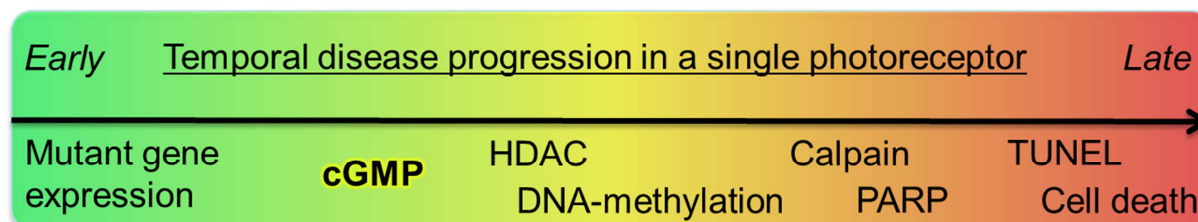
1208 3. 6. On the temporal progression of cell death

1209 The above chapters focused on the question as to **what** happens during neuronal cell
1210 death, yet, a key question that is frequently overlooked is **how long** the cell death process
1211 takes in an individual cell. Although this is a rather simple question, to address it
1212 experimentally has proven to be a difficult task (Henson and Hume, 2006; Skommer et al.,
1213 2010), especially when it comes to investigations in complex neuronal tissues such as the
1214 retina. Since the onset of neuronal cell death in a diseased tissue in most cases follows a
1215 stochastic, non-synchronised process over the entire cell population (Clarke et al., 2000a),
1216 there will be a widespread distribution of different death stages that may be hard to
1217 disentangle. During many of our studies on the mechanisms behind RD, we have therefore
1218 tried to analyse co-appearances (or lack thereof) of various markers, in order to focus on
1219 and understand the degeneration sequence for individual photoreceptors (Ekstrom et al.,
1220 2014; Farinelli et al., 2014; Paquet-Durand et al., 2006; Paquet-Durand et al., 2007; Sancho-
1221 Pelluz et al., 2010), rather than for the retina as a whole. In turn this has enabled us to
1222 suggest a chronology, or "order of appearance", for the degeneration components in a given
1223 photoreceptor (Figure 7). While this alone does not reveal the time needed for the

1224 degeneration (but see below) it can provide important information on causalities.

1225 The question on the duration of cell death is obviously connected to the underlying
 1226 mechanisms, especially as different cell death pathways run on different timescales. For
 1227 instance, necrosis is seen as a fairly rapid destruction of the cell, taking between a few
 1228 minutes and 1-2h to complete (Zong and Thompson, 2006), whereas apoptosis as a
 1229 program driven and orderly disintegration of the cell, is much slower, taking 6-18h to
 1230 complete (Oppenheim, 1991; Wong and Hughes, 1987). Importantly, information on the
 1231 time-course and sequence of degenerative events will define the temporal window-of-
 1232 opportunity, with strong implications for future therapeutic strategies.

1233



1235

1235 **Figure 7: Players and supposed temporal order in *rd1* degeneration.**

1236 Overactivation of the cGMP system triggered by mutations in different genes promotes the
 1237 disease. High cGMP triggers the execution of cell death, likely via concurrent activation of
 1238 histone deacetylase (HDAC), calpain, DNA-methylation, and poly-ADP-ribose polymerase
 1239 (PARP). Since the rise in cGMP occurs early in the degenerative process, targeting of the
 1240 cGMP system gives a chance for protection in time. TUNEL refers to a technique detecting
 1241 dying cells based on chromatin fragmentation.

1242

1243 In a previous study, we used the PDE6 inhibitor zaprinast (Zhang et al., 2005) in wild-type
 1244 retina explant cultures to induce cGMP-dependent photoreceptor cell death in a relatively
 1245 synchronized fashion (Sahaboglu et al., 2013). Curiously, even though zaprinast started to
 1246 exert its inhibitory effect almost immediately after drug application (Wei et al., 2012b), it took
 1247 about 36-48h before a clear rise in photoreceptor cGMP levels could be observed
 1248 (Sahaboglu et al., 2013). This suggests that a photoreceptor cell can sustain PDE6
 1249 inactivation for 1-2 days, keeping cGMP levels within physiological limits, possibly via the
 1250 cGMP – Ca²⁺ feedback regulation detailed in Figure 4 (Burns et al., 2002; Olshevskaya et
 1251 al., 2002). Prolonged PDE6 inactivity may then alter photoreceptor metabolism in a way that
 1252 leads to a catastrophic rise of cGMP, eventually causing the cell to die. Even so, after the
 1253 strong rise in cGMP levels it took another 40h for a photoreceptor to activate DNA
 1254 fragmentation (visualized by the TUNEL assay) and eventually disappear. With a period of
 1255 about 80h – from initiation, to cGMP accumulation, to TUNEL positive reaction, to clearance
 1256 – the time an individual cell needs to die is remarkably long in comparison with the times
 1257 indicated above (a few minutes to 1-2h for necrosis, 6-18h for apoptosis), which therefore
 1258 points towards execution of non-necrotic, non-apoptotic, and also relatively slow, cell death
 1259 mechanisms in RD.

1260

1261 4. Targeting cGMP-signalling for therapy development

1262 The finding that cGMP-dependent cell death may be the prevalent pathogenic
1263 mechanism in a large subset of RD patients raises the possibility to target this pathway for
1264 gene- and mutation-independent therapeutic purposes. Generally speaking, drugs that target
1265 cGMP-signalling have been marketed for decades (e.g. nitro-glycerine, Viagra) illustrating
1266 the feasibility of the concept. However, currently available drugs usually work by *raising*
1267 intracellular cGMP-levels, while the treatment of RD would in most cases require *inhibiting*
1268 cGMP-signalling. Prospective RD drugs could either inhibit cGMP synthesis directly or target
1269 cGMP-signalling indirectly by inhibiting its effectors. Furthermore, such drugs would have to
1270 do this in a highly photoreceptor specific manner, ideally discriminating between rods and
1271 cones.

1272

1273 4.1. Inhibiting cGMP synthesis

1274 In a situation where too much cGMP causes cell death, an ideal treatment approach might
1275 be to reduce the synthesis of cGMP. However, because of the general importance of cGMP
1276 signalling in almost all cells of the body (Pilz and Broderick, 2005), a prospective drug will
1277 need to be highly specific for retGC in photoreceptors, without affecting other GCs
1278 elsewhere. In RP and other rod-cone dystrophies, this would mean that only rod GC should
1279 be inhibited, while cone GC should be unaffected. The key substrate that retGC requires for
1280 cGMP synthesis is GTP and in principle inhibitory GTP analogues such as *Rp*-GTP α S
1281 exhibit a very high specificity for retGC on *in vitro* enzyme preparations (Garger et al., 2001;
1282 Gorczyca et al., 1994). However, such triphosphates, carrying three negative charges, are
1283 highly membrane impermeable. To drive such compounds into a photoreceptor cell, *in vivo*,
1284 will require a very specific and highly efficient drug delivery system (DDS), something that
1285 may still not be available for triphosphates (but see 4.2 below for monophosphate cGMP
1286 analogues). Thus, currently, the direct targeting of retGC for therapeutic purposes does not
1287 appear to be feasible as long as no suitable DDS has been generated.

1288 An alternative approach to reducing cGMP synthesis could be to inhibit upstream
1289 enzymes in the GTP synthesis pathway. One such target is IMPDH1, which catalyses the
1290 rate-limiting step of GTP production and likely produces most of the photoreceptor GTP
1291 (Aherne et al., 2004). IMPDH1 can be inhibited selectively by the registered
1292 immunosuppressive drug mycophenolate (Allison and Eugui, 2000). Accordingly,
1293 mycophenolic acid was suggested to reduce photoreceptor cGMP and to have protective
1294 effects in the *rd1* and *rd10* mouse models (ARVO2018 abstract (Yang et al., 2018)). If
1295 confirmed further, mycophenolate, or similar IMPDH1 targeting drugs, could potentially be
1296 developed into effective RD treatments, with the wealth of already available clinical data
1297 likely facilitating such repurposing.

1298

1299 4.2. Blocking the effects of cGMP-signalling

1300 Other than inhibiting cGMP synthesis, a therapeutic agent may instead block the targets
1301 of cGMP-signalling, without reducing cGMP-levels. As stated above (chapters 2.5 and 2.7),
1302 in the photoreceptor these targets include the disease drivers PKG and CNGB1 (Paquet-
1303 Durand et al., 2011; Paquet-Durand et al., 2009). For the inhibition of CNGB1 the same
1304 conditions as for targeting retGC would apply, namely a drug should be able to discriminate

1305 between rod and cone CNGC, so as to preserve the functionality of cone phototransduction
1306 (chapter 2.5). PKG, on the other hand, is not known to exist in specific rod or cone variants
1307 (Ekstrom et al., 2014). In a situation where cGMP levels are very high in rods, but normal in
1308 cones, dampening PKG activity, with its signal amplified by downstream kinases and
1309 transcriptional activity (Pilz and Broderick, 2005), may provide for stronger leverage
1310 compared to drugs targeting retGC or CNGC. Inhibition of PKG additionally has the
1311 advantage that it is unlikely to interfere with phototransduction (Vighi et al., 2018b).

1312 Intriguingly, the photoreceptor cGMP targets CNGC and PKG can be inhibited with very
1313 high specificity and selectivity by analogues of cGMP (Butt et al., 1990; Vighi et al., 2018b;
1314 Wei et al., 1998). Compared to triphosphate GTP analogues, cGMP analogues carry only
1315 one negative charge and, by adding electronegative or lipophilic substituents, can be
1316 designed to have a high membrane permeability, enabling *in vivo* applications even without
1317 the use of dedicated DDS (Rapoport et al., 1982; Werner et al., 2011; Zhuo et al., 1994).

1318 However, for a long time an important obstacle for the clinical development of cyclic
1319 nucleotide analogues was their rapid clearance via the kidney and their correspondingly very
1320 low bioavailability (Coulson et al., 1983; Schwede et al., 2000). Thus, for drugs aimed at
1321 photoreceptor proteins it is essential to use a suitable DDS that can deliver such compounds
1322 across the different retinal barriers to the photoreceptors, for prolonged periods of time
1323 (Himawan et al., 2019). With such DDS technology now becoming increasingly available,
1324 clinical development of cGMP analogues also becomes feasible. For instance, the European
1325 DRUGSFORD project (*i.e.* “drugs for RD”; www.drugsford.eu) generated over 80 novel
1326 inhibitory cGMP analogues and tested these together with a liposomal DDS to enable
1327 efficient and sustained delivery to the neuroretina. Notably, this combination resulted in the
1328 morphological and functional preservation in different pre-clinical RD models (Vighi et al.,
1329 2018b).

1330 While these results highlight the potential of inhibitory cGMP analogues in forms of RD
1331 connected to high photoreceptor cGMP levels, a clinical proof-of-concept may be several
1332 years away still. Importantly, for all patients in such future clinical trials, the causative
1333 mutations and their connection to high photoreceptor cGMP must be clearly established prior
1334 to their enrolment (see chapter 5.2.1). To turn around the perspective, cGMP analogues
1335 activating PKG were shown to kill healthy, wild-type photoreceptors (Paquet-Durand et al.,
1336 2009), and have furthermore displayed significant anti-proliferative capacities in certain
1337 cancer cell lines (Hoffmann et al., 2017; Vighi et al., 2018a). It thus remains to be seen
1338 whether such activatory analogues could instead have protective capacity in situations
1339 where photoreceptor cGMP is too low, as may be the case in *GUCY2D* or *IMPDH1* loss-of-
1340 function mutations (Aherne et al., 2004; Williams et al., 2006).

1341

1342 5. The future of retinal degeneration research

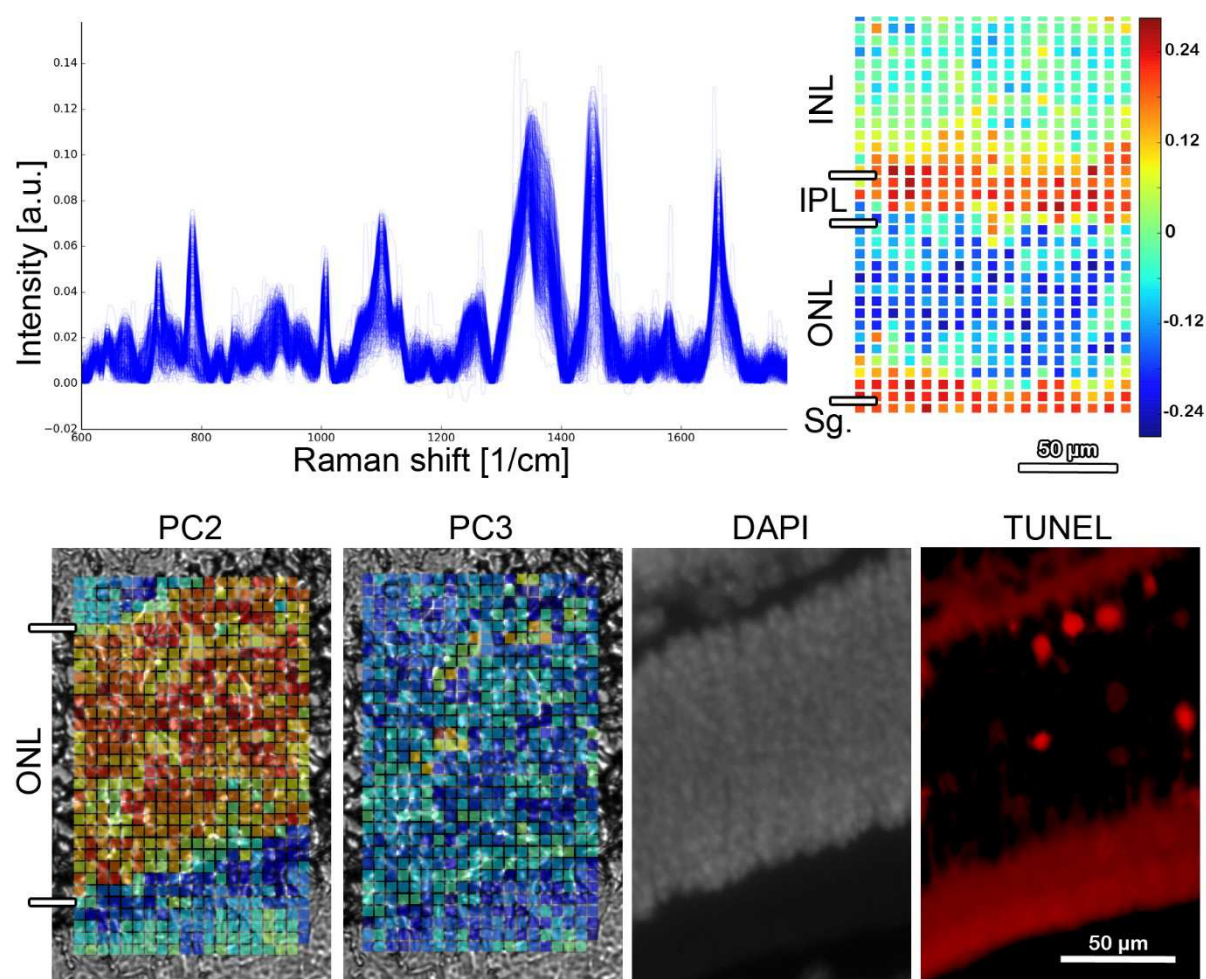
1343 In the final chapter of this review, we give an overview of the technological and
1344 methodological developments that we feel will be needed to forward a deeper understanding
1345 of the complexity of cell death and hereditary retinal degeneration. Another point will be to
1346 advance clinical testing, notably to develop clinical test strategies that fully consider the
1347 genetic heterogeneity of RD-type diseases and exploit it to enable faster and more
1348 meaningful clinical test results.

1349 5. 1. What kind of methods do we need in the future for the study of
1350 photoreceptor cell death mechanisms?

1351 A key problem of all current technical approaches is that these do not allow for a
1352 temporo-spatial resolution of the multitude of metabolic processes happening during cell
1353 death at the level of an individual dying cell. While single-cell RNA sequencing allows to
1354 study gene expression profiles in different cell types within a tissue (Peng et al., 2019),
1355 similar insight into cellular biochemistry is far more difficult to obtain.

1356 To capture the processes governing photoreceptor cell death in their entirety, we will
1357 need to be able to analyse single cell metabolism, ideally following the fate of a single cell
1358 over prolonged periods of time. Single cell analysis at this level will likely require the
1359 development and use of novel technology, collectively referred to as hyperspectral imaging.
1360 Among the techniques that may be useful here in the near future are near-infrared (NIR)
1361 spectroscopy, fluorescence life-time-imaging (FLIM) (Dysli et al., 2018) and the combination
1362 of Raman spectroscopy with laser-confocal microscopy or Raman imaging (Gaifulina et al.,
1363 2016; Manley, 2014). Another technique that may resolve individual metabolites on retinal
1364 preparations is matrix-associated-laser-desorption/ionization (MALDI) mass-spectrometry
1365 imaging (MSI) (Bowrey et al., 2016; Ly et al., 2015). However, MALDI-MSI is destructive,
1366 and its spatial resolution is currently still limited to approx. 30-50 μm , *i.e.* too large to resolve
1367 individual cells.

1368 Raman microscopy is an attractive analytical choice since it is a non-destructive
1369 technique in which single cells or entire tissues may be sampled many times over (Karuna et
1370 al., 2019). It is a label-free technique that provides chemical information about the metabolic
1371 status of biological samples, detecting structural changes within the major macromolecules
1372 such as proteins, lipids, carbohydrate and nucleic acids. Each molecule contributes to a
1373 spectral pattern that is considered as a fingerprint of the analysed cell. Raman spectra
1374 enable discrimination, for example, between healthy and diseased or between living and
1375 dead cells (Brauchle et al., 2014). Furthermore, Raman microscopy can reach a high lateral
1376 and spatial resolution of about 1 μm^3 measurement volume using a confocal setup. Raman
1377 data can be transformed to the corresponding heat-maps and pseudo color-coded images
1378 using multivariate analysis methods. In these constructed images, clusters with similar
1379 spectra are grouped and coded with the same colour (Miljkovic et al., 2010), making it
1380 possible to depict cell types and different cell states within the Raman spectral image.



1381

1382 **Figure 8: Raman imaging detects dying photoreceptors:** Top left: Raman spectra
 1383 collected on cross-sections of *rd1* retina at post-natal day 11, a time of active degeneration
 1384 in this animal model. Top right: Principal component analysis (PCA) of Raman spectra
 1385 obtained by scanning with a confocal Raman microscope. PC2 identifies outer nuclear layer
 1386 (ONL) photoreceptor nuclei (negative values, dark blue). Membranous structures in
 1387 photoreceptor segments (Sg.) and inner plexiform layer (IPL) show positive PC2 values
 1388 (red). PC2 also distinguished neurons in inner nuclear layer (INL, green-yellow). Bottom
 1389 panel: PCA analysis and subsequent DAPI and TUNEL staining. PC2 labels photoreceptor
 1390 nuclei (orange-red) and conforms to a DAPI staining performed on the same specimen. PC3
 1391 colocalizes with TUNEL assay identifying dying cells. Note: retinal specimens may have
 1392 been distorted by staining procedures.

1393

1394 In a pilot study, Raman imaging was performed using the Raman microscope system
 1395 BioRam® (CellTool GmbH Tutzing, Germany). We found that the diversity of neuronal cells
 1396 in the retina can easily be depicted based on their Raman spectroscopic fingerprints.
 1397 Excitingly, a comparatively simple principal component analysis (PCA) readily identified
 1398 dying photoreceptors in RD mutant retina (Figure 8). While still very early, these results
 1399 highlight the promise of label-free and non-destructive hyperspectral imaging for future
 1400 studies of neuronal cell death mechanisms within the retina.

1401 Further development of this technology may allow the identification of individual
 1402 metabolites based on the Raman spectra obtained from pure reference compounds and the
 1403 informatic mixing of a large number of reference spectra (Scheier et al., 2014). Eventually,

1404 this may enable us to temporally and spatially resolve key metabolites (Gaifulina et al., 2016)
1405 relevant for cellular metabolism. Comparing the metabolism of healthy and dying cells will
1406 lead to the construction of the “activitome” (also referred to as “reactome”) of cell death,
1407 promising to deliver a wealth of new data on cell death mechanisms.

1408

1409 5. 2. From bench to bedside: Why is clinical translation so difficult?

1410 The lack of treatments for RD is in part due to an incomplete understanding of the
1411 underlying photoreceptor cell death mechanisms and a lack of targets that could be
1412 exploited for therapy development, especially for pharmacology-based therapies. The
1413 missing mechanistic knowledge furthermore makes it difficult to develop biomarkers for early
1414 disease diagnosis and an assessment of treatment efficacy. Nevertheless, the wealth of new
1415 information on degenerative mechanisms that has become available within the last decade
1416 promises to change this situation (Scholl et al., 2016).

1417

1418 5. 2. 1. Genetic heterogeneity combined with rarity: A unique problem with unique 1419 opportunities

1420 RD-type diseases show an enormous genetic heterogeneity with disease-causing
1421 mutations in more than 270 genes (see chapter 1). Since each of these disease genes can
1422 carry from several dozens to several hundred or more individual mutations (Athanasidou et
1423 al., 2018; Messchaert et al., 2018), we may, at present, estimate the total number of disease
1424 mutations to amount to several tens of thousands. This situation severely hinders the design
1425 of clinical trials as the numbers of patients carrying a specific disease-causing mutation will
1426 be small, even in a best-case scenario. However, a careful choice of the patients to be
1427 included in a clinical trial, with precisely known genotypes, is critical for success during
1428 clinical testing. The typically very slow progression of RD-type diseases over the course of
1429 many decades makes it difficult to identify patients that will be in a suitable stage of the
1430 disease (Iftikhar et al., 2019). Moreover, in preventive treatment trials the slow disease
1431 progression and the lack of biomarkers, that can identify treatment effects early on, will likely
1432 require very long clinical trial timelines, multiplying the associated clinical development costs.

1433 Interestingly, RD-type diseases may allow to solve this problem in a way that is not
1434 available in other, common neurodegenerative diseases of the retina such as diabetic
1435 retinopathy or age-related-macular degeneration, and even less so for diseases of the brain
1436 such as Alzheimer’s, or Parkinson’s. RD-type diseases of the RP and LCA group are not
1437 only closely connected genetically but the causative mutations typically affect the same
1438 biochemical pathways. An example for this closeness may be mutations in the *AIPL1* gene,
1439 which cause fast progressing LCA (den Hollander et al., 2008; Ramamurthy et al., 2004),
1440 and mutations in *PDE6* genes, which produce the relatively slow progression RP phenotype
1441 (Gopalakrishna et al., 2017). Since *AIPL1* is needed to functionally assemble the *PDE6* α - β
1442 dimer, mutations in both genes cause an excessive accumulation of cGMP and
1443 photoreceptor degeneration. In some cases, mutations in the very same gene may produce
1444 a very rapid LCA-type degeneration, while a different mutation results in the less aggressive
1445 RP-type disease progression. An example for this situation are mutations in the *PRPH2*
1446 gene, which can give rise to both LCA and RP depending on where in the gene exactly the
1447 mutation resides (den Hollander et al., 2008; Gill et al., 2019).

1448 This interconnection between LCA and RP enables a clinical trial strategy which has the
1449 potential to significantly shorten clinical test-timeframes. Clinical testing must typically first
1450 establish safety and tolerability in adult subjects in a phase 1 or phase 1/2a trial. Once this
1451 has been demonstrated, a phase 2 (2b) trial can move to children suffering from rapid
1452 progression LCA, to establish clinical efficacy in the relatively short timeframe of 6 to 12
1453 months. If efficacy in LCA can indeed be demonstrated, then there will be sufficient rationale
1454 to perform (and fund) a similar long-term clinical trial also in slow progressing adult RP
1455 patients. Besides, the numbers of patients required for such clinical trials, and even as far as
1456 market registration, can be rather small thanks to special rare disease regulations and
1457 legislation. Thus, as opposed to the situation in most other neurodegenerative diseases, the
1458 interrelation of RD-type diseases provides a unique opportunity to accelerate clinical testing,
1459 at comparatively moderate development costs.

1460

1461 5. 2. 2. Biomarkers for retinal degeneration

1462 Another important problem for clinical translation is a lack of *in vivo* biomarkers that could
1463 be used for the rapid assessment of treatment efficacy. Ideally biomarkers should allow for a
1464 live, non-invasive visualization of cell death in the retina, using techniques such as scanning
1465 laser ophthalmoscopy (SLO) (Beck et al., 2010; Paquet-Durand et al., 2019) or adaptive
1466 optics SLO (AO-SLO) (Walters et al., 2019). For instance, the binding of the protein annexin-
1467 5 to phosphatidylserine, may be utilized for *in vivo* detection of apoptotic cells (Kurosaka et
1468 al., 2003). Such methodology has been developed for studies on glaucoma and retinal
1469 ganglion cell death, initially in a mouse model (Cordeiro et al., 2010) and eventually in a
1470 clinical trial (Cordeiro et al., 2017), using intravitreal injection of a fluorescently labelled
1471 derivative of annexin-5. Similar methodology may be applicable also to the *in vivo* cell death
1472 detection in RD-type diseases. A future combination of non-invasive retinal imaging, such as
1473 SLO, with label-free cell death detection techniques, such as Raman spectroscopy (see
1474 chapter 5. a.), could advance pre-clinical and clinical examinations even further.

1475 Recent developments in the field of magnetic resonance imaging (MRI) suggest that it
1476 may be possible to non-invasively observe oxidative stress, or production of free radicals, in
1477 for instance the retina (Berkowitz, 2018), at least in experimental animals. While this
1478 approach, coined QUEST-MRI, would perhaps not detect ongoing photoreceptor
1479 degeneration as such, it may, once transferred to the clinic, still be useful in providing a
1480 snapshot on the oxidative stress load, and could serve as an index, or surrogate marker, on
1481 the photoreceptor status.

1482 As an example of potential blood-based parameters, it is interesting to note that there are
1483 reports on increased cGMP in blood from RP patients compared to healthy counterparts
1484 (Camara et al., 2013; Kjellstrom et al., 2016). This may be related to the exaggerated cGMP
1485 levels in the photoreceptors of many RP types, *i.e.* the phenomenon discussed in several of
1486 the sections above, especially since at least some of the patients had mutations in the
1487 *PDE6A* gene (Kjellstrom et al., 2016). When connected with other measurements and
1488 parameters obtained from at least certain cohorts of RP patients, a future blood-test may
1489 allow to assess the retinal status and disease progression in genetically defined RP patient
1490 cohorts (Lains et al., 2019).

1491

1492

1493 5. 2. 3. Ocular barriers and retinal drug delivery

1494 The translation of research findings from the lab to the clinic faces another important
1495 hurdle, namely the blood-retinal barrier (BRB) and other ocular barriers that prevent
1496 therapeutic agents from reaching the photoreceptor cells in the retina. As laid out in chapter
1497 1 the retina is shielded against external agents (e.g. toxins, pathogens) by the inner and
1498 outer blood-retinal barrier. To overcome this barrier, a variety of different technical
1499 approaches have been pursued, using different routes of administration, suprachoroidal
1500 injection (Yeh et al., 2018), subretinal injection (Ochakovski et al., 2017), injection into the
1501 capsule of Tenon (Ohira et al., 2015), and intravitreal injection (Meyer et al., 2016). Each of
1502 these administration routes have specific advantages and disadvantages, but whichever
1503 administration route is chosen, the drug formulation and the delivery system used will be
1504 critical for successful treatment development.

1505 Different drug delivery systems (DDS) have been developed to allow therapeutic agents
1506 to reach the retinal photoreceptors. This includes, light-responsive polymers for non-invasive
1507 triggering of intraocular drug release (Huu et al., 2015), polymeric nanoparticles (Koo et al.,
1508 2012), or glutathione-conjugated liposomes originally intended for drug delivery to the brain
1509 (Birngruber et al., 2014; Vighi et al., 2018b). Indeed liposomes – decorated or not with
1510 polyethylenglycol (PEG) chains – may be used for direct compound administration to the
1511 vitreous (Bochot and Fattal, 2012) where, for instance, non-PEGylated liposomes have
1512 shown improved cargo delivery to photoreceptors (Asteriti et al., 2015).

1513 Targeted downregulation of crucial BRB components has also been suggested for drug
1514 delivery to the retina. A proof-of-concept for this approach has been delivered with an
1515 siRNA-mediated transient knock-down of a claudin-5, a protein needed to form tight
1516 junctions in the BRB (Campbell et al., 2013). With this approach, a compound may be
1517 applied systemically via the blood stream, and could, within a specific time-frame penetrate
1518 the retina, as long as BRB component remains open (Campbell et al., 2018).

1519 In terms of retinal drug delivery, the last ten years have seen an important development
1520 and the appearance of many innovative materials, designs, and technologies. Still, efficient
1521 and sustained drug delivery to the photoreceptors remains a major challenge. Importantly,
1522 each compound or therapeutic agent may require highly adapted DDS, which additionally
1523 must comply with regulatory requirements (Himawan et al., 2019). Therefore, future research
1524 into new treatments for RD should take the retinal delivery problem into consideration as
1525 early as possible and synchronize compound and delivery development.

1526

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1532

1533 **6. References**

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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.