Abstract

Cone photoreceptors (cones) are essential for high-resolution daylight vision and colour perception. Loss of cones in hereditary retinal diseases has a dramatic impact on human vision. The mechanisms underlying cone death are poorly understood, and consequently, there are no treatments available. Previous studies suggest a central role for calcium (Ca^{2+}) homeostasis deficits in photoreceptor degeneration; however, direct evidence for this is scarce and physiological measurements of Ca^{2+} in degenerating mammalian cones are lacking.

Here, we took advantage of the transgenic HR2.1:TN-XL mouse line that expresses a genetically encoded Ca^{2+} biosensor exclusively in cones. We cross-bred this line with mouse models for primary (“cone photoreceptor function loss-1”, cpfl1) and secondary (“retinal degeneration-1”, rd1) cone degeneration, respectively, and assessed resting Ca^{2+} levels and light-evoked Ca^{2+} responses in cones using two-photon imaging. We found that Ca^{2+} dynamics were altered in cpfl1 cones, showing higher noise and variable Ca^{2+} levels, with significantly wider distribution than for wild-type and rd1 cones. Unexpectedly, up to 21% of cpfl1 cones still displayed light-evoked Ca^{2+} responses, which were larger and slower than wild-type responses. In contrast, genetically intact rd1 cones were characterized by lower noise and complete lack of visual function. Our study demonstrates alterations in cone Ca^{2+} dynamics in both primary and secondary cone degeneration. Our results are consistent with the view that higher (fluctuating) cone Ca^{2+} levels are involved in photoreceptor cell death in primary (cpfl1) but not in secondary (rd1) cone degeneration. These findings may guide the future development of therapies targeting photoreceptor Ca^{2+} homeostasis.

Introduction

Human vision depends primarily on cone photoreceptors, which mediate high-resolution, day-light colour vision. Rod photoreceptors (rods), on the other hand, are responsible for vision under dim-light conditions, i.e. at night. Cone loss can occur as a consequence of a mutation in the cone itself (primary cone degeneration) or as a “side-effect” of rod photoreceptor loss (secondary cone degeneration). A rapid cone loss as a consequence of mutations in cone-specific Pde6c (cGMP-specific phosphodiesterase) is present in the cpfl1 mouse, an animal model for primary cone degeneration (1–3). Secondary cone degeneration, on the other hand, is observed as a consequence of primary rod photoreceptor cell death in diseases like Retinitis Pigmentosa (4,5). The rd1 mouse carries a rod-specific Pde6b mutation and is a well-known model for...
secondary cone degeneration (6). We used cpfl1 and rd1 mouse lines to investigate the role of Ca\(^{2+}\) homeostasis in primary and secondary cone degeneration, respectively.

In the intact photoreceptor, Ca\(^{2+}\) has many important functions (Fig. 1A and B), including modulating the transduction cascade in the outer segment (OS), and triggering the transmitter release from the axon terminals (7–9). In the dark, high cGMP levels in the OS activate cyclic nucleotide-gated (CNG) channels and allow for Ca\(^{2+}\) influx (10–12). In the light, Ca\(^{2+}\) levels drop due to cGMP hydrolysis by phosphodiesterase 6 (PDE6) and the subsequent closure of CNG channels. Low Ca\(^{2+}\) levels allow dissociation of Ca\(^{2+}\) from guanylate cyclase-activating proteins (GCAPs), inhibition of guanylate cyclase (GC), restoring CNG channels. This close coupling between cGMP and Ca\(^{2+}\) levels, together with the finding that mutant photoreceptors show an abnormal cGMP accumulation (13–15), led to the hypothesis of cGMP-mediated Ca\(^{2+}\) "overload" triggering degeneration (16).

Many studies support this "high Ca\(^{2+}\) hypothesis" (15,17–20), however, there are also several contradicting results: For instance, treatments with Ca\(^{2+}\) channel blockers, aimed at reducing Ca\(^{2+}\) overload and, thus, preventing or at least delaying photoreceptor degeneration, produced inconsistent results for primary rod degeneration (21–24). Furthermore, data from GNGA3-deficient mice show that in the absence of cone CNG channels (and CNG channel-mediated Ca\(^{2+}\) influx) cones still degenerate (25). Additionally, a functional study in Pde6cw59 mutant zebrafish found no increase in cone Ca\(^{2+}\) levels, but instead a suppression of (spontaneous) Ca\(^{2+}\) transients (26). Finally, also an alternative hypothesis has been promoted, which is based on the idea that photoreceptor death might be triggered by too low Ca\(^{2+}\) (i.e. the "low Ca\(^{2+}\) hypothesis") (27).

Here, we set out to study Ca\(^{2+}\) dynamics in degenerating cones using a transgenic mouse line (HR2.1:TN-XL) that expresses a genetically encoded ratiometric Ca\(^{2+}\) biosensor selectively in cones and allows monitoring of light-evoked Ca\(^{2+}\) signals in individual cone axon terminals (28–31). In healthy photoreceptors, opening of CNG channels in the dark depolarizes the cone membrane. Voltage-gated Ca\(^{2+}\) channels (VGCCs) in the synaptic terminal translate this depolarization into a Ca\(^{2+}\) influx, which in turn triggers synaptic vesicle fusion with the membrane and glutamate release (32–35). Thus, the presynaptic Ca\(^{2+}\) level in photoreceptors can serve as a proxy for upstream CNG channel activation in the OS (28).

To study cone Ca\(^{2+}\) in primary and secondary cone degeneration, we crossedbred the HR2.1:TN-XL mouse line with the cpfl1 and rd1 PDE6 mutant lines, respectively. Ca\(^{2+}\) signals were compared between these cpfl1 and rd1 mutant crossbreds and the "wild-type" retina of HR2.1:TN-XL mice. We observed differential changes in Ca\(^{2+}\) in the two mutant lines: In primary cone degeneration, Ca\(^{2+}\) levels were more variable, noisy, and tended to be higher than in wild-type (wt). Notably, a substantial fraction of these cones showed robust light-evoked Ca\(^{2+}\) responses. On the other hand, in secondary cone degeneration, cone Ca\(^{2+}\) levels appeared to be lower and less variable compared to wt and did not show light-evoked Ca\(^{2+}\) responses. Our data are consistent with the idea of higher Ca\(^{2+}\) levels being involved in primary (cpfl1), but not in secondary (rd1) cone degeneration.
Materials and Methods

Animals

The transgenic mouse line HR2.1::TN-XL (C57BL/6j background) expresses the Ca\textsuperscript{2+} biosensor TN-XL (36) under the control of the human red opsin promoter (HR2.1) selectively in cone photoreceptors (28) (Fig. 1C). To study primary and secondary cone degeneration, we crossed the biosensor line with the original rd1 and cpfl1 mutant animals to generate the HR2.1::TN-XL x cpfl1 (C57BL/6j background) and HR2.1::TN-XL x rd1 (C57BL/6j x C3H background) lines. Using PCR amplification and Ndel digestion, we verified that these lines were free of the rd8 (Crb1) mutation (37). While a previous study on C3H rd1 and congenic C3H ut animals, the latter of which show normal electroretinographic responses, had in principle ruled out the presence of the Nob5 (Gpr179) mutation (38), we additionally performed a PCR-based analysis employed recently in a survey on C3H mouse lines (39). As shown in Supplementary Material, Figure S1, the Nob5 mutation is absent in C3H rd1 mice used in our study. For simplicity, in the later parts of the manuscript, we refer to the biosensor lines as ut, cpfl1, and rd1. For Ca\textsuperscript{2+} imaging experiments (see below), we used mice in two time windows (postnatal days 18-20 (P18-20) and P30-33), to which we refer to as P18+ and P30+, respectively. See results for a justification for the use of these time-frames. In general, we used mice irrespective of gender.

Prior to Ca\textsuperscript{2+} imaging, the mice were dark-adapted for approx. 2 hours and then anesthetized using isoflurane (CP Pharma, Burgdorf, Germany). For immunostainings, we used P20 and P30 as analysis time points and the animals were anesthetized with CO\textsubscript{2}. In both cases, anesthetized mice were killed by decapitation. All procedures were performed in accordance with the law on animal protection issued by the German Federal Government (Tierschutzgesetz) and approved by the institutional animal welfare committee of the University of Tübingen.

Immunohistochemistry and fluorescence microscopy

Eyes were marked on the nasal side prior to enucleation. They were fixed in 4% PFA in 0.1 M phosphate buffer saline (PBS, pH 7.4) (~1 hour) and cryo-protected in 30% sucrose in PBS at 4°C overnight. Then, eyes were embedded in Tissue-Tek OCT compound (Sakura Finetek Europe, Alphen aan Den Rijn, Netherlands) and stored at ~20°C until cryo-sectioning into 12 μm thick vertical sections. Sections were rehydrated with PBS, permeabilized in 0.1% Triton X-100 in PBS containing blocking solution (10% goat or donkey serum, 1% BSA). As primary antibodies, we used rabbit anti-GFP (1:600; Millipore, Darmstadt, Germany) or mouse anti-GFP (1:500; Abcam, Cambridge, UK); sheep anti-cGMP (1:500, from Harry W.M. Steinbusch, Maastricht University, Maastricht, Netherlands) and guinea pig anti-glycogen phosphorylase (1:1000, kind gift from B. Pfeiffer-Maastricht University, Maastricht, Netherlands) as described previously (28,31). In brief, eyes were enucleated and dissected in carboxygenated (95% O\textsubscript{2}, 5% CO\textsubscript{2}) artificial cerebrospinal fluid (ACSF) solution, which contained (in mM): 125 NaCl, 2.5 KCl, 2 CaCl\textsubscript{2}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 0.5 L-glutamine, and 20 glucose; maintained at pH 7.4 using carboxygen. Consecutive vertical slices (~200 μm thick) were cut using a tissue chopper (41) and then transferred to the recording chamber of a two-photon microscope, where they were constantly perfused with warmed (~37 °C) ACSF. The microscope (for details, see (42)), consisted of a customized MDM (movable objective microscope; designed by W. Denk, MPI of Neurobiology, Martinsried; purchased from Science Products/Sutter Instruments, Novato, USA), equipped with a mode-locked Ti:Sapphire laser (MaiTai-HP DeepSee; Newport Spectra-Physics, Darmstadt, Germany) tuned to 860 nm, two fluorescence detection channels (483 band-pass (BP) 32; 535 BP 50; AHF, Tübingen, Germany), and a 20x water-immersion objective (XLUMPlanFL, 0.95 NA; Olympus, Hamburg, Germany). The system was controlled by the image acquisition software ScanM (by M. Müller, MPI of Neurobiology, Martinsried, Germany, and T.E.) running under IGOR Pro 6.3, or CfNT (M. Müller, MPI, Martinsried, Germany). A custom-built dichromatic light stimulator (29), equipped with two band-pass filtered LEDs (UV filter: 360 BP 12; green: 578 BP 10) and mounted below the recording chamber, was used to present temporally-modulated, full-field light stimuli (approx. 2mm in diameter) to the retinal slices. Ca\textsuperscript{2+} recordings were performed with a constant background illumination of 10\textsuperscript{7} Ps\textsuperscript{-1} (photo-isomerization rate) for at least 15 seconds. Light stimuli consisted of a series of 1-second bright flashes with 4-second intervals. Flashes evoked similar photo-isomerization rates in both medium (M-) and short (S-)

Analysis of immunodata

The data were obtained from at least three different animals and for each animal at least three immunostained vertical sections were quantified, using mosaic images acquired at 20× magnification. For analysis, the area of the outer nuclear layer (ONL) and the length of the retinal sections were determined using the Zeiss Axiovision software. CGMP positive cells were counted in both biosensor and non-biosensor lines and were expressed as percent positive cells in the ONL (rd1 retina), or as percent positive cones (ut, rd1 and cpfl1). To estimate cone density (number of somata per 10 μm) for P20, P24 and P30 in both biosensor and non-biosensor lines, cones were labelled using glycogen phosphorylase (40) and counted in defined areas. Cones were only included if at least the inner segment (IS) and the soma could be clearly identified. To quantify dying cells, the in situ TUNEL assay was also performed on vertical sections (Supplementary Material, Fig. S2). We measured the area of the ONL, divided it by the total number of cells (obtained from DAPI staining) in that area and determined the fraction of TUNEL-positive nuclei (Supplementary Material, Fig. S3). Statistical comparisons were made using the Wilcoxon rank-sum test using IGOR Pro (Wavemetrics, Lake Oswego, USA).

Two-photon Ca\textsuperscript{2+} imaging

The preparation of retinal slices for Ca\textsuperscript{2+} imaging was performed as described previously (29,31). In brief, eyes were enucleated and dissected in carboxygenated (95% O\textsubscript{2}, 5% CO\textsubscript{2}) artificial cerebrospinal fluid (ACSF) solution, which contained (in mM): 125 NaCl, 2.5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 0.5 L-glutamine, and 20 glucose; maintained at pH 7.4 using carboxygen. Consecutive vertical slices (~200 μm thick) were cut using a tissue chopper (41) and then transferred to the recording chamber of a two-photon microscope, where they were constantly perfused with warmed (~37 °C) ACSF. The microscope (for details, see (42)), consisted of a customized MDM (movable objective microscope; designed by W. Denk, MPI of Neurobiology, Martinsried; purchased from Science Products/Sutter Instruments, Novato, USA), equipped with a mode-locked Ti:Sapphire laser (MaiTai-HP DeepSee; Newport Spectra-Physics, Darmstadt, Germany) tuned to 860 nm, two fluorescence detection channels (483 band-pass (BP) 32; 535 BP 50; AHF, Tübingen, Germany), and a 20x water-immersion objective (XLUMPlanFL, 0.95 NA; Olympus, Hamburg, Germany). The system was controlled by the image acquisition software ScanM (by M. Müller, MPI of Neurobiology, Martinsried, Germany, and T.E.) running under IGOR Pro 6.3, or CfNT (M. Müller, MPI, Martinsried, Germany). A custom-built dichromatic light stimulator (29), equipped with two band-pass filtered LEDs (UV filter: 360 BP 12; green: 578 BP 10) and mounted below the recording chamber, was used to present temporally-modulated, full-field light stimuli (approx. 2mm in diameter) to the retinal slices. Ca\textsuperscript{2+} recordings were performed with a constant background illumination of 10\textsuperscript{7} Ps\textsuperscript{-1} (photo-isomerization rate) for at least 15 seconds. Light stimuli consisted of a series of 1-second bright flashes with 4-second intervals. Flashes evoked similar photo-isomerization rates in both medium (M-) and short (S-)

a 63x oil immersion objective (1.4 NA; cf. Fig. 2A, rd1), and AxioVision (v.4.8.1.0) software (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Figures were assembled either in Adobe Photoshop CS5 (Adobe Systems, San Jose, USA) or Canvas 11 (ACD Systems International Inc., Seattle, USA).
wavelength-sensitive cones (green LED: 6.7; UV LED: 6.5). At these light levels, rods are expected to be saturated and, therefore, light-evoked responses should originate in cones and not in electrically coupled rods. To record Ca\(^{2+}\) levels and light-evoked Ca\(^{2+}\) responses in cone axon terminals, we captured image series (128 x 16 pixels at 31.25 Hz), aligned with the outer plexiform layer (OPL) and covering an area of \(\frac{2475 \times 10}{\text{mm}^2}\) (Fig. 1D).

We recorded both fluorescence channels, as TN-XL allows ratiometric measurements via FRET between its fluorophores enhanced cyan fluorescent protein (eCFP, donor) and citrine (acceptor). In a subset of experiments, absolute Ca\(^{2+}\) concentrations were measured using an “ex-vivo slice calibration” procedure (31). We recorded the fluorescence ratio (see Analysis of Ca\(^{2+}\) imaging data) first in a standard ACSF solution \(R_{\text{base}}\), see below and Fig. 1D), then in Ca\(^{2+}\)-free ACSF supplemented with ionomycin (5 μM) and EGTA (10 mM) to determine the minimum ratio \(R_{\text{min}}\), and finally in ACSF supplemented with ionomycin (5 μM) and high Ca\(^{2+}\) (2.5 mM) to determine the maximum (saturating) ratio \(R_{\text{max}}\). The absolute Ca\(^{2+}\) concentration was estimated using \(R_{\text{min}}, R_{\text{max}}\) the donor fluorescence with a saturating Ca\(^{2+}\) level \(F_D, \text{Ca-bound}\) and under Ca\(^{2+}\)-free conditions.

![Figure 2](https://academic.oup.com/hmg/article-abstract/25/17/3729/2525831)

Figure 2. cGMP accumulation in photoreceptors of Pde6 mutant mice expressing the HR2.1/TN-XL Ca\(^{2+}\)-biosensor. (A) Cross-sections of wt (left), cpfl1 (centre), and rd1 (right) outer retinas immunostained for cGMP (magenta) at P20 (top) and P30 (bottom). The biosensor TN-XL is shown in green. (B) Cone density (in # cells/10 μm) in rd1 animals is significantly reduced at P20, while at P30 both cpfl1 and rd1 retinas show cone loss. (C) The percentage of cGMP-positive cones is strongly increased in cpfl1 retina at P20 and P30, while wt and rd1 cones are negative for cGMP. Scale bars: a, 10 μm. P20 data obtained from 9 sections from 3 wt mice (9/3); cpfl1, 11/3; rd1, 9/3; P30: wt, 14/4; cpfl1, 11/3; rd1, 9/3; *** \(P < 0.001\). OPL, outer plexiform layer, ONL, outer nuclear layer.
(FD, Ca-free), as well as the in-vivo dissociation constant ($K_d = 0.77$, see (43)) for TN-XL:

$$[Ca^{2+}] = K_d \frac{R - R_{min}}{R_{max} - R} \frac{F_{D,Ca-free}}{F_{D,Ca-bound}}$$

Analysis of Ca\textsuperscript{2+} imaging data

All Ca\textsuperscript{2+} data were analysed using custom scripts for IGOR Pro 6.3 (WaveMetrics). To extract cone Ca\textsuperscript{2+} signals, regions of interest (ROIs) were drawn manually around the cone terminals (Fig. 1D) and the pixels within ROIs were averaged for each image frame. An ROI placed outside the OPL was used to determine the baseline fluorescence in both detection channels for background subtraction. The relative Ca\textsuperscript{2+} level was then determined as the ratio (R) between acceptor and donor fluorescence ($F_{A}/F_{D}$) signals after background subtraction. For each ROI, stimulus trials were averaged and the mean trace was filtered (boxcar, 320 ms filter-width).

Relative resting Ca\textsuperscript{2+} level ($R_{base}$) was defined as the mean prior to the light flash (cf. Fig. 3A). Further, we quantified the area between the base line and light response ("response area", $R_A$), response amplitude (AR), response rise ($t_{0.0-0.8}$). The latter was determined by fitting a sigmoid to the response rise to determine the duration between the time points when 20% and 80% AR were reached. As a measure for the "slowness" of a light response, we determined a "response lag" ($t_{lag}$), which were defined as the duration between $t_{50\%}$ and $t_{dec}$ the time point when the Ca\textsuperscript{2+} level started to recover (= to increase). In addition, we quantified response noise ($R_{noise}$), defined as s.d. of the traces (trials) after subtracting the low-pass-filtered mean response (cf. Fig. 6A).

Previous studies showed that during slicing, cells close to the surface might be mechanically damaged and show abnormal Ca\textsuperscript{2+} ratios (28). We tried to address this issue by recording from cones at least 20-50 min in the slice and by excluding cones with unstable baseline and/or extreme noise level. We used the noise distribution of the wt P30\+- dataset and determined from the histogram the noise level ($R_{noise,max}$) that excluded the 5% cones with the highest noise (95\% percentile). Then, from all datasets we excluded cones with $R_{noise} > R_{noise,max}$ unless they displayed clear light responses ($R_A > 10 R_{noise,noise}$, with $R_{noise}$ defined as the s.d. over the whole 5-s trace times the trial duration).

To compare responsiveness to light stimuli between the different data sets, we categorized the cones into “responsive” and “non-responsive”. To include only cones with robust light responses into the "responsive" category, we again used the wt P30\+- dataset: We determined the value ($R_{50\%}A$) that divided the $R_A$ histogram (cf. Fig. 4C) into halves and defined the 50% cones with the larger $R_A$ values as responsive. This $R_{50\%}A$ was then applied to the other datasets. Note that cones for which the response curve could not be fitted (to determine timing parameters, see above) were not classified as “responsive”, even if they fulfilled the $R_A > R_{50\%}A$ criterion.

Statistics

In total, we analysed $n = 2,678$ (89% of 3,007 cells) wt, $n = 1,992$ (81% of 2,469 cells) cpfl1, and $n = 1,696$ (92% of 1,845 cells) rd1 cones. To compare Ca\textsuperscript{2+} level distributions statistically, we fitted the distribution of $R_{base}$ for each line and time point with a Gaussian that was convoluted with an exponential (ExpModGauss in IGOR Pro) and used $R_{base}$ at the distribution peak to centre the histograms for comparison between data sets (Fig. 3B). $R_{base}$ distributions were compared using the Kolmogorov-Smirnov test (K-S test, IGOR Pro) for an alpha level of 5%. To compare light responses sizes statistically, we first normalized $R_A$ of the responsive cells for all the datasets to the mean $R_A$ of ut cones at P30\+. To compare noise levels, we normalized $R_{noise}$ measured in the mutant and at other time windows to $R_{noise,max}$ (see above). The Wilcoxon rank-sum test (as implemented in IGOR Pro; 5% alpha level) was used to compare $R_A$, $t_{lag}$ and $R_{noise}$ between mouse lines and time windows. These data are shown as box-and-whisker plots (top whisker: 90\% percentile; bottom whisker: 10\% percentile).

Results

Normal ut cones and Ca\textsuperscript{2+} biosensor-expressing ut cones were previously found to be functionally equivalent, showing no differences in electroretinographic (ERG) recordings and opsin expression (28–30). To ascertain that the expression of the genetically encoded Ca\textsuperscript{2+} sensor TN-XL in mutant photoreceptors would not alter the degeneration phenotype, we first assessed degeneration markers in HR2.1:TN-XL crossbred mutants. For our analysis, we focussed on two time-windows, postnatal days (P) 18-20 and P30-33, to which we refer to as P18\+ and P30\+, respectively. In both rd1 and cpfl1 genotypes, these two periods correspond to times just beyond the onset of cone degeneration (P18\+) and when roughly 50% of all cones are gone (P30\+) (3,44). Presence of biosensor does not alter Pde6 mutant phenotype

Cone morphology in the wt HR2.1:TN-XL retina (control) appeared normal, whereas HR2.1:TN-XL x cpfl1 cones were characterized by misplaced somata (Fig. 2A), as described before for non-biosensor cpfl1 cones (3). Due to primary rod loss, HR2.1:TN-XL x rd1 retina showed a marked thinning of the outer nuclear layer (ONL) (45), accompanied by dramatic changes in cone morphology (rounded somata with strongly altered neurite morphology; Fig. 2A), virtually identical to what has been described in the (non-biosensor) rd1 mouse (5). We counted cones in the three mouse lines and found cone densities to be significantly reduced over time in HR2.1:TN-XL x cpfl1 and HR2.1:TN-XL x rd1 (Fig. 2B), indicating severe cone degeneration compared to wt HR2.1:TN-XL. Cone densities were within the ranges reported for non-biosensor cpfl1 and rd1 (Table 1). In addition, we performed TUNEL assays to evaluate the number of dying cells at P20 and P30 (Supplementary Material, Fig. S2) and found the fraction of TUNEL-positive cells in HR2.1:TN-XL x cpfl1 and HR2.1:TN-XL x rd1 lines to be similar to that reported for cpfl1 and rd1 before, respectively (Table 1) (3,38).

Next, we assessed cGMP accumulation in the outer retina (3,15): In wild-type retina, whether expressing the TN-XL biosensor or not, essentially no cGMP accumulation could be observed in the ONL, at P20 and P30 (Fig. 2A and C) (3). In rd1 retina, the overall percentage of cGMP positive cells in the ONL at P20 was similar for biosensor and non-biosensor expressing retina (rd1: 7.44 ± 1.09% SD, $n = 9$ vertical sections from 3 mice; HR2.1:TN-XL x rd1: 6.91 ± 0.91% SD, $P = 0.5$, $n = 9$ vertical sections from 3 mice) and all available evidence suggests that this cGMP accumulation is restricted to rods exclusively. Since degenerating cones may lose the expression of conventional markers during degeneration, we introduced a novel cGMP biosensor, termed cpfl1 HR2.1:TN-XL x rd1, targeting specifically rod cGMP (Fig. 2A). We confirmed that this expression was even stronger in our rd1 retina (Fig. 2A and C), as expected, since rd1 retina is characterized by a high level of cGMP accumulation (3,44). Thus, we have the first evidence for a CNG channel expression in rod cGMP accumulation in rd1 retina (3,44). This observation is consistent with the previously reported expression profile of the CNG channel subunit, CNGA1. Therefore, we believe that our results provide compelling evidence for the expression of CNG channels in rod cGMP accumulation in rd1 retina (3,44).
the final stages of cell death, in non-biosensor rd1 retina there is a
degree of uncertainty as to whether rd1 cones do show cGMP
accumulation or not. However, in HR2.1:TN-XL × rd1 retina, no
cGMP positive cones could be detected, indicating that rd1 cGMP
accumulation was indeed restricted to rods only (Fig. 2A and C).
In non-biosensor cpfl1 retina, approximately 50% of the cones
were positive for cGMP (3,15), similar to what was found in
HR2.1:TN-XL × cpfl1 cones (Fig. 2C).

Resting Ca²⁺ is very heterogeneous within cone
populations and differs between mutants
To evaluate potential differences in Ca²⁺ homeostasis between
mutant and wt lines, we recorded Ca²⁺ levels from TN-XL-expressing cones in acute vertical retinal slices using
two-photon Ca²⁺ imaging (Fig. 1C and D; see Methods). We first
assessed relative resting Ca²⁺ (R_base, Fig. 3A) and found that at
the beginning of cone degeneration, at P18+, resting Ca²⁺ level
distributions were comparable in wt and cpfl1, whereas the dis-
tribution for rd1 was more narrow (Fig. 3B, left column). At P30+, when cone degeneration had progressed, the Ca²⁺ level distribution
in rd1 remained narrow, whereas the distributions for both
wt and cpfl1 broadened (Fig. 3B, right column). As some broaden-
ing was also observed in the wt, it may partially reflect
developmental changes. Despite this, the effect was more pro-
nounced in cpfl1. Taken together, these data suggest that the
mutations in cpfl1 and rd1 have opposite effects on cone Ca²⁺
esting levels: As the degeneration progressed, cpfl1 cone
Ca²⁺ levels became increasingly heterogeneous, whereas rd1 cone Ca²⁺ levels were (and remained) more homogenous across
the population.

In a subset of experiments (n = 9 slices from 9 mice), we esti-
ated the absolute resting Ca²⁺ concentrations in cones using an
ex vivo calibration approach in retinal slices (31,46). Because the
difference in relative Ca²⁺ level distribution between wt and the two
mutants was more prominent at P30+, we performed the calibra-
tion experiments at this age window. In wt cones, we determined
an absolute cone Ca²⁺ concentration of 200 nM (median; n = 15
cones in 2 slices from 2 mice), which is in agreement with the liter-
ature (35,47). In cpfl1 cones, the Ca²⁺ concentration was 339 nM
(n = 14 cones in 4 slices from 4 mice), with a higher variability com-
pared to wt (Fig. 3C). While these differences were not statistically
significant, the higher variability in cpfl1 Ca²⁺ concentration
was consistent with the results from the relative Ca²⁺ distributions
(Fig. 3B). Because of the low number of remaining cones in the rd1
retina at P30+, we could only get a rough estimate for the absolute
Ca²⁺ level in rd1 cones (180 nM; n = 4 cones in 3 slices from 3 mice;
Fig. 3C). However, since most Ca²⁺ values in rd1 cones were below
the detection limit of the TN-XL biosensor, the actual intracellular
Ca²⁺ levels in rd1 cones are likely to be much lower, similar to those
measured under Ca²⁺-free conditions.

Unexpected, robust light evoked Ca²⁺ responses in cpfl1
cones
Previous ERG studies have reported very weak responses of cpfl1
retina to bright-field light flashes but could not pinpoint the
origin of these responses (2). To test whether cpfl1 cones respond to light, we recorded cone Ca\(^{2+}\) signals and presented 1-s full-field light flashes on a constant background (cf. Fig. 1D). In wt cones, a light-evoked a decrease in the fluorescence ratio (\(F_\text{A}/F_\text{D}\)) indicates a decrease in Ca\(^{2+}\) level due to a light-induced hyperpolarization of the cone (Fig. 4A). To quantify Ca\(^{2+}\) response sizes, we determined the response area (\(R_\text{A}\)). Light-evoked Ca\(^{2+}\) responses were detected in wt, and to our surprise, also in cpfl1 cones but not in rd1 cones (Fig. 4B). In general, response size tended to increase as resting Ca\(^{2+}\) levels rose.

As expected, we found the highest percentage of cones that met our conservative “responsiveness” criterion (Methods) in wt retina. The percentage of responsive wt cones increased from 36% at P18+ to 50% at P30+, (Fig. 4C and D), possibly due to postnatal maturation of mouse retina (48,49). In cpfl1 retina, we found a substantial percentage of cones that displayed light responses comparable to those measured in wt. At P18+, 8% of the recorded cpfl1 cones were responsive; later this fraction increased to 21%. Moreover, the response size dramatically increased in cpfl1 cones at P30+ (Fig. 4E).

Table 1. Cone density and percentage of TUNEL-positive cells in the studied mouse lines

<table>
<thead>
<tr>
<th>Cone density ([10 µm(^{-1})]</th>
<th>TUNEL positive cells [%]</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>P20</td>
</tr>
<tr>
<td>&quot;wild-type&quot; HR2.1:TN-XL</td>
<td>1.28 (1.19)</td>
</tr>
<tr>
<td>HR2.1:TN-XL x cpfl1</td>
<td>1.09 (1.14)</td>
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<tr>
<td>HR2.1:TN-XL x rd1</td>
<td>0.81 (0.80)</td>
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<tr>
<td>wt</td>
<td>1.4 [P21] (60)</td>
</tr>
<tr>
<td>cpfl1</td>
<td>1.12 [P24] (13)</td>
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<tr>
<td>rd1</td>
<td>0.75 [62]</td>
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For calculation of cone density and % TUNEL positive cells, see Methods. To allow for a comparison with earlier literature, values shown in this table are expressed as mean, with median (where available) in parenthesis. In cases where P20/P30 values were not available for comparison, the actual postnatal age is given in square brackets. cpfl1 at P24+ median 1.13 ± 0.18 SD, 14 vertical sections from 5 mice.

1value given ("50% of wt") was estimated using data given in [61]. References in superscript brackets.
of cpfl1 degeneration, about half of the cones were already lost. In comparison, in the secondary cone degeneration model rd1, where cones are genetically intact, less than 1% of the recorded cones were responsive.

Next, we wanted to see if the light-evoked Ca\textsuperscript{2+} response recorded in cpfl1 cones differed from those in wt cones. Here, we focused on response kinetics, as responses appeared “slower” in cpfl1 compared to wt (cf. Fig. 4B). First, we determined the “response lag” \( \Delta t_{\text{lag}} \) defined as the time period between the time when 20% of the response amplitude was reached \( (t_{20\%}) \) and the time when the Ca\textsuperscript{2+} level started to recover for a 1-s stimulus \( (t_{\text{decay}}) \). We found that \( \Delta t_{\text{lag}} \) was significantly longer in cpfl1 than in wt cones at both ages (Fig. 5B). Moreover, \( \Delta t_{\text{lag}} \) in cpfl1 cone responses increased significantly between P18+ and P30+. Taken together, our data suggest that even at P30+, a substantial fraction of cpfl1 cones generate large, although sluggish light-evoked responses.

**Cpfl1 cones are noisier than wt and rd1 cones**

We noticed that Ca\textsuperscript{2+} traces recorded in cpfl1 cones appeared noisier compared to those in wt cones (Fig. 6A and B). This noise may reflect higher (spontaneous) activity caused by fluctuations in cGMP levels, possibly related to the cGMP accumulation we detected in cpfl1 cones (50) (cf. Fig. 2A). To include rd1 cones in this comparison, we analysed light-responsive and non-responsive cones separately (Fig. 6C and D). At P18+, noise levels were not significantly different between responsive cpfl1 and wt cones, whereas non-responsive cpfl1 cones were noisier than their wt counterparts. At P30+, both responsive and non-responsive wt cones became noisier, but much less so than cpfl1. The dramatic increase in Ca\textsuperscript{2+} signal noise recorded in cpfl1 cones may indicate that cGMP fluctuations progressively increased in responsive cones. In contrast, rd1 cones showed significantly lower noise than wt in both time windows. The small increase in rd1 cone noise at P30+ may relate to the remodelling of the outer retina connectivity (5). In fact, this notion is supported by a study showing that the oscillatory activity that arises in the outer rd1 retina (P30 and later) at least partially depends on voltage-gated Ca\textsuperscript{2+} channel activity in cone terminals (45).

**Discussion**

In many inherited retinal diseases, mutations that affect the photoreceptor OS lead to cell death, and the pathways associated with this are often linked to aberrant Ca\textsuperscript{2+} signalling. Here, we monitored Ca\textsuperscript{2+} dynamics directly during both primary and secondary cone degeneration, to show that Ca\textsuperscript{2+} signalling was indeed altered. Remarkably, although cpfl1 cones suffer from primary Pde6c mutations, a subpopulation was found to be functional, while genetically intact rd1 cones were entirely dysfunctional. Our results raise new questions as to the possible involvement of Ca\textsuperscript{2+} noise and random fluctuations, which could explain the stochastic nature of cone cell death (51–53). In addition, our study highlights the importance of using in vivo single-cell imaging techniques to improve our understanding of the intracellular processes leading to retinal degeneration.

**Cone Ca\textsuperscript{2+} dynamics are altered in primary cone degeneration**

Cpfl1 cones carry Pde6c mutations (1), exhibit cGMP accumulation, and are expected to show altered Ca\textsuperscript{2+} homeostasis (3,16). In line with previous studies, we found that the percentage of cones showing cGMP accumulation remained constant between P20 and P30. High cGMP is predicted to cause changes in Ca\textsuperscript{2+} homeostasis, as evidenced indirectly by the increased activity of Ca\textsuperscript{2+}-dependent calpain-type proteases (3,15). Our study confirms this prediction: Compared to wt, we found that cpfl1 cones showed higher relative resting Ca\textsuperscript{2+} levels. In line with this, we found in our calibration experiments that cpfl1 cones tended to have elevated absolute Ca\textsuperscript{2+} concentrations. In addition, cpfl1 cones had larger light responses, their resting Ca\textsuperscript{2+} was much more heterogeneous, and they displayed increased Ca\textsuperscript{2+} noise levels. Overall, our results on primary cpfl1 cone degeneration support the high Ca\textsuperscript{2+} hypothesis.

These results are interesting in the context of previous studies aimed at developing treatments for hereditary retinal degeneration. The use of inhibitors of the synaptic L-type VGCCs induces a delay in rd1 photoreceptor degeneration (21), which implied that photoreceptor Ca\textsuperscript{2+} overload (16) would occur primarily via the synaptic axonal terminal. We show that Ca\textsuperscript{2+} levels in the axon terminals of PDE6 mutant cones may indeed be increased; however, these changes are tightly linked to primary alterations of Ca\textsuperscript{2+} in the cone OS, as evidenced by our light stimulation experiments. Thus, when therapeutic strategies aimed at blocking either Ca\textsuperscript{2+}-permeable channels in the synapse (i.e. VGCCs) or in the OS (i.e. CNG-gated channels) (38,54) are compared, the latter appear to be a more suitable target.
Cone Ca\(^{2+}\) dynamics in secondary cone degeneration

In contrast to cpfl1 cones, rd1 cones are genetically intact and did not show cGMP accumulation. Yet, major cone loss was evident already at P20, progressing rapidly at P30. In rd1, cone OS and overall morphology changed dramatically in the absence of supporting rods as the photoreceptor layer thinned out. In addition, virtually no rd1 cone responded to light, in line with previous ERG studies (23,38,54–56).

In this scenario, a modulation of cone terminal Ca\(^{2+}\) via the OS seems questionable. The resting Ca\(^{2+}\) distribution in the rd1 cone population was significantly different at both P18\(^-\) and P30\(^-\) compared to wt, and rd1 cone noise levels were significantly lower at both time points. When Ca\(^{2+}\) was washed out of rd1 cones using ionomycin, the resting Ca\(^{2+}\) levels hardly changed at all. These results altogether point at a very low resting Ca\(^{2+}\) in cones upon loss of rods and structural disruption of the outer retina, indicating that rd1 secondary cone cell death may be caused by exceedingly low Ca\(^{2+}\) levels (57); discussed in (11,27). This conclusion would have important implications for therapeutic developments, which often aim at lowering intracellular Ca\(^{2+}\). While such an approach may be viable for primary cone degeneration (see above) it could be detrimental in secondary cone degeneration. To better understand Ca\(^{2+}\) dynamics in secondary cone degeneration, it may be necessary to study further animal models that exhibit a slower cone loss, not overlapping with postnatal retinal development, such as the Pde6b-mutant rd10 mouse (4,15).

Cpfl1 cones show light evoked responses

To our surprise, a substantial number of cpfl1 cones responded to light stimulation. These Ca\(^{2+}\) responses were larger and slower compared with wt cones, possibly due to low remnant PDE activity. Interestingly, cones treated with the PDE6 inhibitor zaprinast exhibited similar changes in response time course (28). Zaprinast application also caused a (reversible) increase in resting Ca\(^{2+}\), consistent with our finding of higher absolute Ca\(^{2+}\) in cpfl1 cones. The cpfl1 allele carries two different mutations, one 116bp intronic insertion between exons 4 and 5 resulting in a splice defect, and one 1bp deletion resulting in a frameshift in exon 7 (1). These two mutations together are expected to abolish PDE6C function completely. Nevertheless, a small proportion of correctly spliced Pde6c transcript carrying only the 1bp deletion appears to be present in cpfl1 cones (1), suggesting that they express lower amounts of PDE6C protein. Due to the severity of the genetic damage, it is unlikely, however, that this protein is functional. On the other hand, cpfl1 mice do show weak responses in photopic ERG recordings (2).

Here, a possible explanation is that cpfl1 cones recruit low levels of rod Pde6a, which would be expected to slow-down cone response kinetics (58), similar to what we observed. Irrespective of the underlying mechanism, our results show that cpfl1 mice do have a residual cone function at least until one month postnatal.

Degenerating cones show altered noise levels

In ut cones, Ca\(^{2+}\) noise levels became significantly higher from P18\(^-\) to P30\(^-\), suggesting changes in PDE/cGMP/Ca\(^{2+}\) signalling with time, as the development and maturation of cones and retinal circuitry progresses (48). Previous studies have found that cone noise depends on variations in CNG channel activity and fluctuations in cGMP levels in the OS (50,59). Moreover, cone noise persists in the downstream circuitry (60).

Our data show altered Ca\(^{2+}\) noise levels in both primary and secondary cone degeneration. At P30\(^-\), in both responsive and non-responsive cpfl1 cones, Ca\(^{2+}\) noise was significantly higher than in their wt counterparts, possibly due to the higher overall Ca\(^{2+}\) levels in cpfl1 cones. It is tempting to speculate that such variations in Ca\(^{2+}\) noise (or Ca\(^{2+}\) fluctuations above or below a critical threshold) over time could contribute to the stochastic nature of photoreceptor cell death. Although at the tissue level, photoreceptor degeneration appears as a well-timed process, the time point when cell death is initiated at the level of the individual photoreceptor is random; it is inherently impossible to predict when a specific photoreceptor is going to die (51–53).

The precise processes that may be triggered by Ca\(^{2+}\) fluctuations remain unknown, but could potentially be linked to activation of Ca\(^{2+}\)-dependent kinases (61,62) with bearings on...
gene expression (63) and/or Ca\(^{2+}\)-dependent calpain-type proteases (3,19). On the other hand, a recent in vivo Ca\(^{2+}\) imaging study followed degenerating cones over a time-course of several hours in intact zebrafish larvae carrying the cpfl1-like Pde6cw59 mutation and reported suppressed Ca\(^{2+}\) fluctuations when compared to ut (26). At present, it is unclear where these differences between mutant cones in zebrafish and mouse arise, but they could be related to developmental stage, experimental conditions, and perhaps species differences. Ca\(^{2+}\) fluctuations may have either beneficial or detrimental consequences. In rd1 cones, on the other hand, Ca\(^{2+}\) noise was significantly lower than in ut, most likely because of the lower Ca\(^{2+}\) baseline levels. An exciting topic for future studies will be to understand if and how increased Ca\(^{2+}\) noise or overall changes in Ca\(^{2+}\) signalling contribute to these degenerative events.

Concluding remarks and future aspects

Several investigations were based on the assumption that altered Ca\(^{2+}\) signalling is involved in or serves as a trigger for rod and cone degeneration (e.g. (21,54,64)). Our results indeed show alterations in the dynamics of Ca\(^{2+}\) signalling in both primary and secondary cone degeneration. For primary cone degeneration, our data is in line with the "high Ca\(^{2+}\) hypothesis", suggesting that cell death may be triggered by Ca\(^{2+}\) overload, possibly via higher random fluctuations in Ca\(^{2+}\) levels. This may have a bearing on the stochastics of cone degeneration (as discussed above) but still leaves open the question whether changes in Ca\(^{2+}\) and/or Ca\(^{2+}\)-dependent enzymes are cause or consequence of primary cone degeneration. Secondary cone degeneration, on the other hand, is very likely associated with low Ca\(^{2+}\) levels and is, thus, more consistent with the "low Ca\(^{2+}\) hypothesis". This may also be true for primary photoreceptor degeneration triggered by mutations that cause low Ca\(^{2+}\) (e.g. CNG channel mutations). Taken together, these findings may have important ramifications for therapeutic approaches focusing on blocking Ca\(^{2+}\) influx in hereditary retinal degeneration. Here, our study may help to reconcile both the high and low Ca\(^{2+}\) hypotheses (16,27) and contribute to resolving a long-lasting controversy on the use of Ca\(^{2+}\) channel antagonists for the treatment of inherited retinal degeneration (21–23).

To gain insight into the precise timing and the role of Ca\(^{2+}\) signalling in the execution of cell death, Ca\(^{2+}\) imaging experiments will need to be combined with cellular activity assays for Ca\(^{2+}\) targets such as kinases, proteases, or histone deacetylases (65,66). On a different note, our study also showed that even genetically impaired cones may show considerable functional activity, highlighting the importance of Ca\(^{2+}\) measurements at single-cell resolution to obtain direct readouts on the functionality and health of cones.

Supplementary Material

Supplementary Material is available at HMG online.

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