Functional Redundancy of R7 RGS Proteins in ON-Bipolar Cell Dendrites

Frank S. Chen, Hoon Shim, Duncan Morhardt, Rebecca Gallman, Elizabeth Krahn, Ludine McWhinney, Anjali Rao, Stephen J. Gold, and Ching-Kang Chen

PURPOSE. In the Gβ5−/− mouse, the electroretinogram (ERG) b-wave is absent, and the R7 subfamily of regulators of G protein signaling (RGS), which includes RGS6, -7, -9, and -11, is downregulated. Mutant mouse strains deficient in RGS7 or -11 were characterized, and the SG711 strain which is deficient in both proteins was examined, to learn whether the loss of some of these RGS proteins causes the absence of the ERG b-wave.

METHODS. Antibodies to RGS7 and -11 were generated to determine their expression levels and localizations in retinas with various genetic backgrounds by Western blot analysis and immunohistochemistry, respectively. The implicit times and amplitudes of ERG a- and b-waves were analyzed to examine photoreceptor and bipolar cell functions.

RESULTS. RGS7 and -11 co-localized to the dendritic tips of the ON-bipolar cells. In the RGS11−/− mouse, the level of RGS7 protein increased. However, the level of RGS11 protein remained unchanged in the RGS7 mutant mouse, where a truncated RGS7 protein was expressed due to the deletion of exon 10. In the SG711 mouse retina, the Gβ5-S protein level was reduced. The ERG b-wave of SG711 mice was markedly delayed. In contrast, RGS11−/− mice showed a moderately delayed b-wave, whereas the RGS7 mutant mice showed normal ERG responses.

CONCLUSIONS. The data demonstrate the presence of a delayed ERG b-wave in SG711 mice and a functionally redundant role for RGS11 and -7 at the tips of ON-bipolar cell dendrites. These results suggest that RGS11 or -7 works as the major physiological GAP (GTPase acceleration protein) for Gαo1 in ON-bipolar cells. (Invest Ophthalmol Vis Sci. 2010;51:686–693) DOI: 10.1167/iovs.09-4084

Vision begins at retinal photoreceptors, and encoded information is relayed to bipolar cells at the retinal outer plexiform layer (OPL). The phototransduction cascade responsible for transducing light into neural signals in photoreceptors is G-protein mediated, as is the metabotropic glutamate receptor.
defects as the result of an arrest in the formation of the triadic ribbon synapses during OPL development. Transgenic restoration of Gß5-L in rods of adult Gß5/– mice rescued neither the OPL morphologic defects nor the absent ERG b-wave, indicating that both phenotypes come from the loss of Gß5-S downstream of the photoreceptors.8 However, it remains uncertain whether a signaling defect in the mGlur6 signaling pathway or the reduced triadic ribbon synapses at the OPL, or both, causes the absence of ERG b-wave in Gß5/– mice. To gain further insight, we recorded ERG responses from mice carrying targeted mutations in both RGS7 and -11 genes. We found that RGS7 and -11 are co-localized at the tips of DBC dendrites and that both are involved in the generation of ERG b-waves in a functionally redundant manner. Because of the presence of a robust, RGS7-11-11+/- ERG b-wave response when both RGS7 and -11 are mutated, our data suggest that OPL morphologic defects, rather than the prolonged mGlub6/Gao1 signal transduction in DBCs, are the major contributing factor to the loss of ERG b-waves in Gß5/– mice.

METHODS

Animals

RGS11 and -7 mutant mice, generated by homologous recombination by Lexicon Pharmaceuticals (The Woodlands, TX), are available from the Mutant Mouse Regional Resource Center. In targeting the RGS11 gene, the first four exons were deleted. The homozygous RGS11 knockout (RGS11/–) mouse has no detectable RGS11 protein and hence is a true null.20 However, in the homozygous RGS7 mutant mouse, the deletion of exon 10 resulted in the production of a transcript encoding a truncated RGS7 protein lacking amino acids S229-Q261. To distinguish it from a true null, we named the homozygous RGS7/– mice rescued neither the RGS7 and Gß5, as previously noted.20 After removal of the cornea and lens, the resulting eye cups were cryoprotected in cold 30% sucrose in 1× phosphate-buffered saline (PBS) at 4°C for 10 to 30 minutes. The short fixation time is crucial for OPL staining of RGS7 and Gß5, which has been described.21 All animals used for immunohistochemistry were killed by CO2 inhalation. A small incision was made in the cornea, and the eyes were immersion fixed with 4% paraformaldehyde in 1× phosphate-buffered saline (PBS) at 4°C for 4 to 10 minutes. The short fixation time is crucial for OPL staining of RGS7 and Gß5, as previously noted.20 21 After removal of the cornea and lens, the resulting eye cups were cryoprotected in cold 30% sucrose in 1× PBS, embedded in a freezing medium (Triangle Biomedical Sciences, Durham, NC), and sectioned at 20°C at 20 μm thickness. The sections were blocked for 1 hour at room temperature with 10% goat serum and 0.3% Triton X-100 in 1× PBS (PBT) and incubated with rabbit primary antibodies at 1:100 dilution for 4 to 8 hours at room temperature. Alexa 563-conjugated goat anti-rabbit antibody (1:2000 dilution; Invitrogen, Carlsbad, CA) was used as a secondary antibody for protein localization. For co-localization studies using antibodies raised in rabbit, a rabbit IgG labeling kit (Zeron Tricolor, Z25360; Invitrogen) was used according to the manufacturer’s instructions. Briefly, 1 μg of affinity-purified antibody was incubated with the labeling reagents in a molar ratio of 1:3 for 5 minutes at room temperature, then stopped by adding the blocking reagent and incubated for an additional 5 minutes. The entire mixture was adjusted with 10% goat serum in PBT and then used in staining with appropriate dilutions unique to each primary antibody. Extensive washing (five times, 30 minutes each) was performed to minimize the background signal. Fluorescent images were acquired with a confocal microscope at the shared EM and microscopy facility of the Department of Anatomy and Neurobiology, Virginia Commonwealth University (LSM510Meta; Carl Zeiss Microimaging, Thornwood, NY). Unmodified *.lsm files were archived and viewed in a separate computer (LSM Image Browser program; Carl Zeiss Microimaging). Images were then exported as *.tif files and opened in image-analysis software (Photoshop; Adobe, San Jose, CA) for cropping. No post hoc image processing, such as contrast and brightness adjustments, was performed during or after image acquisition.

Immunoblot Analysis

Retinal extracts (10 μg) were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 10% dry milk in TBST buffer containing 25 mM Tris (pH 7.5), 137 mM NaCl, and 0.05% Tween-20. For the detection of both forms of Gß5, CT215 was used at 1:10,000 dilution. To detect RGS7, 7RC1 or H-190 was used at 1:20,000 and 1:5,000 dilution, respectively. For RGS11, goat anti-RGS11 or VCU008 was used at 1:5000 and 1:2000 dilution, respectively. The anti-GAPDH antibody was used at 1:200,000 dilution and the GAPDH signal was used as an internal lane-loading control. Species-specific secondary antibodies were used at 1:25,000 dilution. The signal was detected by enhanced chemiluminescence with an extended-duration substrate kit (SuperSignal West Dura;

Antibodies

A rabbit polyclonal antibody for RGS11 (VCU008) was raised against a synthetic peptide (CQSSTPRAATSSPEGADGE) coupled to keyhole limpet hemocyanin and the antibody was affinity purified on the corresponding peptide column (Yenzyem, Burlingame, CA). Similarly, a rabbit polyclonal antibody for RGS7 (VCU015) was raised and purified against a synthetic peptide (CKTLTSKRLTSLVQS; GenScript, Piscataway, NJ). Rabbit anti-Gß5 antibody CT-215 was graciously provided by Mel Simon (California Institute of Technology, Pasadena, CA).20 Goat anti-RGS11 antibody was generously provided by Kirill Marremyanov (University of Minnesota, Minneapolis, MN).20 Rabbit anti-RGS7 antibody 7RC1 was kindly provided by William Simonds (National Institute of Diabetic and Digestive and Kidney Diseases, Bethesda, MD).22 Rabbit anti-RGS7 antibody H-190 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-RGS6 antibody IMG-554 from Imgenex (San Diego, CA); rabbit anti-GAPDH antibody from Cell Signaling Technologies (Danvers, MA); and HRP-conjugated secondary antibodies from Santa Cruz Biotechnology.
Thermo Fisher Scientific, Rockford, IL), and was captured and quantified using an imaging system with accompanying 1-D image-analysis software (IS440; Eastman Kodak, Rochester, NY).

Electroretinography

Before ERG recording, the mice were dark-adapted overnight. They were anesthetized with ketamine/xylazine (150/10 mg/kg; IP), and the pupils were dilated in the dark for a minimum of 10 minutes with topical eye drops of 1% tropicamide and 2.5% phenylephrine (Bausch & Lomb, Tampa, FL). The body temperature was monitored by a rectal probe (Braintree Scientific, Braintree, MA) and maintained at 35°C to 37°C using a plastic heating coil with 43.5°C circulating water. ERG from both eyes was recorded simultaneously (UTAS E-3000 system; LKC Technologies, Gaithersburg, MD) under scotopic conditions, as previously described. Typically, the difference in a-wave amplitudes between the two eyes of an animal was <10%. For the study of light sensitivity, responses from 5 to 20 flashes were averaged at different flash intensities. The interstimulus interval (ISI) was changed incrementally from 5 to 180 seconds with increasing stimulus intensity to ascertain the dark-adapted state of the animals. For double-flash recordings, the interval between trials was empirically determined to be 120 seconds. A typical recording session lasted 2 to 3 hours, and the body temperature was monitored by a rectal probe (Braintree Scientific, Braintree, MA) and maintained at 35°C to 36°C using a plastic heating coil with 37.5°C circulating water. ERG was recorded simultaneously from both eyes using a Goldmann stimulus probe (Braintree Scientific, Braintree, MA). The probe was connected to a Grass 7p24 stimulator and used to deliver a rectangular flash of 300 ms per second with increasing stimulus intensity. The recorded signal was amplified and filtered before being stored for further analysis. The signal was then reprocessed using a customized exponential function to fit the early descending portion of the ERG a-waves initiated by data-analysis software (IgorPro; Wavemetrics, Lake Oswego, OR) by ensemble-fitting the data in the analysis software (IgorPro; Wavemetrics) using a customized exponential function: \( f(x) = 1 - \exp \left\{ -a \cdot \exp \left[ \frac{b - x}{t} \right] \right\} \), where \( a \) is a coefficient, \( b \) is the time at which there is no recovery, and \( t \) is an exponential decay time constant, the time required for 50% recovery is determined for each of the four test flash intensities and used in the Pepperberg plot in which the ISI at 50% recovery (ordinate) vs. the natural log of photoisomerizations (\( R^* \)) per flash per rod per second (abscissa) was drawn. The slope of the linear regression line revealed the dominant recovery time constant (\( \tau_R \)). The number of \( R^* \) produced in each test flash was calculated according to the empirically determined conversion factor.

Determination of the Pepperberg Constant

The recovery phase of rod phototransduction was determined by double-flash techniques as previously described. The relationship between ISI (in milliseconds) and \( 1 - R(t)/R_{\text{max}} \) where \( R(t) \) is the a-wave amplitude at time \( t \), \( R_{\text{max}} \) is the amplitude when \( t = 1 \) second, and the probe (second) flash intensity was fixed at 1.63. By plotting \( 1 - R(t)/R_{\text{max}} \) versus ISI and ensemble-fitting the data in the analysis software (IgorPro; Wavemetrics) using a customized exponential function: \( f(x) = 1 - \exp \left\{ -a \cdot \exp \left[ \frac{b - x}{t} \right] \right\} \), where \( a \) is a coefficient, \( b \) is the time at which there is no recovery, and \( t \) is an exponential decay time constant, the time required for 50% recovery is determined for each of the four test flash intensities and used in the Pepperberg plot in which the ISI at 50% recovery (ordinate) vs. the natural log of photoisomerizations (\( R^* \)) per flash per rod per second (abscissa) was drawn. The slope of the linear regression line revealed the dominant recovery time constant (\( \tau_R \)). The number of \( R^* \) produced in each test flash was calculated according to the empirically determined conversion factor.

**FIGURE 1.** The level of R7 RGS proteins in retinas of various genetically modified mouse lines. (A) Western blot analysis of wild-type (lane 1) and GB5<sup>−/−</sup> (lane 2) mice probed with the following antibodies: CT215 (anti-GB5), VCU008 (anti-RGS11), 7RG-1 (anti-RGS7), and IMG554 (anti-RGS6). (B) Western blot analysis showing the level of three R7 RGS proteins in retinal extracts derived from wild-type (lane 1) and GB5<sup>−/−</sup> (lane 2), RGS11<sup>−/−</sup> (lane 3), and SG711 (lane 4) mice. Arrows: GAPDH signal used as an internal loading control.
We and others have reported that RGS7 and -11 proteins localize to the tips of DBC dendrites.8,9 However, the degree to which they co-localize in individual dendritic tips has not been assessed. Using the rabbit IgG labeling kit (Zenon; Invitrogen), we investigated the distribution patterns of RGS7 and -11 in retinal OPL using two well-characterized rabbit antibodies against RGS7 (7RC-1)20,22 and RGS11 (VCU008, see the Methods section). The specificity of the two antibodies was verified by using retinal extracts derived from wild-type, ods section). The specificity of the two antibodies was verified with these two antibodies revealed that RGS11 and -7 are co-localized to the tips of bipolar cell dendrites (Fig. 2). Because RGS7 and -11 both require interaction with Gβ5-S for stability,19 the co-localization suggests a possible redundant function for these two R7 RGS proteins at the tips of DBC dendrites.

**Upregulation of RGS7 in the RGS11 Knockout Retinas**

Because the localization of RGS7 and -11 overlaps in the OPL, we examined how the level of each RGS proteins is influenced by the targeted mutation of the other. As seen in Figure 3, whereas the overall concentration of Gβ5-S remained unchanged in the RGS11−/− retinal extracts, the RGS7 level increased an average of 60% in the RGS11−/− mice. In contrast, we did not observe detectable changes in the RGS11 level in the SG7 mice (Fig. 1B). The changes in RGS7 levels were specific because the level of RGS6 was not affected in either the RGS11−/− or the SG7 mice, even though, similar to other R7 RGS members, it was downregulated below detection level in the Gβ5−/− mice (Fig. 1A). These data support a selective, compensatory upregulation of RGS7 in retinas of RGS11−/− mice, which would be predicted for proteins with redundant functions. Failure to observe an increase in RGS11 in SG7 mice may be explained by the incomplete genetic knockout in SG7 mouse.

**The Decrease of Gβ5-S Level in the SG711 Mice**

RT-PCR analysis of RNA derived from homozygous RGS7 mutant mouse retinas revealed RGS7 transcripts with exon-10 deletion, which leads to a loss of 33 amino acids (S229-Q261) with the first 27 residues in the interdomain and the last 6 residues in the GGL domain required for interaction with Gβ5-S. This truncated RGS7 protein was not stable when ectopically expressed in insect H5 cells (data not shown). However, in the retina, the truncated RGS7 were detectable and appeared as multiple bands in the immunoblots (Figs. 1, 4). Most of the truncated RGS7 bands migrate faster than the wild-type RGS7 when longer electrophoretic time was used.
We have frequently obtained more than one band of RGS7 by immunoblots, and similarly we have found more than one RGS7 transcript by RT-PCR. The relationship between the multiple transcripts and the proteins is still under investigation. To compare the levels of the normal and mutant RGS7 in wild-type and SG7 mice, we took into account the ensemble signals from all bands and found an average 50% reduction in the level of truncated RGS7 in SG7 mice when compared with full-length RGS7 in wild-type, suggesting that the mutant RGS7 protein is also less stable in the retina. These results did not depend on the nature of the antibody used since similar results were obtainable with an independent, commercially available RGS7 antibody, H-190, and a custom-made antibody named VCU015, which targets a C-terminal peptide of mouse RGS7 (data not shown). Thus, these results support the conclusion that RGS7 is an unstable, truncated protein in retinas of SG7 mice.

We next examined how the level of retinal Gβ5-S protein changes when both RGS7 and -11 genes are targeted. Figure 5 shows that the Gβ5-S protein level decreased by ~30% in SG711 mice. However, the reduction of Gβ5-S in the SG711 retina is not the result of the elimination of Gβ5-S in the OPL as it can still be found at the dendritic tips of DBCs in SG711 mice (data not shown). We reason that the truncated RGS7 protein lacking S229 to Q261 can still weakly associate with Gβ5-S to protect it from degradation.

**Comparable Phototransduction Properties in the Control and Mutant R7 RGS Mice**

The abundant expressions of all members of R7 RGS proteins in the retina indicate their involvement in various retinal responses. To assess whether the loss of RGS11 protein or the truncation of RGS7 protein affects phototransduction, we recorded and analyzed ERG a-waves from these mice and determined the two major photoreceptor-derived properties, the amplification factor and the dominant recovery time constant (Table 1). We found similar maximum a-wave amplitude, amplification factor, and dominant recovery time constant (the Pepperberg constant) among the different genotypes. Therefore, we conclude that photoreceptor functions are normal when both RGS7 and -11 are targeted. This finding is consistent with the fact that RGS9-1 is the major R7 RGS protein in photoreceptors.

**FIGURE 4.** Exon10 deletion of RGS7 reduced both the size and the level of RGS7 in SG7 mouse retinas. (A) A representative immunoblot showing the level of the truncated mutant RGS7 protein in SG7 versus WT mouse retinas. (B) Quantification of the level of reduction in RGS7 using GAPDH signal as a normalization factor (n = 8). A significant 50% decrease of overall RGS7 level was found in the SG7 retinas, whereas the level of RGS11 remained unchanged (data not shown).

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**FIGURE 5.** Downregulation of Gβ5-S in SG711 mouse retinas. (A) Representative immunoblots showing the level of Gβ5-S and -L in serially diluted retinal extracts (10, 5, and 2.5 μg protein/well from high to low) derived from WT and SG711 mice. (B) Quantification of Gβ5-S versus Gβ5-L signals between WT and SG711 mice (n = 8). A significant ~30% decrease in Gβ5-S level is found in SG711 mice.
Prolonged ERG b-Wave Implicit Time in the RGS11/H11546 and SG711 Mice

Despite the normal photoreceptor functions, we noticed a clear difference in ERG responses between the control, RGS11/H11002/H11002, and SG711 mice. The ERG b-wave implicit time significantly increased in the RGS11/H11002/H11002 and SG711 mice, whereas the overall b-wave amplitudes remained similar among the four genotypes (Figs. 6, 7; Table 1). Notably, the rise time of the ERG b-wave in the RGS11−/− mice was delayed at all flash intensities tested when compared with the wild-type control (Fig. 7A). In addition, further delays in the ERG b-wave implicit time were observed in the SG711 mice, especially when flash strength was above 1 cd s m⁻², an intensity that elicits mixed rod/cone responses (Fig. 7A). Because we found

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<th>Table 1. Properties of ERG a- and b-Waves in Different Genotypes</th>
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<tr>
<td>Wild-Type</td>
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<tr>
<td>a-Wave amplitude, µV*</td>
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<td>A5t²‡</td>
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<td>b-Wave implicit time, ms¶</td>
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* Elicited by a flash intensity of 245 cd s m⁻².
† Mean ± SEM (sample size).
‡ The amplification factor of phototransduction, obtained by curve-fitting the descending arms of a-waves according to the Lamb and Pugh model.
§ The dominant recovery time constant determined by Pepperberg plots.
¶ Elicited by 89-cd sec m⁻² flash intensity, represented by the bottom traces in Figure 6.
# P < 0.05 as compared to wild-type; Tukey HSD test.

Figure 6. Delayed ERG b-wave in RGS11−/− and SG711 mice. Representative scotopic ERG responses from the four genotypes are shown. Flash intensity is indicated at left (in log cd s m⁻²).
Mechanism of RGS Modulation of ERG b-Wave

In the dark, mGluR6 at the dendritic tips of DBCs is nearly saturated by glutamate, and the ERG b-wave reflects the light-induced reopening of cation channels in DBCs. Thus, the observed increase in implicit time of the ERG b-wave of RGS11−/− mice suggests a defect in the shutoff phase of the Gβ5-β1 signaling pathway in DBCs. This unique ERG phenotype was further augmented in SG711 mice (Figs. 6, 7), in which only the truncated and partial loss-of-function RGS7 deletion may affect GGL domain function, which is essential for Gβ5 interaction and protein stability. Second, we found that the truncated RGS7 protein is less stable with approximately 50% of the wild-type level in retinas of SG7 mice (Fig. 4). Together, these data suggest that deletion of the 33 amino acids results in a partial loss-of-function RGS7 protein in vivo. This loss-of-function hypothesis is consistent with the measurable behavioral phenotypes of SG7 mice (available online from the Jackson Laboratory, Bar Harbor, ME at http://www.informatics.jax.org/external/ko/lexicon/1011.html), which includes hypoactivity and decreased exploratory responses in the open field. Of interest, RGS11−/− mice show no phenotype in the open-field test.

The shortcoming of using a partial loss-of-function mutant such as the SG7 mouse is that the incomplete deletion limits the conclusions that can be drawn regarding the role of RGS7. For example, we found that SG711 mice generated in this study do not exhibit an obvious weight defect during early postnatal development (data not shown) as reported for Gβ5−/− mice. Because the SG7 mouse is not a null, we cannot conclude whether the weight defects of Gβ5−/− mice are the consequence of simultaneous loss of RGS7 and -11. Similarly, we find it difficult to determine the exact contribution of RGS7 in the generation of ERG b-wave because there is no significant difference in response kinetics between SG7 and control mice. Therefore, efforts to generate and characterize a true RGS7 null mouse line are necessary for a firmer understanding of RGS7 function. Despite this gap, the significant differences in ERG responses in RGS11−/− and SG711 mice when compared with control mice (Figs. 6, 7) strongly support the involvement of both RGS11 and -7 in the generation of a normal ERG b-wave.

DISCUSSION

The deletion of exon 10 of the RGS7 gene in the SG7 mouse results in the expression of a truncated RGS7 protein instead of a null. Using this SG7 line to understand RGS7 function has both advantages and shortcomings. First, by mapping the deleted residues to those of RGS9-1, whose crystal structure is known, we found that the deletion spans an upstream linker region and the first six residues of the GGL domain. This

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protein was present in the OPL. Because mutant RGS7 by itself had no effect, but exacerbated the effect of RGS11 inactivation on ERG b-wave responses, we conclude that both RGS7 and -11 are involved in the mGlul6/Gao1 signaling pathway in DBCs and that they each function in the deactivation of Gao1. However, it is noteworthy that the time course of the ERG b-wave in S7711 mice (in milliseconds) was still much faster than the GTP turnover rate of Gao1 (in seconds) assayed in vitro. Because several members of the RGS family are capable of accelerating GTP hydrolysis by Gao1 in vitro, the specific impact of RGS7 and/or -6 proteins on Gao1 deactivation in DBCs and the possible involvement of other non-R7 RGS proteins require further investigation, which could be performed by examining the ERG responses of a true RGS7 null mouse individually in combination with other relevant RGS knock-out mice. Although an explanation for the missing ERG b-wave in Gβ5−/− mice remains elusive, this study clearly identified an overlapping expression pattern of RGS7 and -11 at the OPL and altered ERG responses in RGS11−/− and S7711 mice. Together, these data firmly establish a redundant involvement of RGS7 and -11 in light-evoked responses of retinal ON-bipolar cells.

**Acknowledgments**

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**References**


31. Stockton RA, Slaughter MM. B-wave of the electroretinogram: a test of mGluR6 signaling pathway in DBCs and the possible involvement of other non-R7 RGS proteins require further investigation, which could be performed by examining the ERG responses of a true RGS7 null mouse individually in combination with other relevant RGS knock-out mice. Although an explanation for the missing ERG b-wave in Gβ5−/− mice remains elusive, this study clearly identified an overlapping expression pattern of RGS7 and -11 at the OPL and altered ERG responses in RGS11−/− and S7711 mice. Together, these data firmly establish a redundant involvement of RGS7 and -11 in light-evoked responses of retinal ON-bipolar cells.

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