Endoplasmic reticulum (ER) Ca\(^{2+}\)-channel activity contributes to ER stress and cone death in cyclic nucleotide-gated channel deficiency

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Endoplasmic reticulum (ER) stress and mislocalization of improperly folded proteins have been shown to contribute to photoreceptor death in models of inherited retinal degenerative diseases. In particular, mice with cone cyclic nucleotide-gated (CNG) channel deficiency, a model for achromatopsia, display both early-onset ER stress and opsin mistrafficking. By 2 weeks of age, these mice show elevated signaling from all three arms of ER-stress pathway, and by 1 month, cone opsin is improperly folded proteins have been shown to contribute to death in a mouse model of CNG-channel deficiency. We examined whether preservation of luminal Ca\(^{2+}\) stores through pharmacological and genetic suppression of ER Ca\(^{2+}\) efflux protects cones by attenuating ER stress. We demonstrated that the inhibition of ER Ca\(^{2+}\)-efflux channels reduced all three arms of ER-stress signaling while improving opsin trafficking to cone outer segments and decreasing cone death by 20–35%. Cone-specific gene deletion of the inositol-1,4,5-trisphosphate receptor type I (IP\(_{\text{R}1}\)) also significantly increased cone density in the CNG-channel-deficient mice, suggesting that IP\(_{\text{R}1}\) signaling contributes to Ca\(^{2+}\) homeostasis and cone survival. Consistent with the important contribution of organellar Ca\(^{2+}\) signaling in this achromatopsia mouse model, significant differences in dynamic intraorganellar Ca\(^{2+}\) levels were detected in CNG-channel-deficient cones. These results thus identify a novel molecular link between Ca\(^{2+}\) homeostasis and cone degeneration, thereby revealing novel therapeutic targets to preserve cones in inherited retinal degenerative diseases.

Cone photoreceptor cyclic nucleotide-gated (CNG) channels are essential for cone phototransduction (1). In darkness or low light, channels are kept open by the binding of cGMP, maintaining steady Ca\(^{2+}\) and Na\(^{+}\) influx into cone outer segments (OS). Light-induced hydrolysis of cGMP by photoreceptor phosphodiesterase (PDE6) results in channel closure and hyperpolarization (1). Cone CNG channels are composed of two structurally related subunits, CNGA3 and CNGB3; the former is the ion-conducting subunit, whereas CNGB3 functions as a modulator. Mutations in these subunits are found in the majority of achromatopsia patients (2).

Studies of mice lacking the functional cone CNG channels, Cnga3\(^{-/-}\) and Cnbg3\(^{-/-}\), have documented early-onset cone degeneration and impaired cone function (3, 4). In addition, mice lacking NRL, a rod-specific neural-retina leucine-zipper transcriptional factor conferring a cone-dominant retina (5), have been cross-bred with CNG-channel-deficient mice, Cnga3\(^{-/-}\);Nrl\(^{-/-}\) and Cnbg3\(^{-/-}\);Nrl\(^{-/-}\), and likewise show early-onset cone degeneration and impaired cone function (6, 7). Cone-dominant Nrl\(^{-/-}\) mouse lines allowed us to examine the mechanisms of cone degeneration at cellular and biochemical levels.

Although permeable to Na\(^{+}\) and Ca\(^{2+}\), photoreceptor CNG channels possess increased Ca\(^{2+}\) permeability necessary for proper function; and cone CNG channels have been shown to have higher Ca\(^{2+}\) affinity than the channel in rods (8). Thus,

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2 The abbreviations used are: CNG, cyclic nucleotide-gated; OS, outer segment; IS, inner segment; PDE6, photoreceptor phosphodiesterase; NRL, neural-retina leucine-zipper transcriptional factor; ER, endoplasmic reticulum; RyR, ryanodine receptor; IP\(_{\text{R}1}\), inositol-1,4,5-trisphosphate receptor; PKG, protein kinase G; UPR, unfolded protein response; 2-APB, 2-aminoethoxyphenyl borate; ERG, electroretinogram; [Ca\(^{2+}\)]\(_{\text{im}}\), endoplasmic reticulum calcium; CHOP, CCAAT/enhancer-binding protein homologous protein; qRT-PCR, quantitative RT-PCR.
changes in CNG-channel activity are expected to cause alterations in [Ca^{2+}], (1) that, given the critical role of Ca^{2+} in photoreceptor physiology (9), may influence cone light response/adaptation and/or survival. Cones lacking functional CNG channels are likely to be especially sensitive to changes in Ca^{2+}-dependent intracellular signaling arising from [Ca^{2+}], reduction. In addition to CNG channels in the OS, photoreceptor [Ca^{2+}] is regulated by Ca^{2+}-sensitive Ca^{2+}-release channels on the endoplasmic reticulum (ER) within the inner-segment/perikaryon regions (10–12), which are implicated in regulating the induction of phototransduction and tonic release of the photoreceptor neurotransmitter glutamate (13, 14). Given that ER Ca^{2+} homeostasis is essential for protein processing, impaired ER Ca^{2+} homeostasis has been linked to ER stress and cell death in multiple cell types (15, 16). Thus, although it could be predicted that CNG-channel deficiency leads to impaired Ca^{2+} homeostasis and ER-stress-associated cone death, the role of Ca^{2+} stores in cone function and disease is currently unclear.

ER Ca^{2+}-release channels are composed of the ryanodine receptor (RyR) and IP_{3}R families (17). Although RyR appears to play less significant roles in mammalian cones (18, 19), IP_{3}R1 was recently shown to be phosphorylated following selective removal of CNG-channel subunits (6) and linked to changes in cGMP-dependent protein kinase G (PKG) activation (20). These findings implicate ER Ca^{2+} channels in ER-stress-related cone death in CNG-channel deficiency; however, the contribution of ER Ca^{2+} release to cone ER-stress activation, protein mislocalization, and degeneration remains poorly understood.

In the current study, we investigated whether ER Ca^{2+}-channel activity contributes to ER stress and cone death in CNG-channel deficiency. Apoptotic and ER-stress markers were significantly reduced after treatment with ER Ca^{2+}-channel inhibitors and following gene deletion of IP_{3}R1 in CNG-channel-deficient mice. Furthermore, cone-opsin localization to OS was significantly improved following treatment with IP_{3}R inhibitors. These results suggest that ER Ca^{2+}-channel activity plays a role in ER-stress activation and contributes to cone degeneration and opsin mislocalization in CNG-channel-deficient cones.

**Results**

**Elevated isoform-specific ER Ca^{2+}-channel expression in Cnga3^{-/-};Nrl^{-/-} mice and localization to photoreceptor inner segment**

Previously, we showed increased phosphorylation of IP_{3}R1 and involvement of the ER-stress pathways in Cnga3^{-/-};Nrl^{-/-} retinas (6, 20). In this study, we investigated whether cone photoreceptors expressed the isoform-specific ER Ca^{2+}-channel protein, IP_{3}R, and whether its expression level was altered in CNG-channel-deficient cones. The data from this study are presented in Fig. 1. Prominent IP_{3}R1 and IP_{3}R3 mRNA expression was detected in Cnga3^{-/-};Nrl^{-/-} retinas, with both isoforms showing significant increases in mRNA expression at postnatal day 15 (P15) and postnatal day 30 (P30), with an ~2–3-fold increase of both isoforms relative to age-matched Nrl^{-/-} controls (Fig. 1A). A comparison of transcript expression in Nrl^{-/-} and Cnga3^{-/-};Nrl^{-/-} mice revealed the IP_{3}R1 mRNA to be ~6–10-fold higher than the expression levels of IP_{3}R3 mRNA at P15, P30, and postnatal day 60 (P60), respectively. IP_{3}R2 mRNA was not detectable in these retinas (data not shown). Western blot analysis of total IP_{3}R1 protein levels only showed a significant increase at P15 with a 1.3-fold increase relative to age-matched Nrl^{-/-} controls (Fig. 1B).

As an ER-resident protein, IP_{3}R1 likely would be expressed in the ER/mitochondrion-rich inner segment (IS) (21, 22). To confirm IP_{3}R1 localization in photoreceptors, we performed immunofluorescence of the specific isoform co-labeled with the OS marker rhodopsin or with the IS marker Na^{+}/K^{+} ATPase α1 at P30 in wild-type (WT) mice. The data presented in Fig. 1C show an absence of IP_{3}R1 co-labeling with rhodopsin but positive co-labeling with Na^{+}/K^{+} ATPase α1, supporting IS localization of IP_{3}R1.

Next, we looked at the isoform-specific expression of the other major ER Ca^{2+}-efflux channel, the RyR; these data are presented in Fig. 2. RyR2 and RyR3 mRNA expression levels were significantly increased at P15 in Cnga3^{-/-};Nrl^{-/-} mice, with an ~2- and 1.8-fold increase, respectively, relative to age-matched Nrl^{-/-} controls (Fig. 2A). RyR1 mRNA expression was significantly elevated only at P60, with an ~2.3-fold increase. In addition, RyR3 mRNA expression levels were significantly increased at P30 and P60 in Cnga3^{-/-};Nrl^{-/-} mice compared with age-matched Nrl^{-/-} controls. However, when comparing isoform expression in Nrl^{-/-} and Cnga3^{-/-};Nrl^{-/-} mice, the RyR2 isoform was ~10–20-fold higher than the expression levels of the RyR1 or RyR3 isoform at P15, P30, and P60 in both genotypes, respectively. Western blot analysis of total RyR2 protein levels showed a significant increase at P15, P30, and P60 with a 1.3-, 1.4-, and 3.4-fold increase relative to age-matched Nrl^{-/-} controls, respectively (Fig. 2B). Similar to the IP_{3}R1 characterization, we performed localization studies of the RyR2 isoform in P30 WT mice. The data presented in Fig. 2C show an absence of RyR2 co-labeling with rhodopsin but positive co-labeling with Na^{+}/K^{+} ATPase α1, supporting IS localization of RyR2. These data identify the IP_{3}R1 and RyR2 calcium release channels as the likely major isoforms expressed during early-onset cone degeneration in CNG-channel deficiency.

**Increased IP_{3}R1 and RyR2 phosphorylation and decreased intracellular Ca^{2+} at early ages in Cnga3^{-/-};Nrl^{-/-} mice**

Cnga3^{-/-};Nrl^{-/-} mice showed enhanced levels of phospho-IP_{3}R1 at P30 (6) and further increased phosphorylation of IP_{3}R1 at P15 following inhibition of cGMP/PKG signaling (20), suggesting perturbed ER Ca^{2+} homeostasis in CNG-channel deficiency. We therefore investigated whether intracellular and ER Ca^{2+} regulation was disturbed at earlier ages of CNG-channel-deficient mice and whether this regulation changes at increasing postnatal ages. The data from this study are presented in Fig. 3. At P15, phosho-RyR2 and phospho-IP_{3}R1 protein levels were significantly increased with an ~1.3- and 2-fold increase, respectively, relative to age-matched Nrl^{-/-} controls (Fig. 3A). Additionally, the protein levels remained significantly elevated at P30, with an ~1.4-fold increase for both
Figure 1. Expression of the IP₃R1 isoform is increased in Cnga3⁻/⁻;Nrl⁻/⁻ mice and is localized to the inner segment in cones. A, retinas prepared from Cnga3⁻/⁻;Nrl⁻/⁻ and Nrl⁻/⁻ mice were analyzed for expression of IP₃R isoforms by qRT-PCR at P15, P30, and P60 following normalization to Hprt1 mRNA controls. B, total IP₃R1 isoform protein levels were measured at P15, P30, and P60 in Cnga3⁻/⁻;Nrl⁻/⁻ and Nrl⁻/⁻ mice. Shown are representative Western blot images with corresponding densitometric analysis following normalization to internal loading control β-actin. C, localization of the IP₃R1 isoform was determined via immunofluorescence co-labeling with rhodopsin or Na⁺/K⁺ ATPase α₁ subunit at P30 in wild-type mice. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Data are presented as mean ± S.D. of at least three independent assays using retinas from at least six mice/group. Unpaired Student’s t test was used to determine significance between CNG-channel-deficient and Nrl⁻/⁻ mice (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
channels relative to age-matched \(Nrt^{-/-}\) controls (Fig. 3A). Interestingly, we also found an \(~50\%\) decrease in intracellular Ca\(^{2+}\) levels in isolated cones in CNG-channel-deficient mice compared with \(Nrt^{-/-}\) controls at P15, but [Ca\(^{2+}\)] was not significantly different at either P30 or P60 (Fig. 3B). RyR2 and IP\(_3\)R1 Ca\(^{2+}\)-efflux activity is regulated by phosphorylation (23–25), and both respond to intracellular Ca\(^{2+}\) levels (26, 27). Therefore, these data support enhanced activity of ER Ca\(^{2+}\) channels on the background of decreased intracellular Ca\(^{2+}\) during early ages of CNG-channel deficiency.

Figure 2. Expression of the RyR2 isoform is increased in \(Cnga3^{-/-};Nrl^{-/-}\) mice and is localized to the inner segment in cones. A, retinas prepared from \(Cnga3^{-/-};Nrl^{-/-}\) and \(Nrl^{-/-}\) mice were analyzed for expression of RyR isoforms by qRT-PCR at P15, P30, and P60 following normalization to \(Hprt1\) mRNA controls. B, total RyR2 isoform protein levels were measured at P15, P30, and P60 in \(Cnga3^{-/-};Nrl^{-/-}\) and \(Nrl^{-/-}\) mice. Shown are representative Western blot images with corresponding densitometric analysis following normalization to internal loading control \(\beta\)-actin. C, localization of the RyR2 isoform was determined via immunofluorescence co-labeling with rhodopsin or Na\(^{+}/K^+\) ATPase \(\alpha1\) subunit at P30 in wild-type mice. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Data are presented as mean \pm S.D. of at least three independent assays using retinas from at least six mice/group. Unpaired Student’s \(t\) test was used to determine significance between CNG-channel-deficient and \(Nrt^{-/-}\) mice (*, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\)).
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Figure 3. Expression of phospho-IP,R1 and phospho-RyR2 is increased and intracellular Ca\(^{2+}\) is decreased at early ages in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice. A, retinal protein expression levels of phospho-RyR2 and phospho-IP,R1 at P15, P30, and P60 in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) and Nrl\(^{+/-}\) mice were analyzed. Shown are representative Western blot images with corresponding densitometric analysis following normalization to internal loading control β-actin. B, basal intracellular Ca\(^{2+}\) levels were also measured in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) and Nrl\(^{+/-}\) mice at P15, P30, and P60. Data are presented as mean ± S.D. of at least three independent assays using retinas from 6–8 mice/group for immunoblotting and 15–33 photoreceptors from 3–5 mice for [Ca\(^{2+}\)]\(_j\) assays. Unpaired Student’s t test was used to determine significance between CNG-channel-deficient and Nrl\(^{-/-}\) mice (*, p < 0.05; **, p < 0.01).

Increased ER-chaperone-protein expression levels and dynamic luminal Ca\(^{2+}\) stores at early ages in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice

Glucose-regulated protein 78/binding immunoglobulin protein (Grp78/BiP) and its association with ER-stress-related and Ca\(^{2+}\) signaling has been well-documented, showing up-regulation when improperly processed proteins, including the rod photoreceptor pigment rhodopsin, accumulate and ER-stress signaling is active (28–31). We have shown previously that ER-stress-marker proteins such as Grp78/BiP are up-regulated in CNG-channel-deficient mice and that they co-localize with S-opsin, indicating improper protein processing and induction of the unfolded protein response (UPR) (6, 20). Additionally, the ER-luminal quality-control chaperone calreticulin has been shown to interact with ER Ca\(^{2+}\)-efflux and -influx channels to modulate Ca\(^{2+}\) signaling within the ER (32–34), as well as aid in protein processing (35–37). Because of their established association with ER stress and protein quality control, we examined these markers to give us better insight into the chaperone and proteostasis status in CNG-channel-deficient mice. The data presented in Fig. 4 show an ~2- and 1.3-fold increase in luminal Grp78/BiP and calreticulin protein levels at P15 in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice compared with age-matched Nrl\(^{-/-}\) controls (Fig. 4A). Furthermore, Grp78/BiP protein levels remained elevated at P30 and P60, although the increase was not as large compared with earlier ages. Interestingly, calreticulin protein levels peaked at P30, with an ~1.6-fold increase, but then showed a 0.3-fold decrease at P60. Together, these changes indicate an enhanced need for protein-processing machinery at early ages in CNG-channel deficiency.

Because proper chaperone function relies on adequate [Ca\(^{2+}\)]\(_i\) (38, 39), we next evaluated intracellular Ca\(^{2+}\) levels following treatment with KCl and ionomycin at increasing ages in isolated cones in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice. Depolarization with KCl was employed to elevate [Ca\(^{2+}\)]\(_i\), by activating the L-type voltage-operated channels within the cone-cell body (40), whereas the ionophore ionomycin depletes ER stores through transient Ca\(^{2+}\) release from the lumen (41). Compared with age-matched Nrl\(^{-/-}\) controls, depolarization with high KCl induced an age-dependent, significant increase in intracellular Ca\(^{2+}\) levels of Cnga3\(^{-/-}\);Nrl\(^{-/-}\) cones, which was followed by decreased [Ca\(^{2+}\)]\(_i\), at P30 and an increase at P60 (Fig. 4B). These data suggest that Ca\(^{2+}\) homeostasis in postnatal Cnga3\(^{-/-}\) cones might involve a time-dependent component. Although not statistically significant, this conclusion is supported by the data trend seen with ionomycin-induced store release, which showed that the pool of store-releasable Ca\(^{2+}\) likewise changes in an age-dependent pattern. Taken together with the Grp78/BiP and calreticulin protein analysis (Fig. 4A), these data suggest that degenerating cones in the CNG-channel-deficiency mouse model show remodeled Ca\(^{2+}\) signaling and an increased demand for protein-processing machinery.

Reduced photoreceptor apoptosis in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice treated with IP,R and RyR inhibitors

Cones in Cnga3\(^{-/-}\) and Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice experience early-onset apoptotic cell death, ER-stress-associated apoptosis, and elevated phosphorylation of IP,R1 (6, 42). Also, our intracellular and intraorganellar Ca\(^{2+}\) measurements show a significant involvement of organellar Ca\(^{2+}\) signaling in CNG-channel deficiency. To determine whether this organellar Ca\(^{2+}\) signaling contributes to cone apoptosis, we examined the effects of IP,R- and RyR-inhibitor treatment on cone apoptotic markers. We treated Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice from postnatal day 8 to postnatal day 15 (P8–P15) with one of two commonly
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![Western blot images](image)

**Figure 4. Expression of ER-chaperone proteins is increased in Cnga3\(^{-/-}\); Nrl\(^{-/-}\) mice and luminal Ca\(^{2+}\) stores undergo dynamic changes.** A, retinas prepared from Cnga3\(^{-/-}\); Nrl\(^{-/-}\) and Nrl\(^{-/-}\) mice were analyzed for protein expression of Grp78/BiP and calreticulin by Western blot analysis at P15, P30, and P60. Shown are representative Western blot images with corresponding densitometric analysis following normalization to internal loading control β-actin. β-actin, 40 intracellular Ca\(^{2+}\) levels were measured following 20 mM KCl or 10 μM ionomycin treatment in Cnga3\(^{-/-}\); Nrl\(^{-/-}\) and Nrl\(^{-/-}\) mice at P15, P30, and P60 to determine relative levels of luminal Ca\(^{2+}\) stores. Data are presented as mean ± S.D. of at least three independent assays using retinas from 6–8 mice/group for immunoblotting and 13–33 photoreceptors from 3–5 mice for [Ca\(^{2+}\)]\(_l\) assays. Unpaired Student’s t test was used to determine significance between CNG-channel-deficient and Nrl\(^{-/-}\) mice (*, p < 0.05; **, p < 0.01).

**Improved cone-opsin trafficking to cone outer segment and reduced protein levels of ER chaperones in Cnga3\(^{-/-}\); Nrl\(^{-/-}\) mice treated with IP\(_3\)R inhibitors**

Retinal cross-sections of Cnga3\(^{-/-}\) mice show decreased levels of opsin localized to cone OS and increased opsin levels in cone IS, outer nuclear layers, and outer plexiform layers (42). Proper protein folding in the ER requires sufficient Ca\(^{2+}\) stores, and depletion of ER-luminal Ca\(^{2+}\) stores impairs protein translation and can lead to impaired protein trafficking (50). Because of the altered activity of IP\(_3\)R1 in CNG-channel-deficient cones, we investigated whether this change in activity contributed to cone-opsin mislocalization in Cnga3\(^{-/-}\) mice. As shown in Fig. 6A, M-opsin localization to the cone OS was significantly increased following both 2-APB and U73122 treatment (P8–P15). The data presented in Fig. 6B show an ~0.21- and 0.22-fold decrease in Grp78/BiP and calreticulin protein levels, respectively, following U73122 treatment in Cnga3\(^{-/-}\); Nrl\(^{-/-}\) retinas compared with age-matched WT controls (presented with age-matched vehicle controls for reference). These results suggest that IP\(_3\)R activity in CNG-channel deficiency may contribute to impaired protein translation and opsin mistrafficking during the early stage of CNG-channel deficiency.

To further test whether IP\(_3\)R activity contributes to improper protein processing, we examined the luminal protein levels of ER chaperones Grp78/BiP and calreticulin following IP\(_3\)R-inhibitor treatment (P8–P15). The data presented in Fig. 6B show an ~0.21- and 0.22-fold decrease in Grp78/BiP and calreticulin protein levels, respectively, following U73122 treatment in Cnga3\(^{-/-}\); Nrl\(^{-/-}\) retinas compared with age-matched, vehicle-treated Cnga3\(^{-/-}\); Nrl\(^{-/-}\) retinas. Similarly, 2-APB treatment resulted in an ~0.38- and 0.32-fold decrease in Grp78/BiP and calreticulin protein levels, respectively, when compared with vehicle controls (Fig. 6B). We concluded from this experiment that inhibition of IP\(_3\)R activity may improve protein processing and trafficking in the ER and thus reduce the need for ER chaperones in CNG-channel deficiency.
Reduced levels of ER-stress-marker proteins in Cnga3+/H11546+/Nrl+/H11546+ mice treated with IP3R and RyR inhibitors

We showed previously that Cnga3+/H11002+/Nrl+/H11002+ retinas are characterized by the activation of all three arms of the ER-stress mechanism, including elevated levels of phospho-eukaryotic-initiation factor 2α (phospho-eIF2α), phospho-serine/threonine-protein kinase/endoribonuclease 1α (phospho-IRE1α), and cleaved activating transcription factor 6 (ATF6) (6, 20). To test the hypothesis that the arms of the ER-stress pathway are sensitive to the activation of the ER calcium-release channel, we exposed them to 2-APB or U73122. Fig. 7 shows that inhibition of IP3R-mediated store release significantly reduces phospho-IRE1α, cleaved ATF6, and phospho-eIF2α protein levels in Cnga3+/H11002+/Nrl+/H11002+ retinas (treatment from P8 to P15) compared with age-matched, vehicle-treated Nrl+/H11002+ controls and correlating quantitative analysis. ONL, outer nuclear layer; INL, inner nuclear layer. Data are represented as mean ± S.D. of at least three independent assays. Unpaired Student’s t test was used to determine significance between the two groups (*, p < 0.05; ***, p < 0.001).

Reduced levels of ER-stress-marker proteins in Cnga3−/−; Nrl−/− mice treated with IP3R and RyR inhibitors

We showed previously that Cnga3−/−;Nrl−/− retinas are characterized by the activation of all three arms of the ER-stress mechanism, including elevated levels of phospho-eukaryotic-initiation factor 2α (phospho-eIF2α), phospho-serine/threonine-protein kinase/endoribonuclease 1α (phospho-IRE1α), and cleaved activating transcription factor 6 (ATF6) (6, 20). To test the hypothesis that the arms of the ER-stress pathway are sensitive to the activation of the ER calcium-release channel, we exposed them to 2-APB or U73122. Fig. 7 shows that inhibition of IP3R-mediated store release significantly reduces phospho-IRE1α, cleaved ATF6, and phospho-eIF2α protein levels in Cnga3−/−;Nrl−/− retinas (treatment from P8 to P15) compared with age-matched, vehicle-treated Cnga3−/−;Nrl−/− retinas. In addition, the potential RyR involvement in elevated ER Ca2+-channel activity during cone degeneration in CNG-channel-deficient mice (Fig. 2) led us to evaluate the possible contribution of this enhanced activity to ER-stress signaling. Similar to IP3R inhibitor results, the data shown in Fig. 8 illustrate a significant reduction in ER-stress activation following RyR inhibition with tetracaine in Cnga3−/−;Nrl−/− retinas (treatment from P8 to P15). The results from this experiment suggest that reducing ER Ca2+-efflux-channel activity may attenuate the ER-stress response in CNG-channel-deficient mice.

Improved cone survival following genetic deletion of IP3R1 in CNG-channel deficiency

To confirm the results from the pharmacological inhibition and specifically to evaluate the effects of ER Ca2+-efflux-channel activity, we evaluated whether cone-specific gene deletion of IP3R1 improved cone survival in CNG-channel-deficient mice. As shown in Fig. 9, retinal cross-sections and whole mounts of 4-month-old Cnga3−/−;Itpr1flox/flox;Hrgp-Cre mice showed increased cone density in both the dorsal and ventral areas compared with 3–4-month-old Cnga3−/− mice (Fig. 9, A
Improved cone survival was also seen at 8 months (data not shown). To address the possible adverse effects of IP₃R1 deletion in cones, we further examined whether IP₃R1 knock-out affected cone function and viability. As shown in Fig. 9, Ipr1lox/lox;Hrgp-Cre mice showed no significant change in cone number (Fig. 9, A and B) or cone or rod function (Fig. 9C) when compared with age-matched WT mice, as measured by

Figure 6. Inhibition of IP₃R activity improved cone-opsin localization to cone outer segment and reduced ER-chaperone protein levels in CNG-channel-deficient mice. A, Cnga3⁻/⁻ mice received IP₃R inhibitor or vehicle treatment for 22 days beginning on P8. At the end of the treatment, M-opsin localization was determined via immunofluorescence on retinal sections. Shown are representative confocal images of immunofluorescence for M-opsin co-labeled with Na⁺/K⁺-ATPase α1 in IP₃R inhibitor and vehicle-treated Cnga3⁻/⁻ mice and control WT mice with quantification. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. B, Cnga3⁻/⁻;Nrl⁻/⁻ mice received IP₃R inhibitor or vehicle treatment for 7 days, beginning on P8, for Western blot analysis. At the end of the treatment, the protein expression levels of Ca²⁺-sensitive ER-chaperone proteins Grp78/BiP and calreticulin were analyzed via Western blot analysis. Shown are representative Western blot images of the IP₃R inhibitor or vehicle-treated Cnga3⁻/⁻;Nrl⁻/⁻ mice with corresponding quantitative analysis following normalization to internal loading control β-actin. Data are presented as mean ± S.D. of at least three independent assays. Unpaired Student’s t test was used to determine significance between IP₃R inhibitor and vehicle-treated Cnga3⁻/⁻;Nrl⁻/⁻ and Cnga3⁻/⁻ mice (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

and B), as measured by counting peanut agglutinin-labeled cones. Improved cone survival was also seen at 8 months (data not shown). To address the possible adverse effects of IP₃R1 deletion in cones, we further examined whether IP₃R1 knock-out affected cone function and viability. As shown in Fig. 9, Ipr1lox/lox;Hrgp-Cre mice showed no significant change in cone number (Fig. 9, A and B) or cone or rod function (Fig. 9C) when compared with age-matched WT mice, as measured by
electroretinogram (ERG) recordings. Both photopic and scotopic a- and b-waves were not significantly affected following IP3R1 deletion in cones. These results indicate that IP3R1 is involved in Cnga3−/− cone cell viability and may be a potential therapeutic target to prevent cone death in CNG-channel deficiency without significantly affecting rod-photoreceptor function or viability.

**Discussion**

This study examined intracellular and intraorganellar Ca2+ levels as cones age in CNG-channel deficiency, as well as ER Ca2+-channel activity to better understand the mechanisms of cone degeneration. CNG channels play a pivotal role in conducting inward Ca2+ currents essential for photoreceptor function and OS health (1, 51); specifically, cone CNG channels must handle the Ca2+ demands of the larger surface area and rapid light adaptation (52, 53). Because cone CNG channels
have relatively higher Ca\textsuperscript{2+} permeability under normal physiological conditions (8), we hypothesized that CNG deletion would alter intracellular Ca\textsuperscript{2+} signaling in these cells. Consistent with the prediction, basal [Ca\textsuperscript{2+}]\textsubscript{i} levels in CNG-channel-deficient cones were \( \approx \)50% lower at P15 compared with age-matched controls, but interestingly they returned to near normal values at ages P30 and P60, suggesting a compensatory response to normalize [Ca\textsuperscript{2+}]\textsubscript{i} that might involve altered function of Ca\textsuperscript{2+} release and/or leak channels (54), stimulation of store-operated channels (41), and/or modulation of Cav1.4 channel gating (55) or trafficking (56). Accordingly, the alteration of the basal [Ca\textsuperscript{2+}]\textsubscript{i} levels in CNG-deficient cones was associated with dynamic changes in the evoked Ca\textsuperscript{2+} signals, including the magnitude of the depolarization-dependent Ca\textsuperscript{2+} increase and the size of the releasable Ca\textsuperscript{2+} pool within intracellular stores. Furthermore, isofrom-specific expression of ER Ca\textsuperscript{2+}-efflux channels was consistently elevated and significantly regulated via phosphorylation at earlier ages, with IP\textsubscript{3}R1 and RyR2 isofoms showing the most significant increases. These data suggest that dysregulation of intracellular-Ca\textsuperscript{2+} homeostasis is an early event in CNG-channel-deficient cone degeneration, which significantly affects isofrom-specific ER Ca\textsuperscript{2+}-channel expression and activity. Our results are consistent with the conclusion that remodeled intracellular-Ca\textsuperscript{2+} signaling is a critical event during disease progression in neurodegenerative diseases. Thus, ER Ca\textsuperscript{2+}-channel activity and changes in [Ca\textsuperscript{2+}]\textsubscript{ER} were linked to the cell death of neurons in mouse models of Alzheimer’s and Huntington’s diseases (57–59), and increased IP\textsubscript{3}R activity has been shown to contribute to neuronal death in familial Alzheimer’s disease models (60).

It is worth noting the contribution of cGMP/PKG signaling in cellular Ca\textsuperscript{2+} homeostasis regulation. CNG-channel-deficient mice display remarkable elevation in cellular cGMP levels and increased PKG activity (61), which reflects a reduction in [Ca\textsuperscript{2+}]\textsubscript{ER}. The cytotoxic levels of cGMP/PKG may adversely influence ER Ca\textsuperscript{2+} signaling via interactions with ER
ER stress and degeneration. Our results suggest that low Ca\(^{2+}\) channels (25, 63). As we have shown previously, retinal phospho-IP_\(_\text{R1}\) levels in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) and Cngb3\(^{-/-}\);Nrl\(^{-/-}\) mice are increased (6) and are further elevated upon cGMP/PKG inhibition (20). Mitochondrial health is also influenced by ER Ca\(^{2+}\) signaling (64, 65), and the photoreceptor IS contains the highest concentration of mitochondria and ER in the cell (21, 66). As CNG-channel-deficient retinas also show mitochondrial insult (6), it is possible that dysregulated ER Ca\(^{2+}\)-signaling links pathological changes in metabolic function and signaling processes in CNG-mutant cones. Moreover, our results identify ER Ca\(^{2+}\)-efflux channels as potential targets to reduce ER stress/mitochondrial insult and cone death in CNG-channel deficiency (6) and likely in other retinal degenerative diseases associated with impaired cellular Ca\(^{2+}\) homeostasis, including those caused by mutations in PDE6 (67, 68) and GUCY2E genes (69, 70).

ER chaperones are known to respond to a variety of stressors, most notably unfolded proteins (37, 50, 71). Previously, we have shown elevated levels of the ER chaperone Grp78/BiP in CNG-channel-deficient cones (6). Increases in Grp78/BiP and calreticulin levels at P15 indicated that CNG-channel-deficient cones require increased protein-processing machinery. Increased Grp78/BiP expression has been shown to preserve photoreceptors in other retinal degenerative disease models (72) and is believed to be a pro-survival component of the UPR (73, 74). The modest Grp78/BiP increases shown at P30 and P60 coincide with significant opsin mislocalization (42). Dysregulation of ER Ca\(^{2+}\) homeostasis may therefore lead to prolonged ER stress (75) and down-regulation/improper function of chaperones (39), which may in turn exacerbate ER Ca\(^{2+}\) release (76), impair protein processing, and lead to protein mistrafficking. Thus, this study has demonstrated for the first time the effects of CNG-channel deficiency on age-related changes in cone intracellular Ca\(^{2+}\) homeostasis and ER-chaperone proteins, providing evidence for the potential consequences of long-term, dysregulated, ER Ca\(^{2+}\) signaling.

Increases in ER Ca\(^{2+}\)-channel activity, specifically IP_\(_R\) activity, have been shown to significantly contribute to neuronal death in Alzheimer’s and Huntington’s disease (77, 78), and pharmacologic and genetic approaches to reduce IP_\(_R\) activity have preserved neurons in disease models (43, 59, 60). In our study, pharmacological inhibition of ER Ca\(^{2+}\)-efflux-channel activity, as well as cone-specific gene deletion of IP_\(_R\), demonstrated significant cone rescue in CNG-channel-deficient mice. Therefore, these data support the view that inhibition of ER Ca\(^{2+}\) release preserves cones and implicates this IP_\(_R\) isoform in pathological Ca\(^{2+}\)-store release and [Ca\(^{2+}\)]\(_\text{ER}\) during cone ER stress and degeneration. Our results suggest that low [Ca\(^{2+}\)], at early ages may trigger subsequent Ca\(^{2+}\) release from intraorganellar stores; however, inhibition of specific isoforms that gate store release may protect cones compromised by CNG-channel deficiency.

An important consideration in pharmacological studies is the off-target effects of the agents used. 2-APB and U73122 have been reported to interact with calcium-permeable transient-receptor-potential channels (79, 80), acting as type-specific agonists or antagonists. Tetracaine has also been reported to block sodium channels (81). Although these interactions may possibly contribute to the findings in CNG-channel-deficient retinas, the results obtained from all IP_\(_R\) and RyR drugs, as well as those found in the IP_\(_R\) genetic knock-out model, specifically suggest that ER Ca\(^{2+}\)-channel activity plays a significant role in disease progression in CNG-channel-deficient mice.

In addition to the possible benefits of ER Ca\(^{2+}\)-channel activity, it is important to note the potential compensatory responses in cones following pharmacological inhibition or gene deletion. Because cone rescue following gene deletion of IP_\(_R\)1 was incomplete, an appealing explanation includes compensatory responses from other IP_\(_R\) isoforms, ER Ca\(^{2+}\) channels, or stromal-interaction-molecule proteins following reduced IP_\(_R\)1 signaling. Our finding that IP_\(_R\)1 deficiency does not affect cone function and survival in WT mice suggests that healthy cones are not sensitive to a lack of IP_\(_R\)1 (at least at the ages examined) and that there may be compensatory expressions of other isoforms of IP_\(_R\) channels or RyR channels or both. To address possible compensatory responses, it would be interesting to determine whether knockdown/knock-out of other IP_\(_R\) isoforms and/or ER Ca\(^{2+}\) channels improves cone survival or enhances cone death.

ER Ca\(^{2+}\) homeostasis and regulation is crucial for maintaining adequate stores to ensure proper protein folding and trafficking. When [Ca\(^{2+}\)]\(_\text{ER}\) stores are depleted, improperly processed proteins accumulate, most likely due to decreased chaperone function, triggering the UPR/ER stress (16, 75), whereas depletion-induced activation of stromal-interaction-molecule proteins could trigger Ca\(^{2+}\) overload through the activation of store-operated Ca\(^{2+}\)-entry (44). In lysosomal storage diseases with characteristic neurodegeneration, ER Ca\(^{2+}\) release is enhanced and triggers UPR-mediated apoptosis of neurons (82, 83). Although initially beneficial, responses that are insufficient to correct the insult or prolonged responses will trigger apoptotic cell death (84, 85). Our finding that CNG-channel-deficient mice show significant reduction in ER-stress signaling following inhibitor treatment is consistent with the important function of ER stress that has been documented in several models of inherited retinal degenerative diseases, including achromatopsia due to CNG-channel deficiency (6, 86). Thus, this study supports the idea that inhibiting ER Ca\(^{2+}\) efflux reduces ER stress and protects cones from prolonged or insufficient UPR signaling in CNG-channel deficiency.

The accumulation of mistrafficked proteins, particularly opsins, has been shown to cause photoreceptor death in inherited retinal degenerative diseases, and clearance of these proteins preserves photoreceptors (87, 88). Because the UPR is initiated via the accumulation of improperly processed proteins in the ER lumen, and attenuation of these pathways can be achieved via ER Ca\(^{2+}\)-efflux-channel inhibition, our experiments investigated the potential improvement in protein processing and trafficking following IP_\(_R\)-inhibitor treatment. The data showed significant improvement in cone-opsin localization to the OS following treatment and significant reduction in cone IS localization. The reduction in luminal Grp78/BiP and calreticulin levels provide additional support for alleviating stress on the ER protein-processing machinery. This study
therefore suggests that inhibiting ER Ca\(^{2+}\)-efflux channels may be a potential therapeutic strategy to reduce ER stress and improve opsin trafficking in retinal diseases with prolonged stress and protein mislocalization.

The connection between the apoptotic cone death observed in this study and its relevance to ER-stress activation has been shown in our previous investigations. ER-stress-associated cone death in CNG-channel-deficient mice was suggested after finding an elevation of all three arms of the ER-stress pathway, phospho-eIF2\(\alpha\), phospho-IRE1\(\alpha\), and cleaved ATF6 (20), as well as increased nuclear localization of CCAAT/enhancer-binding protein homologous protein (CHOP), a critical protein that mediates ER-stress-associated apoptosis (6, 89, 90). Furthermore, our previous work has shown up-regulation of the cysteine-protease calpains and cleavage of caspase-12 and caspase-7 in CNG-channel-deficient retinas (6), which are additional indicators of ER-stress-associated apoptosis (91–93). Also, we showed previously that treatment with the chemical chaperone tauroursodeoxycholic acid, which has proven effective in reducing photoreceptor death in inherited retinal degenerative diseases characterized by enhanced ER-stress signaling (87, 94, 95), reduced ER stress and improved cone survival in CNG-channel-deficient mice (7). To strengthen the connection between ER stress and apoptosis, we treated Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice with tauroursodeoxycholic acid and showed reduced levels of TUNEL-positive cells, suggesting reduced apoptosis following treatment (supplemental Fig. 1).

We also examined the gene expression of CHOP, T-cell death-associated gene 51 (TDAG51), and c-Jun N-terminal protein kinase (JNK), which are additional proteins mediating ER-stress-associated apoptosis (96–99) in CNG-channel-deficient mice. CHOP and JNK expression levels were significantly elevated at P30 in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice compared with Nrl\(^{-/-}\) controls, but TDAG51 expression remained unchanged in CNG-channel-deficient mice (supplemental Fig. 2A). However, CHOP expression was ∼15- and 8-fold higher than JNK and TDAG51 expression, respectively, in Cnga3\(^{-/-}\);Nrl\(^{-/-}\) mice at P30 (supplemental Fig. 2, B and C). These data indicate that CHOP may be the most significant contributor to ER-stress-associated apoptotic signaling in CNG-channel-deficient cones. Together, these data highlight the importance of ER-stress-mediated cell death in CNG-channel deficiency and suggest that reducing ER-stress signaling through the use of chaperones or inhibition of CHOP signaling represents a promising strategy to improve cone survival.

In summary, the loss of functional CNG channels results in impaired cone function and likely decreased cone OS Ca\(^{2+}\) levels, which may influence the ER/mitochondrial responses in cone IS to restore [Ca\(^{2+}\)]\(_i\). In addition, cytotoxic levels of cGMP/PKG may adversely influence ER Ca\(^{2+}\) signaling via interactions with ER Ca\(^{2+}\) channels. Enhanced ER Ca\(^{2+}\)-channel activity may restore OS Ca\(^{2+}\), but chronic signaling may contribute to remodeled Ca\(^{2+}\) signaling that in turn enhances ER stress and cell death through the disruption of ER-chaperone function and subsequent protein mistrafficking. Because altered Ca\(^{2+}\) signaling and ER-stress-associated cell death are common throughout multiple neurodegenerative diseases, it is critical to understand how these dynamic signaling pathways interact, how they direct cell fate, and which components contribute to pathophysiologic.

Experimental procedures

**Mice, antibodies, and other reagents**

The Cnga3\(^{-/-}\) (3), Cnga3\(^{-/-}\);Nrl\(^{-/-}\) (6), Itpr1\(^{flox/flox}\) (100), and Hrgp-Cre (101) mouse lines were generated as described previously. The Nrl\(^{-/-}\) line (5) was provided by Dr. Anand Swaroop (National Institutes of Health, NEI, Bethesda, MD). The Itpr1\(^{flox/flox}\);Hrgp-Cre and Cnga3\(^{-/-}\);Itpr1\(^{flox/flox}\);Hrgp-Cre lines were generated by cross-breeding. The wild-type (C57BL/6) line was obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under cyclic, 12-h light-dark conditions, with ∼7 foot-candles of illumination during the light cycle. Animal maintenance and experiments were approved by the local Institutional Animal Care and Use Committee (University of Oklahoma Health Sciences Center, Oklahoma City, OK) and conformed to the guidelines on the care and use of animals accepted by the Society for Neuroscience and the Association for Research in Vision and Ophthalmology (Rockville, MD). Mice of either sex were used in the experiments. The primary-antibody information is listed in Table 1.

**Table 1 Primary-antibody information**

| Antibody          | Provider                                      | Catalog no. | Dilutions used in IB* or IP* labeling |
|-------------------|-----------------------------------------------|-------------|--------------------------------------|
| M-opsin           | Dr. Cheryl Craft, Keck School of Medicine, USC, Los Angeles |             |                                      |
| IP_5_1            | EMD Millipore, Billerica, MA                  | 07-1213     | 1:2000 (IB)                          |
| Biotinylated PNA* | Vector Laboratories, Burlingame, CA           | B-1075      | 1:200 (IB)                          |
| Na\(^{+}/K\(^{-}\) ATPase \(\alpha\) | Developmental Studies Hybridoma Bank, University of Iowa | a5-s        | 1:100 (IB)                          |
| Rhodopsin 1D4     | Dr. Robert Molday, University of British Columbia, Canada | 3398        | 1:500 (IB)                          |
| Phospho-eIF2\(\alpha\)| Cell Signaling Technology, Beverly, MA         | 3760        | 1:250 (IB)                          |
| Calreticulin      | Active Motif, Carlsbad, CA                    | 40962       | 1:250 (IB)                          |
| ATf6              | Abcam, Inc., Cambridge, MA                    | ab-6276     | 1:2000 (IB)                         |
| Phospho-IRE1\(\alpha\)| Cell Signaling Technology, Beverly, MA         | ab-48187    | 1:100 (IB)                          |
| Grp78/8/Bip       | Cell Signaling Technology, Beverly, MA         | ab-59225    | 1:500 (IB)                          |
| Phospho-RyR2      | Cell Signaling Technology, Beverly, MA         | ab-2827     | 1:500 (IB), 1:100 (IF)              |

* IB, immunoblotting.
  † IF, immunofluorescence.
  * PNA, peanut agglutinin.
obtained from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD), and fluorescence-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Invitrogen. Other reagents were obtained from Sigma-Aldrich, Bio-Rad, Invitrogen, Abcam, and Tocris Biosciences.

**ER Ca^{2+} channel inhibitor treatment**

We used two IP_{3}R inhibitors, the receptor antagonist 2-APB (Tocris Biosciences) and the phospholipase D inhibitor U73122 (Tocris Biosciences) (41, 45, 46), and two RyR inhibitors, tetracaine (Abcam) and dantrolene (Sigma) (47–49), in this study. Starting at P8, Cnga3−/−;Nri−/− mice received 2-APB (300 μg/day, i.p.), U73122 (80 μg/day, i.p.), tetracaine (400 μg/day, i.p.), dantrolene (50 μg/day, i.p.), or vehicle for 7 days for the apoptosis, ER stress, and ER-chaperone studies (treatment from P8 to P15) or 2-APB or U73122 for 22 days for the opsin-localization study (P8–P30). Retinas and eyes were collected at the end of the experiments for analysis.

**RNA isolation and quantitative real-time PCR**

Total RNA was isolated from mouse retinas and reverse-transcribed as described previously (61). qRT-PCR was performed to detect mRNA levels of mouse IP_{3}R (isoforms 1, 2, and 3), RyR (isoforms 1, 2, and 3), CHOP, TDAG51, JNK, and hypoxanthine guanine phosphoribosyltransferase 1 (HPRT1). The primers used are listed in Table 2. Relative gene expression, using the Hprr1 gene as internal control, was determined by the ΔΔCT method as described previously (102). Proper amplification of PCR products was verified via dissociation-curve analysis and agarose gel electrophoresis, and assays were repeated with at least eight different animals of each genotype.

**Retinal protein preparation, SDS-PAGE, and Western blot analysis**

Retinal protein preparation, SDS-PAGE, and Western blot analysis were performed as described previously (20). Briefly, retinas were homogenized in homogenization buffer A (0.32 M sucrose, 20 mM HEPES, pH 7.4, and 3 mM EDTA containing protease and phosphatase inhibitors (Roche Applied Science, catalogue no. 0493159001 and 04906837001, respectively), and homogenates were centrifuged at 3000 × g for 10 min at 4 °C. The resulting supernatant was then centrifuged at 13,000 × g for 35 min at 4 °C to separate cytosolic (supernatant) and membrane (pellet) fractions. Resulting membrane pellet was resuspended in homogenization buffer B (0.32 M sucrose, 20 mM HEPES, pH 7.4, 3 mM EDTA, and 0.1% Triton X-100 containing protease and phosphatase inhibitors as described above), sonicated twice for 10 s on ice at medium speed using a M460 ultrasonic cell disruptor with a 30-s recovery between disruptions, and incubated for 1 h at 4 °C with gentle agitation. After incubation, the homogenate was centrifuged at 13,000 × g for 35 min at 4 °C, and the resulting supernatant was used as the membrane fraction. All protein concentrations were determined using a protein-assay kit from Bio-Rad. Retinal protein samples were then subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were subsequently blocked in 5% nonfat milk (or 5% BSA, if detecting phosphorylated protein) for 1 h at room temperature. Immunoblots were incubated with primary antibody overnight at 4 °C (see Table 1 for antibody information). After washing in Tris-buffered saline with 0.1% Tween 20, the immunoblots were incubated with HRP-conjugated secondary antibody (1:20,000) for 1 h at room temperature. Chemiluminescent substrate (ThermoFisher, catalogue no. 34076) was used to detect primary antibodies binding to respective cognate antigens. Either a Li-Cor Odyssey machine and Li-Cor software (Li-Cor Biosciences, Lincoln, NE) or high-resolution autoradiography films (Denville Scientific, Inc., Metuchen, NJ) and ImageJ software were used for detection and densitometric analysis, respectively.

**Intracellular Ca^{2+} measurements**

A complete description of these methods is found elsewhere (103). Briefly, photoreceptors loaded with 2–5 μM fura-2 AM (fura 2-acetoxymethyl ester, Molecular Probes, Carlsbad, CA) for 10 min followed by a 20-min wash were imaged, and the fluorescent signals were measured as described previously (40). Intracellular organelle Ca^{2+} stores were estimated by measuring fluorescence following KCl treatment and fitting the measurements to exponential and linear curves to approximate [Ca^{2+}], decrease and rise times, respectively (40). For all measurements, the data were pooled according to genotype and presented as mean ± S.E.

**Eye preparation, immunofluorescence labeling, and confocal microscopy**

Mouse eyes were prepared for retinal whole mount or cross-sections for immunofluorescence analysis as described previously (104). For whole mount, eyes were enucleated and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature followed by removal of the cornea and lens. The eyes were then fixed in 4% paraformaldehyde in PBS for 4–6 h at room temperature. Retinas were isolated, and the superior portion was

### Table 2

| Gene symbol | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| Hprr1       | GCAAGCTTCCTGCTTTCCTGTTT | CAAGGCGTCCCCACCAACAC |
| Hpr1        | AGATTGCCATGACCTGCGT | TTTCTCACTCTGAGACAGG |
| Hpr2        | GCTCTTGGCTTTGCACAGGAT | CACCTTCCTCTTCTGAGGCC |
| Hpr3        | CCTGCTCCTTGTGGACCTTCTC | GCTGCTCCTCTTCTGAGCC |
| Ryr1        | AGCTGCAACGCCCTCCACCACATC | GCTGCTCCTCTTCTGAGCC |
| Ryr2        | TCACCGGACACCTCCACGATACCT | GCAAAAGACGAGTACGAGG |
| Ryr3        | GAGGAGGTTCTCTGAGAAGTG | AGTCTGCTCTTCTGAGG |
| Chop        | TGGTTGAGAAGACCCGCTT | AAGGTTGAGAAGACCCGCTT |
| Tdag51      | CCGTACCAACTCCACAGCAC | GCTCTCCTCCTCCTCCCG |
| Ink         | TCAAGACCCCATCCTACACAC | CTCTCATCTCATTAGCTGCC |

**ER Ca^{2+}--channel activity in CNG-channel deficiency**
marked for orientation with a small cut. For cross-sections, the superior portion of the cornea was marked with green dye prior to enucleation. Eyes were then fixed with Prefer (Anatech Ltd., Battle Creek, MI) for 25–30 min at room temperature. After fixation, eyes were transferred to 70% ethanol at 4 °C until ready for paraffin embedding. A Leica microtome (Leica Biosystems, Buffalo Grove, IL) was used to cut 5-μm-thick sections vertically through the retina (along the vertical meridian passing through the optic nerve head), and immunofluorescence labeling was performed as described previously (104). Briefly, retinal whole mounts or sections were blocked with Hank’s balanced salt solution containing 5% BSA and 0.5% Triton X-100 for 1 h at room temperature or overnight at 4 °C. Prior to blocking, antigen retrieval was performed in 10 mM sodium citrate buffer, pH 6.0, in a 70 °C water bath. Primary-antibody incubation was performed for 2 h at room temperature or overnight at 4 °C (see Table 1 for antibody information). Slides were mounted and coverslipped after fluorescence-conjugated secondary-antibody incubation and wash steps. Immunofluorescence labeling was then imaged using an Olympus FV1000 confocal laser-scanning microscope and FluoView imaging software (Olympus, Melville, NY).

**TUNEL assay**

Terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) was performed to analyze photoreceptor apoptosis as described previously (20) using paraffin-embedded retinal sections and an in situ cell death fluorescence detection kit (Roche Applied Science). Immunofluorescence labeling was imaged using an Olympus FV1000 confocal laser-scanning microscope, and TUNEL-positive cells in the outer nuclear layer passing through the optic nerve were counted and averaged from at least three sections/eye from at least four animals/condition.

**Scotopic and photopic ERG recordings**

Full-field ERG recordings were carried out as described previously (4). Briefly, after overnight dark adaptation, mice were anesthetized by intraperitoneal injection of 85 mg/kg ketamine and 14 mg/kg xylazine. ERGs were recorded using an Espion visual electrophysiology system with a Ganzfeld ColorDome system (Diagnosys LLC, Lowell, MA). Potentials were recorded using a gold-wire electrode to contact the corneal surface through a layer of 2.5% hydromellose (Gonak™, Akorn). For assessment of scotopic responses, a stimulus intensity of 2.20 log cd s m⁻² was presented to dark-adapted dilated mouse eyes. To evaluate photopic responses, mice were adapted to a 1.48 log cd s m⁻² light for 5 min, and then a light intensity of 1.89 log cd s m⁻² was given. Responses were differentially amplified, averaged, and analyzed using Espion 100 software (Diagnosys).

**Statistical analysis**

One-way analysis of variance and unpaired Student’s t test were used to evaluate significant differences between multiple groups and two groups, respectively. Statistical analyses and graph generation were performed using GraphPad Prism® software (GraphPad Software, San Diego) for Windows.

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