Mol Neurobiol (2008) 38:253–269
DOI 10.1007/s12035-008-8045-9

Photoreceptor Cell Death Mechanisms in Inherited Retinal Degeneration

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Received: 20 June 2008 / Accepted: 16 October 2008 / Published online: 4 November 2008

Abstract Photoreceptor cell death is the major hallmark of a group of human inherited retinal degenerations commonly referred to as retinitis pigmentosa (RP). Although the causative genetic mutations are often known, the mechanisms leading to photoreceptor degeneration remain poorly defined. Previous research work has focused on apoptosis, but recent evidence suggests that photoreceptor cell death may result primarily from non-apoptotic mechanisms independently of AP1 or p53 transcription factor activity, Bcl proteins, caspases, or cytochrome *c* release. This review briefly describes some animal models used for studies of retinal degeneration, with particular focus on the *rd1* mouse. After outlining the major features of different cell death mechanisms in general, we then compare them with results obtained in retinal degeneration models, where photoreceptor cell death appears to be governed by, among other things, changes in cyclic nucleotide metabolism, downregulation of the transcription factor CREB, and excessive activation of calpain and PARP. Based on recent experimental evidence, we propose a putative non-apoptotic molecular pathway for photoreceptor cell death in the *rd1* retina. The notion that inherited photoreceptor cell death is driven by non-apoptotic mechanisms may provide new ideas for future treatment of RP.

Keywords AIF · Calcium · Calpain · cGMP · CREB · Oxidative stress · PARP · Retina · *rd1* · *rd2* · rds

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Introduction

The retina consists of highly specialized, multilayered neuronal tissue with the unique function of light perception and first order signal processing. The retinal pigment epithelium harbors parts of the retinoid acid cycle and is involved in the continuous regeneration of visual pigments. The outer nuclear layer (ONL) of the retina contains rod and cone photoreceptor nuclei which are responsible for vision in dim lighting and for color and daylight vision, respectively. The cells of the inner nuclear layer (INL) are responsible for trophic support, signal processing, and signal transmission to the ganglion cell layer. Finally, axons of ganglion cells form the optic nerve, which transmits visual information to the brain [1].

Certain inherited forms of retinal degeneration, commonly referred to as retinitis pigmentosa (RP), result in selective photoreceptor cell death. They usually follow a two-stage process in which first the rod-type photoreceptors degenerate, then the cone-type photoreceptors, even though the latter may not primarily be affected by the disease. The progressive loss of these cells produces characteristic clinical symptoms: night blindness, constriction of the visual field (tunnel vision), and finally the loss of central vision [2]. In the developed nations, RP is the prevalent

cause of blindness in the working age population [3] and currently no treatment is available [4]. So far, RP causing mutations have been identified in more than 35 genes (Retnet database, www.sph.uth.tmc.edu/retnet, information retrieved in May 2008).

Genetic mutations triggering photoreceptor degeneration often affect the retinoid acid cycle or the phototransduction cascade [5, 6]. Phototransduction begins with a light-induced, conformational change of 11-*cis*-retinal to all-*trans*-retinal. This in turn activates opsin molecules, causing sequential activation of transducin and cyclic guanosine monophosphate (cGMP)–phosphodiesterase-6 (PDE6) and subsequent decreases in intracellular cGMP concentration. High cGMP levels maintain cyclic nucleotide-gated (CNG) cation channels in the open state, allowing a calcium (Ca^{2+}) influx [4]. Reduction of cGMP levels closes CNG channels, causing cell hyperpolarization and signal transmission to second order neurons in the INL [7].

The genetic causes for RP are known in many cases; hence, gene therapy would in principle be the first choice for treatment [8]. However, gene therapy is severely hampered by the variety of genes and mutations, and therefore therapy would in many cases have to be tailor made for each patient. The situation is further complicated by the fact that many patients still have unidentified mutations and that the affected cells carrying genetic mutations in RP quickly degenerate and thus become unavailable for gene therapy. Since it can be assumed that different genetic mutations eventually trigger identical or similar photoreceptor cell death mechanisms [9–11], it may be possible to treat a number of genetically different types of degeneration using similar pharmacological interventions. However, the mechanisms which cause photoreceptor cell death are still unknown and a thorough understanding of these is essential for the development of effective therapies for RP. Although previous studies have suggested that photoreceptor cell loss in RP is driven by apoptosis [9, 12, 13], recent studies indicate the involvement of other cell death pathways [14–16].

The objective of the present review is to compare the features of different cell death mechanisms in light of recent results concerning photoreceptor degeneration obtained in animal model studies of RP. Taken together, the evidence available to date suggests that non-apoptotic mechanisms are at play during photoreceptor cell death; this may have important implications for the development of therapies for RP.

Animal Models

Because of limited experimental possibilities when working with human RP patients, studies of retinal degeneration mechanisms rely heavily on the use of corresponding animal models. While a relatively large number of retinal degeneration models are available [17, 18], we will concentrate for the purposes of this review on the animal model that has so far been studied the most, the *rd1* mouse. In specific cases, we will also refer to less well-studied models such as the *rd2* mouse or certain rhodopsin mutant mice.

Genetic Models

The *rd1* mouse, first described by Keeler in 1924 [19], is also known as the "rd" ("retinal degeneration") mouse, and is to date the best-characterized animal model of RP, which is caused in it by a mutation in exon 7 of the beta subunit of the rod photoreceptor PDE6 gene, producing a non-functional PDE6 [20] and leading to accumulation of cGMP [21]. In this mouse, rod photoreceptor degeneration starts around postnatal (PN) day 10 [12, 22, 23], peaks at PN12–14 [24, 25], and is almost completed by PN21, when only one to two rows of, predominantly, cone photoreceptors remain in the ONL [26]. Cone photoreceptors, although unaffected by the mutation, also degenerate secondarily within the first two to six postnatal months [7, 27]. Since mutations in the PDE6 beta gene are responsible for RP in about 4–5% of human RP patients [28], this makes the *rd1* mouse a relevant model for human RP. In addition, PDE6 alpha mutations are responsible for another 4–5% of human RP cases [29].

PDE6 mutations in general are also found in other animal models of retinal degeneration, such as the *rd10* mouse, which carries a missense mutation in exon 13 of the PDE6 beta gene [17, 30], causing a slower degeneration than in the *rd1* mouse. In *rd10* animals, rod cell death begins at PN16, peaks around PN20–25, and is completed by PN45 [30–32]. A cone-specific PDE6 mutation characterized by selective cone photoreceptor loss is the genetic basis for retinal degeneration in the cone photoreceptor function loss (cpfl1) mouse [17, 33].

Retinal degeneration in the *rd2* mouse (also referred to as "retinal degeneration slow" or rds) is caused by a mutation in the gene coding for peripherin/rds (Prph2) [34, 35], which may function as a multifunctional scaffolding protein essential for the formation of rod photoreceptor outer segments [36]. In the *rd2* mouse, photoreceptor cell loss starts at 2 weeks after birth but progresses much more slowly than in the *rd1* mouse and is completed only 1 year after birth [37]. Mutations in the Prph2 gene are found in approximately 5% of human RP cases [38], making the *rd2* mouse another relevant model for the study of RP. Interestingly, peripherin may indirectly regulate PDE6 activity [39], and perhaps cause cGMP accumulation and degeneration mechanisms similar to those observed in the *rd1* retina.

The single most important gene as far as RP in humans is concerned is rhodopsin [40]. More than 120 point mutations in the human rhodopsin gene have been identified (Retnet database), giving rise to approximately 10% of human RP cases [41]. A correspondingly large number of different animal models for rhodopsin exist, with highly variable onset and progression of the disease [42–44]. These rhodopsin mutants, although genetically very heterogeneous, can be roughly categorized into two groups: loss-of-function (as seen for instance in the rhodopsin knock-out) [45, 46] or gain-of-function (such as the K296E mutation) [47, 48]. Gain-of-function mutations may cause an increased and sometimes constitutive phototransduction activity, leading to low intracellular cGMP levels, while loss-of-function mutations may reduce phototransduction activity and raise cGMP levels [44].

Light-induced Damage Model

Photoreceptor degeneration induced by excessive illumination has been widely used as a model for RP [49]. This model has the major advantage of a synchronized onset of rod and cone cell death combined with the possibility of regulating damage levels by adjusting light intensity and exposure duration [50, 51]. The progression of the light-induced degeneration is rather quick, usually taking less than 10 days from onset to complete disappearance of rods and cones [51]. Different apoptotic mechanisms have been suggested as the cause of light-induced photoreceptor degeneration [51, 52], but it remains unclear how this model compares with genetically induced RP.

Cell Death Mechanisms

A variety of different concepts have been introduced in the past 30 years to characterize neuronal cell death mechanisms. Terms such as apoptosis, necrosis, programmed cell death (PCD), or autophagy are frequently used to describe forms of cell death that display more or less distinct morphological, biochemical, or genetic features [53]. Cell death during inherited or induced retinal degeneration is usually referred to as apoptosis [9, 10, 51].

Apoptosis was first described as an active process requiring protein synthesis and the availability of energy-containing substrates [54]. As a form of PCD, it is characterized by a sequence of events triggered by the cell itself in which dysfunctional or superfluous cells remove themselves without damaging adjacent, healthy tissue [53, 55]. The sequence of apoptotic events starts with extra- or intracellular signaling that leads to increased transcription and translation of proapoptotic genes and proteins [56, 57]. Among the transcription factors behind initial proapoptotic transcriptional changes are activator protein complex-1 (AP1), a heterodimer composed of c-fos and c-jun proteins [58, 59], and p53 [60]. The progression of apoptosis leads to morphological changes, including membrane blebbing, condensation of the nuclear chromatin and cytoplasm, fragmentation of the nucleus, and budding of the whole cell to produce membrane-bound bodies in which organelles are initially intact [55, 61]. These bodies are disposed of without inducing inflammation. The biochemical mechanisms used to execute apoptosis result in a number of hallmark features, including cytoskeletal proteolysis, disruption of mitochondrial function, and orderly DNA fragmentation. These processes are often driven by caspases, a family of cysteine proteases. Consequently, caspase activity is considered central to most definitions of apoptosis [56, 57] although in some experimental systems neuronal apoptosis has also been found to be caspase independent [62, 63]. As an active process, apoptosis requires energy-containing substrates such as ATP to support transcriptional and translational changes and to mediate activation of the executioner caspases [53, 64]. Hence, mitochondria play a central role in apoptosis, since they supply the energy necessary for its execution. Proteins involved in the apoptotic cascade may originate from or target the mitochondria. These proteins can be grouped into two classes: pro- (such as Bax, Bak, and Bok) and anti-apoptotic members of the Bcl-2 family (such as Bcl-2, Bcl-XL, Bcl-W, and Mcl-1) and factors that may be released during apoptosis, like apoptosis inducing factor (AIF) and cytochrome *c* [65].

The terminal deoxynucleotidyl transferase dUTP nick-end labeling method (TUNEL) [66] is often used to characterize apoptotic cell death *in situ*. However, TUNEL stains cells in many different forms of cell death, including in necrosis [67, 68]. In fact, most techniques used to typify apoptosis have also been reported to label necrosis, possibly because late events are similar in both types of cell death [69].

Necrosis is often seen as the conceptual counterpart of PCD and hence apoptosis as well. It is defined as a passive form of cell death that can only be prevented by removal of the inducing stimulus [53, 70]. As such, necrosis is not a cell death "mechanism" in the proper sense of the word, because it is entirely independent from metabolic pathways [71]. However, there are forms of cell death often referred to as "necrosis-like" that require some level of metabolic activity, and hence are active processes, that nevertheless share a number of morphologic features with necrosis. These features include electron-translucent cytoplasm, swelling of cellular organelles, and loss of plasma membrane integrity [70]. In contrast to apoptosis, necrosis induces an inflammatory reaction due to the release of intracellular contents into the extracellular space [55, 64].

At the biochemical level, necrosis—or necrosis-like PCD—has been associated with calpain activation, as opposed to caspase activation which is seen as a hallmark of apoptosis [62, 72]. While apoptosis as an active process relies on the presence of energy-containing substrates and functional biosynthesis [53, 64], necrosis-like processes can occur quickly and are associated with a bioenergetic failure [70, 73].

Although the role of apoptosis in developmental processes—in the mammalian retina, for instance—is well established [74–76], its relevance for pathological processes is less clear [53, 62]. Apart from apoptosis, a number of other PCD mechanisms have been described such as caspase-independent apoptosis, autophagy, and necrosis-like PCD [53, 62, 70, 72, 77, 78]. These may be relevant in the context of retinal photoreceptor degeneration, where pharmacological inhibition or genetic manipulation of characteristic apoptotic processes, for instance the caspase cascade, does not promote survival [78, 79]. The form of cell death initiated in retinal degeneration might be governed by energy availability in the sense that a mutation-induced imbalance in a photoreceptor cell's metabolic function could push it towards utilizing more necrosis-like mechanisms [57, 71, 80].

Secondary Cell Death of Photoreceptors

Different mechanisms are discussed as possible causes of mutation-independent secondary cell death of cone photoreceptors [6, 81]. These include loss of structural support [82, 83] and/or loss of trophic support, i.e., rod-derived cone viability factor [84, 85] when rod cells are degenerating and disappearing. Furthermore, dying rods may release toxic substances that negatively affect the neighboring cells ("innocent bystander" effect) [86, 87]. Yet another hypothesis concerning secondary cell death focuses on the importance of changes in oxygen consumption levels as a function of rod degeneration: loss of rods, and hence oxygen consumption, may induce hyperoxia, with a concomitant, eventually fatal increase in oxidative stress in surviving cells [88–90].

Although RP was originally described as an inflammation of the retina (hence the name "retinitis"), there is no clear evidence for inflammatory processes during inherited retinal degeneration. Nevertheless, a chronic activation of microglial cells has been described in animal models of RP [91–93]; moreover, in other retinal diseases such as AMD, diabetic retinopathy, and glaucoma, activation of the immune system seems to contribute to cell death [94–97]. An immune response might possibly enhance degenerative processes and could in part be responsible for the secondary loss of cone photoreceptors which are not affected by the primary genetic mutation [98, 99].

Cell Death in Retinal Degeneration

A number of diverse processes are at play during retinal degeneration in RP. Mutation-induced rod cell death is followed by mutation-independent cone cell death [25, 26, 100]. The degeneration of both rods and cones in turn triggers a remodelling of retinal circuitry and tertiary degeneration of inner retina neurons [101]. This picture is further complicated by developmental photoreceptor cell death, which occurs in the first four postnatal weeks [22, 74, 75].

Depending on the animal model and time point studied, four different degenerative processes may be at play simultaneously in different cell types and retinal layers during RP (Fig. 1). These processes are most likely driven by different cell death mechanisms, making the situation difficult to analyze, particularly when tissue-based methods like microarray, ELISA, or Western Blot are used. The inconsistent and sometimes contradictory results concerning cell death mechanisms reported in the literature (compare [9, 102, 103] with [11, 78, 104]) may in part be due to variations in methods, models or study time points, and, in particular, the lack of cellular resolution. In order to avoid such pitfalls, an analysis of the complex situation in the retina requires the use of methods that can identify degenerative processes at the cellular level [16]. Examples for such methods are shown in Fig. 2 and include immunofluorescence, *in situ* hybridization, and *in situ* enzyme activity assays. Further development of these and related techniques may considerably expand our knowledge of the causes of cell death in RP.

Photoreceptor Cell Death Mechanisms in Inherited Retinal Degeneration

Photoreceptor cell death is often regarded as an apoptotic mechanism [6, 9, 12]. While light-induced photoreceptor degeneration seems to follow this pattern [51], no clear relevance of events characteristic for apoptosis has yet been demonstrated in inherited retinal degenerations. In the following, we will therefore review the existing knowledge about factors involved in retinal degeneration, including some not normally associated with apoptosis. At the same time, we will also point out that factors normally regarded as hallmarks of apoptosis do not seem to be involved in photoreceptor cell death. This will be followed by an attempt to identify an alternative cell death pathway that matches the evidence gained to date.

Cyclic Nucleotide Signaling

The cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)

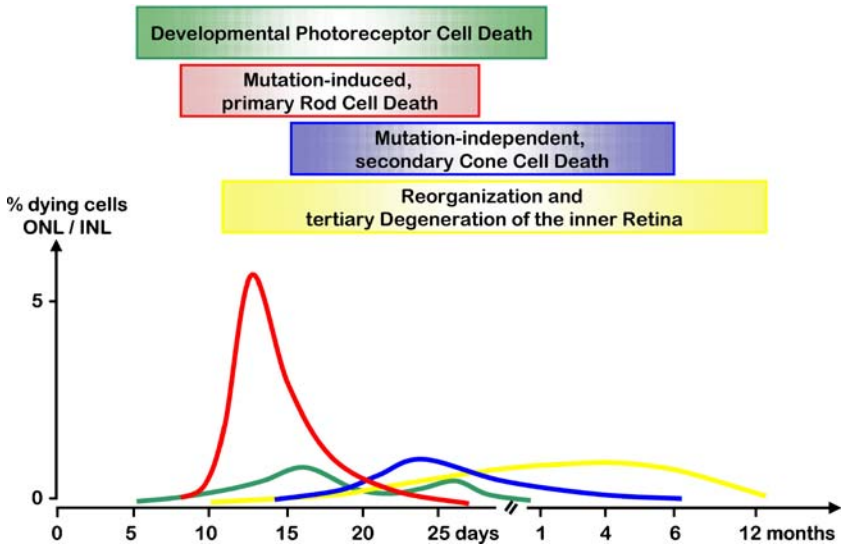


Fig. 1 Cell death mechanisms during rd1 mouse retinal degeneration: developmental photoreceptor cell death, probably driven by apoptotic processes, occurs in a time period ranging approximately from PN5–30. *rd1* mutation-induced rod photoreceptor cell death starts around PN10, peaks at PN13, and ends around PN25. This process induces a secondary cone degeneration which starts shortly after the beginning of rod degeneration. Depending on the area of the retina, cone cell death may take anywhere from 2 to 6 or more months to be completed. Both rod and cone cell death lead to tertiary changes in the inner retina, resulting in an extensive remodelling and cell death of various inner retina cell types. Up to four different degeneration processes, probably governed by different mechanisms, may be active at the same time. This makes it very difficult to analyze retinal degeneration at the tissue level and may explain some of the contradictory results reported in the past. In fast degeneration models such as the *rd1*, it may be possible to partially distinguish different degeneration processes by an appropriate choice of analysis time points. This is almost impossible in slower retinal degeneration models such as the *rd2*, where events are more protracted, with lower intensities, lower signal-to-noise ratios, and a higher degree of parallel activity on different cell death pathways. The above graph was compiled using data from [25, 26, 74, 75, 101, 228]. The relative proportions of different degeneration processes are indicative only and may have been affected by different methodologies used

occupy a center stage in intracellular signaling and the regulation of pathways promoting cell survival or death [105, 106].

The importance of cGMP for photoreceptor survival is illustrated by the fact that genetic mutations in enzymes involved in cGMP metabolism appear in some cohorts of human RP patients and also evoke retinal degeneration in corresponding animal models. For instance, mutations affecting the cGMP-hydrolyzing PDE6 are seen in up to 10% of human RP cases and form the basis for several different animal models for RP, including the *rd1* mouse (Fig. 2) [20, 29, 32, 107, 108]. Along the same lines, gain-of-function mutations in the gene encoding for photoreceptor guanylate cyclase GC [109] or the guanylate cyclase activating protein (GCAP) [110] are likely to yield excessive production of cGMP, and are known to cause photoreceptor cell death. Conversely, loss-of-function mutations in either GC1 or GCAP can be expected to lead to strongly decreased cGMP production, again resulting in selective photoreceptor cell death [111, 112].

cAMP produced by Ca^{2+}-activated adenylate cyclase (AC) appears to be equally important for cellular survival. It mediates the activation of protein kinase A (PKA), which is able to phosphorylate and activate cAMP response element binding protein (CREB), a transcription factor important for neuronal survival [113, 114]. Several different retinal degeneration models, including *rd1*, *rd2*, and certain rhodopsin mutants, coincide with abnormally high cAMP levels [115–117] which may negatively affect CREB activity [118, 119]. Likewise, inadequate cAMP production is also known to cause a downregulation of CREB and subsequent cell death [120].

Taken together, the evidence indicates that a deregulation of either cAMP or cGMP (or both at the same time), leading to either insufficient or excessive levels of these cyclic nucleotides, causes cell death. The mechanisms leading to such a deregulation of intracellular cyclic nucleotide levels and their downstream effects are still unknown; however, it is essential to elucidate them, since they are likely to involve key target proteins with a bearing on gene transcription [105, 120].

Gene Transcription

Inherited forms of retinal degeneration are usually accompanied by extensive changes in gene transcription, as shown in the example of the *rd1* mouse retina [14, 121, 122]. A number of transcription factors that may mediate differential gene regulation are also involved in the regulation of cell survival and cell death. Among these is

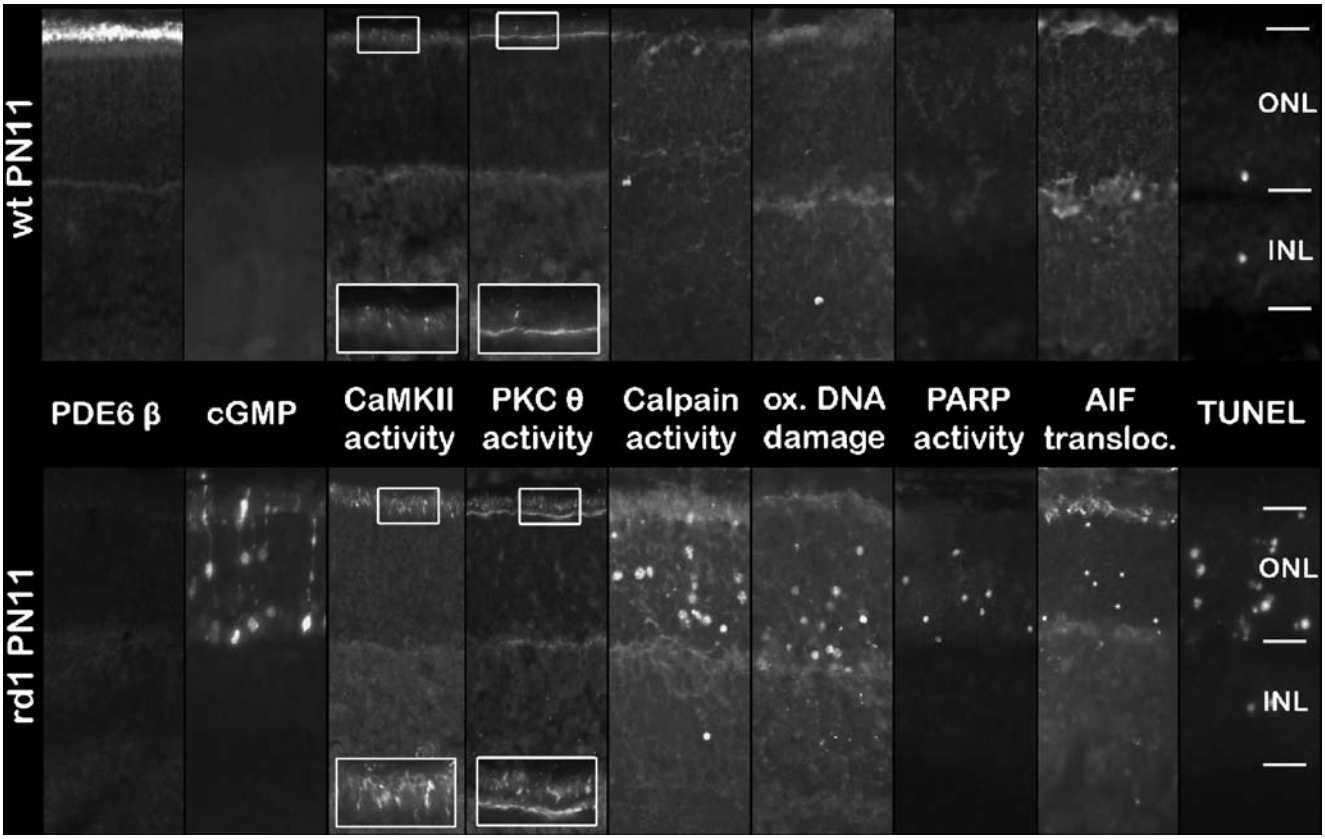


Fig. 2 Differential regulation of markers at the onset of rd1 retinal degeneration: In *rd1* photoreceptors, the PDE6 beta mutation leads to a dramatic accumulation of cGMP at postnatal (PN) day 11 when compared with wild type (wt). cGMP accumulation coincides with increased activation of CaMKII and PKC θ in *rd1* photoreceptor segments. In the *rd1* situation, a subpopulation of photoreceptor cell bodies shows strong increases in calpain activity, oxidatively damaged DNA, and PARP activity. Degenerating *rd1* photoreceptors also show nuclear translocation of AIF and a positive reaction in the TUNEL assay. The stains shown here are representative of at least three different specimens from each genotype

p53, a transcription factor important for the course of classical apoptosis [123]. Regulation of p53 may be partially involved in *rd2* photoreceptor degeneration, since its knock-out has been shown to cause minor delays [124]. In the *rd1* situation, however, the absence of p53 neither prevented nor delayed degeneration [125].

A second transcription factor that is closely associated with apoptosis is the activator protein complex-1 (AP1), a heterodimer composed of c-fos and c-jun proteins [58]. Light-induced photoreceptor degeneration in mice is prevented by ablation of the c-fos gene, demonstrating a crucial role of c-fos for cell death in this experimental model [126]. Although *rd1* photoreceptor degeneration is accompanied by a strong transcriptional upregulation of c-fos [14, 122], the c-fos knock-out phenotype does not prevent *rd1* mutation-induced cell death [127].

The transcription factor CREB is of utmost importance for neuronal survival and neuroprotection. It may be phosphorylated in a Ca^{2+}-dependent fashion either via the AC/cAMP/PKA pathway (see above) or via calmodulin kinases (CAMK) [128]. Ca^{2+}-independent activation of CREB may be mediated by other kinases such as PKC theta, PKG, Akt, or ERK in response to cGMP or neurotrophic factors such as CNTF, BDNF, FGF2, NGF, or IGF [113, 129].

A large number of genes relevant for neuronal survival are under the transcriptional control of CREB (see CREB target gene database: http://natural.salk.edu/CREB); this includes calpastatin, the endogenous inhibitor of calpains (see below) [130], OGG1 [131], an enzyme engaged in the repair of oxidative DNA damage, and the anti-apoptotic protein Bcl-2 [129]. However, it is unlikely that *rd1* degeneration is due to a downregulation of the Bcl-2 pathway, since upregulation of Bcl-2 did not prevent or delay the degeneration [132].

CREB binding protein (CBP) is another CREB target which acts as a transcriptional co-activator of CREB but also displays histone acetyltransferase activity. Fine tuning of histone acetylation/deacetylation appears to be important for neuroprotection [133, 134].

Activation of CREB is normally seen as neuroprotective [113, 128]; accordingly, disruption of CREB function leads to neurodegeneration [114, 129, 135]. Moreover, we have recently identified a transcriptional downregulation and reduced activation of CREB in the degenerating *rd1* mouse retina [136]. On the other hand, we have also observed abnormal activation of CREB kinases in *rd1* photoreceptors, notably Akt kinase [137], CaMKII [138], PKC theta (Fig. 2) [122], and also ERK1/2 throughout the retina [139]. This contrasting evidence—increased activity of CREB kinases combined with downregulation of CREB activity—indicates an involvement of negative feedback mechanisms in photoreceptor degeneration. Negative feedback of CREB gene transcription is possibly mediated by inducible cAMP early repressor (ICER) [140], the endogenous CREB antagonist, which may be dependent on PKA activity [141]. ICER seems to play an important role in neuronal cell death *in vivo* as well as *in vitro* [119, 142], but so far its relevance for retinal degeneration mechanisms is unclear.

Role of Calcium, Energetic Status, and the Endoplasmatic Reticulum (ER)

Neuronal cell death is frequently associated with Ca^{2+} influx, membrane depolarization, and energetic collapse caused by a depletion of adenosine 5′-triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) [143, 144]. Excessive Ca^{2+} influx has long been regarded as a major factor in photoreceptor degeneration [51, 145–147], and strong activation of Ca^{2+}-dependent enzymes has been observed in *rd1* photoreceptors (Fig. 2) [136, 138]. However, the role of Ca^{2+} influx on cell death mechanisms is still under debate. On the one hand, Ca^{2+} is seen as a trigger of apoptosis through the inactivation of mitochondria and subsequent activation of apoptotic machinery [148]. On the other hand, Ca^{2+} influx causes ATP depletion by activating Ca^{2+} extrusion mechanisms such as Ca^{2+}-ATPases or the Na-ATPases required to drive Na^{+}/Ca^{2+} exchangers, while at the same time inhibiting mitochondrial ATP synthesis [149]. This, however, would mean that energy sources necessary for apoptosis are unavailable and should cause necrosis instead [70, 73, 80, 150]. Because of the very high ATP affinity of Ca^{2+}-ATPases [149], strong Ca^{2+} influx is almost synonymous with a depletion of energy-containing substrates and is hence unlikely to be associated with apoptosis.

To date it is unclear whether Ca^{2+} influx from extracellular sources is responsible for photoreceptor cell death since evidence concerning the efficacy of measures that block Ca^{2+} influx is contradicting (e.g., compare [102, 147] with [104, 151, 152]). Indeed, it is conceivable that the observed rise in retinal Ca^{2+} levels [145] is only secondary to the degeneration process and it is not even clear whether it occurs in photoreceptors or in other retinal cell types such as Müller glial cells [153]. Furthermore, fluctuations in cytosolic Ca^{2+} may be caused not only by Ca^{2+} influx from extracellular sources but also by intracellular release from either the mitochondria, photoreceptor discs, or the endoplasmatic reticulum (ER) [148, 154].

The ER performs crucial cellular functions including synthesis, folding, and post-translational modification of proteins. An accumulation of incorrectly processed proteins may disturb ER functions and initiate the so-called unfolded protein response (UPR), which can be seen as a way to protect the ER from stress. However, if the challenge on the ER exceeds the protective capacity of the UPR, the increasing ER stress might trigger cell death by means of various mediators including caspase-12 [155, 156]. Neurons may be particularly sensitive to the presence of misprocessed, i.e., erroneously folded, proteins, and the involvement of ER stress in neurodegenerative diseases is currently attracting much interest [157]. This involvement appears to include forms of retinal degeneration, since rats carrying mutated rhodopsin display an abnormal expression of BiP and CHOP, which are genes connected with ER stress [158]. In addition, Sanges [159] has shown that caspase-12 translocates from the inner segments to the nuclei of the photoreceptors in the degenerating *rd1* retina. Caspase-12 was also found to localize to apoptotic nuclei in photoreceptors obtained from *in vitro* differentiated, *rd1* ciliary margin-derived neurospheres; this was linked in various ways with Ca^{2+} mobilization and calpain activity. A recent report from Yang [160] confirms the caspase-12 connection in *rd1* cell death and also suggests the involvement of further ER stress-related factors in this context, such as GRP78/BiP, EIF2a, and PERK. The potential importance of ER stress for RP is reflected in the very recent suggestion that silencing gene therapy, aimed at the mutated gene in order to avoid the production of misfolded and hence stress-generating proteins, will be a very crucial component in future forms of RP treatment [161].

In summary, Ca^{2+}, the energetic status, and ER stress are different factors that appear to be interconnected in cell death. It is therefore tempting to speculate that a primary rise in intracellular Ca^{2+} triggered by ER stress could be followed by mitochondrial dysfunction, an energetic collapse, and a secondary wave of extracellular Ca^{2+}.

Calpain Activity

Calpains are a group of calcium-activated proteases with 14 known isoforms [162] that have been implicated in neuronal degeneration in a number of different neuronal tissues including the retina [53, 77, 136]. Calpains are inhibited by the highly specific, endogenous calpain

inhibitor, calpastatin [162–164]. Dissociation of calpastatin allows for a Ca^{2+}-dependent conformational change and autolytic cleavage that gives rise to the active protease [162, 164, 165]. Overexpression of calpastatin has been correlated with an increased resistance to injury and reduced neuronal cell death [166–168]. In the retina, pharmacological inhibition of calpains blocked cell death in different experimental systems [169–172].

Previously, we have demonstrated that calpains are strongly activated in degenerating *rd1* mouse photoreceptors, in contrast to those of their wild-type counterparts (Fig. 2). Calpain activation was concomitant with the already-mentioned transcriptional downregulation of CREB and calpastatin [136], suggesting that decreased CREB activity may lead to decreased calpastatin transcription, thus indirectly causing calpain activation.

The functions of calpains both under normal physiological conditions and during cell death are still largely unknown, and for many of the potential substrates [16, 163, 173], a biological role still needs to be determined. The only photoreceptor-specific calpain substrate known hitherto is arrestin [174], a component of the phototransduction cascade that is responsible for blocking the interaction of rhodopsin with transducin. Other calpain substrates with a suggested involvement in photoreceptor degeneration are PARP [175] and AIF [168] (see below).

Calpain activity may result in an increased production of reactive oxygen species (ROS) and accumulation of oxidative DNA damage by abolishing AIF's oxidoreductase activity and OGG1-dependent DNA repair activity [168, 176], since both of these enzymes are calpain substrates. In conjunction with ROS production, calpain activity has been suggested as a cause of lysosomal membrane disruption with the resultant leakage and activation of various hydrolytic enzymes, in particular cathepsins [72]. Increased cathepsin activity has been demonstrated in the *rd1* retina [177, 178] but to date it is not clear whether this activity resides in degenerating photoreceptors or in other parts of the retina.

Oxidative Stress

ROS generation and oxidative stress are normal byproducts of mitochondrial metabolism; this is counterbalanced by an intricate network of protective mechanisms that include chemical and enzymatic antioxidants and even repair mechanisms [179, 180]. These have been confirmed to be active in the human retina as well [181]. This complex of protective mechanisms against oxidative and/or nitrosative stress has been called 'the antioxidant network' [182–184]. In certain neurodegenerative disorders, however, an increase of oxidative stress or a downregulation of defense mechanisms may lead to pathological changes in cellular metabolism which eventually result in cell death [185, 186]. An involvement of oxidative stress in inherited photoreceptor degeneration was recently found in the *rd1* mouse model for both mutation-induced rod cell death (Fig. 2) [187] and secondary cone cell death [100]. Protective effects of antioxidants were also found in *in vitro* studies of photoreceptor degeneration [188].

Increased oxidative stress may originate from either excessive energy demand, impairment of oxidative phosphorylation, or decreased antioxidant defenses. It has been suggested that activation of mitochondrial calpain-10 by Ca^{2+} inactivates mitochondrial complex-1 and causes mitochondrial permeability transition pores (MPTP) to open [189]; this could explain the observed calpain-dependent translocation of AIF from mitochondria to the nucleus [159, 190]. AIF release might also be mediated—independently of MPTP opening—by calpain-1, which has been shown to translocate to the inner mitochondrial membrane during neurodegeneration [168]. Since AIF has considerable redox activity and is essential for mitochondrial complex-1 function [191], calpain-induced depletion of mitochondrial AIF would result in mitochondrial dysfunction and increased oxidative stress, which would explain the apparent link between calpain activity and oxidative stress-induced cell damage [72].

It remains unclear whether oxidative stress is upstream from calpain activity [168, 192] or downstream from it [193–195]. Since antioxidants and calpain inhibitors have synergistic protective effects [186], it is even possible that oxidative stress and calpain activation go hand in hand. Paradoxically, oxidative stress may also prolong neuronal survival by inactivating calpains and caspases through oxidation of the cysteine residue that is essential for their activity [185, 196]. Further experimental studies will be required to identify the relation between oxidative stress and calpains by investigating their interplay in the context of photoreceptor cell death.

Downregulation of defensive mechanisms against oxidative stress such as the action of glutathione-*S*-transferase and glutathione peroxidase has been demonstrated in the *rd1* retina [177, 182], where such downregulation is likely to enhance the deleterious effects of ROS. Although ROS may interact with many different cellular components, a major product of ROS reactions in the DNA is the oxidated nucleotide 8-oxo-guanosine (8-oxoG) [197]. Because of its ability to pair with either adenine or cytosine, 8-oxoG is highly mutagenic [198]. Such mismatches are corrected by a specific DNA repair enzyme called 8-oxoG DNA glycosylase (OGG1) [176]. OGG1 gene transcription is regulated by CREB [131] and, as mentioned, we have found a transcriptional downregulation of CREB and OGG1 in the *rd1* retina [122]. Since OGG1 can be cleaved and inactivated by calpains [176],

the increase in calpain activity [136], together with the downregulation, would severely compromise the cells' ability to repair oxidative stress-induced DNA damage. This in turn would result in the continuous activation of other enzymes engaged in DNA repair such as poly-ADP-ribose polymerase (PARP).

PARP Activity

DNA damage is known to trigger the activation of specific repair enzymes such as PARP [199]. PARP uses NAD to generate poly-ADP-ribose polymers and add them for instance onto DNA binding histones. The repulsive force generated by negatively charged poly-ADP-ribose polymers opens up the tightly packed chromatin structure to allow for an access of other DNA repair enzymes such as OGG1 [200]. Since it promotes repair of damaged DNA and thus continuously protects the cell, PARP activity is often seen as beneficial. However, excessive PARP activity and consumption of NAD indirectly causes ATP depletion, increases oxidative stress, and eventually causes mitochondrial depolarization and opening of mitochondrial permeability transition pores (MPTP) [201, 202]. This in turn may lead to a leakage of mitochondrial proteins into the cytosol [77] which, in the case of AIF may further translocate to the nucleus and precipitate cell death [203]. PARP is therefore regarded as an important factor in neurodegeneration and other severe diseases [204–206].

We were recently able to show a strong activation of PARP in degenerating *rd1* photoreceptors (Fig. 2) [25]. PARP activity coincided and co-localized with stains for poly-ADP-ribose (PAR), the product of PARP activity, and in part with markers for cell death; this was indicated, for example, by the TUNEL assay and nuclear translocation of AIF. The latter confirmed earlier findings by Sanges [172]. PAR also co-localized with oxidative DNA damage, suggesting that PARP activation was triggered by oxidative stress. Inhibition of PARP rescued *rd1* photoreceptors *in vitro*, indicating that PARP activity was a contributor to their demise. How exactly PARP activity drives cell death is still unclear. Apart from excessive NAD consumption [201, 202], production of poly-ADP-ribosylated proteins [207], or free PAR polymers have also been proposed to cause neuronal degeneration [208].

In classical apoptosis, PARP activity is completely abolished by caspase-dependent cleavage within PARP's DNA binding domain [209, 210]. Interestingly, PARP is a calpain substrate [175, 210–212], and it has also been suggested that calpain-dependent cleavage of PARP ablates PARP activity [168, 213, 214]. This, however, does not harmonize with the observation that calpain and PARP activity co-localize to the same degenerating photoreceptors (FPD, unpublished observations). The four known calpain-specific cleavage sites are within the automodification domain of PARP [175], and cleavage will thus separate the catalytic domain from the DNA binding domain that is normally required for activation. It is tempting to speculate that calpain-dependent PARP cleavage turns the enzyme into a constitutively active form that no longer requires binding to damaged DNA for activity. Even though the activity of the catalytic domain alone may be lower than that of the full-length PARP enzyme [215], a constitutive activation will most likely result in higher activity levels over time.

By contrast, PARP activity was responsible for calpain activation in a Bcl-2 dependent fashion in genotoxically challenged mouse embryonic fibroblasts [216], but whether a similar sequence of activation could take place also in *rd1* photoreceptors is unclear, since their degeneration is independent of Bcl-2 expression [132]. The question of whether PARP activity during retinal degeneration is triggered by calpain activity or *vice versa* thus remains unanswered to date and may be highly dependent on the experimental system used.

AIF Nuclear Translocation + DNA Fragmentation

AIF is a flavoprotein that is normally localized in the mitochondrial intermembrane space, where it appears to have oxidoreductase activity [217]. The importance of AIF for mitochondrial metabolism, and thus cell survival, is illustrated by the fact that animals with genetic ablation are not viable [218]. Moreover, low AIF expression levels in the Harlequin mouse lead to increased oxidative stress and late onset retinal degeneration [219]. These findings thus suggest a pro-survival role for AIF, and this has indeed been observed [219]. On the other hand, AIF has decidedly cell death-promoting effects under pathological conditions when it translocates to the nucleus and triggers activation of endonucleases and widespread DNA fragmentation [203]. AIF-induced DNA fragmentation and subsequent cell death can be detected relatively easily with the TUNEL method (Fig. 2) [66].

AIF nuclear translocation is mediated at least in part by PARP activity [63, 207, 220], and we have recently suggested that overactivated PARP triggers AIF-mediated death of *rd1* rod photoreceptors (Fig. 2) [25]. Furthermore, cleavage by calpain-1 may also cause nuclear translocation of AIF [168]. The importance of this is indicated by the fact that AIF nuclear translocation and cell death in retinal progenitor-derived, photoreceptor-like cells is prevented by calpain inhibition as well as by siRNA mediated knock-down of AIF [172]. Taken together, these results suggest that AIF nuclear translocation is located downstream of PARP and calpain activation, at a point just prior to DNA fragmentation and the final stages of cell death.

Molecular Cell Death Pathways in *rd1* Photoreceptors

In the *rd1* mouse and in other animal models of inherited photoreceptor degeneration, cell death is independent of such hallmark features of apoptosis as mitochondrial cytochrome *c* release [77], regulation/cleavage of Bcl-2 and Bcl-XL [132], activation of transcription factors c-fos/AP-1 [127] and p53 [125], and activation of caspases [78, 79]. Along the same lines, PDE6 inhibition-induced cone cell death was found to be independent of protein biosynthesis [221], which is otherwise crucial for upregulation of apoptotic enzymes. Furthermore, the use of Ca^{2+}-channel blockers to prevent retinal degeneration in the *rd1* mouse has so far produced contradictory findings (compare [102, 146] with [104, 151, 152]).

On the other hand, a number of factors that are atypical for classical apoptosis have been unequivocally shown to be involved with retinal photoreceptor degeneration. Among these factors are alterations in cyclic nucleotide signaling [21, 115], calpain activity [16], PARP activity [25], and cathepsin activity [177, 178], all of which are commonly associated with alternative cell death pathways such as necrosis-like PCD or autophagy [64, 70, 72, 80]. Other hallmark events of photoreceptor degeneration, such as oxidative stress [100, 187], AIF nuclear translocation [172], and DNA fragmentation [9, 25], have been associated with apoptotic as well as non-apoptotic forms of cell death [10]. Taken together, the evidence to date is suggestive of an alternative, non-apoptotic cell death mechanism during inherited photoreceptor cell death.

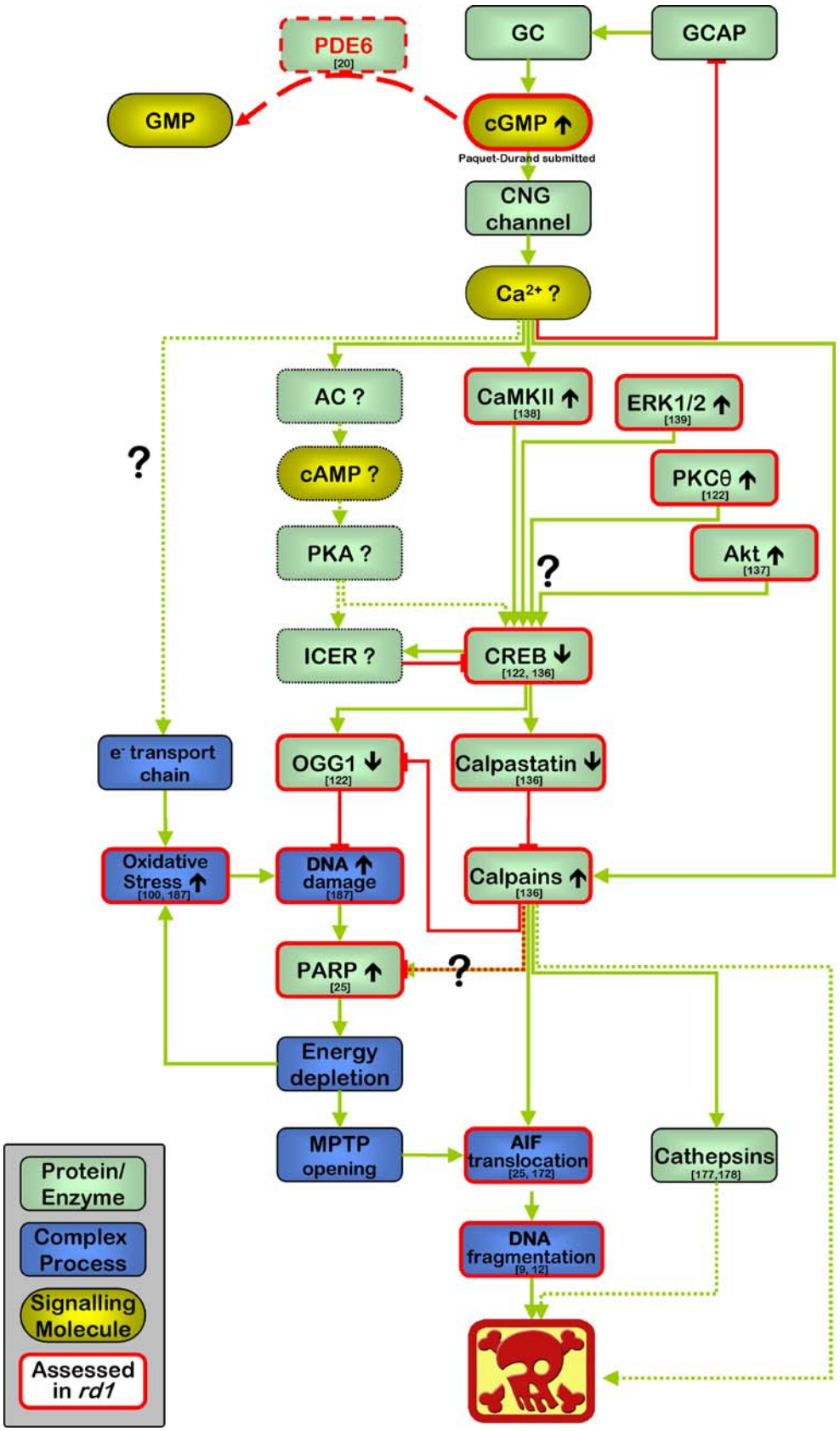
In the following, we will try to construct a map of the molecular pathways leading to photoreceptor death on the basis of the evidence gathered to date, with special reference to the *rd1* mouse, which is so far the best-studied model of retinal degeneration (Fig. 3). The proposed model partly relies on correlative data that sometimes lack established causal connections to photoreceptor-specific events. By necessity, the character of this scheme is therefore somewhat provisional and will require regular updating and extensions when new information is available. Nevertheless, it provides a conceptual framework for future studies into the mechanisms of photoreceptor cell death and may open up new perspectives for the rational development of therapies for RP and related diseases.

Deregulation of the intracellular cGMP levels [115] (Paquet-Durand submitted) induced by the PDE6 mutation is the starting point for photoreceptor degeneration in the *rd1* mouse [20]. High cGMP levels lead to constitutive activation of cGMP-gated channels [145] and triggers a series of Ca^{2+}-dependent processes. High intracellular Ca^{2+} inhibits GCAP [112] and thus cGMP production; this negative feedback mechanism should in principle prevent an excessive rise in intracellular cGMP. At present, however, it is not clear why this feedback mechanism is ineffective in degenerating *rd1* photoreceptors.

High intracellular levels of Ca^{2+} activate AC, leading to the production of cAMP. Elevated cAMP levels have been found during *rd1* degeneration [115], in the *rd2* retina [117], and in Pro347Ser rhodopsin mutants [116]. However, it is at present not clear whether this increase in cAMP occurs in photoreceptors or in other parts of the retina. cAMP in turn activates PKA, which may then activate the transcription factor CREB. Ca^{2+}-dependent activation of CaMKII was observed in *rd1* photoreceptors [138], along with the activation of several other known CREB kinases, notably Akt kinase [137], PKC theta [122], and ERK1/2 [139]. Activation of upstream kinases should lead to increased activity and expression of the transcription factor CREB; paradoxically, however, we have observed a down-regulation of CREB both at the transcriptional level and at the phosphorylated protein level [122, 136]. This finding is suggestive of a negative feedback mechanism which might be triggered by the transcriptional repressor of CREB, ICER [140].

Downregulation of CREB corresponds to reduced transcription of CREB target genes, including calpastatin

Fig. 3 Molecular pathways during retinal photoreceptor degeneration: PDE6 mutation-induced deregulation of cGMP may cause Ca^{2+} ion influx through CNG ion channels and subsequent activation of Ca^{2+}-dependent enzymes. While Ca^{2+}-induced activation of CaMKII and calpain has been demonstrated in *rd1* photoreceptors, activation of adenylate cyclase (AC), cAMP production, and PKA-dependent phosphorylation has not yet been shown in these cells. Activation of a number of CREB kinases (CaMKII, ERK1/2, Akt, PKC θ) has been observed; this should lead to increased CREB expression and activity. Paradoxically, CREB activity and expression is reduced in the *rd1* mouse, suggesting the presence of a negative feedback mechanism such as the one mediated by ICER. Downregulation of the CREB target genes calpastatin and OGG1 coincides with increased calpain activity and the occurrence of oxidative DNA damage; this in turn may cause the activation of repair enzymes such as PARP. In fact, strong activation of PARP was observed in degenerating photoreceptors. Excessive activation of PARP, however, may lead to energy depletion, mitochondrial depolarization, opening of the mitochondrial permeability transition pore (MPTP), and subsequent translocation of AIF from the mitochondria to the nucleus. AIF translocation may also be mediated by calpain, independently of MPTP opening. In the nucleus, AIF may activate specific endonucleases which cause DNA fragmentation, effectively killing the cell. Excessive calpain activity is known to cause cell death, and a number of calpain targets such as PARP and cathepsins are known. However, their relevance for photoreceptor degeneration has not yet been clearly established. *Green boxes* = proteins/enzymes; *yellow boxes* = small signaling molecules; *blue boxes* = complex processes; *black arrows in boxes* = increased/decreased expression/activity/concentration; *green arrows* = activating interaction; *red, blunt-ended lines* = inhibitory interaction; *dotted lines* = uncertain interaction. Parameters surrounded with *thick red lines* represent results of measurements in the retina or photoreceptors at the mRNA, protein, or activity level. Only *rd1* retina-specific references are shown


PDE6
[20]
GC
GCAP
GMP
cGMP ↑
Paquet-Durand submitted
CNG channel
Ca2+ ?
AC ?
CaMKII ↑
[138]
ERK1/2 ↑
[139]
?
cAMP ?
PKCθ ↑
[122]
PKA ?
Akt ↑
[137]
?
ICER ?
CREB ↓
[122, 136]
e- transport chain
OGG1 ↓
[122]
Calpastatin ↓
[136]
Oxidative Stress ↑
[100, 187]
DNA ↑ damage
[187]
Calpains ↑
[136]
PARP ↑
[25]
?
Energy depletion
MPTP opening
AIF translocation
[25, 172]
Cathepsins
[177,178]
DNA fragmentation
[9, 12]
Protein/ Enzyme
Complex Process
Signalling Molecule
Assessed in rd1

[136] and OGG1 [122]. Reduced expression of calpastatin will facilitate the Ca^{2+}-mediated activation of calpains, as has been shown in *rd1* photoreceptors [136].

Decreased expression of OGG1 will compromise the repair of oxidatively damaged DNA [195]; in combination with excessive calpain activity and cleavage of OGG1 [176], this will be further reduced. The presence of damaged DNA will result in activation of the DNA repair enzyme PARP [200]. Strongly increased PARP activity has been shown in *rd1* photoreceptors [25] and may lead to excessive consumption of energy-containing substrates [202], causing even higher levels of oxidative stress and eventually mitochondrial depolarization and MPTP opening. At present, it is unclear whether the observed translocation of AIF from mitochondria to the nucleus [25] is due to MPTP opening or direct cleavage of AIF by calpain-1 [168]. AIF translocation co-localizes with widespread DNA fragmentation and cell death.

Apart from AIF and OGG1, calpain may have a number of other intracellular targets (such as PARP) which may be relevant for the degeneration process. Although it has been suggested that calpain-mediated activation of cathepsins plays an important role in cell death [72], and increased cathepsin activity was detected in the *rd1* situation [177, 178], a relevance of cathepsin activity for photoreceptor degeneration has so far not been clearly established.

Although the cGMP-dependent molecular cell death pathway presented here primarily reflects the *rd1* situation, there are a number of reasons to believe that similar pathways are followed in photoreceptor degeneration induced by different genetic mutations. This does not only concern mutations in other PDE6 genes directly affecting PDE6 function such as PDE6 alpha [29], PDE6 gamma [222], or cone-specific PDE6 alpha prime [17] but also mutations that affect PDE6 activity only indirectly. Notable examples for this category are mutations in the aryl hydrocarbon receptor-interacting protein-like (AIPL1) gene that are associated with Lebers congenital amaurosis [223] and mutations/knock-out of genes encoding for GC1 and GC2 [224]. These mutations are associated with reduced PDE6 expression and photoreceptor cell death. Furthermore, cGMP accumulation may also be caused independently of PDE6 activity by excessive production of cGMP due to gain-of-function GCAP mutations [112, 225] or pathological elevation of nitric oxide synthase (NOS) activity and subsequent activation of soluble GC [226].

Apart from oxidative stress and AIF nuclear translocation/DNA fragmentation which may also occur during apoptosis, the pathway proposed here shares none of the hallmark features of classical apoptosis. A major gap of knowledge exists concerning the sequence and time scale of degenerative events. In order to fully establish the sequence and causal connections of degeneration pathways, it will be necessary to identify temporal sequences along these molecular pathways with cellular or even subcellular resolution. High-resolution time-lapse imaging using specific markers for degeneration processes is one technique that might make such studies possible in the future. Furthermore, in the past, most studies on retinal degeneration mechanisms have focused on the expression of genes or proteins. However, in recent years, it has become increasingly evident that actual enzyme activity can be far more important than expression. It will therefore be important to develop enzymatic assays that can detect activities at the cellular level and characterize metabolic pathways *in situ*.

Summary and Conclusion

Cell death in inherited retinal photoreceptor degeneration has often been understood as apoptosis [9, 12, 227]. However, there are considerable differences between classical apoptosis and photoreceptor cell death, suggesting the action of non-apoptotic cell death mechanisms. Based on evidence collected in recent years, we propose an alternative molecular pathway as a cause of photoreceptor cell death. Importantly, this pathway shares none of the major characteristics of classical apoptosis but is instead characterized by a deregulation of cyclic nucleotide metabolism, downregulation of the transcription factor CREB, oxidative stress, and excessive activation of calpain and PARP. This may have far-reaching implications for the design of therapies for diseases that, like RP, affect photoreceptors. While therapeutic approaches aimed at classical apoptosis have so far been only moderately successful at best, the recognition that non-apoptotic processes are involved may open up new avenues and provide a number of new therapeutic targets. Since the retina is an integral part of the central nervous system, many of the characteristics of cell death in the retina and in photoreceptors in particular may be relevant for neuronal cell death in general.

Acknowledgements We apologize to those colleagues working on retinal degeneration whose work was not cited in this review due to space constraints. Parts of the work presented here have been supported by grants from the Kerstan foundation, the German research council (DFG), the European Union (RETNET, MRTN-CT-2003-504003; EVI-GENORET, LSHG-CT-2005-512036; NEUROTRAIN, MEST-CT-2005-020235), Foundation Fighting Blindness (FFB), Petrus och Augusta Hedlunds Stiftelse, Dutch Retina Foundation, Kronprinsessan Margaretas Arbetsnämnd för synskadade (KMA), Synfrämjandets Forskningsfond, Torsten och Ragnar Söderbergs Stiftelser, Vetenskapsrådet medicin (VRM), the Crafoord Foundation, and Fundación Oftalmológica del Mediterráneo (FOM).

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