cGMP/Protein Kinase G Signaling Suppresses Inositol 1,4,5-Trisphosphate Receptor Phosphorylation and Promotes Endoplasmic Reticulum Stress in Photoreceptors of Cyclic Nucleotide-gated Channel-deficient Mice*

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Photoreceptor cyclic nucleotide-gated (CNG) channels play a pivotal role in phototransduction. Mutations in the cone CNG channel subunits CNGA3 and CNGB3 are associated with achromatopsia and cone dystrophies. We have shown endoplasmic reticulum (ER) stress-associated apoptosis in CNG channel deficiency. This work investigated whether cGMP/PKG signaling regulates ER stress and IP3R1 phosphorylation in CNG channel-deficient cones. Treatment with PKG inhibitor or deletion of GC1 effectively reduced apoptotic cone death, increased expression of cone proteins, and decreased activation of Müller glial cells. Furthermore, we observed significantly increased phosphorylation of IP3R1 and reduced ER stress. Our findings demonstrate a role of cGMP/PKG signaling in ER stress and ER Ca2+ channel regulation and provide insights into the mechanism of cone degeneration in CNG channel deficiency.

Results: Suppressing cGMP/PKG signaling enhances inositol 1,4,5-trisphosphate receptor 1 (IP3R1) phosphorylation and inhibits endoplasmic reticulum stress and cone death.

Conclusion: cGMP/PKG signaling regulates IP3R1 activity and promotes endoplasmic reticulum stress in CNG channel deficiency.

Significance: Understanding of the mechanism(s) of photoreceptor degeneration is essential for therapeutic strategy development.

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specific neural retina leucine zipper transcriptional factor, promotes differentiation of rods, and NRL deficiency produces a cone-only retina (14). Because cones include only 2–3% of the total photoreceptor population in the wild-type mouse retina, the use of the Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> and Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mouse lines enabled us to explore the cellular alterations and biochemical events in CNG channel-deficient cones to understand the mechanism(s) of cone degeneration.

CNG channel-deficient mice display early-onset apoptotic cone death, with cone degeneration evident in the 2nd postnatal week (8, 12). We have shown that apoptotic cone death is associated with endoplasmic reticulum (ER) stress, manifested by elevated ER stress marker proteins, including Grp78/Bip, and CCAAT/-enhancer-binding protein homologous protein (CHOP), up-regulated cysteine protease calpains, and increased processing of caspase-12 and caspase-7 (12, 13). We have also shown increased phosphorylation of the ER Ca<sup>2+</sup> channel inositol 1,4,5-trisphosphate receptor 1 (IP<sub>3</sub>R1) (12). Reduced cone death following the treatment with the ER chaperone tauroursodeoxycholic acid (TUDCA) (13) suggests a contribution of ER stress to cone death.

In addition to ER stress, CNG channel deficiency is associated with elevation of cellular cGMP levels. Retinal cGMP levels in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice sharply increased at postnatal day 8 (P8), peaked around P10–15, remained high through P30–60, and returned to near control levels at P90 (15). The cGMP elevation pattern correlated with apoptotic cone death (8, 12). Using Cnga3<sup>−/−</sup>/Gucy2e<sup>−/−</sup> mice lacking retinal guanylyl cyclase 1 (retGC1), an enzyme responsible for biosynthesis of cGMP in photoreceptors, we showed that cGMP accumulation contributed to cone death in the absence of CNG channels. Cone density and expression levels of cone proteins were significantly increased in Cnga3<sup>−/−</sup>/Gucy2e<sup>−/−</sup> mice, compared with Cnga3<sup>−/−</sup> mice (15). We also showed that the activity and expression levels of cGMP-dependent protein kinase (protein kinase G, PKG) were significantly increased, suggesting a potential role of cGMP/PKG signaling in cone death.

In this study, we investigated whether cGMP/PKG signaling contributes to ER stress and regulates IP<sub>3</sub>R1 phosphorylation in CNG channel-deficient cones. Treatment with PKG inhibitors and deletion of retGC1 effectively reduced apoptotic cone death and Müller glial cell activation and increased expression levels of cone proteins in CNG channel-deficient mice. Furthermore, inhibition of cGMP/PKG signaling significantly increased phosphorylation of IP<sub>3</sub>R1 and reduced ER stress. Findings from this work support a cGMP/PKG-regulated, IP<sub>3</sub>R1-associated ER stress/apoptosis in CNG channel-deficient cones.

**Experimental Procedures**

**Mice, Antibodies, and Other Materials**—The Cnga3<sup>−/−</sup> (9), Nrl<sup>−/−</sup> (14), Gucy2e<sup>−/−</sup> (16), and Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> (12, 13) mouse lines were generated as described previously. Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup>/Gucy2e<sup>−/−</sup> line was generated by cross-breeding. All mice were maintained under cyclic light (12-h light-dark) conditions. During the light cycle, cage illumination was ∼7 foot-candles. All 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 adopted by the Society for Neuroscience and the Association for Research in Vision and Ophthalmology (Rockville, MD).

Primary antibodies used in this study are listed in Table 1. Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Fluorescent goat anti-rabbit and goat anti-mouse antibodies were obtained from Invitrogen. All other reagents were purchased from Sigma, Bio-Rad, and Invitrogen.

**PKG Inhibitor Treatment**—We used two PKG inhibitors, KT5823 (Sigma) and (R<sub>y</sub>)-8-Br-cGMPS (Santa Cruz Biotechnology, Dallas, TX), in this study (17–19). Starting at P7, Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice received KT5823 (1.0 µmol/kg body weight/day, i.p.) or (R<sub>y</sub>)-8-Br-cGMPS (5.0 µmol/kg body weight/day, i.p.) or vehicle for 8 days for the apoptosis and ER stress study, or for 20 days for the cone protein expression level study. Retinas and eyes were collected at the end of the experiments for PKG activity, TUNEL labeling, caspase-7 cleavage, phospho-eIF2α levels, and expression levels of cone proteins.

**PKG Activity Assay**—PKG activity in the retinal lysate was assayed using the CycLex cGMP-dependent protein kinase assay kit (purchased from MBL International, Woburn, MA) as described previously (15). This immune colorimetric assay analyzes PKG activity by measuring the levels of phosphorylated PKG substrate, which is phosphorylated by PKG family members PKGI and PKGII. The phosphorylated substrate is detected by the phospho-specific monoclonal antibody 10H11 that recognizes the phosphothreonine 68/119 residues on the substrate. Briefly, retinal proteins (100 µg) in kinase buffer (200 µl) were added to the assay plates coated with recombinant PKG substrate containing threonine 68/119 residues. The plates were incubated in the presence of Mg<sup>2+</sup> and ATP for 30 min at 30 °C to phosphorylate the substrate. After washing, 100 µl of the HRP-conjugated phosphospecific antibody was added to each well, and samples were incubated for 1 h at room temperature. The wells were washed and substrate reagent was added, leading to a color change from colorless to blue catalyzed by HRP. The reaction was stopped with stop solution, which changes the color from blue to yellow, and the absorbance at 450 nm was measured with a SpectraMax 190 microplate spectrophotometer (Molecular Devices). Each reaction was performed in duplicate. Results are an average of three to four independent experiments using retinas prepared from five to six mice.

**Eye Preparation, Immunofluorescence Labeling, and Confocal Microscopy**—We prepared mouse eye cross-sections for immunohistochemical analysis as described previously (11). Briefly, euthanasia of mice was performed by CO₂ asphyxiation, and mouse eyes were enucleated and fixed with Prefer (Anatech Ltd., Battle Creek, MI) for 25–30 min at room temperature. The superior portion of the cornea was marked with a green dye for orientation before enucleation. Fixed eyes were then trans-
Reduced Photoreceptor Apoptosis in Cnga3−/−/Nrl−/− Mice Treated with PKG Inhibitor—We previously showed that cones of Cnga3−/− and Cnga3−/−/Nrl−/− mice undergo early-

TABLE 1
List of antibodies used in this study

| Antibody | Provider | Catalog no. | Dilutions used in immunoblotting (IB) or immunofluorescence (IF) labeling |
|----------|----------|-------------|--------------------------------------------------|
| M-opsin  | Dr. Cheryl Craft, Keck School of Medicine, University of Southern California | | 1:2000 (IB) |
| S-opsin  | Dr. Muna Naash, University of Oklahoma Health Sciences Center | | 1:1000 (IB) |
| Cone arrestin | Dr. Cheryl Craft, Keck School of Medicine | | 1:2000 (IB) |
| GADD 153 (CHOP-10) | Santa Cruz Biotechnology Inc., Santa Cruz, CA | sc-575 | 1:100 (IB) |
| Phospho-CREB | Cell Signaling Technology, Beverly, MA | sc-7978 | 1:500 (IF) |
| Phospho-eIF2α | Cell Signaling Technology, Beverly, MA | 3398 | 1:500 (IB) |
| Phospho-IP, R | Cell Signaling Technology, Beverly, MA | 3760 | 1:250 (IB) |
| Caspase-7 | Santa Cruz Biotechnology Inc., Santa Cruz, CA | 9492 | 1:250 (IB) |
| Histone 3 (H3) | Active Motif, Carlsbad, CA | 4499 | 1:2000 (IB) |
| ATF-6 | Dako Denmark A/S, Glostrup, Denmark | 40962 | 1:250 (IB) |
| GFAP | Dako Denmark A/S, Glostrup, Denmark | 20334 | 1:500 (IF) |
| β-Actin | Abcam, Inc., Cambridge, MA | ab-6276 | 1:2000 (IB) |
| Phospho-IRE1α | Abcam, Inc., Cambridge, MA | ab-48187 | 1:100 (IB) |

\( \text{Nrl} \rightarrow \text{NCX} \to \text{PKG} \rightarrow \text{cGMP} \to \text{GAP} \to \text{NFAT} \to \text{Atf} \to \text{NF-κB} \to \text{AP-1} \)
onset apoptosis (8, 12). Retinas of these mice also showed a remarkable elevation of [cGMP] and increased PKG activity (12, 15, 22). Furthermore, we provided evidence that the cGMP accumulation contributed to cone degeneration (15). To determine whether the beneficial effects of cGMP reduction are associated with PKG signaling, we examined the effects of PKG inhibition on apoptotic cone death. We used two commonly used PKG inhibitors, KT5823 and (R)-8-Br-cGMPS (17–19) in our study. We found that treatment with PKG inhibitor significantly reduced photoreceptor apoptosis in Cnga3−/−/Nrl−/− mice. As shown in Fig. 1A, PKG activity in Cnga3−/−/Nrl−/− retinas was strongly reduced by PKG inhibitors. Subsequently, the increased TUNEL labeling was significantly reduced in KT5823-treated mice and was nearly completely abolished in (R)-8-Br-cGMPS-treated mice (Fig. 1, B and C). In addition, caspase-7 cleavage was abolished (Fig. 1D), and CHOP expression was reduced (Fig. 1E) in Cnga3−/−/Nrl−/− mice treated with PKG inhibitor, compared with vehicle-treated controls.

We also examined the effects of PKG inhibition on the activity of cAMP-response element-binding protein (CREB), which is a known substrate of PKG. We found that the level of phospho-CREB was significantly reduced in Cnga3−/−/Nrl−/− mice treated with (R)-8-Br-cGMPS, compared with vehicle-treated controls (Fig. 1F), supporting the effectiveness of PKG inhibitor treatment.

Reduced cGMP Level, PKG Activity, and Photoreceptor Apoptosis in Cnga3−/−/Nrl−/−/Gucy2e−/− Mice—Cnga3−/−/Gucy2e−/− mice showed increased cone density and expression levels of cone proteins, compared with those in Cnga3−/−/Nrl−/− mice (15). This work examined whether deletion of RetGC1 reduces apoptotic cone death. We generated a Cnga3−/−/Nrl−/−/Gucy2e−/− mouse line to perform biochemical assays on a cone-dominant retina lacking a CNG channel and RetGC1. Retinal cGMP levels, PKG activity, and TUNEL labeling were examined in P15 Cnga3−/−/Nrl−/−/Gucy2e−/− mice and compared with those in age-matched Cnga3−/−/Nrl−/− and Nrl−/− mice. As shown in Fig. 2, the cGMP level, PKG activity, and TUNEL-positive cells in Cnga3−/−/Nrl−/−/Gucy2e−/− mice increased by about 70-, 2.5-, and 2.4-fold, respectively, compared with Nrl−/− mice. Deletion of RetGC1 completely abolished cGMP (Fig. 2A) and nearly completely abolished elevated PKG activity (Fig. 2B) and TUNEL-positive labeling (Fig. 2, C and D).

Increased Expression Levels of Cone Proteins in Cnga3−/−/Nrl−/− Mice Treated with PKG Inhibitor and in Cnga3−/−/Nrl−/−/Gucy2e−/− Mice—To evaluate whether suppressing PKG signaling improves cone survival, we examined expression levels of cone opsin in Cnga3−/−/Nrl−/− mice treated with PKG inhibitor. As shown in Fig. 3, A and B, M-opsin and S-opsin levels increased by about 28 and 23%, respectively, in Cnga3−/−/Nrl−/− mice treated with KT5823, compared with vehicle-treated controls. We also examined the expression levels of cone proteins in Cnga3−/−/Nrl−/−/Gucy2e−/− mice. Cone opsin and cone arrestin expression levels significantly increased in P60 Cnga3−/−/Nrl−/−/Gucy2e−/− mice, compared with those in age-matched Cnga3−/−/Nrl−/− mice (Fig. 3C).

Reduced Müller Glial Cell Activation in Cnga3−/−/Nrl−/− Mice Treated with PKG Inhibitor and in Cnga3−/−/Nrl−/−/Gucy2e−/− Mice—Müller glia are known to activate in response to retinal stress, including photoreceptor death and injury, by profound up-regulation of GFAP in intermediate filaments. GFAP immunolabeling is commonly used to assess Müller glial cell activation in retinal degeneration (23–25). We previously showed increased GFAP expression in Cnga3−/−/Nrl−/− retinas (8). In this study, we examined GFAP expression in Cnga3−/−/Nrl−/− mice treated with the PKG inhibitor to determine whether the overall retinal stress is reduced. Fig. 4A shows GFAP immunofluorescence labeling of retinal sections prepared from Cnga3−/−/Nrl−/− mice treated with (R)-8-Br-cGMPS or vehicle and the corresponding quantification of immunofluorescence intensity. The retinal sections from vehicle-treated mice showed strong up-regulation of GFAP across the retina, with immunostaining mainly detected in the inner and outer plexiform layers and the ganglionic layer (Fig. 4A, upper panels). In contrast, the retinal sections prepared from (R)-8-Br-cGMPS-treated mice showed significantly reduced GFAP immunoreactivity (Fig. 4A, lower panels). Similar results were obtained from mice treated with KT5823 (Fig. 4B). The reduction of Müller glial cell activation was also observed in Cnga3−/−/Nrl−/−/Gucy2e−/− mice, compared with that in Cnga3−/−/Nrl−/− mice (Fig. 4C).

Reduced ER Stress and Increased IP3R1 Phosphorylation in Retinas of Cnga3−/−/Nrl−/− Mice Treated with PKG Inhibitor—We previously showed that the levels of the ER stress marker proteins, including phospho-eIF2α, which represents a well characterized arm/pathway of ER stress (26, 27), were enhanced in Cnga3−/−/Nrl−/− retinas (12). In this study, we first investigated whether inhibition of PKG suppresses PERK/eIF2α pathway and ER stress. We found that PKG inhibitor treatment significantly reduced levels of phospho-eIF2α in Cnga3−/−/Nrl−/− retinas. The level of phospho-eIF2α was about 1.3-fold higher in Cnga3−/−/Nrl−/− mice than in Nrl−/− mice. Treatment with (R)-8-Br-cGMPS reduced the level of phospho-eIF2α by about 25%, compared with vehicle-treated Cnga3−/−/Nrl−/− controls (Fig. 5A), whereas treatment with KT5823 reduced it by about 40%, compared with vehicle-treated controls (Fig. 5B).

IP3R plays a critical role in the cellular Ca2+ regulation, including ER Ca2+ homeostasis and responses to cellular Ca2+ perturbation and stress. We have shown an increased IP3R1 phosphorylation in Cnga3−/−/Nrl−/− retinas (12). In this study, we examined the effects of PKG inhibitor on IP3R1 phosphorylation. The level of phospho-IP3R1 was about 30% higher in Cnga3−/−/Nrl−/− mice than in Nrl−/− mice (Fig. 5, A and B). Following treatment with the PKG inhibitor, the level of phospho-IP3R1 increased further. The level of phospho-IP3R1 in Cnga3−/−/Nrl−/− mice increased by about 33% following treatment with (R)-8-Br-cGMPS (Fig. 5A) and increased by about 12% following treatment with KT5823 (Fig. 5B), compared with vehicle-treated Cnga3−/−/Nrl−/− controls. Thus, inhibition of PKG activity reduced ER stress and increased IP3R1 phosphorylation in CNG channel-deficient mice.

In this work, we also examined the activity of the other arms/pathways of ER stress in CNG channel-deficient mice and the effects of PKG inhibition. These arms include the activating
FIGURE 1. Reduced photoreceptor apoptosis in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice treated with PKG inhibitor. A, reduced PKG activity in retinas of Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice treated with KT5823 (left panel) or (R)<sub>p</sub>-8-Br-cGMPS (right panel). The relative PKG activity in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice was normalized to the values in Nrl<sup>−/−</sup> mice. B and C, reduced TUNEL labeling on retinal sections of Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice treated with KT5823 or (R)<sub>p</sub>-8-Br-cGMPS. Shown are representative confocal images showing TUNEL-positive cells in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice treated with KT5823 (B, upper panels) or (R)<sub>p</sub>-8-Br-cGMPS (B, lower panels), and corresponding quantitative analysis (C). D, reduced caspase-7 cleavage in retinas of Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice treated with KT5823. Shown are representative images of the Western blot detection of caspase-7. E, reduced expression level of CHOP in retinas of Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice treated with (R)<sub>p</sub>-8-Br-cGMPS. Representative images of Western blot detection of CHOP are shown. F, reduced phospho-CREB level in retinas of Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice treated with (R)<sub>p</sub>-8-Br-cGMPS. Shown are representative confocal images of the phospho-CREB immunofluorescence labeling. ONL, outer nuclear layer; INL, inner nuclear layer; RGC, retinal ganglion cell; Rp-8-Br, (R)<sub>p</sub>-8-Br-cGMPS. Data are represented as mean ± S.E. from 3 to 4 assays using eyes/retinas prepared from 5 to 6 mice. Unpaired Student’s t test was used to determine the significance of differences (*, p < 0.05; ***, p < 0.001).
transcription factor 6 (ATF6) and the serine/threonine-protein kinase/endoribonuclease (IRE1) pathways. Activation of the ATF6 arm is characterized by cleavage of the pro-protein and production of the cleaved form. Using the anti-ATF6 antibody that recognizes both the pro-form and the cleaved form of the protein, our analysis showed the presence of the cleaved ATF6 in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> retinas, which was barely detected in Nrl<sup>−/−</sup> retinas (Fig. 6A). The cleaved form of ATF6 in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> retinas was increased by about 4.5-fold, compared with Nrl<sup>−/−</sup> retinas (Fig. 6A). Moreover, treatment with PKG inhibitor significantly suppressed cleavage of ATF6 (Fig. 6A). Activity of the IRE1 arm was evaluated by examining the expression levels of phospho-IRE1α using anti-phospho-IRE1α antibody. As shown in Fig. 6B, the level of phospho-IRE1α in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> retinas was increased by about 1.8-fold, compared with Nrl<sup>−/−</sup> retinas. Treatment with the PKG inhibitor reduced its phosphorylation, but the differences did not reach statistical significance (Fig. 6B).

Reduced ER Stress and Increased IP<sub>3</sub>R1 Phosphorylation in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup>/Gucy2e<sup>−/−</sup> Retinas—We next examined whether ER stress and IP<sub>3</sub>R1 phosphorylation are altered in retinas of Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup>/Gucy2e<sup>−/−</sup> mice. Similar to our findings from the treatment with PKG inhibitor, the level of phospho-eIF2α was significantly reduced, and the level of phospho-IP<sub>3</sub>R1 was further increased in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup>/Gucy2e<sup>−/−</sup> mice, compared with that in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice. As shown in Fig. 7, the level of phospho-eIF2α in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup>/Gucy2e<sup>−/−</sup> mice decreased by about 37% and the level of phospho-IP<sub>3</sub>R1 increased by about 17%, compared with that in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice.

Discussion

Contribution of cGMP/PKG Signaling to Apoptotic Cone Death—This study using a PKG inhibitor and deletion of retGC1, which effectively abolished PKG activation, establishes a role for PKG signaling in cone death. Suppressing
cGMP production and inhibiting PKG activity significantly decreased apoptotic cone death, caspase-7 cleavage, and Müller glial cell activation in Cnga3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice. The effectiveness of PKG inhibitor treatment was evident by the strong suppression of the enzyme’s activity and by effective reduction of the activation of CREB (Fig. 1). Thus, our exper-
Experimental data support the view that cGMP/PKG signaling activated in CNG channel deficiency contributes to cone death and that the toxicity of elevated cGMP is primarily mediated via PKG signaling.

The contribution of PKG signaling to photoreceptor death was previously shown in the rd1 mouse, a model of recessive retinitis pigmentosa caused by a nonsense mutation in the rod Pde6b gene. These mice also display ER stress-associated photoreceptor apoptosis (28, 29). cGMP accumulation has been shown to cause cell death in rd1 mice by overactivating the CNG channel, Ca\(^{2+}\) overload (30, 31), and by activating PKG signaling. Treatment of rd1 retina with PKG inhibitor reduced apoptotic photoreceptor death and improved rod survival (32). Thus, excessive activation of PKG signaling is harmful to photoreceptors, and our work provides insights into how cGMP/PKG signaling contributes to photoreceptor death.

**FIGURE 5. Reduced levels of phospho-eIF2\(\alpha\) and increased levels of phospho-IP3R1 in retinas of Cnga3\(^{-/-}\)/Nrl\(^{-/-}\) mice treated with PKG inhibitor.** Shown are representative images of the Western blot detection of phospho-eIF2\(\alpha\) and phospho-IP3R1 in retinas of Cnga3\(^{-/-}\)/Nrl\(^{-/-}\) mice treated with (R.p)-8-Br-cGMPS (A) or KT5823 (B) and corresponding densitometric analysis. The relative expression levels in Cnga3\(^{-/-}\)/Nrl\(^{-/-}\) mice were normalized to the values in Nrl\(^{-/-}\) mice. Data are represented as means \(\pm\) S.E. of measurements from four assays using retinas prepared from 4 to 5 mice. Unpaired Student’s t test was used for determination of the significance of differences between the drug-treated and vehicle-treated mice (*, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\)).

Contribution of cGMP/PKG Signaling to ER Stress—CNG channel-deficient mice display early-onset ER stress, shown by studies at the protein and mRNA expression levels. The expression levels of phospho-eIF2\(\alpha\) and CHOP were increased in CNG channel-deficient mice (12). Gene expression profiling studies demonstrated up-regulation of EIF2/ER stress pathways (13). In addition to examining the levels of phospho-eIF2\(\alpha\), we examined the activity of the other two arms/pathways of ER stress and detected increased cleavage of ATF6 and increased expression levels of phospho-IRE1\(\alpha\) in Cnga3\(^{-/-}\)/Nrl\(^{-/-}\) retinas, compared with Nrl\(^{-/-}\) retinas. The relevance of ER stress in cone degeneration is highlighted by a recent report that links mutations in ATF6 gene with achromatopsia (33). This is the first nonphotoreceptor-specific gene thus far identified to associate with achromatopsia. ER stress was also indirectly supported by increased levels of calpains, Bcl-2/Bcl-x,
and caspase-12/caspase-7 cleavage (12). Thus, the activation of all three ER stress pathways and processing of the ER stress-associated caspases suggest that cone death in CNG channel deficiency is associated with ER stress. Experiments with the ER chemical chaperone TUDCA and the molecular chaperone 11-cis-retinal further support this view. Treatment with TUDCA effectively suppressed caspase-7 cleavage and phosphorylation of eIF2α/H9251 and increased cone-specific protein expression levels (13). Treatment with 11-cis-retinal significantly improved cone survival, manifested as increased levels of cone proteins (data not shown). To understand the mechanism of ER stress and determine whether the elevated cGMP/PKG signaling contributes to ER stress, we examined the effects of PKG inhibitor and Gucy2e deletion. We found that inhibition of cGMP production and PKG activity effectively reduced ER stress in CNG channel-deficient mice, supporting a contribution of cGMP/PKG signaling to ER stress. Involvement of cGMP/PKG signaling in ER stress and apoptosis was previously shown in pancreatic β cells and myocardial cells (34, 35). This study for the first time demonstrated the regulatory role of cGMP/PKG signaling in ER stress in neural retinal cells.

**Potential ER Ca²⁺ Dysregulation and Impaired ER Function in CNG Channel Deficiency**—As a Ca²⁺ store, the ER is responsible for sequestering excess intracellular Ca²⁺ and releasing ER luminal Ca²⁺ stores when intracellular Ca²⁺ levels are low. ER Ca²⁺ homeostasis is crucial for cellular Ca²⁺ signaling and the ER's function as a site of cellular protein processing. ER requires high levels of luminal Ca²⁺ to maintain proper protein folding and exportation. Low luminal Ca²⁺ is known to cause an accumulation of incorrectly folded proteins and the subsequent unfolded protein response and ER stress (36–39). ER Ca²⁺ homeostasis is regulated by three ER Ca²⁺ channels/pumps as follows: the IP₃R and ryanodine receptors for Ca²⁺ efflux out of the ER into the cytosol, and the sarco/endoplasmic reticulum Ca²⁺-ATPase for Ca²⁺ influx into the ER (Fig. 8). The activity of these channels/pumps is primarily regulated by cellular Ca²⁺ levels, their respective ligands, and numerous signaling molecules/pathways. As nonselective cation channels, CNG channels are the main source of the Ca²⁺ inward currents in the outer segments of photoreceptors and play a pivotal role in the light response/adaptation and cellular Ca²⁺ homeostasis. Cones lacking functional CNG channels likely suffer from cellular Ca²⁺ perturbation/cytosolic Ca²⁺ reduction. In mouse rods, shutting down the influx through the CNG channels in the light lowers free cytoplasmic Ca²⁺ nearly 10-fold (40). The lowered cytosolic Ca²⁺ level in CNG channel deficiency is also supported by the remarkable elevation in cellular cGMP levels, because cGMP production in photoreceptors is tightly nega-
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CNG channel deficiency promotes Ca\(^{2+}\) release from the ER, and subsequently causes ER Ca\(^{2+}\) depletion. The suppressive regulation of IP\(_{3}\)R phosphorylation, leading to excess Ca\(^{2+}\) release from the ER, and subsequently causes ER Ca\(^{2+}\) reduction/ER malfunction. How cGMP/PKG signaling suppresses phosphorylation of IP\(_{3}\)R1 in CNG channel-deficient cones remains to be determined and may involve complex mechanisms. Numerous molecules/pathways, including PKA (53, 54), PKC (49), PKG (47), AKT (48, 55), and ERK (52), have been reported to regulate IP\(_{3}\)R phosphorylation. Furthermore, PKG has multiple targets, and cross-talks among pathways exist.

It is important to emphasize that the level of phospho-IP\(_{3}\)R1 in Cnga3\(^{-/-}\)/Nrl\(^{-/-}\) mice always increased relative to Nrl\(^{-/-}\) mice (Figs. 5 and 7) (12). A possible explanation for this observation is compensatory regulation. The Ca\(^{2+}\) release from the ER in CNG channel deficiency is likely increased (and Ca\(^{2+}\) entry is probably decreased) to compensate for the reduced cytosolic Ca\(^{2+}\) level, which could potentially lead to a reduction of ER Ca\(^{2+}\) level. Indeed, alteration of ryanodine receptors, including increased phosphorylation and expression level, can be detected in CNG channel-deficient retinas (data not shown). As a consequence, the function of IP\(_{3}\)R could be suppressed to protect the ER from excess Ca\(^{2+}\) release/Ca\(^{2+}\) depletion. The suppressive regulation of IP\(_{3}\)R has been shown to protect hippocampal neurons from apoptosis both in vitro and in an animal model of ER stress (56). The protective effects of cGMP/PKG signaling inhibition on ER stress and cone death, accompanied with increased IP\(_{3}\)R phosphorylation, suggest that the regulatory suppression of IP\(_{3}\)R1 in CNG channel deficiency is insufficient to compensate, likely due to the elevated cGMP/PKG signaling. Thus, the elevated cGMP/PKG signaling in CNG channel deficiency may interfere with the compensatory inhibition of IP\(_{3}\)R1, leading to excess Ca\(^{2+}\) release from the ER and ER stress. Based on our findings, we postulate that the reduced cytosolic Ca\(^{2+}\) and increased cGMP/PKG signaling in CNG channel deficiency promotes Ca\(^{2+}\) release from the ER, leading to ER Ca\(^{2+}\) dysregulation/ER stress and apoptotic death (Fig. 8).
Presence of a Nonapoptotic cGMP/PKG-independent Death Mechanism in CNG Channel Deficiency—We found that TUNEL-positive staining was almost completely abolished in Gucy2e-deleted mice and in PKG inhibitor-treated Cnga3/H11002/H11002/Nrl/H11002 mice (see Figs. 1 and 2). However, the cone rescue was only partial (Fig. 3) (15). These findings suggest that apoptosis may not be the only mechanism in cone death; a nonapoptotic, cGMP/PKG-independent mechanism may exist. The early-onset apoptotic cone death appears to be mediated mainly by cGMP/PKG signaling. The correlation between the time course of cGMP elevation and the TUNEL labeling (15) also supports this view.

In summary, this study shows that treatment with the PKG inhibitor or deletion of Gucy2e suppressed cone apoptosis and improved cone survival in CNG channel-deficient mice, demonstrating a role of cGMP/PKG signaling in cone death. ER stress was decreased by treatment with the PKG inhibitor and deletion of Gucy2e, indicating cGMP/PKG signaling dependence. Moreover, suppressing cGMP/PKG signaling increased IP$_3$R phosphorylation, suggesting a role of ER Ca$^{2+}$ channel regulation in ER stress and cone death. This work provides insights into ER Ca$^{2+}$ channel regulation/ER stress in cone death and improves our understanding of the mechanism of cone degeneration in CNG channel deficiency.

FIGURE 8. Mechanism of ER stress in CNG channel deficiency: potential involvement of the ER Ca$^{2+}$ channels. In CNG channel deficiency, decreased cytosolic Ca$^{2+}$ and increased cGMP/PKG signaling, which is a consequence of the reduced cytosolic Ca$^{2+}$ level, activates ER Ca$^{2+}$-releasing channels to remedy the cytosolic Ca$^{2+}$ perturbation. As a consequence, the decreased ER Ca$^{2+}$ level interferes with ER function/protein folding, which causes opsin mistrafficking/mislocalization and triggers unfolded protein response (UPR) and ER stress. Suppressing cGMP/PKG signaling by Gucy2e deletion or by PKG inhibitor inhibits IP$_3$R, decreases ER Ca$^{2+}$ release, and reduces ER stress. RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca$^{2+}$-ATPase.

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