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GUCY2D Cone–Rod Dystrophy-6 Is a “Phototransduction Disease” Triggered by Abnormal Calcium Feedback on Retinal Membrane Guanylyl Cyclase 1

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The Arg838Ser mutation in retinal membrane guanylyl cyclase 1 (RetGC1) has been linked to autosomal dominant cone–rod dystrophy type 6 (CORD6). It is believed that photoreceptor degeneration is caused by the altered sensitivity of RetGC1 to calcium regulation via guanylyl cyclase activating proteins (GCAPs). To determine the mechanism by which this mutation leads to degeneration, we investigated the structure and function of rod photoreceptors in two transgenic mouse lines, 362 and 379, expressing R838S RetGC1. In both lines, rod outer segments became shorter than in their nontransgenic siblings by 3–4 weeks of age, before the eventual photoreceptor degeneration. Despite the shortening of their outer segments, the dark current of transgenic rods was 1.5–2.2-fold higher than in nontransgenic controls. Similarly, the dim flash response amplitude in R838S rods was larger, time to peak was delayed, and flash sensitivity was increased, all suggesting elevated dark-adapted free cGMP in transgenic rods. In rods expressing R838S RetGC1, dark-current noise increased and the exchange current, detected after a saturating flash, became more pronounced. These results suggest disrupted Ca2+ phototransduction feedback and abnormally high free-Ca2+ concentration in the outer segments. Notably, photoreceptor degeneration, which typically occurred after 3 months of age in R838S RetGC1 transgenic mice in GCAP12+/− or GCAP12+/- backgrounds, was prevented in GCAP12+/− mice lacking Ca2+ feedback to guanylyl cyclase. In summary, the dysregulation of guanylyl cyclase in RetGC1-linked CORD6 is a “phototransduction disease,” which means it is associated with increased free-cGMP and Ca2+ levels in photoreceptors.

Key words: calcium; cGMP; guanylyl cyclase; photoreceptors; phototransduction; retinal degeneration

Significance Statement

In a mouse model expressing human membrane guanylyl cyclase 1 (RetGC1, GUCY2D), a mutation associated with early progressing congenital blindness, cone–rod dystrophy type 6 (CORD6), deregulates calcium-sensitive feedback of phototransduction to the cyclase mediated by guanylyl cyclase activating proteins (GCAPs), which are calcium-sensor proteins. The abnormal calcium sensitivity of the cyclase increases cGMP-gated dark current in the rod outer segments, reshapes rod photoreponses, and triggers photoreceptor death. This work is the first to demonstrate a direct physiological effect of GUCY2D CORD6–linked mutation on photoreceptor physiology in vivo. It also identifies the abnormal regulation of the cyclase by calcium-sensor proteins as the main trigger for the photoreceptor death.

Introduction

Retinal membrane guanylyl cyclase (RetGC1), one of the essential enzymes in photoreceptor signaling, enables photosensitivity of rods and cones by opening cGMP-gated channels in the outer segment plasma membrane. The partial depolarization of photoreceptors caused by the inward current of Na+ and Ca2+ through cGMP-gated channels in the dark becomes reversed when light activates the hydrolysis of cGMP by phosphodiesterase 6 (PDE6) and suppresses the influx of Na+ and Ca2+ (Tau and Hardie, 2009;
Arshavsky and Burns, 2012; Koch and Dell’Oro, 2015). While Ca\(^{2+}\) influx is suppressed upon light stimulation of rods, its extrusion via Na\(^{+}/Ca\(^{2+}\), K\(^{+}\) exchanger 1 (NCX1; Reiländer et al., 1992) still carries on to produce a decrease in rod outer segment Ca\(^{2+}\) concentration. This upregulates the negative Ca\(^{2+}\) feedback on RetGC (Koch and Styer, 1988; Pugh et al., 1991; Burns et al., 2002), which is mediated by guanylyl cyclase-activating proteins (GCAPs), which are Ca\(^{2+}/Mg\(^{2+}\) sensors (Palczewski et al., 1994; Dizhoor et al., 1994, 1995, 2010; Imanishi et al., 2004; Makino et al., 2012). As a result of the light-induced decrease in Ca\(^{2+}\), GCAPs convert to a Mg\(^{2+}\)-liganded state that stimulates RetGC activity. Upon the return of photoreceptors to darkness, PDE6 is inactivated, the influx of Ca\(^{2+}\) through the cGMP-gated channels resumes, and Ca\(^{2+}\) concentration returns to its dark-adapted level. Subsequently, GCAPs convert back to Ca\(^{2+}\)-liganded state and decelerate production of cGMP.

Two isozymes of RetGC are present in photoreceptors RetGC1, the predominant isozyme in rods and cones, and RetGC2, an ancillary isozyme in rods (Dizhoor et al., 1994; Lowe et al., 1995; Yang et al., 1999; Peshenko et al., 2011; Xu et al., 2013). Multiple mutations in the human GUCY2D gene, coding for the RetGC1 isozyme, have been linked to different types of congenital blindness. Some mutations cause Leber’s congenital amaurosis (LCA), which is typically a nondegenerative disease causing severe recessive blindness resulting from loss of RetGC1 activity and or activation that renders rods and cones nonfunctional from birth (Perrault et al., 2000; Stone, 2007; Jacobson et al., 2013). Unlike GUCY2D LCA, autosomal-dominant GUCY2D cone–rod dystrophy 6 (CORD6) is an early-onset progressing degeneration of photoreceptive cones and rods, frequently linked to substitutions Cys, Ser, Pro, His, or Gly replacing Arg838 in the dimerization domain of RetGC1 (Ramamurthy et al., 2001; Ito et al., 2004; Udar et al., 2003; Hunt et al., 2010; Garcia-Hoyos et al., 2011), a part of the GCAP-regulated interface on the enzyme (Peshenko et al., 2015a,b). A number of previous in vitro studies using recombina-
Figure 2. Expected increase in guanylyl cyclase activity in R838S^+ rods due to the change in its Ca^{2+} sensitivity. A, Guanylyl cyclase activity, mean ± SD, in retinal homogenates from dark-adapted wild-type (closed triangles, △) and R838S^+ littermates (open circles, ○) was assayed as described in Materials and Methods. The activities were normalized as the percentage of maximal catalytic activity of the cyclase in each preparation and fitted with the Hill function, \( A = (A_{\text{max}} - A_{\text{min}})/(1 + ([Ca]/K_{Ca})^n) + A_{\text{min}} \), where \( A_{\text{max}} \) and \( A_{\text{min}} \) are the respective maximal and minimal activity, \( [Ca] \) is free Ca^{2+} concentration, \( K_{Ca} \) is free Ca^{2+} concentration causing 50% inhibition of the cyclase activity, and \( n \) is the Hill coefficient. The arrows indicate the approximate range of free-Ca^{2+} change in mouse rods between light-adapted and dark-adapted state (Woodruff et al., 2002; Osheva et al., 2004). B, Altered Ca^{2+} feedback on the cyclase predicted to increase cGMP production and Ca^{2+} influx in the dark does not upregulate NOS1 and NOS2 expression in R838S^+ retinas. Protein fractions extracted from R838S^+ and wild-type littermates at 3 weeks of age were equalized by total protein concentration and 10, 20, and 30 μL of the samples were separated using 7% SDS-PAGE. Western immunoblot was probed with anti-NOS1 (top), anti-NOS2 (middle), and β-actin (bottom) antibodies. Chemiluminescence for NOS1 was proportional to the sample load (right) and was quantified from the immunoblot on R838S^+ retinas as 1.1 ± 0.2 (SD), N = 3, of the wild-type level. Signal-to-background ratio on immunoblots with NOS1 did not allow for reliable quantification, but the observed band intensities gave no indication of upregulation.

SuperSignal chemiluminescence substrate (Pierce/Thermo Fisher Scientific). Chemiluminescence image was acquired using a Fotodyne Luminous FX instrument.

Retinal morphology. Mice were perfused through the heart as above with PBS and then with 2.5% glutaraldehyde in PBS. The eyes were surgically removed and fixed overnight in 2.5% glutaraldehyde/2.5% formaldehyde at 4°C. The fixed eyes were washed three times for 15 min each in PBS, soaked in PBS overnight, processed for paraffin embedding, sectioned, and stained with hematoxylin/eosin (AML Laboratory). The outer segment length was measured from a confocal differential interference contrast image. The retina morphology was analyzed using images taken by an Olympus Magnafire camera mounted on an Olympus BX21 microscope. The photoreceptor nuclei in the outer nuclear layer of the retina were counted from a 425 μm fragment of the retina, midway between the optic nerve and the periphery of the retina, and the densities of the nuclei per 100 μm distance were averaged for several frames for each retina.

Guanylyl cyclase assay. Mice were dark-adapted overnight and their retinas were dissected and assayed under infrared illumination as previously described (Peshenko et al., 2011, 2016). Briefly, the assay contained in 25 μL of 30 mm 3-(N-morpholino)propanesulfonic acid (MOPS)-KOH, pH 7.2, 60 mm KCl, 4 mm NaCl, 1 mm DTT, 0.5 mm ATP, 4 mm cGMP, 1 mm GTP, 10 μm creatine phosphate, 0.5 μM of creatine phosphokinase (Sigma-Aldrich), 1 μM of [α-32P]-GTP, 0.1 μM of [β,γ-32P]-GTP (PerkinElmer), phosphodiesterase inhibitors zantrapant and dipyradionc, and 2 mm Ca^{2+}/EGTA buffer, with MgCl2 added to maintain variable [Ca^{2+}]free at 0.9 mm free Mg^{2+} (Peshenko and Dizhoor, 2006). Where indicated, myristoylated recombinant mouse GCAP1 expressed in Escherichia coli and purified as previously described (Peshenko and Dizhoor, 2006; Peshenko et al., 2011) was added to the assay.

Electrophysiology. Single-cell suction recordings were done as previously described (Wang et al., 2014). Mice were dark-adapted overnight and killed by 5 min CO2 inhalation. Then their eyes were enucleated under dim red light using forceps. Retinas were then isotoped into Locke’s solution (112.5 mm NaCl, 3.6 mm KCl, 2.4 mm MgCl2, 1.2 mm CaCl2, 10 mm HEPES, 20 mm NaHCO3, 3 mm sodium succinate, 0.5 mm sodium glutamate, 0.02 mM EDTA, 10 mM glucose, 0.1% minimum essential media vitamins, and 0.2% minimum essential media amino acids, pH 7.4) using microsyringes and fine forceps under a stereomicroscope fit with infrared image converters. A retina was chopped by a razor blade into small pieces and transferred into the recording chamber perfused with 33-37°C Locke’s solution equilibrated with 95%O2/5%CO2. A single rod outer segment was then drawn into a microglass pipette under microscope (IX51, Olympus) with infrared illumination and camera. The pipette was filled with electrode solution (140 mm NaCl, 3.6 mm KCl, 2.4 mm MgCl2, 1.2 mm CaCl2, 3 mm HEPES, 0.02 mM EDTA, 10 mm glucose, pH 7.4 with NaOH) and controlled by micromanipulator (MP-225, Sutter Instrument). Test flashes of 500 or 505 nm were generated in a calibrated LED light stimulus system and delivered to the recording chamber through a custom-made optical system. Flash intensity and duration were controlled by an LED driver (LDC210, Thorlabs) con-
Krohn-Hite), digitized at 1 kHz (Digitida 1322A, Molecular Devices), stored and analyzed on a computer using pClamp9.

Experimental design and statistical analysis. All animal experiments indiscriminately used male and female mice. Since the magnitude of the expected effects, by the nature of the experiments, was not feasible to predict a priori, the size of the animal groups was not explicitly planned in advance and the statistical analysis was applied to the data collected upon the completion of the experiments. Unless noted otherwise, two-tailed unpaired Student’s t test (Origin8, OriginLab) was used to test for the significance of differences in the mean values of two sample groups. P values of < 0.05 were considered to be statistically significant. Where indicated, one-way ANOVA/Bonferroni test at α = 0.01 (KaleidaGraph, Synergy Software) was applied for comparison of photoreceptor loss between several animal groups.

Results

The R383S RetGC1 expression dysregulates cyclical modulation by Ca2+

Two mouse lines expressing the R383S RetGC1 under control of rod opsin promoter, Lines 362 and 379, have been shown to undergo rapid early-onset loss of rod electroretinography (ERG) concomitant with the degeneration of rods. This degeneration is more severe in Line 362 (Dizhvor et al., 2016). To determine whether the R383S RetGC1 transgene in the fast-degenerating line 362 is expressed in rod photoreceptor outer segments, the line was outcrossed to RetGC1−/− background, which eliminates interference from the endogenous mouse RetGC1 ortholog (Yang et al., 1999). Immunolabeling of retinal sections demonstrated the robust and uniform expression of RetGC1 in rod outer segments of R383S RetGC1−/− mice (Fig. 1). Thus, R383S RetGC1 was specifically expressed in Line 362 transgenic rods. This result, together with the previous finding that the mutant cyclical is expressed in the rods of Line 379 (Dizhvor et al., 2016), enabled investigation of the function of individual rods expressing R383S RetGC1 in the two transgenic lines. The experiments described further were conducted using the original R383S mouse lines, propagated in RetGC1−/− background.

The guanylyl cyclical activity measured in the retina using our standard assay (Peshenko et al., 2011) directly assesses Ca2+ sensitivity of the photoreceptor-specific RetGC activity (Olshevskaya et al., 2004, 2012; Woodruff et al., 2007; Makino et al., 2008, 2012). Despite the fast degeneration of R383S rods in Line 362 (Dizhvor et al., 2016), their cyclical activity was still measurable at 3 weeks of age, allowing for a comparison of RetGC Ca2+ sensitivity between transgene-

Figure 3. Shortening of the outer segment in R383S rods occurs at early ages in Lines 362 (A) and 379 (B). Top, Differential interference contrast images of sections from wild type and R383S littermates perfused and fixed with glutaraldehyde/paraformaldehyde solution at 3.5 and 4.5 weeks, respectively. Bottom, Length distribution for the outer segments of wild type and identifiable R383S rods. The horizontal bars represent the mean average for each group: RPE, Retinal pigment epithelium; ROS, rod outer segments; RIS, rod inner segments; ONL, outer nuclear layer; ***, p < 0.0001, t-test.
positive and transgene-negative littermates (Fig. 2A). The CORD6-linked R8385 mutation in RetGCI has a dual effect on the cyclase regulatory properties in the retina. It increases EC50 values for the inhibitory effect of Ca2+ (from 76 nM in wild-type littermates to 123 nM in the transgene-positive mice of Line 362) and causes nearly twofold reduction of the Hill coefficient (from 1.73 to 0.99). As a result, the cyclase activity remained markedly elevated at the free-Ca2+ concentrations typical for the normal dark-adapted mouse photoreceptors (Olshevskaya et al., 2004; Woodruff et al., 2007; Fig. 2A).

We hence reasoned that if the altered Ca2+ sensitivity of the cyclase measured in retinal homogenate in vitro translates into a corresponding increase of free cGMP in living R8385+ rods, then one would expect a larger fraction of the channels to remain open in dark-adapted R8385+ rods, producing a larger inward dark current. To adequately interpret any potential changes in the dark current, we also verified that dysregulation of the cyclase does not result in a compensatory upregulation of the NCGI channel in the transgenic retinas (Fig. 2B).

Figure 5. Increased dark current in Line 379 R8385+ rods. A–D, Representative response families to flash stimuli recorded by suction electrode from a wild-type (A) and Line 379 R8385+ rods (B). Data were obtained at 4–4.5 weeks of age. Flash strength ranged from 1 to 3200 photons·μm−2 with 0.5 log unit steps and flash duration was 2 or 20 ms. Bold traces represent the responses of the two rods to a flash of 32 photons·μm−2. C, D, Intensity–response curves of wild type filled circles and Line 379 R8385+ (open diamonds). ○: rods shown in absolute (C) or normalized (D) values. Data are shown as mean ± SEM, N = 11 (wild type) and 24 (R8385+). In D, the slope parameter from the Naka–Rushon equation (fitting curves not shown) was not significantly different between wild type (1.20 ± 0.07) and R8385+ (1.23 ± 0.04); P value for two-tailed unpaired test was 0.74.

The presence of a CORD6 RetGCI in rods elevates their dark current and delays their response kinetics

The outer segment of degenerating R8385+ rods were still present at a young age for both Lines 362 and 379, despite showing nearly twofold and 1.5-fold reduction in length at 3.5 weeks of age (Fig. 3A) and 4.5 weeks of age (Fig. 3B) respectively, which preceded their pronounced degeneration at later stages (Dizhoor et al., 2016; see Fig. 9). This made possible the functional analysis of R8385+ rods before their degeneration by recordings with a suction pipette electrode (Figs. 4–8; Tables 1, 2). Notably, despite the reduction of their outer segment length, R8385+ rods from both mouse lines exhibited significantly elevated dark current compared with their respective nontransgenic littermates. The increase in dark current was twofold in Line 362 (Fig. 4; Table 1) and 1.5-fold in Line 379 (Fig. 5; Table 2) at 4 weeks of age. Taking into account the reduced length of the outer segment (Fig. 3), such increase in the dark current indicates an even more drastic increase in the cGMP-gated current density in R8385+ rods. This result is consistent with the inefficient suppression of the RetGCI activity at dark-adapted Ca2+ levels in R8385+ rods (Fig. 2). The larger fraction of open cGMP-gated channels in the dark and the slower activation

Figure 6. Delayed onset of dim flash response recovery in R8385+ rods. A–D, Averaged normalized (A, C) and fractional (B, D) dim flash responses from wild-type (black traces) and R8385+ (red traces) rods from Line 362 (A, B) and Line 379 (C, D) mice. Fractional responses (photons·s−1·μm−2) were obtained by dividing dim flash responses by dark current (pA) and flash intensity (photons·μm−2). Flash strength in A and B were 10 photons·μm−2 for wild type and 3 or 10 photons·μm−2 for R8385+. Flash strength in C and D were 3 or 10 photons·μm−2 for wild type and 1, 3, or 10 photons·μm−2 for R8385+. All with a flash duration of 2 or 20 ms. The specific flash intensity was selected depending on the sensitivity of each rod. Data are shown as mean ± SEM, N = 6 (wild type in Line 362 panel), 17 (R8385+ Line 362), 11 (wild type in Line 379 panel), and 24 (R8385+ Line 379).
of RetGC due to reduced cooperativity for Ca$^{2+}$ (Fig. 2A) can explain the increased flash sensitivity of mutant rods, such that a larger response amplitude was produced by a given flash in Lines 362 and 379 in comparison with their respective wild-type littermates (Fig. 4C; Table 1; Fig. 5C; Table 2, respectively). The half-saturation flash strength, $I_{1/2}$, which is the same, and were shipped together with their respective R838S* siblings. As a result, the differences between wild-type and R838S* transgenic rods in each case would be independent of other factors and reflect the change in rod function caused by the expression of R838S RetGC.

The shape of dim flash responses in R838S* rods also changed in a manner consistent with elevated free-cGMP content: the amplitude of the flash response rose and reached its peak later in both Line 362 (Fig. 6A,B) and Line 379 (Fig. 6C,D). However, the rod dim flash recovery time constant, dominated by the inactivation of transducin (Kispe et al., 2006), was not significantly different between transgenic and wild-type littermate controls (Fig. 6 Tables 1, 2), indicating normal transducin/PDE6 complex inactivation. Similarly, the rising phase of the fractional response was unaffected by the expression of R838S RetGC in both Line 362 (Fig. 6B) and Line 379 (Fig. 6D). Thus, the activation of the phototransduction cascade remained unaltered in the mutant rods.

The current carried through the NCKX1 exchanger can be observed following the rapid closing of all cGMP-gated channels by a saturating flash (Yau and Nakatani, 1984). Notably, this exchange current was more pronounced in R838S* rods than in wild-type littermates (Fig. 7A). Direct comparison of the exchange currents, measured from the point of saturation of the cGMP-gated channels revealed that it is ~3-fold larger in R838S* rods compared with rods of wild-type controls (Fig. 7B). We found no indications of the NCKX1 exchange being upregulated in R838S* rods by Western immunoblotting (Fig. 2B). Therefore, the more pronounced exchange current in R838S* rods is consistent with their larger dark current and argues that the free-Ca$^{2+}$ concentration in their outer segments becomes elevated.

Figure 7. Increased NCKX1 exchange current in R838S* rods. A, Averaged saturating flash responses of wild-type (black) and R838S* (red) rods from Line 362. Each trace was set to 0 pA at 0.08 s after the 3200 photons μm$^{-2}$ flash stimulus when the cGMP-gated current becomes fully blocked. B, Magnified view of the peaks of the responses from A. Data are shown as mean ± SEM, N = 10 (wild type) and 16 (R838S*).

The presence of a CORD6 RetGC1 in rods increases their dark noise

During the recordings from R838S* rods, we noticed that the baseline of the recordings in darkness had frequent "bumps." Such increase in dark noise was observed in rods from both Lines 362 and 379 but not in control wild-type rods (Fig. 8A,B). This increase is reminiscent of the increased dark noise in GCAP−/− mouse rods (Burns et al., 2002). In that case, the lack of proper phototransduction feedback and slow Ca$^{2+}$-mediated inactivation renders the discrete events caused by spontaneous activation of visual pigment 4–5 times larger than normal, making them readily observable in the dark. To test whether the increased dark noise in R838S* rods was caused by abnormal Ca$^{2+}$-mediated feedback on rod phototransduction, we first evaluated the instrumental noise in R838S* rods under saturating background light. In this condition, the cGMP-gated channels are closed so that phototransduction signals, including those from the spontaneous activation of the pigment, become undetectable. Notably, the discrete bumps were not observed in bright-light recordings (Fig. 8A,B, bottom traces), indicating that they were not derived from the instrumental noise, but rather were generated by rod phototransduction. We next analyzed the power spectrum of the dark noise (Fig. 8C,D). Its phototransduction component was extracted by subtracting the instrumental noise spectrum from the total dark-noise spectrum. The difference spectra thus obtained were well fitted with the scaled power spectrum of the averaged dim flash response for both Line 362 (Fig. 8E) and Line 379 (Fig. 8F), demonstrating that the origin of the discrete dark noise in R838S* rods is the spontaneous activation of visual pigments. Thus, similar to the case in GCAP−/− rods (Burns et al., 2002), the abnormal Ca$^{2+}$-mediated feedback on the cyclase resulted in increased dark noise in both lines expressing the mutant R838S RetGC.

R838S* rod degeneration is triggered by dysregulation of the Ca$^{2+}$ feedback via GCAPs

Mouse photoreceptors express only two homologs of GCAPs—GCAP1 and GCAP2 (Howes et al., 1998). To test whether the abnormal regulation of the RetGC1 harboring CORD6 mutation was the main event triggering the downstream processes eventu-
ally leading to the photoreceptor death, we compared the rate at which R838S⁺ rods degenerated in mice having normal (GCAP1,2⁺/⁺) or partially reduced (GCAP1,2⁺/-) content of GCAPs versus mice completely lacking both GCAPs (GCAP1,2⁻/-; Mendez et al., 2001). The respective genotypes were bred by repetitively crossing Line 362 to the GCAP1,2⁻/- double knock-out mice. In contrast to the normal (GCAP1,2⁺/⁺) and hemizygous (GCAP1,2⁺/-) backgrounds, a complete lack of GCAPs to medi-
To further verify that the CORD6 cyclase mutant expressed in the R838S+GAP1,1/2−/− rods still retained its propensity for abnormal regulation by GCAPs, we compared the Ca cooperate sensitivity of the cyclase in GCAP1,1/2−/− mice harboring normal complement of RetGCI and RetG2 (Mendez et al., 2001; Pesheko et al., 2011) and in R838S+GAP1,1/2−/− mice, by reconstituting the homogenates of their dark-adapted retinas with purified recombinant mouse GCAP1. The Ca cooperate sensitivity of the cyclase in R838S+GAP1,1/2−/− retinas in the presence of the added GCAP1 was shifted toward higher Ca cooperate concentrations in a manner similar to the original Line 362 (Fig. 9D). We also bred four R838S+GAP1,1/2−/− mice (two males and two females), subsequently verified as having normal (similar to the C57Bl/6 wild type) retinal histology at 4 months and confirmed that the retinas of their R838S+GAP1,1/2−/− progeny underwent rapid degeneration after 2 months of age (data not shown). Hence, only the lack of abnormal Ca cooperate feedback modulation via GCAPs prevented the CORD6 RetGCI mutant present in the R838S+GAP1,1/2−/− rods from triggering the photoreceptor death.

Discussion

Among the R838 substitutions in RetGCI linked to the CORD6 (Kellsell et al., 1998; Gregory-Evans et al., 2000; Weigell-Weber et al., 2000; Downes et al., 2001; Payne et al., 2001; Ramamurthy et al., 2001; Kitiratschky et al., 2008; Udar et al., 2003), the R838S mutation, usually present in combinations with other substitutions not directly related to the phenotype, causes one of the most severe forms of GUCY2D CORD6 (Ramamurthy et al., 2001). Expression of R838S RetGCI in mouse rods (Dizhoo et al., 2016), unlike overexpression of normal RetGCI (Boyce et al., 2013, 2015), results in a rapid decline of rod ERG responses due to progressive degeneration of rod photoreceptors. Notably, photopic ERG responses from cones, which by design of the model do not express the CORD6 cyclase mutant (Fig. 1), remain strong in R838S+ mice, even after the loss of rod ERG (Dizhoo et al., 2016). The clinical symptoms of CORD6 (Kellsell et al., 1998; Gregory-Evans et al., 2000; Hunt et al., 2010) include early onset and decline of vision in childhood due to progressive degeneration of functional photoreceptors. Consistently with that, individual R838S+ rods in the mouse model remain highly functional at the early stage preceding the massive retinal degeneration, albeit with markedly changed shape of photoresponses (Figs. 4–7).

Variability of the photoresponse between different R838S+ rods (Tables 1, 2) likely originates from a nonuniform cell-to-cell transgene expression, and the larger variability in Line 379 would...
be consistent with a larger variation in $R8385^+$ rod outer segment length for this line (Fig. 3). The variability of expression between different rods in these two lines could also explain why, after the initial rapid decline, both rod ERG and rod nuclei count in Line 379 reach a plateau after 3 months of age and then the remaining rods survive for much longer (Dizhoor et al., 2016), while in Line 362 $R8385^+$ all rods die out after 3 months of age (Fig. 9).

Photoreceptor degeneration in the CORD6 R8385 RetGC1 model resembles the phenotype caused by mutations in GCAP1, which associate with dominant cone and cone-rod degenerations that are clinically distinct from CORD6 (Olshesvaya et al., 2004; Woodruff et al., 2007). Mutations, such as Y99C or E155G, render GCAP1 less sensitive to Ca$^{2+}$ and hence shift Ca$^{2+}$ sensitivity of the cyclase in a manner similar to the R8385 RetGC1 mutation (Dizhoor et al., 1998; Sokal et al., 1998; Wilkie et al., 2001). Also, similarly to the R8385$^+$ mice, cGMP-gated channel activity and Ca$^{2+}$ influx in rods expressing mutant GCAP1 increase and the abnormal Ca$^{2+}$ feedback results in photoreceptor death (Olshesvaya et al., 2004; Woodruff et al., 2007). Nonetheless, the molecular events underlying the pathology are different between the GCAP1 and RetGC1 mutations. While the mutations in GCAP1 directly affect its Ca$^{2+}$ sensor function by reducing GCAP1 affinity for Ca$^{2+}$, the Ca$^{2+}$ sensor properties of GCAPs per se remain unaffected in CORD6. Instead, the R8385 mutation in RetGC1 biases its binding to Mg$^{2+}$-liganded versus Ca$^{2+}$-liganded GCAP, even in the presence of a larger Ca$^{2+}$ GCAP pool (Ramamurthy et al., 2001; Peshenko et al., 2004). As a result, the R8385 mutant RetGC1 remains in its activated state at higher than normal Ca$^{2+}$.

The longer time to peak and the increased amplitude of dim flash response (Fig. 6), as well as the more prominent dark noise in the $R8385^+$ rods (Fig. 8), all indicate that the dynamic regulation of the cyclase by Ca$^{2+}$ becomes altered, suppressing its activation in response to the reduction in free Ca$^{2+}$ after illumination. On the other hand, the increased exchange current in $R8385^+$ rods demonstrates more robust efflux of Ca$^{2+}$ in dark-adapted conditions, indicative of higher intracellular Ca$^{2+}$ concentrations (Iwamoto et al., 2000). However, the increased dark current also suggests that the elevation of Ca$^{2+}$ levels in these rods does not reach the levels sufficient for complete inhibition of the mutant cyclase in the dark. If the cyclase activity is not fully inhibited in the dark and merely stabilizes a higher RetGC/PDE6 activities equilibrium, then a less cooperative (Fig. 2A) response of the cyclase to the decrease of free-Ca$^{2+}$ concentrations could make its light-driven acceleration slower than normal.

Conforming with the hypothesis that the CORD6 mutation of cyclase leads to elevation of free cGMP and Ca$^{2+}$ in the outer segments (Tucker et al., 1999; Ramamurthy et al., 2001; Peshenko et al., 2004), the abnormalities in $R8385^+$ rod photoreponses can be attributed to the change in Ca$^{2+}$ sensitivity of the cyclase regulation. Indeed, the larger dark current carried by Na$^+$ and Ca$^{2+}$ through the cGMP-gated channels in $R8385^+$ rods indicates elevated free-cGMP concentrations in outer segments (Figs. 4, 5), consistent with the right shift in the Ca$^{2+}$ sensitivity of the mutant cyclase and the reduced cooperativity of its Ca$^{2+}$-dependent deceleration (Fig. 2A). The more prominent exchange current produced by Ca$^{2+}$ efflux through the NCKX1 (Fig. 7), under the conditions of the experiment limited by the NCKX1 affinity for Ca$^{2+}$ (Iwamoto et al., 2000), indicates that the free-Ca$^{2+}$ concentration is also increased. Hence, both elevated cGMP and/or Ca$^{2+}$ in the diseased photoreceptors, both being considered likely apoptotic agents (Olshesvaya et al., 2004; Xu et al., 2013), could trigger the photoreceptor death.

Our results directly demonstrate that the abnormal Ca$^{2+}$ feedback on the cyclase via GCAPs is the principal initiating event leading to CORD6. $R8385^+$ rods preserved in GCAP-deficient mice further support this evidence (Fig. 9). The dramatic rescue of degeneration in the absence of GCAPs also suggests that processes other than abnormal Ca$^{2+}$ feedback, such as, for example, potential unfolded protein response directly provoked by the mutant cyclase, are unlikely to create the apoptotic trigger in this case. However, the exact pathway(s) through which the apoptotic progression builds up remains to be established. The elevated free-cGMP content detectable by the increased $R8385^+$ rod photocurrent could conceivably lead to apoptosis via activation of cGMP-dependent protein kinase (PKG or cGK) in photoreceptors (Paquet-Durand et al., 2009; Xu et al., 2013). Both isoforms of the kinase (PKG1 and PKG2, or cGK1 and cGKII) are present in photoreceptors (Gamm et al., 2000; Feil et al., 2005). Deletion of a single isoyme, PKG1, does not affect the severity of rod degeneration in the rd1 mouse model, in which cGMP rises due to the lack of PDE6 activity (Wang et al., 2017). However, PKG2 could not be deleted in that study because rd1 and the Prk2g gene coding for PKG2 are located on the same chromosome. This leaves open a possibility that PKG2 can specifically mediate the apoptotic pathway or does so in a compensatory fashion in the absence of PKG1. Therefore, any future studies of the degeneration mechanism in $R8385^+$ rods will have to address the possible effects of PKG1 and PKG2 individual and double-gene knockouts to delineate or exclude a possible PKG-activated apoptotic pathway in CORD6.

Increase in Ca$^{2+}$ flux could provide another strong possibility for triggering apoptosis, similar to that in rd1, where deletion of the rod CNG1 channel slows the pace of degeneration (Wang et al., 2017). A larger cGMP-gated current in the outer segments would cause stronger depolarization of $R8385^+$ rods in the dark. Therefore, the increased Ca$^{2+}$ influx through cGMP-gated channels in the outer segment and/or the voltage-gated Ca$^{2+}$ channels in the synaptic terminals of the $R8385^+$ rods could overload the Ca$^{2+}$ scavenging capacity of the mitochondria (Giarmaco et al., 2017) and set the apoptosis in motion. One could also speculate that the increased Ca$^{2+}$ flux elevates tonic release of glutamate from the synapse in the dark and thus affects the synaptic transmission. However, the early onset of morphological changes and rod degeneration in $R8385^+$ mice reduce scotopic ERG b-waves even at a young age (Dizhoor et al., 2016), making this issue difficult to address. We reason that studies of the degenerative processes in $R8385^+$ rods should help us better understand general mechanisms of CORD6 pathology and, over the long term, could facilitate development of gene therapy approaches to treat the disease.

References


that determine Ca\(^{2+}\) sensitivity of photoreceptor guanylyl cyclase kinetic analysis of the interaction between the Ca\(^{2+}\)-bound and the Ca\(^{2+}\)-free guanylyl cyclase activating proteins (GCAs) and recombinant photoreceptor guanylyl cyclase 1 (RetGC-1). Biochemistry 43:13796–13804. CrossRef MedLine


