Guanylate Cyclase Activating Protein 2 Contributes to Phototransduction and Light Adaptation in Mouse Cone Photoreceptors

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Running title: GCAP1 and GCAP2 regulate mouse cone phototransduction

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ABSTRACT

Light adaptation of photoreceptor cells is mediated by Ca2+-dependent mechanisms. In darkness, Ca2+ influx through cGMP-gated channels into the outer segment of photoreceptors is balanced by Ca2+ extrusion via Na+/Ca2+ K+ exchangers (NCKXs). Light activates a G protein signaling cascade, which closes cGMP-gated channels and decreases Ca2+ levels in photoreceptor outer segment because of continuing Ca2+ extrusion by NCKXs. Guanylate Cyclase Activating Proteins (GCAPs) then upregulate cGMP synthesis by activating retinal membrane guanylate cyclases (RetGCs) in low Ca2+. This activation of RetGC accelerates photoresponse recovery and critically contributes to light adaptation of the nighttime rod and daytime cone photoreceptors. In mouse rod photoreceptors, GCAP1 and GCAP2 both contribute to the Ca2+-feedback mechanism. In contrast, only GCAP1 appears to modulate RetGC activity in mouse cones because evidence of GCAP2 expression in cones is lacking. Surprisingly, we found that GCAP2 is expressed in cones and can regulate light sensitivity and response kinetics as well as light adaptation of GCAP1-deficient mouse cones. Further, we show that GCAP2 promotes cGMP synthesis and cGMP-gated channel opening in mouse cones exposed to low Ca2+. Our biochemical model and experiments indicate that GCAP2 significantly contributes to the activation of RetGC1 at low Ca2+ when GCAP1 is not present. Of note, in wild-type mouse cones, GCAP1 dominates the regulation of cGMP synthesis. We conclude that under normal physiological conditions, GCAP1 dominates the regulation of cGMP synthesis in mouse cones, but if its function becomes compromised, GCAP2 contributes to the regulation of phototransduction and light adaptation of cones.

INTRODUCTION

Guanylate Cyclase Activating Proteins (GCAPs) are EF-hand proteins that regulate cGMP synthesis by retinal membrane Guanylate (Guanylyl) Cyclases (RetGCs) in a Ca2+-dependent manner (1-6). In low Ca2+, when the active EF-hand sites of the GCAP protein are not occupied by Ca2+, GCAPs activate RetGCs and promote the synthesis of cGMP. High Ca2+ blocks the activation of GC by GCAPs and only a low basal level of cGMP synthesis is maintained in the cells. The presence of several GCAP isoforms in photoreceptor cells has
been well established (7-10). The diversity of GCAPs is particularly apparent in fish photoreceptors, where at least seven different GCAP genes are expressed (7). Human photoreceptors express GCAP1-3 whereas only GCAP1 and GCAP2 are present in mouse photoreceptor cells (8,10). Several mutations in the GUCA1A gene encoding for GCAP1 cause severe hereditary blinding diseases, including Leber Congenital Amaurosis (LCA), Macular Dystrophy (MD) and cone-rod dystrophies (11-18). Although significant advances have been made in understanding the etiology of these diseases, it is still not clear why mutations in GUCA1A preferentially lead to cone, rather than rod, dystrophies and loss of daytime vision.

GCAPs-mediated regulation of cGMP synthesis in the photoreceptors has been shown to be the single most important Ca\(^{2+}\)-mediated pathway of light adaptation (19,20). In darkness, steady state cGMP concentration in photoreceptor outer segments is maintained by a low basal synthesis of cGMP by RetGCs and its hydrolysis by phosphodiesterase (PDE6). Light activates a G protein signaling cascade leading to the increased hydrolysis rate of cGMP by PDE6 and a decline of the rod and cone outer segment cGMP concentration. Consequently, cGMP-gated channels in the outer segment plasma membrane close, leading to a decreased inflow of Na\(^+\) and Ca\(^{2+}\) into the outer segments (reviewed in (21)). As Ca\(^{2+}\) ions are continuously extruded from outer segments by Na\(^+\)/Ca\(^{2+}\), K\(^+\) exchangers (22-25), Ca\(^{2+}\) level drops and Mg\(^{2+}\) replaces Ca\(^{2+}\) in the Ca\(^{2+}/\)Mg\(^{2+}\) binding sites of GCAPs (26). The Mg\(^{2+}\)-GCAPs activate GCs to accelerate cGMP synthesis, promoting the recovery of the photoreceptor cell to its dark-adapted state after a transient light stimulus or preventing a closure of all cGMP-gated channels during continuous illumination.

It is believed that mouse rods express both GCAP1 and GCAP2 whereas mouse cones express only GCAP1 in their outer segments (9,10). In rods, GCAP1 and GCAP2 regulate the cGMP synthesis in a relay fashion. Early in the photoresponse or at dim background light, when Ca\(^{2+}\) level is only slightly lower than in darkness, GCAP1-mediated feedback dominates. Later in the photoresponse or at brighter background light, when Ca\(^{2+}\) drops to lower levels, GCAP2-mediated feedback is also engaged (19,27). This model is consistent with the higher Ca\(^{2+}\) affinity of GCAP2 compared to GCAP1 (18,28,29). Although previous studies have suggested that GCAP2 may not be substantially present in normal or Nrl\(^{−/−}\) mouse cone outer segments (10,30,31), direct genetic and functional approaches have not been used to test whether GCAP2 has any physiological role in mouse cones. Here, we aimed to determine the contribution of GCAP1 and GCAP2 in mouse cone phototransduction and light adaptation by using a comprehensive electrophysiology, genetic, biochemistry and single-cell immunohistochemistry study.

RESULTS

GCAP1 and GCAP2 are expressed in mouse cones

GCAP1 is expressed in outer segments of vertebrate rods and cones from zebra fish to human (8,9). However, the expression pattern of GCAP2 varies among different species (9). Previous studies have shown contradicting results about its presence in mouse photoreceptors (9,10,32). Thus, we sought to determine the expression pattern of GCAP2 in mouse cones by single-cell immunohistochemistry in retinas from wild-type (WT) control, Gcap1\(^{−/−}\), Gcap2\(^{−/−}\) and GCAP1/2 double knockout (Gcaps\(^{−/−}\)) mice. The top two panels of Fig. 1 demonstrate expression of GCAP2 in WT control and Gcaps\(^{−/−}\) cones based on the co-localization of mouse cone arrestin (mCAR, green) and GCAP2 (red) antibodies. Additional GCAP2 signal around the cones is from rod photoreceptors that were sometimes surrounding the cones even after the mechanical cell isolation (see Experimental Procedures). We observed overlap between the cone arrestin and GCAP2 signals in both WT control and Gcap1\(^{−/−}\) cones, suggesting that GCAP2 is expressed in mouse cones. As expected, the GCAP2 signal was not observed in Gcap2\(^{−/−}\) or Gcaps\(^{−/−}\) cones (Fig. 1 bottom two panels), thereby confirming the specificity of the GCAP2 antibody (33). Together, these results clearly demonstrate that GCAP2 is expressed in mouse cones.
GCAP1 and GCAP2 regulate the kinetics and sensitivity of mouse cone phototransduction

To determine the specific roles of GCAP2 and GCAP1 in mouse cone phototransduction, we compared light responses of dark-adapted cones from WT control, Gcap1<sup>−/−</sup>, Gcap2<sup>−/−</sup>, and Gcaps<sup>−/−</sup> mice using ex vivo Electroretinography (ERG) recordings. To isolate the cone photoreceptor component of the ex vivo ERG signal, we used synaptic blockers and Ba<sup>2+</sup> to remove b- and c-waves and rod-saturating background light. Key experiments and the light adaptation studies were also done in Gnat1<sup>−/−</sup> genetic background to remove the rod component of the ERG signal. As has been shown previously (19), simultaneous deletion of GCAP1 and GCAP2 slowed down light response recovery and increased the sensitivity of cones to light flashes (see Fig. 2d and Table 1). Removal of GCAP1 alone increased time-to-peak (t<sub>p</sub>) of the responses elicited by dim light (Fig. 2d, h and Table 1) and increased the sensitivity of cones almost as much as the deletion of both GCAP1 and GCAP2 (Fig. 2d and Table 1). However, the recovery kinetics of the late tail phase of the responses in Gcap1<sup>−/−</sup> cones was not decelerated for both dim flashes (Fig. 2d) and bright saturating flashes (Fig. 2e). Afterdepolarization, or response recovery overshoot, which was often present both in control and GCAP-deficient cones prevented us from fitting exponential function to the late tail phase of the responses to estimate the response recovery time constant (t<sub>rec</sub>). However, the faster overall kinetics of Gcap1<sup>−/−</sup> cone dim flash responses as compared to that of Gcaps<sup>−/−</sup> cones was demonstrated by their shorter integration time when compared to Gcaps<sup>−/−</sup> mice (Table 1). Isolating the cone component of the response by using Gnat1<sup>−/−</sup> mice confirmed that the responses from GCAP1-deficient cones are still substantially faster than these of cones lacking both GCAP1 and GCAP2 (Fig. 2f, g). These results suggest that GCAP2 can shape the light response kinetics specifically in brighter light, at least in the absence of GCAP1. On the other hand, the sensitivity of dark-adapted cones to dim light flashes appears to be mediated mainly by GCAP1 (2h and Table 1). We also recorded light responses from Gcap2<sup>−/−</sup> mice (Fig. 2c) but did not find any significant changes of response kinetics or light sensitivity of GCAP2-deficient cones compared to WT controls (Fig. 2d, e, h and Table 1). Thus, we conclude that GCAP1 can support normal cone photoresponses in the absence of GCAP2.

GCAP2 promotes GMP synthesis in low Ca<sup>2+</sup> in mouse cones

Biochemical experiments have demonstrated that GCAP proteins activate cGMP synthesis of RetGCs in low Ca<sup>2+</sup> (1,6). Here, we asked whether GCAP2 could promote cGMP synthesis in intact mouse cones. To assess the Ca<sup>2+</sup>-mediated acceleration of cGMP synthesis in cones, we determined the change of the maximal saturated cone photoresponse amplitude (r<sub>max</sub>) when the retinas were switched from normal perfusion solution with 1.2 mM [Ca<sup>2+</sup>]<sub>e</sub> to low ~30 nM [Ca<sup>2+</sup>]<sub>e</sub> in ex vivo ERG experiments. Such a treatment causes rapid reduction in the level of Ca<sup>2+</sup> in photoreceptor outer segments and the subsequent GCAPs-mediated upregulation of cGMP synthesis (41). The t<sub>max</sub> is proportional to the cGMP-mediated channel current, and thus, increased cGMP concentration caused by accelerated cGMP synthesis rate is expected to increase r<sub>max</sub>. We determined t<sub>max</sub> from saturated cone responses elicited by periodic bright test flashes in dark-adapted mouse retinas before and after low Ca<sup>2+</sup> exposure. In control Gnat1<sup>−/−</sup> retinas, t<sub>max</sub> increased about 4-fold after a low Ca<sup>2+</sup> exposure (Fig. 3, black squares), demonstrating the upregulation of cGMP synthesis and subsequent opening of the cGMP-gated channels. However, the cells could not maintain such a high CNG channel current for long and eventually t<sub>max</sub> declined under low Ca<sup>2+</sup>. When cones lacking both GCAP1 and GCAP2 (from Gcaps<sup>−/−</sup>Gnat1<sup>−/−</sup> retinas) were exposed to low Ca<sup>2+</sup>, a much more subtle increase of t<sub>max</sub> was observed (Fig. 3, green squares), consistent with the lack of upregulation of cGMP synthesis in low Ca<sup>2+</sup>. In the absence of both GCAPs. Notably, when we exposed Gcap1<sup>−/−</sup>Gnat1<sup>−/−</sup> retinas to low Ca<sup>2+</sup>, we observed substantial increase in r<sub>max</sub> that was comparable to that in control Gnat1<sup>−/−</sup> mice (Fig. 3, red squares). These results demonstrate that Ca<sup>2+</sup>-feedback mediated by GCAP2 can promote acceleration of the cGMP synthesis in intact mouse cones in the absence of GCAP1.

GCAP2 contributes to mouse cone light adaptation in bright background light

GCAP-mediated Ca<sup>2+</sup> feedback dominates the regulation of rod and cone photoreceptor
sensitivity in response to fast increments or decrements of background light (19, 34). However, the distinct contributions of GCAP1 and GCAP2 to the light adaptation capacity of mouse cones is not known. To address this question, we determined how the sensitivity of cones is regulated by background light in isolated retinas from control mice expressing both GCAPs, and from mice lacking either both GCAPs or only GCAP1. All mice were on Gnat1<sup>−/−</sup> background to eliminate rod signaling and facilitate the quantification of cone light adaptation. When mouse cones are exposed to a step of light, they produce an initial hyperpolarizing response peak, followed by partial relaxation to a plateau (Fig. 4a-c). This relaxation was attenuated after removal of both GCAPs (Fig. 4b), consistent with the dominant role of the GCAPs-mediated feedback in cone light adaptation. Notably, GCAP1-deficient cones exhibited prominent relaxation after the peak of the response comparable to that in control cones, indicative of efficient light adaptation. We quantified the relaxation magnitude and kinetics by fitting a sum of two exponential functions from the peak to the plateau of the step responses using Eq. 3 (see Fig. 4a-c). Although we used two exponential function, the relaxation was dominated by the exponential term with the faster of the two time constants (τ<sub>1</sub>). Thus, we used τ<sub>1</sub> to assess the kinetics of relaxation, and the amplitude from peak to the plateau (A) normalized by the peak amplitude (A<sub>p</sub>) to assess the magnitude of relaxation (see Eq. 3). The A<sub>1p</sub> was similar between control (76 ± 1%) and Gcap<sup>−/−</sup> (80 ± 1%) mice but significantly smaller in the absence of both GCAP1 and GCAP2 (27 ± 5%). This result demonstrates that the expression of GCAP2 in GCAP1-deficient cones was sufficient to promote robust light adaptation as demonstrated by the substantial relaxation of their response in steady background light. However, the kinetics of the relaxation was decelerated significantly by the deletion of GCAP1 alone (from 165 ± 30 ms in control to 495 ± 20 ms in Gcap<sup>−/−</sup> mice) whereas the value for τ<sub>1</sub> was not statistically different in Gcaps<sup>−/−</sup> cones (423 ± 30 ms) as compared to that in Gcap<sup>−/−</sup> cones. This result is consistent with the dominant role of GCAP1 in driving the rapid light adaptation of mouse cones.

To quantify the efficiency of light adaptation, we measured the sensitivity of cones to light flashes at 4.5 s after the step onset at different background light intensities. The sensitivity normalized to the sensitivity in darkness declined steeper in Gcaps<sup>−/−</sup> cones than in control or Gcap<sup>−/−</sup> cones (Fig. 4d). As expected, based on their higher sensitivity in darkness, the operating range of Gcap<sup>−/−</sup> cones was shifted to dimmer background light intensities. However, the slope of the adaptation curve was not changed, and the adaptation capacity was clearly better in Gcap<sup>−/−</sup> mice than in Gcaps<sup>−/−</sup> mice. These results indicate that GCAP2 can contribute to the light adaptation of mouse cones in the absence of GCAP1. We did not have Gcap<sup>−/−</sup> Gnat1<sup>−/−</sup> mice. Thus, in an effort to investigate the role of GCAP2 in light adaptation, we compared light adaptation between Gcap<sup>−/−</sup> and WT mice (on Gnat1<sup>−/−</sup> background). In those experiments, we did not observe any change in light adaptation caused by the deletion of GCAP2 (data not shown), consistent with our flash response data showing only negligible phenotype in GCAP2-deficient cones (Fig. 2c-e and h).

**GCAP1 and GCAP2 compete for activation of RetGC1 in low Ca<sup>2+</sup>**

Our results demonstrate that GCAP2 is expressed in mouse cones and that it can contribute to the Ca<sup>2+</sup>-dependent activation of RetGCs and phototransduction feedback in their outer segments. However, it remained unclear whether the expression level of GCAP2 in WT cones is sufficient to contribute to the overall Ca<sup>2+</sup> feedback. A simple biochemical model (see Experimental Procedures for details) predicts that quite small concentration of GCAP2, ~0.1 – 0.5 µM in the cone outer segment, could explain the ~4-fold increase of r<sub>max</sub> in low Ca<sup>2+</sup> observed in Gcap<sup>−/−</sup> cones (see Fig. 3). Based on the model prediction, we designed a biochemical experiment to assess the extent of activation of the native RetGC1 (the predominant guanylate cyclase isozyme expressed in the cones (30,31,35)) by recombinant GCAP1 and GCAP2. We used photoreceptor membranes from Gcaps<sup>−/−</sup> RetGC2<sup>−/−</sup> mouse retinas retaining only RetGC1 isozyme to measure cGMP synthesis by RetGC1 in low Ca<sup>2+</sup> at normal physiological 0.9 mM Mg<sup>2+</sup> (36). Consistent with our model, the low basal activity of RetGC1 was significantly increased by addition of either 0.5 µM GCAP2 (derived from the biochemical model) or 3 µM GCAP1 (the estimated GCAP1 concentration in mouse rods (29)) (Fig. 5).
Next, we assessed whether GCAP2 can contribute to the regulation of RetGC1 activity in the presence of GCAP1. To test this, we used M26R GCAP1, a mutant form that can bind to RetGC1 like the wild type GCAP1, but does not activate it (37,38). In the presence of 3 µM GCAP1, addition of M26R GCAP1 started to decrease RetGC1 activity at ~0.3 µM (Fig. 5, red circles), its near-physiological concentration (28,29), and reached half-maximal inhibition at 1 µM. At the same concentration of M26R GCAP1, the activation of RetGC1 by 0.5 µM GCAP2, which in the absence of GCAP1 would be sufficient to effectively accelerate RetGC1 in vivo (Fig. 2, black circles), became almost completely suppressed (Fig. 5, black circles).

DISCUSSION
Ca2+-dependent regulation of cGMP synthesis by GCAP2 in mouse cone photoreceptors
Our experiments clearly demonstrate that GCAP2 is expressed in mouse cones (Fig. 1). To address the possible functional role of GCAP2 in cones, we investigated its ability to upregulate cGMP synthesis in low Ca2+ and to mediate light adaptation in cones lacking GCAP1. As previous studies have suggested that GCAP1 and RetGC1 dominate the synthesis of cGMP in the mouse cone outer segments, we expected that Gcap1+/− retinas would respond to low Ca2+ exposure similarly to Gcaps−/− retinas (9,10,30,31,35). However, Gcap1+/− cones were able to boost their maximal response amplitude in low Ca2+ as much as control WT cones (Fig. 3). Based on our model presented in Experimental Procedures, as low as 0.1 µM GCAP2 in the outer segments of Gcap1+/− cones could explain the ~4-fold increase of their maximal response amplitude in low Ca2+. This is more than 10-fold lower concentration than the known GCAP1 or GCAP2 concentration in mouse rod outer segments (28,29). The quantitative power of these experiments might be limited due to the cooperativity of the CNG channel for cGMP (39,40) or the transient nature of the increase in photoreceptor response amplitude in low Ca2+ (41-45). Yet, despite the quantitative limitations of our study, our results clearly demonstrate that GCAP2 can activate RetGC in mouse cone photoreceptor cells when GCAP1 has been deleted.

Similarly, when we examined light adaptation in Gcap1+/− cones, we found that the slope of the light adaptation curve was comparable to that in control cones. In addition, the adaptation capacity of GCAP1-deficient cones was substantially better than that of Gcaps−/− cones (Fig. 4). Together, these results demonstrate that GCAP2 is able to upregulate cGMP synthesis and to mediate light adaptation in cones in the absence of GCAP1.

The role of GCAP1 and GCAP2 in cone phototransduction and light adaptation
The relative contribution of GCAP1 and GCAP2 in rod physiology has been established in mouse rod photoreceptors (27). There, GCAP1 is more important in determining the peak amplitude of the dim flash response, whereas GCAP2 shapes the response recovery kinetics after the peak amplitude. These results are consistent with the known biochemical properties of GCAP1 and GCAP2. Namely, GCAP2 has a higher affinity to Ca2+ (KCa = 50 nM) as compared to that of GCAP1 (KCa = 130 nM) (18,28,29). In darkness, Ca2+ concentration in mouse rod outer segment is ~250 nM, and it declines to ~20 – 50 nM in bright light (46). Hence, after a dim flash, Ca2+ dissociates first from GCAP1 and the GCAP1-mediated feedback dominates over the GCAP2-mediated pathway. Later, when Ca2+ has dropped to a lower level, it can also dissociate from GCAP2, upregulating the GCAP2-mediated feedback to contribute to the recovery phase kinetics of the dim flash response. Notably, the primary target for GCAP1 in mouse photoreceptors is RetGC1 while regulation of the ancillary isoform RetGC2 is carried out mostly by GCAP2 (47). Hence, in mouse rods activation of the cyclase after the flash of light occurs first as activation of RetGC1 by GCAP1 followed by additional activation of RetGC1 and RetGC2 by GCAP2 (27). Here, we compared dark-adapted cone flash responses from WT, Gcaps−/−, Gcap1+/− and Gcap2+/− mice to understand the relative contributions of GCAP1 and GCAP2 in determining the sensitivity and response kinetics of mammalian cones (Fig. 2). We found that deletion of GCAP1 causes a comparable increase of the sensitivity and dim flash response amplitude as the deletion of both GCAP1 and GCAP2 (Fig. 2d, h and Table 1). Thus, just as in rods, GCAP1 seems to dominate the upregulation of cGMP synthesis up to...
the peak of the dim flash response, and the sensitivity of cones is set almost completely by GCAP1.

Comparison of saturated bright flash responses from WT, Gcaps\(^{-/+}\), Gcap1\(^{-/-}\), and Gcap2\(^{-/-}\) mice revealed that deletion of both GCAP1 and GCAP2 significantly delays the escape of cones from saturation, whereas the deletion of GCAP1 had a much less dramatic effect, and the deletion of GCAP2 has almost no effect at all on the recovery kinetics (Fig. 2e). Notably, the recovery kinetics of Gcap1\(^{-/-}\) cones were not slower than those of WT cones so that cone responses from Gcap1\(^{-/-}\) mice recovered to the baseline level at the same time as these of WT and Gcap2\(^{-/-}\) mice (Fig. 2d). Thus, it appears that both GCAP1 and GCAP2 can compensate for the lack of the other isoform in accelerating the recovery of bright flash responses (Fig. 2e). These results are also consistent with the idea that a larger drop in Ca\(^{2+}\) caused by brighter light is required to activate the GCAP2 pathway. In support of this notion, we observed deviation between Gcap1\(^{-/-}\) and Gcaps\(^{-/-}\) mouse light adaptation only at brighter background light. This, again, suggests that GCAP2 is more important under brighter illumination and at lower Ca\(^{2+}\).

Although our data clearly shows that GCAP2 contributes significantly to the physiology of mouse cones in Gcap1\(^{-/-}\) mice, it is not clear if GCAP2 plays a role in the phototransduction and/or light adaptation of healthy WT cones. Evidently, GCAP2 is present in native mouse and Nrl\(^{+/+}\) cones at much lower levels than in rods while GCAP1 expression in cones is very strong (5,10,31). However, our functional data from Gcap1\(^{-/-}\) mice could be explained even by a rather low 0.1-0.5 \(\mu\)M GCAP2 concentrations in the absence of GCAP1. On the other hand, our biochemical experiments assessing the relative contribution of the two GCAP isoforms show that even at equal concentrations of the two GCAP isoforms, GCAP1 effectively outcompetes GCAP2 from RetGC1 (see Fig. 5 and (28,38)). Assuming further that GCAP2 expression in mouse cones is lower than that of GCAP1, we conclude that under normal physiological conditions GCAP1 would dominate the regulation of cGMP synthesis in mouse cones. However, if the function of GCAP1 becomes compromised, GCAP2 should be able to effectively regulate the phototransduction feedback and light adaptation of cones.

**EXPERIMENTAL PROCEDURES**

**Ethical approval**

All experimental procedures were in accordance of Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees (IACUC) at Washington University in St. Louis, Salus University, and University of Southern California.

**Animals**

Wild-type (WT) C57Bl/6J control and age-matched adult mice devoid of Guanylate Cyclase Activating Protein 1 (Gcap1\(^{-/-}\), (48)) or 2 (Gcap2\(^{-/-}\), (49)), or both (Gcaps\(^{-/-}\), (19)) were used in this study. The mutant strains were bred to the control C57Bl/6J background for several generations but were not siblings of the control mice. For some electrophysiology experiments, Gcap1\(^{-/-}\) and Gcaps\(^{-/-}\) mice were bred into Gnat1\(^{-/-}\) background to remove the rod-driven light responses (50). Mice were kept under 12/12 hour light/dark cycle and had free access to regular mouse chow and clean water.

**Single-cell immunohistochemistry**

Freshly dissected retinas from C57, Gcap1\(^{-/-}\), Gcap2\(^{-/-}\), and Gcaps\(^{-/-}\) mice were washed in Ames media, placed on ice-cooled glass slide with a few drops of cold Ames buffer, and chopped with razor blade. Dissociated cells and small cell clumps were collected into 8-chamber slides (Lab-Tek\® #177445) that were pre-coated with wheat germ agglutinin (100 \(\mu\)M wheat germ agglutinin were added to the wells and incubated for 1h). After cells were collected into wells, equal volumes of formaldehyde (4% in PBS) were added. The slides were centrifuged for 10 min at 168 g to attach the cells to the glass surface. Cells were washed in PBS and blocked with 5% goat serum, 0.1% triton x-100 in PBS for 1 h and incubated overnight with a rabbit polyclonal anti-mCAR antibody (51), 1:700 in blocking buffer). The next day, slides were washed in PBS and incubated with a secondary anti-rabbit antibody to visualize mCAR-labeled cones. Following PBS washes, cells were incubated with biotinylated anti-GCAP2 antibody ((33), 1:300 of
1mg/ml in blocking buffer). The GCAP2 signal was visualized by Texas red-avidin (1:200, Vector laboratories). The cells were mounted in Vectashield with DAPI (Vector laboratories). Fluorescent images were acquired using a Zeiss Axioskope microscope using the same settings and exposure times for the different genotypes.

**Ex vivo electroretinography**

We used ex vivo electroretinography (ERG) to assess the function of mouse cone phototransduction and light adaptation (52). Either a background light of 70,000 photons (530 nm) µm² s⁻¹ or Gnat1⁻/⁻ genetic background (53) was used to remove the rod component of the ERG signal. The Gnat1⁻/⁻ mouse rods do not respond to light but maintain normal morphology. The background light needed to fully saturate rods was surprisingly high and would have been expected to bleach a significant amount of pigments during our experiments. However, after about 10 min. exposure to the background light the cone responses remained stable for up to at least 2 hours (the longest experiment) potentially due to a balance between pigment bleaching and regeneration via the Müller cell (54) visual cycle pathway. Retinas were dissected from dark adapted eyes under IR illumination and mounted to a custom-build ERG specimen holder described in Vinberg et al. 2014 (52). Flashes and steps of light were provided by green LEDs (530 nm, Luxeon Rebel LED SR-01-M0090) via an inverted microscope light path where the condenser was replaced by a 10X objective forming a homogenous 2.35 mm spot of light at the sample. The intensity of the light stimulus was calibrated at the level of the sample by a photometer (Model 211, UDT Instruments). Retinas were perfused at 1 mL/min. at 37 °C with bicarbonate-buffered Locke’s solution containing in mM: NaCl, 112; KCl, 3.6; MgCl2, 2.4; 1.2, CaCl2; HEPES, 10; NaHCO3, 20; Na2-succinate, 3; Na-glutamate, 0.5; glucose, 10. The solution was equilibrated with 95%O2/5%CO2 at 37 °C. Low Ca²⁺ solution was prepared by using 0.1 mM CaCl2 instead of 1.2 mM and adding 0.4 mM EGTA. Addition of EGTA caused acidification of the medium and we used NaOH to equalize the pH of our normal Locke’s and low Ca²⁺ media. We estimate that the free [Ca²⁺] of the low Ca²⁺ medium is ~30 nM in the presence of 2.4 mM [Mg²⁺] (55).

Differential amplifier (DP-311, Warner Instruments) and Bessel filter (model 3382, Krohn-Hite Corporation) together with DigiData 1440 digitizer and pCLAMP software (Axon Instruments) were used to acquire data at 10 kHz with 300 Hz low-pass filter. Clampfit (Axon Instruments), Origin 9.0.0 (Originlab) and Excel (Microsoft) software were used to analyze and graph the data. A Naka-Rushton function

\[
\frac{r}{r_{\text{max}}} = \frac{I_F}{I_{1/2} + I_F}
\]  

was fitted to the response amplitude (r) data. There, \(r_{\text{max}}\) is the maximal saturated response amplitude, \(I_F\) is flash intensity and \(I_{1/2}\) is the light intensity (in photons µm²) required to elicit a half-maximal response. A modified Weber-Fechner function

\[
\frac{S_F}{S_{F,D}} = \frac{I_0^n}{I_0^n + I^n}
\]  

was fitted to light adaptation data. There, \(S_F\) is the sensitivity of cones to a flash of light (\(I_F\) that elicits \(r < 0.2r_{\text{max}}\)) defined as \(r/I_F\), \(S_{F,D}\) is the sensitivity in darkness, \(I\) is the background light intensity (in photons µm² s⁻¹), \(I_0\) is the background light intensity in which \(S_F = 0.5S_{F,D}\), and \(n\) is a factor determining the steepness of the adaptation curve.

A sum of two exponential functions was used to quantify the kinetics and magnitude of light response relaxation after the initial peak during light steps

\[
r(t) = r_0 + A_1(1 - e^{-\frac{t-t_0}{\tau_1}}) + (A - A_1)(1 - e^{-\frac{t-t_0}{\tau_2}})
\]  

where \(r_0\) is peak amplitude measured at \(t_0\), \(A\) is amplitude measured from the peak to the steady state plateau of the step response, \(A_1\) is the fraction of recovery covered by the time constant \(\tau_1\) and \((A-A_1)\) is the fraction of the recovery covered by the time constant \(\tau_2\).

**Biochemical model of RetGC1 activation by GCAP2**

We used the following equations to model binding of Ca²⁺ to GCAP2 and binding/activation of RetGC1 by Ca²⁺-free GCAP2. The parameter values have been taken from Peshenko et al. 2011 (28).
where \( K_{Ca} = 50 \text{ nM} \) is the apparent dissociation constant of \( \text{Ca}^{2+} \) from GCAP2. We model the activation of RetGC1 by GCAP2 by assuming that only \( \text{Ca}^{2+} \)-free GCAP2 can activate the RetGC1:

\[
\text{GC1} + \text{GCAP2} \underset{K_{a}}{\overset{K_{s}}{\rightleftharpoons}} \text{GC1-GCAP2} \quad (6)
\]

\[
\Rightarrow \text{GC1} - \text{GCAP2} = \frac{\text{GCAP2}}{K_{GC1} + \text{GCAP2}} \frac{G_{C1}}{G_{Total}}, \quad (7)
\]

where \( K_{GC1} = 1.25 \mu \text{M} \) and \( G_{C1Total} = 3.2 \mu \text{M} \). Cyclase activity (\( \alpha \), in \( \mu \text{M s}^{-1} \)) is

\[
\alpha = k_{a} \text{GC1} + k_{s} \text{GC1-GCAP2} \quad (8)
\]

if we assume that GTP (the substrate) \( >> K_{m,\text{GTP-GC}} \) (dissociation constant of the GTP from RetGC1).

We assume that the basal RetGC1 activity \( k_{a} = 2.6 \text{ s}^{-1} \) and for the activated RetGC1 \( k_{s} = 33 \text{ s}^{-1} \) (28).

Concentrations of GCAP2-free GC1 and GCAP2-bound GC1-GCAP2 in a specific \([\text{Ca}^{2+}]\) and \([\text{GCAP2}]\) can be calculated from the equations (5), (6), and (7).

At steady state

\[
\alpha = \beta \text{ cGMP},
\]

\[
\Rightarrow \text{cGMP} = \frac{cG}{\beta}, \quad (10)
\]

where \( \beta = 4.1 \text{ s}^{-1} \) is spontaneous cGMP hydrolysis activity of rod PDE in darkness (56). The CNG channel current (57)

\[
J_{eG} = J_{max} \frac{cG^3}{cG^3 + (20\mu\text{M})^3}, \quad (11)
\]

where \( J_{max} \) is the CNG channel current at high [cGMP]. Assuming that [\( \text{Ca}^{2+} \)] is 250 nM in a dark adapted mouse cone outer segment under normal extracellular \( \text{Ca}^{2+} \) and declines to 25 nM during our low \( \text{Ca}^{2+} \) exposure (see above), as low as 0.1 \mu M total concentration of GCAP2 in the cone outer segment is predicted to cause a 4.4-fold increase of \( J_{eG} \) when switched from normal (1.2 mM) \( \text{Ca}^{2+} \) to low \( \text{Ca}^{2+} \).

**Expression and purification of GCAPs**

We used recombinant mouse myristoylated GCAP1 (E6S) and GCAP2 were expressed from pET11d vector (Novagen/Calbiochem) in E. coli strain harboring yeast N-myristoyl transferase as described previously (28). GCAP2 was purified using urea extraction from the inclusion bodies and size-exclusion chromatography (26,58). GCAP1 was purified using urea extraction, hydrophobic and size-exclusion column chromatography as previously described to reach the final protein 95% purity by SDS-PAGE (28,59). The M26R bovine GCAP1 mutant was produced and purified as previously described (37,38).

**RetGC assays**

The native mouse RetGC1 activity was assayed under infrared illumination in dark-adapted Gcaps\(^{-/-}\)/RetGC2\(^{-/-}\) triple-knockout mouse retina homogenates isolated as previously described(28).

Briefly, the assay mixture (25 \muL) containing retinal homogenate, 30 \text{mM} MOPS–KOH (pH 7.2), 60 \text{mM} KCl, 4 \text{mM} NaCl, 1\text{mM} DTT, 2 \text{mM} EGTA, 0.9 \text{mM} free \text{Mg}^{2+}, 0.3 \text{mM} \text{ATP}, 4 \text{mM} \text{cGMP}, 1 \text{mM} \text{GTP}, and 1 \muCi of \text{[\( \alpha^{32P}\)]GTP}, 100 \muM zaprinast and dipryridamole, 10 \text{mM} creatine phosphate and 0.5 unit of creatine phosphokinase was incubated at 30°C for 8 min and the reaction was stopped by heat-inactivation at 95°C for 2 min.

The resultant \text{[^32P]cGMP} product was separated by TLC using fluorescently-backed polyethyleneimine cellulose plates (Merck) developed in 0.2 \text{M} \text{LiCl}, eluted with 2 \text{M} \text{LiCl} and the radioactivity was counted using ScintiSafe liquid scintillation cocktail (Thermo Fisher Scientific) with addition of 20% ethanol.
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Conflict of interest
Author declare no competing interests.

Author contributions
F.V., A.M.D., J.C., and V.J.K. conceptualized and designed the research and wrote the manuscript. F.V. (electrophysiology), I.V.P. (biochemistry), and J.C. (immunohistochemistry) conducted the experiments and analyzed the data.
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Table 1.

| Genotype | r<sub>max</sub> (µV) | t<sub>p</sub> (ms) | T<sub>i</sub> (ms) | I<sub>1/2</sub> (photons µm<sup>-2</sup>) | I<sub>0</sub> (photons µm<sup>-2</sup>) | n |
|----------|----------------|--------------|-------------|------------------|------------------|----|
| WT       | 144 ± 15       | 53 ± 1       | 51 ± 0.1    | 3,200 ± 130       | 14,500 ± 3,000   | 1.0 ± 0.1 |
| Gcaps<sup>-/-</sup> | 156 ± 26     | 76 ± 3*      | 90 ± 9*     | 1,900 ± 150*      | 3,600 ± 800*     | 1.5 ± 0.03* |
| Gcap1<sup>-/-</sup> | 135 ± 6       | 66 ± 3*      | 64 ± 4*<sup>❄</sup> | 2,100 ± 320*     | 6,900 ± 900*     | 1.0 ± 0.02<sup>❄</sup> |
| Gcap2<sup>-/-</sup> | 125 ± 18      | 52 ± 2<sup>❄</sup> | 49 ± 2<sup>❄</sup> | 4,000 ± 450<sup>❄</sup> | NA               | NA              |

Table 1. Light (flash) response parameters from WT, Gcaps<sup>-/-</sup>, Gcap1<sup>-/-</sup>, and Gcap2<sup>-/-</sup> mouse cones. All recordings were from Gnat1<sup>+/+</sup> mice, except for the light adaptation parameters I<sub>0</sub> and n, which were obtained from Gnat1<sup>-/-</sup> mice. Retinas were exposed to constant 70,000 photons (530 nm) µm<sup>-2</sup> s<sup>-1</sup> background light to suppress the rod component of the response except in the light adaptation experiments that were from Gnat1<sup>-/-</sup> mice. r<sub>max</sub>, saturated photoresponse amplitude (I<sub>F</sub> = 183,000 photons µm<sup>-2</sup> at 530 nm); t<sub>p</sub>, time-to-peak (I<sub>F</sub> = 220 photons µm<sup>-2</sup> at 530 nm); T<sub>i</sub>, integration time defined as an area under a dim flash response divided by the amplitude of the response; I<sub>1/2</sub>, light flash intensity eliciting a response with peak amplitude r = 0.5r<sub>max</sub>, determined by fitting Eq. 1 to the response amplitude data; I<sub>0</sub>, background light intensity at which cone sensitivity is 50% of that in darkness determined by fitting Eq. 2 to the light adaptation data; n, steepness factor determined by fitting Eq. 2 to the light adaptation data * and ▫ indicates statistically significant difference as compared to the WT control and Gcaps<sup>-/-</sup> mouse cones, respectively (p < 0.05, two-tailed Student t-test).
Figure legends

Figure 1. GCAP2 is expressed in the mouse cones. Dissociated retinal cells from mice of the indicated genotypes were incubated with the mCAR antibody (green) to label cones followed by incubation with GCAP2 antibody (red). Arrowheads point to the positions of the cones in each field. Nuclei were stained with DAPI (blue). Images shown are representative of 38 cones from 14 fields (C57), 26 cones from 10 fields (Gcap1−/−), 10 cones from 7 fields (Gcap2−/−) and 9 cones from 3 fields (Gcaps−/−). Scale bar = 20 µm.

Figure 2. GCAP1 and GCAP2 regulate mouse cone phototransduction. Responses of dark-adapted cones to 1 ms flashes of light with intensity I_r ranging from 220 to 183,000 photons (530 nm) µm^{-2} in the presence of rod-saturating background light from isolated wild type control (a), Gcap1−/− (b), and Gcap2−/− (c) mouse retinas. (d) Averaged responses of control (black), Gcap1−/− (red), and Gcap2−/− (blue), and Gcaps−/− (green) mouse cones to a 220 photons µm^{-2} flash normalized with r_{max}. (e) Saturated responses of control (black), Gcap1−/− (red), and Gcap2−/− (blue), and Gcaps−/− (green) mouse cones to the 183,000 photons µm^{-2} flash normalized with r_{max}. Normalized dim flash (f) and saturated (g) light responses recorded from dark adapted retinas of control (black), Gcap1−/− (red) and Gcaps−/− (green) mice that were bred on Gnat1−/− background. (h) The smooth traces plot Eq. 1 with I_{LO} of 3,200, 1,900, 2,100, and 4,000 photons µm^{-2} fitted to the average response amplitude data (r/r_{max} as a function of I_r) of each genotype. Error bars give SEM. N = 3 mice (6 retinas) for each genotype.

Figure 3. GCAP2 promotes CNG channel current in low Ca^{2+}. Normalized r_{max}, the saturated photoresponse amplitude of dark-adapted cones, of control Gnat1−/− (black), Gcaps−/− Gnat1−/− (green), and Gcap1−/− Gnat1−/− (red) mice in normal Ca^{2+} (at t = 0 s) and during low Ca^{2+} exposure (t > 0 s). The values for r_{max} were normalized to their respective value in normal Ca^{2+} just before the switch to low Ca^{2+} at t = 0 s. N = 3 mice (6 retinas) for each genotype. Error bars give SEM.

Figure 4. GCAP1 and GCAP2 contribute to the light adaptation capacity of cones. Responses of cones to 7 s steps of light with a 1 ms flash delivered 4.5 s after the step onset from isolated retinas of control Gnat1−/− (a), Gcaps−/− Gnat1−/− (b), and Gcap1−/− Gnat1−/− (c) mice, obtained using ex vivo ERG recordings. Smooth grey traces plot Eq. 3 with best-fitting parameters A_1, t_1, and t_2. See text for numerical values and statistical analysis. (d) Sensitivity (S_E) normalized with the dark-adapted sensitivity (S_{F0}) of cones as a function of background light intensity (I) in control Gnat1−/− (black), Gcaps−/− Gnat1−/− (green), and Gcap1−/− Gnat1−/− (red). Smooth lines plot Eq. 2 with I_0 of 13,500 photons µm^{-2} s^{-1} (n = 1), 6,700 photons µm^{-2} s^{-1} (n = 1.4), and 4,100 photons µm^{-2} s^{-1} (n = 1) for control Gnat1−/− (black), Gcaps−/− Gnat1−/− (green), and Gcap1−/− Gnat1−/− (red). N = 3 mice (4 retinas) for each genotype. Error bars give SEM.

Figure 5. GCAP1 and GCAP2 compete for the activation of RetGC1. The native RetGC1 activity in Gcaps−/−RetGC2−/− mouse retinas was assayed as described under Experimental Procedures in the absence of GCAPs (▲) or in the presence of 3 µM mouse GCAP1 (●) or 0.5 µM GCAP2 (■). Variable concentrations of the competing bovine M26R GCAP1 were added to the assay as indicated. The data, average ± SD of two to four independent measurements, were fitted assuming a sigmoidal Hill function.
Figure 1.
Figure 3.

$\text{Gnat}1^{-/-}$

![Graph showing normalized amplitude over time for Control, Gcap1^{-/-}, and Gcaps^{-/-}.]
Figure 4.

(a) *Gnat1*<sup>-/-</sup>

(b) *Gcaps*<sup>-/-</sup> *Gnat1*<sup>-/-</sup>

(c) *Gcap1*<sup>-/-</sup> *Gnat1*<sup>-/-</sup>

(d) Norm. Sensitivity vs. Background light.

- Control
- *Gcap1*<sup>-/-</sup> *Gnat1*<sup>-/-</sup>
- *Gcaps*<sup>-/-</sup> *Gnat1*<sup>-/-</sup>
