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Potential Contribution of Ryanodine Receptor 2 Upregulation to cGMP/PKG Signaling-induced Cone Degeneration in Cyclic Nucleotide-gated Channel Deficiency

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Abstract

Photoreceptor cyclic nucleotide-gated (CNG) channels regulate Ca^{2+} influx in rod and cone photoreceptors. Mutations in cone CNG channel subunits CNGA3 and CNGB3 are associated with achromatopsia and cone dystrophies. Mice lacking functional cone CNG channel show endoplasmic reticulum (ER) stress-associated cone degeneration. The elevated cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase (PKG) signaling and upregulation of the ER Ca^{2+} channel ryanodine receptor 2 (RyR2) have been implicated in cone degeneration. This work investigates the potential contribution of RyR2 to cGMP/PKG signaling-induced ER stress and cone degeneration. We demonstrated that the expression and activity of RyR2 were highly regulated by cGMP/PKG signaling. Depletion of cGMP by deleting retinal guanylate cyclase 1 or inhibition of PKG using chemical inhibitors suppressed upregulation of RyR2 in CNG channel deficiency. Depletion of cGMP or deletion of *Ryr2* equivalently inhibited unfolded protein response/ER stress, activation of the CCAAT-enhancer-binding protein homologous protein, and activation of the cyclic adenosine monophosphate response element binding protein, leading to early-onset cone protection. In addition, treatment with cGMP significantly enhanced *Ryr2* expression in cultured photoreceptor-derived Weri-Rb1 cells. Findings from this work demonstrate the regulation of cGMP/PKG signaling on RyR2 in the retina and support a role of RyR2 upregulation in cGMP/PKG signaling-induced ER stress and photoreceptor degeneration.

Keywords

CNG channel; cone photoreceptors; retinal degeneration; ryanodine receptor; cGMP/PKG signaling

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Author Contribution

FY and HM performed research and analyzed data; FY and XQD designed research and wrote the manuscript. MRB contributed to research design and manuscript writing.

All authors declare that they have no conflict of interest.

Introduction

Photoreceptor cyclic nucleotide-gated (CNG) channels play a pivotal role in rod and cone phototransduction. In darkness or dim light, the CNG channel is kept open by binding of cyclic guanosine monophosphate (cGMP), maintaining a steady Ca^{2+} and Na^{+} influx. Light induces hydrolysis of cGMP by photoreceptor phosphodiesterase 6, resulting in closure of the channel and hyperpolarization of the cell (1, 2). The cone photoreceptor CNG channel is composed of two structurally related subunits, CNGA3 and CNGB3. CNGA3 is an ion-conducting subunit, whereas CNGB3 functions as a modulator (2). Mutations in *CNGA3* and *CNGB3* genes are associated with achromatopsia, progressive cone dystrophies, and early-onset macular degeneration (3–5).

Cone loss in patients with achromatopsia and cone dystrophies associated with CNG channel mutations has been well documented by optical coherence tomography (6–12). Impaired cone function and endoplasmic reticulum (ER) stress-associated cone apoptosis/progressive cone degeneration have also been characterized in *Cnga3*^{-/-} and *Cngb3*^{-/-} mice (13–16) and in *Cnga3*^{-/-}/*Nrl*^{-/-} mice with CNG channel deficiency on a cone-dominant *Nrl*^{-/-} background (17, 18). One typical cellular alteration in CNG channel deficiency is the remarkable elevation of cellular cGMP level and increased activity of the cGMP-dependent protein kinase (protein kinase G, PKG) (17, 19, 20). Retinal cGMP levels in *Cnga3*^{-/-}/*Nrl*^{-/-} 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 (19). The cGMP elevation pattern correlated with apoptotic cone death (13, 17, 19). The contribution of elevated cGMP/PKG signaling to ER stress/cone death was demonstrated by genetic deletion of retinal guanylate cyclase 1 (retGC1), an enzyme responsible for biosynthesis of cGMP in photoreceptors, and inhibition of PKG using chemical inhibitors (19, 21). How cGMP/PKG signaling induces ER stress and cone death, however, remains unidentified.

Along with elevated cGMP/PKG signaling, CNG channel-deficient cones show cellular calcium/ER calcium dysregulation. Cellular calcium/ER calcium homeostasis is essential to normal cellular signaling, including photoreceptor light adaptation and protein folding. Perturbation of cellular calcium has been associated with cellular dysfunction, ER stress, and cell death (22–26). As non-selective cation channels, CNG channels play a pivotal role in cellular calcium homeostasis/ER calcium homeostasis. Measurements of intracellular Ca^{2+} levels showed reduced cytosolic Ca^{2+} level and altered cellular Ca^{2+} dynamics in CNG channel-deficient cones (27). ER calcium homeostasis is primarily regulated by two ER calcium channels, inositol-1,4,5-trisphosphate receptor (IP₃R) (28) and ryanodine receptor (RyR) (29), which efflux calcium out of the ER, and sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) (30), which influxes calcium into the ER. Three RyR isoforms have been identified; RyR1, RyR2, and RyR3. All three isoforms of RyR are expressed in photoreceptors, and RyR2 has been identified as the major form in photoreceptors and is localized in the inner segments and outer nuclear layer (27, 31–33). CNG channel-deficient retinas show elevated RyR2 expression/activity and treatment with RyR inhibitor or deletion of *Ryr2* reduced ER stress/cone death and improved cone protein outer segment localization (27, 34).

In the current study, we examined the regulation of cGMP/PKG signaling on RyR2 and investigated whether RyR2 contributes to cGMP/PKG signaling-induced ER stress/cone death. We demonstrated that the expression and activity of RyR2 were highly regulated by cGMP/PKG signaling. Depletion of cGMP or inhibition of PKG abolished upregulation of RyR2 in CNG channel-deficient retinas. Depletion of cGMP or deletion of *Ryr2* equivalently inhibited unfolded protein response (UPR)/ER stress, activation of the CCAAT-enhancer-binding protein homologous protein (CHOP), and activation of the cyclic adenosine monophosphate response element binding protein (CREB), leading to early-onset cone protection. In addition, treatment with cGMP significantly enhanced *Ryr2* expression in cultured photoreceptor-derived Weri-Rb1 cells. Our findings demonstrate a tight regulation of cGMP/PKG signaling on RyR2 in the retina, and support a role of RyR2 upregulation in cGMP/PKG signaling-induced ER stress and cone degeneration. This work provides insight into the mechanism of photoreceptor degeneration in CNG channel deficiency and provides a better understanding of how cGMP/PKG signaling induces photoreceptor cell death.

Materials and Methods

Mice, antibodies, and other reagents

The *Cnga3*^{-/-} (14), *Nrl*^{-/-} (35), *Gucy2e*^{-/-} (36), and *Hrgp*^{cre} (transgenic mice expressing Cre recombinase directed by the human red/green pigment [HRGP] gene promoter) (37) mouse lines were generated as described previously. The *Ryr2*^{flox/flox} mouse line (38) was obtained from Jackson Laboratory (Bar Harbor, ME). The *Cnga3*^{-/-}/*Nrl*^{-/-} (17), *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-} (21), and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} (34) mouse lines were generated by cross-breeding. Lack of expression of CNGA3 in the retina was shown by immunofluorescence labeling using anti-CNGA3 antibody (Supplementary Fig. 1). Genotyping was conducted to confirm the knockout/expression of the specific genes, including *Nrl*, *Gucy2e*, *Ryr2*^{fl/fl}, and *Hrgp*^{cre} (Supplementary Fig. 2). NRL is a basic-motif leucine zipper transcription factor essential for the normal development of rods. Mice lacking the *Nrl* gene have no rods, but have increased numbers of cones, functionally manifested as a loss of rod function coupled with super-normal cone function (35). Morphologically, *Nrl*^{-/-} photoreceptors have a cone-like nucleus, short and disorganized outer segments, and frequently form rosette-like structures in the retina (35). Electrophysiological studies demonstrate the expression of functional S- and M-opsins (39, 40), whereas gene expression studies confirm the transformation of all rods into cones in *Nrl*^{-/-} retinas (41). As a valuable mammalian cone-dominant model (cones comprise only 2-3% of the total photoreceptor population in the wild-type mouse retina), *Nrl*^{-/-} mouse line has been commonly used for studies of biochemical alterations in cones (17, 42, 43). The present study used *Cnga3*^{-/-}/*Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice to explore the regulation of cGMP/PKG signaling on RyR2 in cones lacking CNG channel. It should be noted that *Cnga3*^{-/-}/*Nrl*^{-/-} mice display the phenotype of loss of cone function, cone degeneration, and apoptotic cone death, similar to that in *Cnga3*^{-/-} mice (17, 18). All mice were maintained under cyclic light (12 h light-dark) conditions. In 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).

The primary antibodies used are listed in Table 1. Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). All other reagents were purchased from Sigma Aldrich (St Louis, MO), Bio-Rad (Hercules, CA), and Thermo Fisher Scientific (Waltham, MA).

PKG inhibitor treatment

Two PKG inhibitors, KT5823 (Sigma Aldrich, St Louis, MO) and (R_p)-8-Br-cGMPS (Santa Cruz Biotechnology, Dallas, TX), were used. (R_p)-8-Br-cGMPS is a cyclic nucleotide analog (an R_p -diastereomer of cGMP), whereas KT5823 is a K-series inhibitor that inactivates the ATP-binding site through competition with ATP (44). These two compounds have been shown to reduce PKG activity in CNG channel-deficient mice and reduce ER stress/cone death (21). Starting at postnatal day 7 (P7), *Cnga3*^{-/-}/*Nrl*^{-/-} mice received intraperitoneal injection of KT5823 (1.0 μ mol/kg body weight/day), (R_p)-8-Br-cGMPS (5.0 μ mol/kg body weight/day), or vehicle for 9 days, as described previously (21). At the end of the treatment, retinas were collected for biochemical evaluations.

Retinal cross-section preparation

Mouse eyes were enucleated (the superior portion of the cornea was marked for orientation before enucleation) and fixed in Prefer (Anatech Ltd., Battle Creek, MI) for 25 min at room temperature. Fixed eyes were then stored in 70% ethanol until processing for sections, as described previously (27). Paraffin-embedded sections (5- μ m thickness) passing vertically through the retina (along the vertical meridian passing through the optic nerve head, to allow an examination of the retina in the dorsal and ventral hemisphere) were prepared with a microtome (Leica, Bannockburn, IL).

TUNEL assay

The terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) was carried out to evaluate photoreceptor apoptotic death, as described previously (45). Paraffin-embedded retinal cross sections and the *In Situ* Cell Death Fluorescein Detection kit (Roche Diagnostics, Mannheim, Germany) were used. The images of the immunohistochemistry were taken with the FV1000 confocal laser scanning microscope (Olympus, Center Valley, PA). The total TUNEL-positive cells in the outer nuclear layer were counted and averaged from at least three sections/eye.

PCR array and quantitative RT-PCR

Total RNA preparation and reverse transcription were performed as described previously (18). The Mouse Unfolded Protein Response RT² ProfilerTM PCR Array (Qiagen, Hilden, Germany), which profiles the expression of 84 genes involved in unfolded protein binding, protein folding, endoplasmic-reticulum-associated protein degradation, and heat shock proteins, was used under the manufacturer's instructions. The quantitative reverse

transcription polymerase chain reaction (qRT-PCR) assays were performed with a real-time PCR detection system (iCycler; Bio-Rad). The primers used are listed in Table 2. The relative gene expression value was calculated based on the Ct method (18).

Retinal protein preparation, SDS-PAGE, and western blot analysis

Retinal protein preparation, SDS-PAGE, and western blot analysis were performed as described previously (21). 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) (Sigma Aldrich), and homogenates were centrifuged at 3000 rpm for 10 min at 4 °C. The resulting supernatant was then centrifuged at 13,000 rpm for 35 min at 4 °C to separate cytosolic (supernatant) and membrane (pellet) fractions. The resulting pellet in the first step and the membrane pellet in the second step were 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 15 s on ice at medium speed using a Masonix XL2000 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 rpm for 35 min at 4 °C. The resulting supernatant was used as the nuclear and 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 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 (Thermo Fisher Scientific, Waltham, MA) was used to detect primary antibodies binding to respective cognate antigens. A Li-Cor Odyssey machine and Li-Cor software (Li-Cor Biosciences, Lincoln, NE) were used for detection and densitometric analysis.

Weri-Rb1 cell culture and cGMP treatment

The Weri-Rb1 (ATCC, Manassas, VA) retinoblastoma cell line was cultured in RPMI-1640 medium (ATCC) with 15% FBS, 2 mM L-glutamine (Mediatech, Inc., Herndon, VA), 0.1% Fungizone (Invivogen, San Diego, CA), 5 μ M plasmocin (Invivogen), 55 μ M β -mercaptoethanol (Invitrogen), and 10 μ g/ml insulin (I5500, Sigma-Aldrich, St. Louis, MO) at 37 °C in a humidified incubator with 5% CO₂, as described (46). For cGMP treatment, cells cultured in RPMI-1640 medium were treated with cGMP (Santa Cruz, Dallas, TX) at various concentrations for 24 hours. At the end of the experiments, cells were harvested for evaluation of gene expression.

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. Results are expressed as means $\pm$ SEM of number of observations. Differences were considered statistically significant when *p* < 0.05. Data were analyzed and graphed using GraphPad Prism® software (GraphPad Software).

Results

Depletion of cGMP suppressed upregulation of RyR2 activity and expression in *Cnga3^{-/-}/Nr1^{-/-}* retinas

To explore the potential regulation of cGMP/PKG signaling and determine whether RyR2 upregulation is a consequence of cGMP/PKG signaling, we analyzed the activity and expression of RyR2 in CNG channel-deficient retinas after cGMP depletion. Genetic deletion of retGC1 (encoded by *Gucy2e*) was used to deplete cGMP in *Cnga3^{-/-}/Nr1^{-/-}* mice (*Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}*). This approach has been shown to abolish the elevation of cGMP level and PKG activity in *Cnga3^{-/-}/Nr1^{-/-}* retina and improve cone survival (19, 21). Retinas of *Cnga3^{-/-}/Nr1^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}*, and *Nr1^{-/-}* mice at P15, P30 and P60 were analyzed for RyR2 activity and expression. The activity of the channel was evaluated by examining the level of phosphorylated protein using immunoblotting (47). Expression was evaluated by examining the mRNA level of the channel using qRT-PCR. We found that depletion of cGMP nearly completely abolished the elevation of phospho-RyR2 in CNG channel-deficient mice. As shown in Fig. 1A, phospho-RyR2 level was doubled in *Cnga3^{-/-}/Nr1^{-/-}* mice, compared with *Nr1^{-/-}* mice, and was reduced by about 50% in *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}* mice. Similarly, RyR2 mRNA level was decreased by about 50% in *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}* retinas, compared with age-matched *Cnga3^{-/-}/Nr1^{-/-}* controls (Fig. 1B).

Treatment with PKG inhibitors suppressed upregulation of RyR2 activity and expression in *Cnga3^{-/-}/Nr1^{-/-}* retinas

In addition to genetic manipulation, we used PKG inhibitors to explore the contribution of PKG signaling. Starting at P7, *Cnga3^{-/-}/Nr1^{-/-}* mice received intraperitoneal injection of PKG inhibitor KT5823 (1.0 μ mol/kg body weight/day), (*R_p*)-8-Br-cGMPS (5.0 μ mol/kg body weight/day), or vehicle for 9 days (21). At the end of the treatment, retinas were collected for evaluation of phospho-RyR2 and RyR2 mRNA levels. We found that the treatment completely abolished the elevation of phospho-RyR2 in CNG channel-deficient mice. The phospho-RyR2 level in *Cnga3^{-/-}/Nr1^{-/-}* mice was reduced by about 65% and 80%, respectively, after KT5823 or (*R_p*)-8-Br-cGMPS treatment, compared with vehicle-treated controls (Fig. 2A). qRT-PCR results showed that the RyR2 mRNA level was decreased by about 30% after treatment with (*R_p*)-8-Br-cGMPS, but not KT5823 (Fig. 2B). Taken together, the results from genetic deletion and pharmacological inhibition experiments showed that cGMP/PKG signaling increased RyR2 activity and expression in *Cnga3^{-/-}/Nr1^{-/-}* retinas.

CaMKII activity was not altered in *Cnga3^{-/-}/Nr1^{-/-}* retinas

Several kinases, including PKG, PKA (protein kinase A, cAMP-dependent protein kinase), and calmodulin-dependent protein kinase II (CaMKII), have been shown to phosphorylate RyR2 *in vitro* and *in vivo* (48–52). We previously showed that the cellular cAMP level was not elevated in CNG channel-deficient mice (19), suggesting that PKA is less likely responsible for the elevated RyR2 activity. In the present study, we examined CaMKII activity in CNG channel-deficient retinas to determine whether this kinase plays a role in RyR2 activation. Retinas of *Cnga3^{-/-}/Nr1^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}*, and *Nr1^{-/-}* mice at

P15, P30, and P60 were analyzed for levels of phospho-CaMKII α and β isoforms by immunoblotting. The analysis showed that the levels of phospho-CaMKII were not different between *Cnga3*^{-/-}/*Nrl*^{-/-} and age-matched *Nrl*^{-/-} controls (Fig. 3), suggesting that this kinase is less likely to be responsible for the upregulation of RyR2. Of note, the levels of phospho-CaMKII were significantly reduced in *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-} retinas, compared with *Cnga3*^{-/-}/*Nrl*^{-/-} and *Nrl*^{-/-} retinas, particularly at P15 and P60 (Fig. 3), suggesting a regulation of cGMP signaling on CaMKII.

Depletion of cGMP and deletion of Ryr2 equivalently suppressed UPR/ER stress in *Cnga3*^{-/-}/*Nrl*^{-/-} retinas

CNG channel-deficient retinas show ER stress (17, 21) and cone protein mislocalization (13, 53, 54), suggesting dysregulation of ER homeostasis and protein folding. Inhibition of cGMP/PKG signaling (21) or RyR2 function (27, 34) suppressed ER stress/improved cone outer segment protein localization. In the present study, we examined the effects of cGMP depletion and *Ryr2* deletion on UPR to determine whether these manipulations exert similar actions on ER homeostasis. Retinas of *Cnga3*^{-/-}/*Nrl*^{-/-}, *Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice at P15 were analyzed for expression of UPR genes using the Mouse Unfolded Protein Response RT² Profiler™ PCR Array. This array profiles the expression of 84 genes involved in unfolded protein binding, protein folding, ER-associated protein degradation, heat shock proteins, and enzymatic regulation of unfolded glycoproteins. We found that the expression of fourteen genes was significantly increased in *Cnga3*^{-/-}/*Nrl*^{-/-} mice (Fig. 4A–B). These fourteen genes were *Atf6b* (encoding activating transcription factor 6 beta), *Bax* (encoding Bcl2-associated X protein), *Calr* (encoding calreticulin), *Cebpb* (encoding CCAAT/enhancer binding protein beta), *Creb3l3* (encoding cAMP responsive element binding protein 3-like 3), *Der1l* (encoding degradation in ER protein 1), *Dnajc3* (encoding DnaJ [Hsp40] homolog, subfamily C, member 3), *Edem1* (encoding ER degradation enhancer, mannosidase alpha-like 1), *Ern2* (encoding ER to nucleus signaling 2), *Ero1lb* (encoding ER oxidoreductase 1 like protein beta), *Ganc* (encoding glucosidase, alpha; neutral C), *Hspb9* (encoding heat shock protein, alpha-crystallin-related, B9), *Srebf2* (encoding sterol regulatory element binding factor 2), and *Uggt2* (encoding UDP-glucose glycoprotein glucosyltransferase 2), with *Ern2* most significantly elevated. These genes are primarily involved in UPR (*Atf6b*, *Calr*, *Hspb9*, and *Dnajc3*), ER-associated protein degradation (*Der1l* and *Edem1*), ER stress-associated cell death (*Cebpb*, *Ern2*, and *Creb3l3*), and reglucosylation of unfolded glycoproteins (*Uggt2*), supporting UPR at the gene expression level. Depletion of cGMP abolished elevation of ten genes among the list (Fig. 4A), while deletion of *Ryr2* abolished elevation of the thirteen genes (Fig. 4B). The expression of Der1-1, Creb3l3, and Uggt2, encoded by *Der1l*, *Creb3l3*, and *Uggt2*, respectively, were further evaluated using immunoblotting. Similar to findings at mRNA levels, the expression levels of Der1-1 and Creb3l3 were increased in CNGA3-deficient retina, and depletion of cGMP or deletion of RyR2 reduced the protein expression levels. However, we did not detect elevation of Uggt2 in CNGA3-deficient retina (Figure 4C). Thus, the data from the gene expression and protein expression levels suggest that depletion of cGMP and deletion of *Ryr2* equivalently suppressed UPR in *Cnga3*^{-/-}/*Nrl*^{-/-} retinas.

We also evaluated the effects of cGMP depletion and *Ryr2* deletion on the ER stress pathway activities. The three hallmarks of ER stress, eIF2 α (eukaryotic translation initiation factor 2 alpha), Ire1 α (serine/threonine-protein kinase/endoribonuclease), and ATF6 (activating transcription factor 6), were examined. At P15, the phospho-Ire1 α and phospho-eIF2 α levels were already increased in *Cnga3*^{-/-}/*Nrl*^{-/-} mice, compared with *Nrl*^{-/-} controls, and the elevations remained at P30 and P60 (Fig. 5A). Depletion of cGMP appeared to differentially affect the pathway activities. The levels of phospho-Ire1 α were reduced in *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-} mice at P15 and P30, but not P60, compared with age-matched *Cnga3*^{-/-}/*Nrl*^{-/-} controls, whereas the levels of phospho-eIF2 α were reduced at P30 and P60, but not at P15 (Fig. 5A). Upon ER stress, ATF6 is cleaved. The cleaved form translocates to the nucleus, where it regulates transcription of ER stress-related genes and subsequent ER stress-related cellular responses (55). The ATF6 cleavage and nuclear localization was significantly increased in *Cnga3*^{-/-}/*Nrl*^{-/-} retinas, and depletion of cGMP or deletion of *Ryr2* effectively inhibited activation of ATF6 (Fig. 5B). The effects of cGMP depletion and *Ryr2* deficiency were further examined by evaluating the expression of CHOP, a member of the C/EBP (CCAAT enhancer-binding protein) transcription factor family induced in ER stress through the Grp78/Bip-phospho-eIF2 α pathway (56). The expression level of CHOP was significantly increased in *Cnga3*^{-/-}/*Nrl*^{-/-} mice. Depletion of cGMP or deletion of *Ryr2* effectively suppressed expression of CHOP (Fig. 5C).

Depletion of cGMP and deletion of *Ryr2* reduced CREB activity in *Cnga3*^{-/-}/*Nrl*^{-/-} retinas

CREB, a 43kDa basic/leucine zipper transcription factor, is activated/phosphorylated by various kinases, including PKG (57). We evaluated the effects of cGMP depletion on CREB activity in CNG channel deficiency. Retinas of *Cnga3*^{-/-}/*Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-}, and *Nrl*^{-/-} mice at P15, P30, and P60 were analyzed for levels of phospho-CREB by immunoblotting. The analysis showed that the phospho-CREB level was increased by one- to two-fold in *Cnga3*^{-/-}/*Nrl*^{-/-} mice, compared with *Nrl*^{-/-} retinas (Fig. 6A). Depletion of cGMP abolished the activation of CREB at P15 and P30, and significantly reduced it at P60 (Fig. 6A), supporting a cGMP-dependent activation of CREB in CNG channel deficiency. CREB activity was also analyzed in *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} retinas. The analysis showed that deletion of *Ryr2* abolished the activation of CREB at P30 (Fig. 6B). However, CREB activity was increased in *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} retinas at P15, compared with age-matched *Cnga3*^{-/-}/*Nrl*^{-/-} controls (Fig. 6B).

Depletion of cGMP and deletion of *Ryr2* led to early-onset cone protection in *Cnga3*^{-/-}/*Nrl*^{-/-} mice

CNG channel-deficient mice display early-onset, progressive cone degeneration (14, 16). In the present study, we evaluated early age cone survival/protection in *Cnga3*^{-/-}/*Nrl*^{-/-} mice after cGMP depletion or *Ryr2* deletion. The expression levels of cone proteins, including cone arrestin (CAR) and M-opsin, and photoreceptor apoptosis were analyzed in mice at P15 and P30. We found that CAR level was increased by about two- and five-fold in *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-} mice, compared with age-matched *Cnga3*^{-/-}/*Nrl*^{-/-} controls, and M-opsin level was increased by about one-fold (Fig. 7A). Similar findings were obtained in mice with *Ryr2* deletion. M-opsin and CAR levels were increased by about one- to two-fold in *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice, compared with age-matched *Cnga3*^{-/-}/*Nrl*^{-/-}

controls (Fig. 7B). The increased expression of CAR after depletion of cGMP or deletion of *Ryr2* was also shown by immunofluorescence labeling (Fig. 7C, the abnormal retinal morphology reflected rosette-like structures formed in mice lacking *Nrl*). Photoreceptor apoptosis/death was evaluated by TUNEL. The number of TUNEL-positive cells detected in the outer nuclear layer (ONL) of *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} retinas was significantly reduced, compared with age-matched *Cnga3*^{-/-}/*Nrl*^{-/-} controls (Fig. 7D). To determine whether microglia cells contribute to the TUNEL-positive labeling, we examined location/activity of these cells in *Cnga3*^{-/-}/*Nrl*^{-/-} and *Nrl*^{-/-} retinas by immunofluorescence labeling using an antibody against the microglia cell marker ionized calcium binding adaptor molecule 1 (Iba1) (58). The assay showed that the Iba1 labeling was mainly observed in the outer plexiform layers (OPL) and the inner plexiform layers (IPL), but not in the ONL, and there was no increased labeling intensity in *Cnga3*^{-/-}/*Nrl*^{-/-} retinas, compared with that in *Nrl*^{-/-} retinas (see Supplementary Fig. 3). These results do not support the contribution of microglia cells to the TUNEL-positive labeling detected in the ONL of *Cnga3*^{-/-}/*Nrl*^{-/-} retinas. Thus, depletion of cGMP or deletion of *Ryr2* effectively reduced early-onset cone degeneration/death in *Cnga3*^{-/-}/*Nrl*^{-/-} mice. Müller glia activate in response to retinal stress with increased expression of glial fibrillary acidic protein (GFAP) in intermediate filaments, and upregulation of GFAP has been previously shown in CNG channel deficient-retinas (13, 21). The present work examined GFAP expression in *Cnga3*^{-/-}/*Nrl*^{-/-} and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice at P30 by immunoblotting. As shown in Fig. 7E, GFAP level was increased by about one-fold in *Cnga3*^{-/-}/*Nrl*^{-/-} mice, compared with controls. This elevation was completely abolished by deletion of *Ryr2*. This observation was in line with the reduced photoreceptor death after *Ryr2* deletion.

Treatment with cGMP increased *Ryr2* expression in cultured photoreceptor-derived Weri-Rb1 cells

The regulation of cGMP signaling on RyR2 was also evaluated in an *in vitro* cell culture system using the photoreceptor-derived Weri-Rb1 cells (45, 59). This approach examines the regulation in a system in which cGMP signaling is artificially elevated. Cells were treated with various concentrations of cGMP for 24 hours and were harvested at the end of treatment for evaluation of *Ryr2* expression by qRT-PCR. We found that treatment with cGMP increased RyR2 mRNA level by 10-to-15-fold (Fig. 8). This treatment did not alter calreticulin (an ER chaperone protein) mRNA level, increased IP₃R1 mRNA level, and decreased SERCA2 mRNA level (Fig. 8).

Discussion

We and others previously demonstrated that CNG channel-deficient retinas undergo remarkable elevation of cellular cGMP and increased PKG activity (17, 19, 20), and further provided evidence that cGMP/PKG signaling contributes to ER stress and cone degeneration (19, 21). We also documented the upregulation of RyR2 in CNG channel deficiency, and its contribution to ER stress/cone degeneration and protein mislocalization (27, 34). In the current work, we tested our hypothesis that upregulation of RyR2 mediates cGMP/PKG signaling-induced ER stress and cone death. We examined the activity and expression of RyR2 in CNG channel-deficient retinas after cGMP/PKG inhibition. We also examined the

effects of cGMP depletion and *Ryr2* deletion on UPR/ER stress pathway activity, downstream signaling activity, and cone survival/death. We demonstrated that RyR2 activity and expression are highly regulated by cGMP/PKG signaling, and depletion of cGMP and deletion of *Ryr2* equivalently suppressed UPR/ER stress and activation of CHOP, resulting in early-onset cone protection. These findings support the view that RyR2/ER calcium regulation is a significant downstream effector of cGMP/PKG signaling, and upregulation of RyR2 contributes to cGMP/PKG signaling-induced UPR/ER stress. Although regulation of RyR by cGMP/PKG signaling has been investigated in other tissues (51, 60, 61), no study investigating this phenomenon in retina/photoreceptors has been reported. This is an important area to explore because perturbation of cGMP/PKG signaling and cellular calcium/ER calcium dysregulation represent significant cellular defects in many distinct types of retinal degenerations (62, 63). Our work for the first time demonstrates the regulation of cGMP/PKG signaling on RyR2 in the retina, and its significance in retinal degeneration.

The regulation of cGMP/PKG on RyR2 activity and expression was demonstrated by cGMP depletion and PKG inhibition. The regulation of RyR2 gene expression by cGMP signaling was also shown in cultured photoreceptor-derived cells. Two interesting findings were obtained. First, the cGMP/PKG signaling regulation of RyR2 was significant and powerful. RyRs have been shown to be primarily regulated by cytosolic Ca^{2+} and protein kinases, including PKG (60, 61, 64, 65). Depletion of cGMP or inhibition of PKG nearly completely abolished the channel upregulation in CNG channel deficiency, although the cellular calcium homeostasis perturbation was still present. Second, cGMP/PKG signaling regulated both activity and expression of the channel. We observed that the two PKG inhibitors had more significant effects on the channel's activity than on gene expression, and Rp-8-Br-cGMPS, but not KT5823, suppressed the gene expression. How KT5823 affected the channel expression differently from (Rp)-8-Br-cGMPS is not known at this time, but the two compounds exert their inhibition of PKG activity through different mechanisms (44). How cGMP/PKG signaling regulates the channel at a gene expression level remains unknown and merits further investigation. It should be pointed out that cGMP/PKG signaling also regulates activity of IP₃R1, the other ER calcium efflux channel, in CNG channel deficiency. Unlike RyR2, IP₃R1 phosphorylation decreases the channel's activity and reduces Ca^{2+} release from the ER, though the reported data were controversial (66–71). Our previous report demonstrated that depletion of cGMP or inhibition of PKG increased phosphorylation of IP₃R1 (21), thereby reducing the channel activity. The mechanisms underlying how cGMP/PKG signaling increases phosphorylation of RyR2 and decreases phosphorylation of IP₃R1 remain to be determined; the former likely results from direct phosphorylation by PKG, and the latter might involve a more complex mechanism, involving a number of regulatory enzymes that directly interact with IP₃R1. The protein kinases PKG, PKA, and CaMKII have been shown to phosphorylate RyRs (48–52). Our previous studies showed no significant elevation of cAMP in CNG channel-deficient retina (19), suggesting a less important role of PKA. In this study, we examined the level of CaMKII in the retinas. We found that the level of phospho-CaMKII was not different between *Cnga3*^{-/-}/*Nrl*^{-/-} and *Nrl*^{-/-} mice, suggesting that the increased level of phospho-RyR2 is less likely to result from CaMKII signaling. However, depletion of cGMP was shown to lower the level of phospho-

CaMKII in the retina, supporting a regulation of cGMP signaling on CaMKII. Nevertheless, data from this study favor the view that CaMKII is less likely to be responsible for RyR2 upregulation in CNG channel deficiency.

Depletion of cGMP and deletion of *Ryr2* suppressed CREB signaling. CREB is a 43kDa basic leucine zipper transcription factor that is expressed in most tissues, and regulates cell proliferation, differentiation, and survival. It is activated by phosphorylation from various kinases, including PKA, PKG, mitogen-activated protein kinase signaling pathway, and CaMKII (57). In particular, PKG was found to phosphorylate CREB in neuronal cells, including in the hippocampus (72) and cerebellar neurons (73). Our data showing reduced CREB activity/phospho-CREB levels after depletion of cGMP support the cGMP/PKG regulation of CREB in the retina. In addition to protein kinases, CREB has been shown to be regulated by other cellular events, including ER stress and calcium signaling/ER calcium signaling (74, 75). Data from the present study showed reduced CREB activity after *Ryr2* deletion, supporting a regulation of RyR2/ER calcium homeostasis on CREB. Thus, the upregulation of CREB in CNG channel deficiency is likely a consequence of both elevated cGMP/PKG signaling and enhanced RyR2 function/ER calcium dysregulation. CREB signaling has been shown to have pro-survival effects (76–78) and anti-survival effects (79–81). Alterations of CREB activity have been implicated in human neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (82), and in animal models of retinal degeneration (83–85). Upregulation of CREB was observed in canine models of retinitis pigmentosa and an N-methyl-D-aspartate-induced rat model of retinal degeneration (83, 84). However, downregulation of CREB has been reported in retinal degeneration 10 (*rd10*) mice (85). In the present work, we showed that CREB activity was significantly elevated in CNG channel-deficient retinas, and depletion of cGMP or deletion of *Ryr2* abolished upregulation of CREB activation. The significance of CREB upregulation in CNG channel deficiency and its role in photoreceptor survival/degeneration are not clear. Because the suppression of CREB upregulation by depletion of cGMP or deletion of *Ryr2* was accompanied by improved cone survival/reduced cone death, one may presume that upregulation of CREB in CNG channel deficiency is likely anti-survival. However, experiments using genetic or chemical approaches to manipulate CREB activity would provide more definitive information.

In summary, we demonstrate the regulation of cGMP/PKG signaling on RyR2 in CNG channel-deficiency. Our findings support the view that upregulation of RyR2 contributes, at least in part, to cGMP/signaling-induced ER stress and photoreceptor degeneration. This work provides insight into how elevation of cGMP/PKG signaling induces ER stress and photoreceptor degeneration in CNG channel deficiency. Because perturbation of cGMP/PKG signaling and cellular calcium dysregulation are common throughout multiple neurodegenerative diseases, including many distinct types of photoreceptor degenerations, it is critical to understand how these dynamic signaling pathways interact and direct cell fate and determine the pathogenesis components.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used:

ATF6	activating transcription factor 6
CaMKII	calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CAR	cone arrestin
cGMP	cyclic guanosine monophosphate
CHOP	CCAAT-enhancer-binding protein homologous protein
CNG	cyclic nucleotide-gated
CREB	cyclic adenosine monophosphate response element binding protein
ER	endoplasmic reticulum
eIF2α	eukaryotic translation initiation factor 2 alpha
GFAP	glial fibrillary acidic protein
HRGP	human red/green pigment
Iba1	ionized calcium binding adaptor molecule 1
IP₃R	inositol-1,4,5-trisphosphate receptor
Ire1α	serine/threonine-protein kinase/endoribonuclease 1α
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RetGC1	retinal guanylate cyclase 1
RyR2	ryanodine receptor 2
SERCA	sarco/endoplasmic reticulum Ca ²⁺ -ATPase
TUNEL	terminal deoxynucleotidyltransferase dUTP nick end-labeling
UPR	unfolded protein response

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Figure 1. Depletion of cGMP suppressed upregulation of RyR2 in *Cnga3*^{-/-}/*Nr1h1*^{-/-} retinas. Expression of phospho-RyR2 and RyR2 mRNA levels in retinas of *Nr1h1*^{-/-}, *Cnga3*^{-/-}/*Nr1h1*^{-/-}, and *Cnga3*^{-/-}/*Nr1h1*^{-/-}/*Gucy2e*^{-/-} mice at P15, P30, and P60 were analyzed by immunoblotting and qRT-PCR. **(A)** Increased phospho-RyR2 level in *Cnga3*^{-/-}/*Nr1h1*^{-/-} retinas and deletion of retGC1 suppressed the upregulation. Shown are representative immunoblotting images of phospho-RyR2 and corresponding quantitative analysis. **(B)** qRT-PCR results showing increased mRNA level of RyR2 in *Cnga3*^{-/-}/*Nr1h1*^{-/-} retinas. Deletion of retGC1 suppressed the upregulation. Data are represented as mean ± SEM 3-4 assays using retinas prepared from 8-10 mice in each group (**P* < 0.05, ***P* < 0.01).

Figure 2. Treatment with PKG inhibitor suppressed upregulation of RyR2 in *Cnga3*^{-/-}/*Nr1h1*^{-/-} retinas.

Cnga3^{-/-}/*Nr1h1*^{-/-} mice received intraperitoneal injection of KT5823 (1.0 μmol/kg body weight/day), (Rp)-8-Br-cGMPS (5.0 μmol/kg body weight/day), or vehicle for 9 days, starting at P7, and were then analyzed for phospho-RyR2 and RyR2 mRNA levels in the retina by immunoblotting and qRT-PCR. **(A)** Increased phospho-RyR2 level in *Cnga3*^{-/-}/*Nr1h1*^{-/-} retinas and reduced level after treatment with PKG inhibitors. Shown are representative immunoblotting images of phospho-RyR2 and the corresponding quantitative analysis. **(B)** qRT-PCR results showing an increased mRNA level of RyR2 in *Cnga3*^{-/-}/*Nr1h1*^{-/-} retinas and decreased expression after treatment with PKG inhibitors. Data are represented as mean ± SEM 3-4 assays using retinas prepared from 6-8 mice in each group (**P* < 0.05, ***P* < 0.01).

Figure 3. Phospho-CaMKII level was not altered in *Cnga3*^{-/-}/*Nrl*^{-/-} retinas. Expression levels of phospho-CaMKII in retinas of *Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-} mice at P15, P30, and P60 were analyzed by immunoblotting. Shown are representative immunoblotting images and corresponding quantitative analysis. Data are represented as mean ± SEM 3-4 assays using retinas prepared from 8-10 mice in each group (**P* < 0.05).

Figure 4. Depletion of cGMP and deletion of *Ryr2* suppressed upregulation of UPR genes in *Cnga3^{-/-}/Nr1^{-/-}* retinas.

Expression of UPR genes were evaluated in retinas of *Nr1^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}*, and *Cnga3^{-/-}/Nr1^{-/-}/Ryr2^{fl/fl}/Hrgp^{cre}* mice by PCR array and immunoblotting. (A-B) Depletion of cGMP (A) or deletion of *Ryr2* (B) suppressed upregulation of UPR genes in *Cnga3^{-/-}/Nr1^{-/-}* retinas. Shown are results of PCR array obtained from retinas of P15 *Nr1^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}*, and *Cnga3^{-/-}/Nr1^{-/-}/Ryr2^{fl/fl}/Hrgp^{cre}* mice. (C) Immunoblotting evaluation of expression levels

of *Derl-1*, *Creb3l3*, and *Ugt2* in the retinas of P15 *Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-} mice. Shown are representative immunoblotting images and the corresponding quantitative analysis. Data are represented as mean $\pm$ *SEM* 3-4 assays using retinas prepared from 6-10 mice in each group (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Figure 5. Depletion of cGMP and deletion of *Ryr2* suppressed ER stress and CHOP activation in *Cnga3^{-/-}/Nr1^{-/-}* retinas.

Expression of ER stress markers and CHOP were evaluated in retinas of *Nrf^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}*, and *Cnga3^{-/-}/Nr1^{-/-}/Ryr2^{fl/fl}/Hrgp^{cre}* mice by immunoblotting. (A) Levels of phospho-eIF2α and phospho-Ire1α were evaluated in retinas of *Nrf^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}*, and *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}* mice at P15, P30, and P60. Shown are representative immunoblotting images and the corresponding quantitative analysis. (B) ATF6 cleavage and nuclear translocation were evaluated in retinas of P15 *Nrf^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}*, *Cnga3^{-/-}/Nr1^{-/-}/Gucy2e^{-/-}*, and *Cnga3^{-/-}/Nr1^{-/-}/Ryr2^{fl/fl}/Hrgp^{cre}*

mice. Shown are representative immunoblotting images and the corresponding quantitative analysis. (C) The expression levels of CHOP were evaluated in retinas of P15 *Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice. Shown are representative immunoblotting images and corresponding quantitative analysis. Data are represented as mean ± SEM 3-4 assays using retinas prepared from 6-10 mice in each group (**P* < 0.05, ***P* < 0.01).

Figure 6. Depletion of cGMP and deletion of *Ryr2* suppressed upregulation of CREB activity in *Cnga3*^{-/-}/*Nrl*^{-/-} retinas.

Expression of phospho-CREB and total CREB were analyzed in retinas of *Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice at P15, P30, and P60 by immunoblotting. **(A)** Depletion of cGMP suppressed upregulation of phospho-CREB in *Cnga3*^{-/-}/*Nrl*^{-/-} retinas. Shown are representative immunoblotting images of phospho-CREB and the corresponding quantitative analysis. **(B)** Deletion of *Ryr2* increased the phospho-CREB level in *Cnga3*^{-/-}/*Nrl*^{-/-} mice at P15, but reduced it at P30. Shown are representative immunoblotting images of phospho-CREB and the corresponding quantitative analysis. Data are represented as mean ± SEM 3-4 assays using retinas prepared from 8-10 mice in each group (**P* < 0.05).

Figure 7. Depletion of cGMP and deletion of *Ryr2* led to early-onset cone protection in *Cnga3*^{-/-}/*Nrl*^{-/-} mice.

Cone survival/death and retinal stress were analyzed in *Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice at P15 and P30. (A) Depletion of cGMP increased expression levels of cone proteins in *Cnga3*^{-/-}/*Nrl*^{-/-} mice. Shown are representative immunoblotting images of CAR and M-opsin and the corresponding quantitative analysis. (B) Deletion of *Ryr2* increased expression levels of cone proteins in *Cnga3*^{-/-}/*Nrl*^{-/-} mice. Shown are representative immunoblotting images of

CAR and M-opsin and the corresponding quantitative analysis. **(C)** Shown are representative confocal images of immunofluorescence labeling of CAR (Arr3) in *Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}/*Gucy2e*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice at P30. **(D)** Deletion of *Ryr2* reduced cone apoptosis in *Cnga3*^{-/-}/*Nrl*^{-/-} mice. Cone apoptosis was evaluated by TUNEL labeling on retinal cross sections of *Nrl*^{-/-}, *Cnga3*^{-/-}/*Nrl*^{-/-}, and *Cnga3*^{-/-}/*Nrl*^{-/-}/*Ryr2*^{fl/fl}/*Hrgp*^{cre} mice at P30. Shown are representative confocal images of TUNEL labeling and the corresponding quantitative analysis. ONL, outer nuclear layer; INL, inner nuclear layer. **(E)** Deletion of *Ryr2* reduced activation of Müller cells in *Cnga3*^{-/-}/*Nrl*^{-/-} mice. Shown are representative immunoblotting images of GFAP and corresponding quantitative analysis in mice at P30. Data are represented as means ± SEM 3-4 assays using retinas prepared from 8-11 mice in each group (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Figure 8. Treatment with cGMP increased expression of RyR2 mRNA in cultured photoreceptor-derived Weri-Rb1 cells.

Cells were treated with various concentrations of cGMP for 24 hours and were harvested at the end of treatment for gene expression evaluation by qRT-PCR. Shown are mRNA expression levels of RyR2, IP₃R1, SERCA2, and calreticulin. Data are represented as mean $\pm$ SEM of three assays from two independent experiments (* P < 0.05, ** P < 0.01, compared with untreated controls).

Table 1.

List of primary antibodies

Antibody	Provider/Reference	Catalog No.	Dilutions used in IB and IF
M-opsin	EMD Millipore, Billerica, MA	AB5405	1: 500 (IB)
Cone arrestin (CAR)	EMD Millipore, Billerica, MA	AB15282	1: 500 (IB) 1:200 (IF)
p-eIF2 α	Cell signaling, Danvers, MA	3398	1: 500 (IB)
p-Ire1 α	Abcam, Cambridge, MA	ab48187	1: 500 (IB)
p-CREB	Cell signaling, Danvers, MA	91985	1: 500 (IB)
CREB	Cell signaling, Danvers, MA	9197	1: 500 (IB)
p-RyR2	Abcam, Cambridge, MA	ab59225	1: 500 (IB)
p-CaMKII	Cell signaling, Danvers, MA	12716	1: 500 (IB)
GFAP	Dako	Z0334	1: 500 (IB)
Derl-1	Abcam, Cambridge, MA	ab176732	1: 500 (IB)
Creb3l3	Novus, Centennial, CO	NBP2-16008	1: 500 (IB)
Uggt2	Thermo Fisher Scientific, Waltham, MA	PA5-27960	1: 500 (IB)
ATF6	Active Motif, Carlsbad, CA	40962	1: 500 (IB)
CHOP	Cell signaling, Danvers, MA	2985	1: 500 (IB)
TBP	Thermo Fisher Scientific, Waltham, MA	MA1-10883	1: 1000 (IB)
Iba1	FUJIFILM Wako Chemicals, Richmond, VA	019-19741	1:200 (IF)
CNGA3	Matveev et al., (43)	-	1:200 (IF)
β -actin	Abcam, Cambridge, MA	ab6276	1: 2000

Table 2.

List of primers used for qRT-PCR

Gene symbols	Species	Forward primer	Reverse primer
<i>Hprt1</i>	mouse	GCAAACCTTTGCTTCCCTGGTT	CAAGGGCATATCCAACAACA
<i>Ryr2</i>	mouse	CTACCCGAACCTCCAGCGATACT	GCAAAAGAAGGAGATGATGGTGTG
<i>Hprt1</i>	human	GCTATAAATTCTTTGCTGACCTGCTG	AATTACTTTTATGTCCCCTGTTGACTGG
<i>Itpr1</i>	human	ACGCTGACATCCTGATTGAG	ATTCCCTCACACTCTTGCTG
<i>Ryr2</i>	human	TGGAGAAATCAGAAGGGCAAG	CTTATCAGTTGAAGACCGGGAG
<i>Serca2</i>	human	ATCTTTGAATCCCCATACCCG	CGACATAGAGGATCAGGAAGTG
<i>Calr</i>	human	CCTATGATAACTTTGGCGTGC	TTGTCCTTCATTGTTTCTCTGC