Retinitis pigmentosa: rapid neurodegeneration is governed by slow cell death mechanisms

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For most neurodegenerative diseases the precise duration of an individual cell’s death is unknown, which is an obstacle when counteractive measures are being considered. To address this, we used the rd1 mouse model for retinal neurodegeneration, characterized by phosphodiesterase-6 (PDE6) dysfunction and photoreceptor death triggered by high cyclic guanosine-monophosphate (cGMP) levels. Using cellular data on cGMP accumulation, cell death, and survival, we created mathematical models to simulate the temporal development of the degeneration. We validated model predictions using organotypic retinal explant cultures derived from wild-type animals and exposed to the selective PDE6 inhibitor zaprinast. Together, photoreceptor data and modeling for the first time delineated three major cell death phases in a complex neuronal tissue: (1) initiation, taking up to 36 h, (2) execution, lasting another 40 h, and finally (3) clearance, lasting about 7 h. Surprisingly, photoreceptor neurodegeneration was noticeably slower than necrosis or apoptosis, suggesting a different mechanism of death for these neurons.

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Neurodegenerative diseases are an increasing health concern in the aging population, but despite massive research two fundamental questions remain: (1) what are the mechanisms of cell death governing neurodegenerative diseases? The seminal work of Kerr et al.1 and the introduction of the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method2 rapidly pinpointed apoptosis as the causative cell death pathway, although the relevance of alternative degeneration mechanisms is becoming increasingly evident.3 (2) How long is the actual cell death process? Although sounding simple, this question has never been answered satisfactorily,4 primarily because it is difficult to test experimentally. The questions are obviously connected, not the least since different cell death pathways run on different timescales. For instance, necrosis is seen as a rapid, chaotic, and unordered destruction of the cell taking between a few minutes to 1–2 h to complete,5 whereas apoptosis refers to a comparatively slow, program driven, and orderly cellular disintegration that may take 6–18 h to complete.6,7 Knowledge on the time course of cell death will define the window-of-opportunity and may thus strongly influence future therapeutic strategies.

To follow cell death, from the first symptoms to the disappearance of a cell, requires sophisticated long-term, live-tissue imaging technology.5 Yet, previous in vivo imaging experiments8 could not determine the precise time frame for cell death, mainly because markers for the beginning of cellular deterioration were lacking, and most knowledge on cell death duration hence comes from dissociated cell cultures.9 The use of intact neuronal tissues for ex vivo analyses presents an alternative and such studies have focused on the late phases of cell death, identified by pyknosis or DNA fragmentation (DAPI or TUNEL staining, respectively) to resolve the time a dying cell takes to completely disappear. This ‘clearance time’ was suggested to range from 1 to 5 h in different models for neurodegeneration.10 However, as pathological alterations in DNA and nuclear structure are detectable only toward the end of the cell death process, the clearance time does not indicate how much time any affected cell has spent going from the initiation to the very end.

We set out to study the duration of neuronal cell death, using the rd1 mouse, a homologous animal model for retinitis pigmentosa (inherited retinal degeneration, RD) with an early, rapid loss of photoreceptors, the light-sensitive neurons of the retina. The rd1 mutation leads to loss-of-activity in rod photoreceptor cyclic guanosine-mono-phosphate (cGMP) phosphodiesterase-6 (PDE6)11 and an accumulation of cGMP, triggering cell death.12,13 The mechanisms behind hereditary photoreceptor neurodegeneration as such are unsettled and have been suggested to involve apoptosis,14 necrosis,15 as well as non-apoptotic cell death.16 Neuronal degeneration models – including the rd1 mouse – often exhibit...
a constant rate of cell death, resembling the exponential decay of radioactive elements.\textsuperscript{17,18} We built on this knowledge and used markers characteristic for different cell death stages to create a mathematical model, which for the first time allowed estimating the temporal duration of photoreceptor neurodegeneration \textit{in vivo}. The model predictions were validated using organotypic retinal \textit{in vitro} culture, demonstrating that the photoreceptor cell death mechanism was considerably slower than both necrosis and apoptosis.

**Results**

**Accumulation of cGMP and photoreceptor cell death in the \textit{rd1} retina.** cGMP accumulation found in \textit{rd1} photoreceptors is seen as the first sign of impending cellular degeneration.\textsuperscript{13} Cell death is easily detected using the TUNEL method, which detects both necrotic and apoptotic cells.\textsuperscript{2,19} A variety of different TUNEL-positive phenotypes were observed: some cells stained only in perinuclear areas, in others the entire nucleus was strongly positive, and yet others showed a very condensed, pyknotic, TUNEL-positive nucleus, all probably relating to different phases of cell death (Figure 1a, Supplementary Figure 1, 2). Interestingly, although high cGMP triggers TUNEL-positive \textit{rd1} cell death,\textsuperscript{12} cGMP did not co-label with TUNEL in photoreceptor cells (Figure 1a). Hence, cGMP and TUNEL labeled two distinct degeneration stages, separated in time by a transition period. Seen from a mechanistic point of view, PDE6 dysfunction caused a temporary rise in cGMP, followed by (yet unidentified) intermediate processes in a transition stage, before the cells turned TUNEL positive to be finally cleared away (Figure 1b). Our methodology thus provided an opportunity to study three different and temporally unique events during an individual photoreceptor cell’s death.

Cellular photoreceptor cGMP accumulation (Figure 2a) was an extremely rare event in wild-type (\textit{wt}) retina, with only few positive cells observed per retinal cross-section, in particular at early post-natal (P) days. In contrast, the number of cGMP-positive photoreceptors was significantly elevated in the outer nuclear layer (ONL) of the \textit{rd1} retina already from P8.

The early post-natal mouse retina displays a measurable amount of developmental photoreceptor cell death,\textsuperscript{20} seen also here by the TUNEL assay in \textit{wt} specimens (Supplementary Figure 1A). Although \textit{rd1} photoreceptor cell death was numerically higher at P9 when compared with \textit{wt}, statistically significant differences were found only from P11 onward (Figure 2a).

The cGMP-dependent cell death under study was negative for caspase activity (Supplementary Figure 2) – indicating a non-apoptotic cell death mechanism – and topologically independent (i.e., no clumping of dying cells), suggesting cell-autonomous, non-necrotic processes. The delay between the significant rise of cGMP at P8, and TUNEL at P11, indicated that photoreceptor death execution could take as long as 2 to 3 days.

The percentage of cGMP-positive ONL cells in the \textit{rd1} retina peaked at P13, coinciding with the peak of cell death (Figure 2a), after which both cGMP and TUNEL-positive cells declined, but cells with high cGMP remained more numerous than TUNEL-positive cells. As the cellular life-time of a marker determines its detection probability, these results proposed that cGMP positivity lasted longer than TUNEL positivity.

The amount of surviving photoreceptor rows showed a minor decrease in \textit{wt} retina (because of developmental processes), whereas in \textit{rd1} retina photoreceptor numbers strongly declined from P13 onward (Figure 2b). These data served as an index of the clearance of cells.

**Modeling photoreceptor cell death kinetics.** Based on the \textit{in vivo} data on cGMP accumulation, TUNEL positivity, and survival of photoreceptors, we constructed a mathematical model for the temporal progression of neurodegeneration in the \textit{rd1} retina (Figure 2b). We reasoned that in addition to three phases defined by cGMP, TUNEL, and clearance of cells, at least two additional transition states must exist: (1) a first one relating to unknown molecular events that, while

![Figure 1](cGMP and photoreceptor degeneration: co-stainings in P13 \textit{rd1} retina showed no colocalization between cGMP and TUNEL (a). These markers hence labeled two different stages in PDE6 dysfunction induced cell death, separated in time by a transition phase (b). The final clearance of cells characterizes an additional stage in cell death. Images shown are representative for at least five different \textit{wt} and \textit{rd1} animals)
cGMP negative, eventually cause unphysiologically high cGMP. (2) A second one in between cGMP and TUNEL positivity, because there was no apparent colocalization between these two markers (Figure 1a). The model also included an initial stage in which the cells were healthy. Thus, in total six different stages were considered, going from healthy (H) to transition-1 (Tr 1) to high cGMP (cGMP") to transition-2 (Tr 2) to TUNEL (TU") to dead (D). These stages were represented by a set of differential equations (1–6), to allow calculating the average life-times for each stage as the inverse of the respective decay constant $k$. The values for these equation constants representing the five cell death stages are given in Table 1, together with approximate average life-times and parameter errors. The decay constant $k_0$ of cGMP was considerably larger than the decay of the upstream stage Tr 1 ($k_0$), hence the average life time of cGMP" was governed by $k_0$, rather than $k_0$. The model reproduces the progression of cell death as evidenced by cGMP accumulation (sum squared error (SQE) = 0.82), TUNEL assay (SQE = 0.79), and loss of photoreceptor rows (SQE = 0.98). Altogether, the average time for an individual cell to die was predicted to take 83.8 ± 9.4 h (Table 1).

As the loss of photoreceptor cells (Figure 2c) did not follow a strictly exponential decay, we tested an alternative mathematical approach in which $k_1$ was defined by an asymmetric, generalized logistic function rather than a step function. This may reflect photoreceptor biology in the sense that toward the end of differentiation, the risk for a photoreceptor to die increases dramatically, to then remain constant once full differentiation is reached. Indeed, this approach allowed for a better model fit to cell loss in the initial stage of the degeneration, but in later stages the model fit was not significantly improved (Supplementary Figure 3).

Another, independent estimate of how long the cell death process took, was obtained by plotting data on cGMP accumulation (Figure 2a) together with the derived photoreceptor survival data (Figure 2c) and fitting Gauss curves to

### Table 1

| Stage            | Constant | Value (1/day) | Error (1/day) | Time (h) | Error (h) |
|------------------|----------|---------------|---------------|----------|-----------|
| H to Tr 1        | $k_1$    | 0.68          | 0.06          | 35.3     | 3.4       |
| cGMP" to Tr 2    | $k_2$    | 0.68          | 0.07          | 35.7     | 4.0       |
| cGMP" to Tr 2    | $k_3$    | 4.24          | 0.69          | 5.6      | 0.9       |
| Tr 2 to TU"      | $k_4$    | 5.54          | 0.98          | 4.3      | 0.7       |
| TU" to D         | $k_5$    | 8.19          | 1.13          | 2.9      | 0.4       |

The table gives measures for the average life-times for the five main stages of cell death. The model constants $k_1$–$k_5$ were optimized using a Nelder–Mead simplex algorithm, the parameter errors were calculated using the Bootstrapping Algorithm (100 000 samples).
because of rod loss at this time point in vivo. In relative terms cone numbers were far higher in rd1 approximately 5% of ONL cells stained positively for GP. In vitro, owing to a somewhat slower retinal development, P20 retinal cultures after 10 d of zaprinast treatment (i.e., P20). The secondary, mutation-independent loss of cone photoreceptor degeneration, in humans, primary rod degeneration is often followed by a secondary, mutation-independent loss of cone photoreceptors. The rd1 mouse also suffers from such a secondary loss of cones. Zaprinast inhibits both rod and cone PDE6 with similar specificity.22 To test how zaprinast treatment affected photoreceptor degeneration, we performed immunostaining for the cone marker glycogen phosphorylase (GP)23 on retinal cultures derived from wt animals; error bars indicate S.E.M.; dotted lines in (a) indicate wt situation.

**Table 2** Constants of fitted Gauss curves

| Constant | Value | Error |
|----------|-------|-------|
| f1       | 0.12  | 0.01  |
| f2       | 0.36  | 0.02  |
| μ1       | 11.91 d | 0.16 d |
| μ2       | 13.16 d | 0.07 d |
| σ1       | 1.02 d | 0.07 d |
| σ2       | 1.58 d | 0.14 d |

The table gives the optimized parameters of the two fitted Gauss curves (see Figure 2d). The parameter errors were calculated using the Fisher information matrix.26

**Progression of zaprinast–cGMP-induced cell death.** To test and validate the model predictions, we used organotypic retinal explant cultures derived from wt animals, exposed to the selective PDE6 inhibitor zaprinast.20 Zaprinast raises intracellular cGMP levels and induces wt photoreceptor degeneration similar to what is seen in rd1 retina.12,21 The effects of zaprinast on cGMP levels and TUNEL positivity were investigated at time points ranging from 8 h to 10 d. A significant rise in cGMP-positive cells was detected after 36 h of zaprinast treatment and at all later time points assessed (Figure 3a). Retinal explantation is a traumatic event and the cultures therefore displayed elevated rates of cell death (TUNEL assay) even under control conditions (Figure 3a). This can be regarded as basal level of cell death. Zaprinast caused a significant rise in cell death, but only after 72 h of treatment.

As a result of the culture situation, the number of photoreceptor rows in the ONL in vitro is decreasing more strongly than it would in vivo, in healthy wt retina (Figure 3b). Yet, zaprinast significantly exacerbated this cell loss from 6 d of treatment onward. The delay of almost 36 h between the zaprinast induced rise of cGMP and the rise of cell death another 36 h later corresponded to both the in vivo findings and the results of mathematical modeling (Figure 4).

**Preservation of cone photoreceptors.** In inherited RD in humans, primary rod degeneration is often followed by a secondary, mutation-independent loss of cone photoreceptors. The rd1 mouse also suffers from such a secondary loss of cones. Zaprinast inhibits both rod and cone PDE6 with similar specificity.22 To test how zaprinast treatment affected rod and cone photoreceptors, we performed immunostaining for the cone marker glycogen phosphorylase (GP)23 on retinal cultures after 10 d of zaprinast treatment (i.e., P20).

Owing to a somewhat slower retinal development, P20 in vitro corresponds to P18 in vivo. At P18 in wt retina in vivo, approximately 5% of ONL cells stained positively for GP. In relative terms cone numbers were far higher in rd1 retina because of rod loss at this time point in vivo (Supplementary Figure 4A, B; quantified in E). Higher numbers of cones were also found in zaprinast-treated versus -untreated in vitro retina (Supplementary Figure 3C, D; quantified in E). Even if the relative effect was much smaller than in vivo, this suggested that zaprinast-induced degeneration of ONL cells affected mostly rods, particularly when absolute numbers of cones at P18 were considered (Supplementary Figure 4F).

**Discussion**

Here, we show for the first time that inherited neuronal cell death in the retina – despite a rapid progression of overall tissue degeneration – is a surprisingly slow process at the level of the individual cell. This affords interesting insights into the underlying mechanism, since a total duration of cell death of approximately 80 h is incompatible with the execution of conventionally assumed necrotic or apoptotic cell death.

**cGMP in photoreceptor cell death.** High levels of cGMP are known to cause photoreceptor degeneration.13 In the rd1 retina accumulation of cGMP is caused by PDE6 dysfunction,11 and this situation can be replicated by pharmacological inhibition of PDE6.13,21 The rd1 mouse seems particularly well suited for studies into the temporal characteristics of cell death, because cGMP accumulation provides a clear label for cell death induction, an event...
cGMP production. Why this control mechanism fails to keep cGMP in check after prolonged elevation of cGMP is unknown, but may be linked to cGMP-dependent activation of PKG and its effects on gene regulation. Indeed, extensive gene expression changes were seen in rd1 retina already at P11. At the same time, excessively high cGMP-levels may precipitate rd1 cell death via over-activation of CNG channels and subsequent influx of Ca$^{2+}$ ions.

**Cell death mechanisms.** A variety of different mechanisms have been connected with neuronal cell death, but necrosis and apoptosis are possibly the best well known and often seen as conceptual counterparts. While necrosis usually is very rapid, taking no more than a few hours to complete, apoptosis is somewhat slower, requiring 6–18 h typically. Characteristic features of necrosis not seen in apoptosis are activation of the immune system and an inflammation, as well as necrotic clumping of dying cells. Photoreceptor cell death during primary RD is topologically independent and generally there is no evidence for inflammation, although an upregulation of innate immunity and secondary infiltration of microglial cells into the ONL has been reported. Both rd1 and wt retina show small numbers of cells with activated caspase-3 – a key feature of apoptosis and likely related to early post-natal developmental cell death.

However, rd1 mutation-induced cell death is caspase-independent and devoid of classical apoptotic features. It is tempting to speculate that instead of necrosis or apoptosis, we may be facing an alternative cell death mechanism, possibly involving metabolic activities of PKG, histone deacetylase (HDAC), poly-ADP-ribose polymerase (PARP), and calpains. Our data indicate that this form of cell death is considerably more time consuming than both necrosis and apoptosis. This is in line with findings in caspase-deficient neurons suggesting that caspase-independent cell death may be slower than caspase-mediated apoptosis.

**The kinetics of photoreceptor death.** Rod photoreceptors in the mouse are born over a 16-d period ranging from 7 d before to 9 d after birth, and one could hypothesize that the lifespan of rd1 photoreceptors might be constant and predetermined by their respective date of birth. On the other hand, rd1 photoreceptor degeneration at the tissue level is governed by a constant rate of cell death and first-order kinetics, with one cell’s death entirely independent from another cell’s death. This is very similar to what is observed in the exponential decay of radioactive elements and hence, the lifespan of an individual photoreceptor cell will be random and governed by probabilistic and stochastic effects. In this model, the average life-span of a cell population will be random and governed by the severity of the (genetic) insult.

The kinetics of cell death presented here are generally compatible with the ‘one-hit-model’. We introduced several novel parameters to this model, including different cell death stages and a variable risk for the initial stage. Indeed, we found that an individual rd1 photoreceptor’s risk to die may...
increase during early post-natal differentiation and reach a constant value only once the fully differentiated state is reached. This would result in an apparent wave of cell death at the time of differentiation and may serve to explain the center to periphery progression of the rd1 degeneration, which follows the pattern of retinal development.

**Zaprinast treatment: simulation of an inherited disease?**

Studies of inherited RDs can be helped by disease simulation on different genetic backgrounds or in different species. Pioneering works by Lolley et al. used the general PDE inhibitor 3-isobutyl-1-methylxanthine to pharmacologically induce selective, cGMP-dependent photoreceptor degeneration in *Xenopus* embryos. In mammalian systems, zaprinast is a highly selective PDE6 inhibitor, which – as used here (100 μM) – causes cGMP accumulation and exclusive photoreceptor death. Interestingly, and very similar to the in vivo characteristics of the rd1 retina, zaprinast affected sub-populations of photoreceptor cells at different times, which also in vitro might be connected to ongoing development of photoreceptors, superseded by stochastic effects.

Unexpectedly, the dramatic rise of photoreceptor cGMP levels was not an immediate effect of zaprinast treatment. Although zaprinast increases Ca²⁺-levels cGMP dependently in mouse photoreceptors within minutes of application, a catastrophic rise of cellular cGMP to levels detectable with immunostaining appeared only after 36 h and beyond. These non-linear kinetics of cGMP accumulation suggest that feedback control mechanisms, such as the CNG-Ca²⁺-GC loop, prevent the rise of cGMP for approximately 1.5 d, before a changing metabolism causes cGMP levels to go unchecked.

Contrary to what might be expected from its inhibitory capacity on both rod and cone PDE6 isoforms, zaprinast did not seem to affect cones. This may point at an increased resistance of cones to higher cGMP levels but could also be due to their later differentiation. Anyhow, within the time frame of our experiments, in vitro application of zaprinast on wt retina faithfully reproduced the selective rod photoreceptor loss seen in rd1 retina in vivo. This approach could prove very useful to study retinal neurodegenerative mechanisms, for instance on non-degenerating knock-out rodent models or on large, non-rodent animal models.

**The slow death of photoreceptors: three phases in cell death.** Our different experimental approaches delineate three major phases in the progression of cell death and give estimates on their duration (Figure 4).

1. **The initiation phase:** this phase of cell death is inherently difficult to study because of our lack of understanding of relevant metabolic processes and suitable markers. Nevertheless, both in vivo and in vitro data suggested that after inhibition or genetic inactivation of PDE6, cGMP levels rise but are maintained within physiological limits by feedback control mechanisms. Possibly, the cell is not yet committed to die at this stage, although toward its end the feedback control is shut down and cGMP rises beyond physiological limits. PKG is involved in the degeneration and has a 100-fold higher sensitivity to cGMP compared with CNG channels. PKG could therefore have a preeminent role during this phase, which our data suggest takes about 36 h.

2. **The execution phase:** once the cellular metabolism has switched to allow for a catastrophic rise of cGMP, the cell likely becomes committed to die and enters the death execution phase. This phase may be characterized by an over-activation of HDAC and subsequently PARP, resulting in chromatin changes and rearrangements. In addition, high cGMP likely acts on CNG channels to cause excessive Ca²⁺ influx and calpain activation. The execution phase may last between 36 and 48 h. Similarly, chick embryo neuronal precursor cells became committed to die approximately 2 d before clear signs of impending cell death were observed.

3. **The clearance phase:** once the cell has reached the final stages of cell death, extensive DNA fragmentation sets in, as evidenced by the TUNEL assay. It is worth mentioning that the TUNEL assay generally labels dying cells, including in necrosis and apoptosis. Our model suggests that it may take about 4 h until a maximal degree of DNA fragmentation is reached, and from then on the nucleus becomes more and more condensed and pyknotic until the cellular debris is completely removed, a stage that may take another 3 h. This is in agreement with studies on clearance of TUNEL-positive cells in rat and mouse neocortex, estimated to last between 1 and 4 h, and clearance of pyknotic ganglion cells during retinal development within 1 h.

Even with cautious extrapolation to the human situation, where RD caused by homologous mutations may take several decades to complete, the observed delays suggest a window-of-opportunity sufficiently large for therapeutic interventions in patients. They also confirm previous observations that photoreceptor metabolism may suffer significantly before evident cell loss leads to first clinical symptoms. With respect to potential neuroprotective treatments, targeting of metabolic processes during initiation or early execution phases may be particularly promising.

**Conclusion**

Our study for the first time provides consistent estimates on the duration of neuronal cell death in a hereditary neurodegenerative disease. With a period of about 80 h – from initiation, to cGMP accumulation, to TUNEL-positive reaction, to clearance – the time an individual cell needs to die is remarkably long and points toward execution of non-necrotic, non-apoptotic, and relatively slow cell death mechanisms. This has clear relevance for the development of potential therapies, for instance in acute neurodegenerative disorders such as stroke or spinal-cord injury, where the time frame for cell death will directly determine the window-of-opportunity and potential therapeutic options. Similarly, for chronic and inherited forms of neurodegeneration, knowledge on the temporal progression of cell death will provide insights into the underlying degenerative mechanisms and again define possible treatment approaches.
The mathematical model presented here may be extended to also include other processes causally involved in cell death, such as enzymatic activities of PKG, HDAC, PARP, and calpain. Combined with the use of transgenic biosensors for cGMP and Ca²⁺, this could allow precise delineation of the temporal progression and interdependence of different metabolic processes causing cell death. Furthermore, the current experimental approach and type of mathematical modeling may be used to study cell death in general, provided at least two temporally distinct degeneration markers can be identified.

Materials and Methods

Animals. C3H rd1/rd1 (rd1) and control C3H wt mice were housed under standard white cyclic lighting, had free access to food and water, and were used irrespective of gender. All procedures were performed in accordance with the local ethics committee at Tübingen University (64 registration from 23 January 2008), and the ARVO statement for the use of animals in ophthalmic and visual research. All efforts were made to minimize the number of animals used and their suffering. Day of birth was considered as post-natal day (P) 0.

Organotypic retinal explant culture. Retinala from P5 rd1 and wt animals were used to generate retinal explants as described before.10 Explants of the retinas were cultured on Millicell HA culture dish filter inserts (Millipore, Carrigtwohill, Cork, Ireland; PIHA03050) with the retinal pigment epithelium facing the membrane. Inserts were put into six-well culture plates and incubated in R16 nutrient medium with supplements at 37 °C. Every second day the full volume of nutrient medium, 1.5 ml per well, was replaced with fresh medium. After 5 d in vitro (i.e., P10), the two retinal explants obtained from one animal were split into two groups, one was exposed to 100 μM zaprinast in DMSO (treatment group), one was exposed to DMSO only (control group); 0.3% DMSO. The culture period was ended by immediate fixation in 4% paraformaldehyde in PBS at post-treatment time points ranging from 8 h to 10 d.

Immunostaining and TUNEL assay. Retinal cryosections obtained either from in vivo animals or following in vitro explant culture, were dried for 30–60 min at 37 °C. Subsequently, the tissue was rehydrated in PBS, and pre-incubated for 1 h at room temperature in blocking solution, containing 10% normal serum, and 0.1% or 0.3% Triton in PBS (PBST). Immunostaining was performed overnight at 4 °C, using primary antibodies against cGMP (provided by Harry Steinbusch, Maastricht University, The Netherlands), Cngb1 (provided by Stylianos Michalakis, Maastricht University, The Netherlands), Cngb1 (provided by Stylianos Michalakis, Maastricht University, The Netherlands), and the ARVO statement for the use of animals in ophthalmic and visual research. All efforts were made to minimize the number of animals used and their suffering. Day of birth was considered as post-natal day (P) 0.

Mathematical models. For modeling cell death progression and estimating its duration, irreversible first-order kinetics were assumed.17 The model consisted of six stages, termed ‘healthy’ (H), ‘transition state-1’ (Tr1), ‘cGMP-positive’ (cGMP ⊕), ‘transition state-2’ (Tr2), ‘TUNEL-positive’ (TU ⊕) and ‘dead’ (D). The changes in the six stages are represented by an ordinary differential equation system:

\[
\frac{d}{dt} H(t) = -k_1 \cdot H(t) \quad (1)
\]

\[
\frac{d}{dt} Tr1(t) = k_1 \cdot H(t) - k_2 \cdot Tr1(t) \quad (2)
\]

\[
\frac{d}{dt} cGMP ⊕(t) = k_2 \cdot Tr1(t) - k_3 \cdot cGMP ⊕(t) \quad (3)
\]

\[
\frac{d}{dt} Tr2(t) = k_3 \cdot cGMP ⊕(t) - k_4 \cdot Tr2(t) \quad (4)
\]

\[
\frac{d}{dt} TU ⊕(t) = k_5 \cdot Tr2(t) - k_6 \cdot TU ⊕(t) \quad (5)
\]

\[
\frac{d}{dt} D(t) = k_6 \cdot TU ⊕(t) \quad (6)
\]

The SOE was calculated using equation (8) where \( y \) represents the measured values of healthy, cGMP-positive, TUNEL-positive and dead cells stages, \( y^p \) represents predicted values of these variables, whereas \( n \) stands for the total number of observations. For the SOE calculation, we summed the predicted values for the healthy, the Tr1 state, because these two stages were indistinguishable experimentally.

\[
SQE = \sum_{i=0}^{n} (y_i - y^p_i)^2 \quad (8)
\]

To fit the parameters, we used the Nelder–Mead Simplex Algorithm ('minsearch') to minimize SQE and to match the model as closely as possible to the observed cell death processes. Initially, decay constants and starting time \( t_0 \) of the system of differential equations were part of the optimization and subsequent bootstrapping. From the probability density function of possible start times, we choose the time with the highest probability below 10 d post-natal, which was 9.84 d. For biological reasons, solutions beyond 10 d were not considered. The final parameters were then optimized with a fixed starting time of 9.84 d.

Microscopy, cell counting, and statistical analysis. Morphological observations and routine light microscopy were performed on a Zeiss Imager Z1 Apotome Microscope (Zeiss, Oberkochen, Germany), equipped with a Zeiss Axiocam digital camera. Images were captured using Zeiss Axiosvision 4.8 software; image overlays and contrast enhancement were done using Adobe Photoshop CS5. Images shown in figures are representative for least three different animals or following in vitro culture, were dried for 30–60 min at 37 °C. Subsequently, the tissue was rehydrated in PBS, and pre-incubated for 1 h at room temperature in blocking solution, containing 10% normal serum, and 0.1% or 0.3% Triton in PBS (PBST). Immunostaining was performed overnight at 4 °C, using primary antibodies against cGMP (provided by Harry Steinbusch, Maastricht University, The Netherlands), Cngb1 (provided by Stylianos Michalakis, Ludwig Maximilian University of Munich, Germany), and GP (provided by Brigitte Pfeiffer-Gugliemi, University of Tübingen, Germany) diluted 1:500 in blocking solution. The tissue was rinsed with PBST, and incubated for 1 h with a corresponding, Alexafluor-488 conjugated, secondary antibody (1:200–1:750, Maastricht University, The Netherlands) diluted 1:200–1:750. Life technologies, Darmstadt, Germany), diluted in PBST. Sections were rinsed in PBS, and mounted in Vectashield with DAPI for nuclear counterstaining (Vector Laboratories Inc., Burlingame, CA, USA). The TUNEL assay was performed on an in situ cell death detection kit conjugated with tetra-methyl-rhodamine or fluorescein isothiocyanate (Roche Diagnostics, Mannheim, Germany). For controls terminal deoxynucleotidyl transferase enzyme was either omitted from the labeling solution (negative control), or sections were pre-treated for 30 min with DNase I (Roche, 3 U/ml) in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA to induce DNA strand breaks (positive control). Although negative control gave no staining, positive control stained all nuclei in all layers of the retina.10,16

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The second model was realized in Microsoft Excel 2010 (Microsoft, Unterschleissheim, Germany) using the ‘Solver’ functionality with genetic algorithms and SQE to fit the functions to the measured data.

Conflict of Interest
The authors declare no conflict of interest.

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1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 25: 239–257.
2. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nucleic DNA fragmentation. J Cell Biol 1992; 119: 493–501.
3. Zong WX, Thompson CB. Necrotic death as a cell fate. Genes Dev 2006; 20: 1–15.
4. Henson PM, Hume DA. Apoptotic cell removal in development and tissue homeostasis. Annu Rev Genomics Hum Genet 2004; 5: 362–373.
5. Oppenheim RW, Flavell RA, Vinsant S, Prevette D, Kuan CY, Rakic P. Programmed cell death during development of the nervous system. Annu Rev Neurosci 1991; 14: 453–501.
6. Arrowood WH, Hughes A. Role of cell death in the topogenesis of neuronal distributions in the developing cat retinal ganglion cell layer. J Comp Neurol 1987; 262: 496–511.
7. Cordeiro MF, Guo L, Coxon KM, Duggan J, Nizari S, Normando EM et al. Imaging multiple phases of neurodegeneration: a novel approach to assessing cell death in vivo. Cell Death Dis 2010; 1: 63.
8. Albizri H, Can S, Holiena P, Scholl C, Lederer E, Kitanovic I et al. Real-time monitoring of cisplatin-induced cell death. PLoS ONE 2011; 6: e19714.
9. Gohike JM, Wright WC, Faustman EM. The role of cell death during neocortical neurogenesis and synaptogenesis: implications from a computational model for the rat and mouse. Brain Res Dev Brain Res 2004; 151: 43–54.
10. Bowes C, Li T, Danigier M, Baster LC, Applebury ML, Farber DB. Retinal degeneration in the rd mouse is caused by a defect in the beta subunit of rod cGMP-phosphodiesterase. Nature 1990; 347: 677–680.
11. Paquet-Durand F, Hauck SM, van Veen T, Ekstrom P, Ueffing M, Ekstroem P. PKG activity causes photoreceptor cell death in development and tissue homeostasis. Trends Immunol 2006; 27: 244–250.
12. Skommer J, Daryniewicz Z, Wlodkowic D. Cell death goes LIVE: technological advances in real-time tracking of cell death. Cell Cycle 2010; 9: 2330–2341.
13. Oppenheim RW. Cell death during development of the nervous system. Annu Rev Neurosci 1991; 14: 453–501.
14. Fox DA, Poblenz AT, He L. Calcium overload triggers rod photoreceptor apoptotic cell death in chemical-induced and inherited retinal degenerations. Ann N Y Acad Sci 1999; 883: 282–285.
15. Paquet-Durand F, Beck S, Michalisik S, Goldmann T, Huber G, Mühlfeld R et al. A key role for cyclic nucleotide gated (CNG) channels in cGMP-related retinal pigments. Hum Mol Genet 2011; 20: 341–347.
16. Azad S, Paquet-Durand F, Medstrand P, van Veen T, Ekstrom PA. Up-regulation and increased phosphorylation of protein kinase C (PKC) delta, mu and theta in the degenerating rd1 mouse retina. Mol Vis Neurosci 2006; 31: 759–773.
17. Murakami Y, Matsuura H, Rito M, Suzuki J, Hisatomi T, Ikeda Y et al. Receptor interacting protein kinase mediates necrotic cone but not rod cell death in a mouse model of inherited degeneration. Proc Natl Acad Sci USA 2012; 109: 14958–14963.
18. Sancho-Pelluz J, Alavi MV, Sahaboglu A, Kustermann S, Fainelli P, Azad S et al. Excessive HDAC activation is critical for neurodegeneration in the rd1 mouse. Cell Death Dis 2010; 1: 1–8.
19. Paquet-Durand F, Sanges D, McCull J, Silva J, van Veen T, Marigo V et al. Photoreceptor rescue and toxicity induced by different calpain inhibitors. J Neurochem 2010; 115: 930–940.
20. Oppenheim RW, Flavell RA, Vinsant S, Prevett D, Kuan CY, Raicic P. Programmed cell death of developing mammalian neurons after genetic deletion of caspases. J Neurosci 2001; 21: 4762–4769.
21. Trifunovic D, Sahaboglu A, Kaur J, Mencic S, Zrenner E, Ueffing M et al. Neuroprotective strategies for the treatment of inherited photoreceptor degeneration. Curr Mol Med 2012; 12: 598–612.
22. Cepko CL, Austin CP, Yang X, Alexides M, Ezredzila D. Cell fate determination in the vertebrate retina. Proc Natl Acad Sci USA 1996; 93: 589–595.
23. Skommer J, Raychaudhuri S, Wlodkowic D. Timing is everything: stochastic origins of cell-cell variability in cancer cell death. Front Biosci 2011; 16: 307–314.
24. Wei T, Schubert T, Paquet-Durand F, Tamitomo N, Chang L, Koeppen K et al. Generation and functional characterization of a transgenic mouse expressing a Ca(2) + /sensore in cone photoreceptors. J Neurosci 2012; 32: 6949–6981.
25. Johnson JE, Jr, Perkins GA, Giddabasappa A, Chaney S, Xiao W, White AD et al. Spatiotemporal regulation of ATP and Ca(2) + dynamics in vertebrate rod and cone ribbon synapses. Mol Vis 2007; 13: 887–919.
26. Szél A, van Veen T, Röhl P, Röhl P. Rod cell cone differentiation. Nature 1994; 370: 336.
27. Lincoln TM, Cornell CWL. Intracellular cyclic GMP receptor proteins. FASEB J 1993; 7: 328–338.
28. Cellebro A, Galli-Resta L, Colombaion L. The dynamics of neuronal death: a time-lapse study in the retina. J Neurosci 2000; 20: RC92.
29. Acosta ML, Shin YS, Ready S, Fletcher EL, Christie DL, Kalbaniats M et al. Retinal metabolic state of the proline-23-histidine rat model of retinitis pigmentosa. Am J Physiol Cell Physiol 2010; 298: C764–C774.
30. Hoops S, Sahle S, Gauges R, Lee C, Pahle J, Simus N et al. COPASI· A Complex Pathway Simulator. Bioinformatics 2006; 22: 3067–3074.
31. Lindner O, Hitzenbichl B. Experimental design for optimal parameter estimation of an enzyme kinetic process based on the analysis of the Fisher information matrix. J Theor Biol 2006; 238: 111–123.

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