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R9AP Stabilizes RGS11-Gβ5 and Accelerates the Early Light Response of ON-Bipolar Cells

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Abstract

The rate-limiting step in the recovery of the photoreceptor light response is the hydrolysis of GTP by transducin, a reaction that is accelerated by the RGS9-Gβ5 complex, and its membrane anchor, R9AP. Similar complexes, including RGS7, RGS11, and Gβ5 are found in retinal ON-bipolar cell dendrites. Here we present evidence that R9AP is also expressed in the dendritic tips of ON-bipolar cells. Immunofluorescent staining for R9AP revealed a punctate pattern of labeling in the outer plexiform layer, where it co-localized with mGluR6. In photoreceptors, R9AP is required for proteolytic stability of the entire RGS complex, and we found that genetic deletion of R9AP also results in a marked reduction in the levels of RGS11 and Gβ5 in the bipolar cell dendrites; the level of RGS7 was unaffected, suggesting the presence of another interaction partner to stabilize RGS7. To determine the effect of R9AP deletion on the response kinetics of ON-bipolar cells, we compared the electroretinogram between wild type and R9AP deficient mice. The electroretinogram b-wave, reflecting ON-bipolar cell activity, was delayed and larger in the R9AP deficient mice. Our data indicate that R9AP is required for stable expression of RGS11-Gβ5 in ON-bipolar cell dendrites. Further, they suggest that the RGS11-Gβ5-R9AP complex accelerates the initial ON-bipolar cell response to light.

Keywords

Electroretinogram; ERG b-wave; RGS proteins; retina

INTRODUCTION

At the first retinal synapse, ON- and OFF-bipolar cells respond with opposite polarities to light-induced changes in synaptic glutamate, which is tonically released from photoreceptor terminals in the dark (Werblin & Dowling, 1969; Bloomfield & Dowling, 1985). ON-bipolar

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cells express a metabotropic glutamate receptor, mGluR6 (Nakajima et al., 1993) that regulates the activity of a non-selective cation channel containing the TRPM1 subunit (Morgans et al., 2009, Shen et al., 2009), via the heteromeric G protein, Go (Dhingra et al., 2000). In the dark, activation of mGluR6 causes the cation channels to close, and the ON-bipolar cells to hyperpolarize. The light-induced decrease in synaptic glutamate shuts off mGluR6 stimulation, thereby causing cation channels to open, and generating the depolarizing light response of ON-bipolar cells.

Termination of G protein signaling cascades requires hydrolysis of bound GTP by the activated Gα subunit. In retinal rods, the rapid recovery of the photoresponse is inconsistent with the slow intrinsic rate of GTP hydrolysis by transducin and requires the action of a GTPase activating protein (GAP) complex. In rods, the GAP complex comprises Gβ5 and a member of the R7 subfamily of regulator of G protein signaling (RGS) proteins, RGS9 (He et al., 1998). Rod photoresponses from transgenic mice lacking RGS9 show normal activation kinetics, but a profoundly slowed deactivation time-course (Chen et al., 2000). RGS9-Gβ5 is tethered to the membrane via the anchoring protein, R9AP (Hu & Wensel, 2002). Rod photoresponses from R9AP deficient mice also have a slowed deactivation that is indistinguishable from RGS9 or Gβ5 deficient mice (Keresztes et al., 2004), indicating that membrane anchoring of the RGS9-Gβ5 complex is essential for its stability and proper function.

In response to illumination of the retina, glutamate release from photoreceptor terminals is reduced, mGluR6 is no longer stimulated, Go is deactivated, and ON-bipolar cells depolarize within 100 msec (Berntson & Taylor, 2000), which is substantially faster than the intrinsic rate of GTP hydrolysis by Go (Higashijima et al., 1987; Lan et al., 1998). Similar to the photoreceptor pathway, rapid inactivation of Go would seem to require a GAP complex to accelerate GTP hydrolysis.

RGS7, RGS11 and Gβ5 co-localize with mGluR6 and Go in the dendritic tips of ON-bipolar cells (Morgans et al., 2007; Rao et al., 2007; Cao et al., 2009). Additionally, R9AP forms a complex with RGS11 both in-vitro (Martemyanov et al., 2005) and in-vivo (Morgans et al., 2007; Song et al., 2007). Furthermore, R9AP colocalizes with RGS11 and enhances the stability of RGS11-Gβ5 (Cao et al., 2009). Functionally, mice mutant for RGS11 have a delayed light response measured from the ERG b-wave (Mojumder et al., 2009; Zhang et al., 2009; Chen et al., 2009) and from patch clamp recording of chemically-simulated light responses of bipolar cells (Zhang et al., 2009). In contrast, Cao et al., (2009) reported no delay in patch clamp recordings of light responses from bipolar cells of RGS11 deficient mice.

However, while RGS11 deficiency results in a reduction of R9AP in ON-bipolar cell dendritic tips, there is little reduction in overall retinal expression levels of R9AP, presumably because the vast majority of R9AP is found in the photoreceptors and is unaffected by deletion of RGS11 (Cao et al., 2009; Zhang et al., 2009). In contrast, R9AP deficiency results in the complete absence of RGS11 from the retina (Cao et al., 2008). In this report, using R9AP deficient mice, we confirm that R9AP is localized to the dendritic tips of ON-bipolar cells and necessary for the stability of RGS11-Gβ5. Furthermore, we examine the kinetics of light response in mice lacking R9AP using the electroretinogram (ERG). We find that in addition to R9AP's previously described role in the recovery of the photoreceptor light response (Keresztes et al., 2004), it also plays a role in regulating the kinetics of the ON-bipolar cell light response. In $R9AP^{-/-}$ mice, the ERG b-wave, which mostly reflects the response of ON-bipolar cells to light, is delayed similarly to that reported in $RGS11^{-/-}$ mice (Mojumder et al., 2009; Zhang et al., 2009, Chen et al., 2009). The

combined results suggest that R9AP-RGS11-Gβ5 complex may be a GAP for Go in ON-bipolar cells.

MATERIALS AND METHODS

Animals

R9AP-deficient heterozygous mice were a generous gift from Vadim Arshavsky (Duke University) and Stefan Heller (Stanford University). They were generated using 129/SvJ embryonic stem cells, and crossed with C57BL/6 mice (Keresztes et al., 2004). Wild-type (WT) and R9AP$^{-/-}$ mice were littermates from heterozygous matings. Mice were maintained and sacrificed in accordance with guidelines provided by the NIH and OHSU and conform to principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience. All experiments were reviewed and approved by the OHSU Institutional Animal Care and Use Committee.

Immunohistochemistry

Adult mice were euthanized with an overdose of pentobarbital. Preparation of retina sections was carried out as previously described (Morgans et al., 2007). Confocal images were acquired on an Olympus Fluoview1000 with a focal plane ≤ 1 μm. The following antibodies (and dilutions) were used in these studies: a rabbit polyclonal antibody (R4612) raised against full-length bovine RGS7 (1:1000) and a goat polyclonal antibody against a Gβ5 peptide (MATDGLHENETLASLKC; 1:1000) both produced by Bethyl Laboratories (Montgomery, TX; (Morgans et al., 2007)); a rabbit polyclonal antibody raised against residues 248–471 of mouse RGS11 (1:1000–1:5000) (Chen et al., 2003); a goat polyclonal antiserum raised against R9AP (1:1000) (Hu & Wensel, 2002); an affinity purified sheep polyclonal antibody against mGluR6 (1:100; Morgans et al. 2006); and a mouse monoclonal antibody (clone MC5) against PKCα (1:5000; Sigma-Aldrich, St. Louis, MO).

HEK293 cell transfection

HEK293 cells were grown, transfected as previously described (Morgans et al., 2006). Cells were transfected with 200–500 ng each of plasmids encoding RGS11, Gβ5, plus or minus R9AP. For immunoprecipitations, transfected cells were solubilized in IP buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 8.0) by incubation on ice for 1 hr, and insoluble material was removed by centrifugation for 30 min at 20 000 × g. Aliquots of 0.5 ml extract containing 20 μg protein were pre-cleared with 20 μl 50% protein A-sepharose beads, and then incubated overnight at 4°C with another 10 μl beads in the presence or absence of 0.5 μl R9AP antiserum. The beads were washed twice with IP buffer, then with IP buffer without detergent, and finally subjected to SDS PAGE and western blotting for RGS11.

Immunoblotting

Two adult retinas each from WT and R9AP$^{-/-}$ mice were homogenized in a buffer containing 125 mM Tris, pH 6.8, 1% Triton X-100, 0.1% SDS, plus 1 Complete Mini-Protease Inhibitor tablet (+EDTA) (Roche, South San Francisco, CA) per 10 ml, and the protein concentration was determined using the BCA reagent (Pierce, Rockford, IL). Ten μg of total protein from each extract was subjected to SDS-PAGE on a 4–12% gradient gel (NuPage, Invitrogen, San Diego, CA). After blotting onto Immobilon (Millipore, Billerica, MA), the membranes were treated with blocking reagent (Rockland Immunochemicals, Gilbertsville, PA), and the blots were incubated for 2–4 hours with the appropriate dilutions of the primary antibodies (affinity-purified anti-RGS7 – 1:5,000; anti-RGS11 antiserum – 1:20,000; anti-R9AP antiserum – 1:10,000; anti-Gβ5 antiserum – 1:20,000). After three

rinses in TBST (Tris-buffered saline containing 0.1% Tween-20), the blots were incubated with appropriate secondary antibodies, which were fluorescently tagged with Alexa680 or Alexa800 (1:10,000 dilution). Blot fluorescence was imaged on a LiCor Odyssey Infrared Imaging System (LiCor, Lincoln, NE).

Electron Microscopy

Posterior eyecups were placed in chilled fixative containing 1% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, and 0.01% calcium chloride in 0.1M phosphate buffer (PB; pH 7.4) for 24 hours. Postfixation was performed with 1% OsO_4 in 0.1M PB. The tissue was then dehydrated through graded ethanol solutions and propylene oxide before embedding in EM-bed 512 resin. Ultrathin sections (90 nm) were collected on copper grids and contrasted with uranyl acetate and lead citrate before viewing on a Tecnai 12 electron microscope using an operating voltage of 80V. Digital micrographs were acquired with an AMT 542 camera (Advanced Microscopy Techniques, Danvers, MA).

Electroretinogram recording

Six mice (3 WT and 3 $R9AP^{-/-}$) were dark-adapted overnight (> 12 hrs.) and prepared for recording under dim red light. Animal preparation and anesthesia for ERG were as previously described (Morgans et al., 2009; Zhang et al., 2009). Full-field scotopic ERGs were recorded to flashes of increasing intensity (−4.5 to 2.2 log scotopic candela-sec/metre^2 [sc cd-s/m^2]). The inter-flash interval ranged from 3 sec for the dimmest intensity up to 10 sec for the flash intensity (−1.9 log sc cd-s/m^2) that produced maximal b-wave amplitude for both WT and $R9AP^{-/-}$ mice. For higher flash intensities (−0.4 – 2.2 log sc cd-s/m^2), the inter-flash interval ranged from 45 to 90 sec for WT mice and 180–300 sec for $R9AP^{-/-}$ mice. The longer inter-flash interval for $R9AP^{-/-}$ mice was necessary in order to achieve complete ERG recovery between successive bright flashes. ERGs were the averages of multiple responses varying from n=15 for the dimmest flash to n=2 for a −1.9 l log sc cd-s/m^2. For higher flash intensities, ERGs were recorded to a single flash. A flash intensity of 1 log sc cd-s/m^2 is equivalent to approximately 2.7 log photoisomerizations (Lyubarsky et al., 2004). ERGs were low pass filtered (−3dB) at 300 Hz for intensities lower than −0.4 log sc cd-s/m^2 and 1000 Hz at higher intensities. ERGs were amplified (2–10k) and high pass filtered (−3 dB at 0.3 Hz) before being sampled at 5 kHz with a 12-bit A/D converter and stored for off-line analysis.

ERG Analysis

In order to study the kinetics of the rising phase of the ERG response, the rate of rise was calculated from the derivative of the filtered ERG (dERG(t)/dt) for flash intensities below −1.5 log sc cd-s/m^2 before intrusion of the a-wave. For higher flash intensities, the derivative was calculated from the isolated P2 response after filtering (see Results). For both the ERG and P2 responses, filtering involved digital isolation and subtraction of the oscillatory potentials. The oscillatory potentials (OPs) were digitally isolated with an anti-causal, Chebychev filter (−3dB at 30 & 300 Hz) using Matlab routines written by one of the authors (BGJ). The lower cutoff of the bandpass filter was set at 30 Hz, which enabled isolation of all OPs but did not introduce distortion into the rising phase of the ERG response (see Fig 5 in Results). The maximum rate of rise was obtained from the peak of the derivative.

To quantify photoreceptor kinetics, a P3 model was ensemble fit to the leading edges of bright flash ERG a-waves recorded for flash intensities from 0.7 to 2.2 log cd-s/m^2 The “leading edge” was determined to be all points with amplitude less than 80% of the a-wave peak for each flash intensity. The 80% value was chosen to avoid the influence of post-receptoral components that may contribute to the a-wave near its peak (Robson & Frishman

1999). During fitting, $Rmax_{P3}$ was fixed at the maximal a-wave amplitude obtained and S and td were varied. The derived parameters were: S ((sc cd-s)$^{-1}$ s^{-2}) the rod sensitivity parameter that scales flash intensity, t_d (msec), the delay due to the filter and finite duration of the flash and $Rmax_{P3}$ (μV), the maximal rod response.

Statistics

Latency and amplitude measurements of both the ERG b-wave and rate of rise functions were compared using a repeated measures (intensity) ANOVA testing for the main effect of mutation v non-mutation. Bonferonni correction was used to account for multiple comparisons. The level of statistical significance was set at $p<0.05$ before Bonferonni correction. Phototransduction parameters were compared using 2-tailed student's t-test.

RESULTS

R9AP is expressed in ON-bipolar cell dendrites

Immunofluorescent staining of R9AP in the mouse retina was detected as discrete puncta in the OPL in addition to the expected strong immunofluorescent staining in outer segments (Fig. 1A). Immunoreactivity was absent in the R9AP$^{-/-}$ retina demonstrating the specificity of the antibody. To determine whether R9AP was localized within ON-bipolar cell dendrites, mouse retinal sections were double labeled with either mGluR6 or PKCα, a marker of rod bipolar cells (Haverkamp et al., 2000). PKCα was distributed throughout bipolar cells, but R9AP was confined to the dendritic tips of the rod bipolar cell dendrites (Fig. 1B top). Double labeling for R9AP and mGluR6 confirmed the co-localization of these two proteins in ON-bipolar cell dendritic tips (Fig. 1B bottom).

In photoreceptors, proper assembly of the RGS9-Gβ5-R9AP complex is required for proteolytic stability of the individual components. Therefore, we examined the effects of R9AP deletion on the expression of potential binding partners Gβ5, RGS11, and RGS7 in the OPL. Genetic deletion of R9AP caused a dramatic reduction of Gβ5 and the total absence of RGS11 immunofluorescence in the OPL (Fig. 2B). In contrast, strong, punctate RGS7 immunoreactivity was present in both WT and R9AP$^{-/-}$ retinal sections (Fig. 2B). There were no ultrastructural abnormalities detectable in electron micrographs from the OPL of R9AP$^{-/-}$ mice (Fig. 2C).

When total retinal expression levels were examined using immunoblots, R9AP and RGS11 were undetectable in retinal extract from R9AP$^{-/-}$ mice (Fig. 2A); RGS9 was also undetectable (data not shown). In comparison, the intensity of the RGS7 band appeared elevated in immunoblots from R9AP$^{-/-}$ mice. Gβ5L, which is expressed specifically in photoreceptor outer segments (Watson et al., 1996), was undetectable in R9AP$^{-/-}$ mice as previously reported (Cao et al., 2008). However, the expression of the short form, Gβ5S, was not reduced in the R9AP$^{-/-}$ retina (Fig. 2A), most likely reflecting its expression in the inner plexiform layer (IPL; Cao et al., 2008), where it may be stabilized by interaction with other proteins. These combined results indicate that R9AP co-localizes with mGluR6 receptors in the dendritic tips of ON-bipolar cells and that R9AP is required for the stable expression of RGS11 and Gβ5 in the OPL, in agreement with a recent report (Cao et al., 2009),.

Binding between R9AP and RGS11 was investigated by transfection of HEK293 cells with plasmids encoding RGS11 and Gβ5, plus or minus an R9AP plasmid. Co-immunoprecipitation of R9AP from extracts of the transfected cells followed by western blotting for RGS11 confirmed binding between RGS11 and R9AP (Fig. 2D).

Elimination of R9AP alters the ERG b-wave

To investigate the effect of genetic ablation of R9AP on the retinal light response, we compared ERGs from WT and R9AP$^{-/-}$ mice. Figure 3A shows mean ERG waveforms from R9AP$^{-/-}$ and WT mice for select flash intensities covering the range used. The peaks of the ERG b-waves were significantly delayed ($p<0.04$) in R9AP$^{-/-}$ mice on average (± S.E.) by 8.2 ± 1.8 msec for lower flash intensities (<=−1.5 log sc cd-s/m^2; Fig. 3A, lower panel). There was also a trend for larger b-waves in these mice although the increase was not significant ($p=0.5$). Analysis of the ERG b-wave at higher flash intensities was completed after subtraction of the ERG a-wave (see below). WT and R9AP deficient mice had essentially identical bright flash ERG a-waves (Fig. 3B); phototransduction parameters derived from the fit of a P3 model to the ERG a-wave were not significantly different between WT ($Rmax_{P3}$ = −770 ± 61 μV, S = 1359 ± 315 (cd-s/m^2)$^{-1}$ s^{-2}) and R9AP$^{-/-}$ ($Rmax_{P3}$ = −776 ± 44 μV, S = 1025 ± 69 (cd-s/m^2)$^{-1}$ s^{-2}) mice. The a-wave results indicate that the delay in the scotopic ERG b-wave of R9AP$^{-/-}$ mice reflects changes downstream of the photoreceptors. However, interpretation of the ERG b-wave delay with respect to ON-bipolar cell signaling is confounded by two factors. First, while the mouse ERG b-wave largely reflects ON-bipolar signaling, other retinal layers may also shape the waveform (Frishman, 2006). Second, recovery of the photoreceptor response is dramatically slowed in R9AP deficient mice (Keresztes et al., 2004), which in turn may affect ERG b-wave kinetics. Since our primary purpose was to ascertain the role of R9AP in shaping the ON-bipolar cell light response, we analyzed the ERG results using two approaches designed to minimize these confounding factors.

In the first approach, we analyzed the ERG over the flash intensity range for which the photoreceptors make little or no contribution to the massed ERG response. To establish the appropriate flash intensity range, we analysed ERGs from TRPM1 deficient mice, which have no ERG b-wave (Shen et al., 2009; Morgans et al., 2009). For TRPM1$^{-/-}$ mice, the amplitude of the ERG a-wave, generated by the photoreceptors was less than 4% of overall ERG amplitude from their WT littermates for flash intensities below −2.5 log sc cd-s/m^2 (Morgans et al., 2009). Figure 4A shows the ERG (top trace) from a R9AP WT mouse in response to a −2.5 log sc cd-s/m^2 flash. The high frequency oscillatory potentials superimposed on the ERG, generated within the proximal retina (Wachtmeister, 1998), were digitally isolated (Fig. 4A, bottom trace), and subtracted from the ERG (see Methods). The kinetics of the resulting filtered ERG b-wave were not altered by OP removal (Fig. 4A, middle trace).

Figure 4B shows mean filtered ERG b-waves (solid lines) ± SEM (dotted lines) from R9AP$^{/-}$ and WT mice in response to a −2.5 log sc cd-s/m^2 flash. Both the onset and peak of the filtered ERG b-wave are clearly delayed in R9AP$^{-/-}$ mice. What is unclear from Figure 4B is whether the delay in the peak of the filtered b-wave results solely from the delay in the onset of the rising phase of the ERG b-wave or whether there is also a decrease in the slope of the rising phase. In order to investigate this issue, the maximum rate of rise (i.e. maximal slope) of the filtered ERG was calculated from the derivative of the response (see Methods). Figure 4C shows the means of the derivatives of the filtered ERGs for the −2.5 log sc cd-s/m^2 flash. Derived parameters were the maximal slope of the rising phase of the ERG, defined by the peak of the derivative (arrows), and the latency to reach this maximal slope. Figure 4D shows the latency to reach maximal slope plotted as a function of flash intensity. The latency to reach maximal slope of R9AP$^{-/-}$ mice was delayed on average (±S.E.) by 10.2 ± 0.6 msec in comparison with WT mice, although the difference did not reach statistical significance ($p=0.07$). There was also no significant difference in maximum slope between R9AP$^{-/-}$ and WT mice ($p=0.38$). The 10.2 msec delay in the time to reach maximal slope in the R9AP$^{-/-}$ mice, while not reaching significance, is similar to the of 8.2 msec delay in the ERG b-wave in these mice (Fig. 3A). These results suggest that the delayed b-

wave peak in the R9AP$^{-/-}$ mice results from a delay in the onset of the b-wave response and not from a decrease in the slope of the rising phase of the ERG b-wave.

While the above analysis eliminates or minimizes photoreceptor contribution to the rising phase of the ERG b-wave, it is possible that the delayed onset of the b-wave in R9AP mice could be due to slowed photoreceptor recovery. To address this possibility we used a second approach in comparing the ERG between R9AP and WT mice. For this approach, we identified the ERG conditions for which the slowed rod recovery of the R9AP$^{-/-}$ mice did not alter the rising phase of the ERG b-wave. To achieve this aim, the following conditions were met for both WT and R9AP$^{-/-}$ mice: First, the flash intensity used was sufficiently bright to rapidly drive rods into saturation, which was maintained throughout the course of the b-wave. This light intensity would terminate release of glutamate from saturated photoreceptors, ensuring that the derived bipolar cell response is solely dependent on the rate of glutamate clearance from the synapse, and/or the rate of shutoff of the mGluR6 mediated cascade. Under these conditions, all ON-bipolar cell responses should rise at the same rate. Second, photoreceptor and proximal retinal contributions were digitally isolated and subtracted from the ERG to derive the ON-bipolar cell response.

Figure 5A shows the derivation of the bipolar cell contributions to the bright flash ERG using the conditions described above. The ERG of a WT mouse (Fig. 5A solid black line) was recorded in response to a bright flash (1.8 log cd-s/m^2). At this flash intensity, rods were driven rapidly into saturation, some 5–10 msec before the onset of the rising phase of the ERG b-wave in the WT and R9AP$^{-/-}$ mice respectively (Fig. 3B). For this bright 1.8 log cd-s/m^2 flash, rods stay saturated for > 1 sec (data not shown) thereby exceeding the time course of the b-wave (Fig. 3A).

The photoreceptor contribution was removed by subtraction of the P3 model (Fig. 5A, blue line) to give the post-receptoral P2 response (Fig. 5A dashed line). Subsequent digital filtering removed all oscillatory potentials without altering the kinetics of the rising phase of P2 (Fig. 5A red line). The resulting P2 response represents the contribution of the bipolar cells to the rising phase of the ERG b-wave. P2 responses derived from four bright flash intensities (1.5, 1.8, 2.0 and 2.2 log cd-s/m^2 flash) all rose in parallel (Fig. 5B), indicating photoreceptors were saturated before onset of the b-wave.

Figure 6A shows the mean derived P2 (solid lines) ± SE (dotted lines) from R9AP$^{-/-}$ and WT mice in response to a 1.8 log sc cd-s/m^2 flash. This result provides further evidence that a delay in ON-bipolar cell signaling underlies the delayed rising phase of the ERG b-wave in R9AP$^{-/-}$ mice. Maximal P2 amplitude was significantly larger ($p<0.04$) in R9AP$^{-/-}$ mice (mean ± SE: 1865 ± 176 μV) compared with WT mice (1225 ± 116 μV). As for dim flash intensities, the maximum slope of the rising phase of the P2 response and latency to this maximum slope were derived from the derivative of the P2 response (Fig. 6B). Figure 6C shows the latency to reach maximal slope of the rising phase of the P2 response as a function of flash intensity. R9AP$^{-/-}$ mice were significantly delayed ($p<0.001$) in comparison with WT mice. By comparison there was no difference in maximum rate of rise of the P2 response between WT and R9AP$^{-/-}$ mice ($p=0.52$). R9AP$^{-/-}$ mice were delayed on average (± S.E.) by 13.2 ± 0.5 msec in comparison with WT mice.

The two approaches used to minimize the confounding factors, stemming from non-bipolar cell contributions to the ERG b-wave and slower photoreceptor recovery in the R9AP deficient mice, produced consistent results. Both approaches indicated a relatively constant delay in the time to reach the maximal rate of rise in R9AP$^{-/-}$ mice but no change in maximal b-wave slope. Additionally, the delay to maximal rate of rise is similar to the delay

in the peak of the b-wave in the R9AP$^{-/-}$ mice. Thus the delay in the ERG b-wave peak in R9AP$^{-/-}$ mice results from a delay in the onset of the ERG b-wave.

DISCUSSION

Within the retina, R9AP is predominantly located in photoreceptor outer segments where it serves to anchor RGS9-Gβ5L to the disc membrane (Hu & Wensel, 2002; Lishko et al., 2002). R9AP also stabilizes the expression of RGS9-Gβ5 (Keresztes et al., 2004) and dramatically increases the ability of RGS9-Gβ5 to stimulate transducin GTPase activity (Hu & Wensel, 2002; Lishko et al., 2002; Baker et al., 2006). Genetic ablation of R9AP in mice dramatically slows recovery of the rod photoresponse (Keresztes et al., 2004). This slowed recovery is likely due to the concurrent loss of RGS9 and Gβ5, since mice lacking either of these proteins also have a delayed recovery of the light response (Chen et al., 2000; Krispel et al., 2003).

Here we confirm that, in addition to photoreceptors, R9AP is also located in the dendrites of ON-bipolar cells in the OPL where it colocalizes with mGluR6 in agreement with the previous report (Cao et al., 2009). Within the retina, RGS9 is not found outside the photoreceptors but the related proteins RGS7 and RGS11 are found in ON-bipolar cell dendrites (Morgans et al., 2007; Rao et al., 2007; Song et al., 2007; Mojumder et al., 2009; Cao et al., 2009; Chen et al., 2009; Zhang et al., 2009). Co-immunoprecipitation studies indicate that both R9AP and Gβ5 form complexes with RGS11 in-vivo (Morgans et al., 2007; Song et al., 2007; Cao et al., 2009) and pull-down assays indicate that GST-tagged R9AP and recombinant RGS11-Gβ5 interact in vitro (Martemyanov et al., 2005). Immunological studies show that R9AP deficiency results in the total absence of RGS11, a substantial reduction in Gβ5 from the OPL, but an increase in RGS7 expression (Fig. 2 and Cao et al., 2009). In contrast, R9AP is not totally absent from the retina of RGS11 deficient mice (Cao et al., 2009; Zhang et al., 2009), but instead is displaced from ON-bipolar cell dendritic tips and no longer anchored to the membrane (Cao et al., 2009)

These combined results indicate that R9AP is required for the stable expression of RGS11-Gβ5 in the OPL. Thus, within the retina R9AP serves a common purpose for the stable expression and anchoring of RGS11-Gβ5 in ON-bipolar cells and RGS9-Gβ5 in photoreceptors. Human subjects with mutations in the gene encoding R9AP (Nishiguchi et al., 2004; Cheng, JY et al., 2007) have visual impairments that may therefore, be attributed to slowing of both rod recovery and the onset of the ON-bipolar cell light response.

ERG b-waves recorded from R9AP deficient mice were indeed delayed by 10–13 msec, similar in magnitude to the delays reported for RGS11 deficient mice (Mojumder et al., 2009; Zhang et al., 2009). The maximum rate of rise of the ERG response corresponding to the rate of ON-bipolar cell depolarization is not altered in either RGS11 (Zhang et al., 2009) or R9AP deficient mice (Figs 4 & 6). The similarities in the ERG results indicate that the RGS11 and R9AP deficient mice are functionally equivalent. Two lines of evidence support the conclusion that the delay in the onset of the ERG b-wave in R9AP and RGS11 deficient mice originates within the ON-bipolar cells. First, the delay in the onset of the ERG b-wave in RGS11 deficient mice remained after inner retinal contributions to the ERG were blocked by intravitreal injection of GABA (Mojumder et al., 2009). Second, patch-clamp recordings of chemically-simulated light responses of rod bipolar cells, which bypass the photoreceptors, confirm a significant delay in the onset of the ON-bipolar cell response in mice deficient in RGS11 and with a deletion mutation in RGS7 (Zhang et al., 2009). While Cao et al., (2009) reported no delay in patch clamp recordings of light responses from bipolar cells of RGS11 deficient mice, closer inspection of their Figure 9B suggests a small delay in the onset of the response from these mice. While ON-bipolar cell responses are

delayed in both R9AP and RGS11 deficient mice, the magnitudes of these delays (<25 msec) are far less than expected if RGS11-Gβ5-R9AP was the predominant GAP complex for Go in ON-Bipolar cells. Instead the delay in the onset of the ON-bipolar cell response in R9AP and RGS11 deficient mice suggests a role for RGS11-Gβ5-R9AP in accelerating the early phase of Go inactivation, and that additional RGS proteins may be involved.

An unexpected finding in our study was an increase in the P2 amplitudes to a bright flash in R9AP-deficient mice compared with the WT controls (Figure 6). Larger P2 amplitudes in the R9AP deficient mice are consistent with larger ERG b-wave amplitudes reported for RGS9 deficient mice (Lyubarsky et al., 2001) and in mice deficient in RGS11, particularly when combined with a deletion mutation in RGS7 (Mojumder et al., 2009; Zhang et al., 2009). The peak of the ERG b-wave/P2 response and the kinetics of the falling phase can be shaped by feedback mechanisms at the level of bipolar cell terminals (Kapousta-Bruneau, 2000; McCall et al., 2002; Mojumder et al., 2009), therefore, further studies will be needed to identify the origin of the increased P2 amplitude in the R9AP deficient mouse.

We have shown here that RGS11-Gβ5-R9AP affects the initial kinetics of the ON-bipolar cell light response, but not the maximal rate of rise. GTP hydrolysis by Go would be expected to limit the kinetics of the ON-bipolar cell light response beyond the first 20 msec, thus it is probable that an additional GAP protein is involved in this later phase. Other RGS proteins that could fulfill this role have been identified in gene profiling studies of fluorescently-tagged ON-bipolar cells and include RGS7, RGS16, RGS19 and RET-RGS1 (Dhingra et al., 2008). RGS7, for example, is co-localized with mGluR6, similar to RGS11 (Morgans et al., 2007; Cao et al., 2008; Mojumder et al., 2009), and its presence in ON-bipolar cell dendrites appears unaffected by the absence of R9AP (Fig. 2). Thus, it is possible that RGS7 can compensate for the lack of RGS11-Gβ5-R9AP complex in ON - bipolar cell dendrites.

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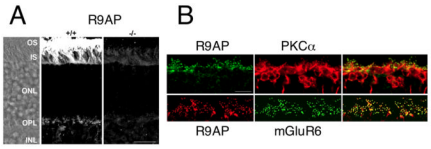

Figure 1. R9AP is localized to the tips of ON-bipolar cell dendrites

A. Vertical cryosections from wild-type (+/+) and R9AP deficient (−/−) mouse retina were labeled for R9AP by immunofluorescence. Left panel: Nomarsky image of the +/+ retina section in the middle panel. Abbreviations: OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. The scale bar represents 10 μm. ***B. Top row***: Mouse retina section double labeled for R9AP (left, green) and PKCα (middle, red).***Bottom row***: OPL of a mouse retina section double labeled for R9AP (left, red) and mGluR6 (middle, green). Merged images are shown on the right; co-localization of the two antigens appears yellow. The scale bar applies to all panels and represents 10 μm.

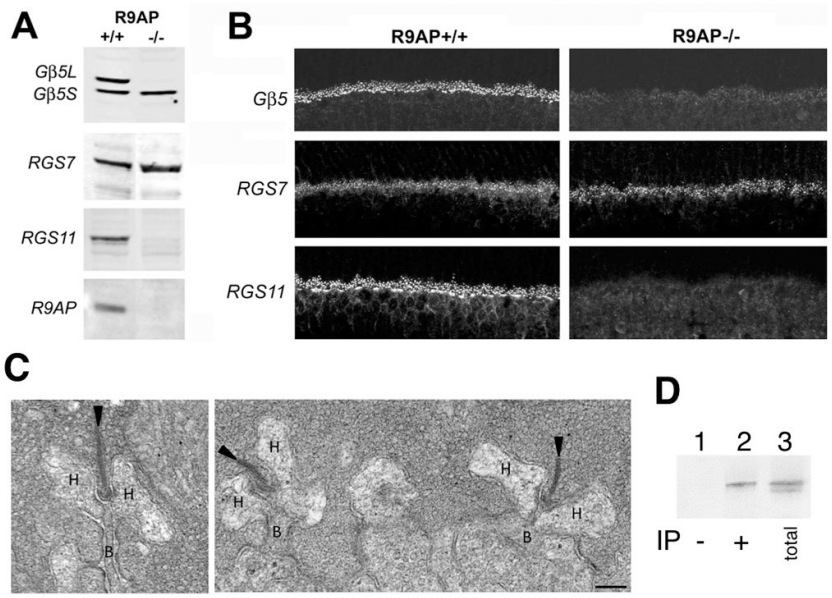

Figure 2. The R9AP$^{-/-}$ retina lacks RGS11 but has a normal OPL ultrastructure

A. Western blots of retinal extracts from R9AP +/+ and −/− littermates were probed for Gβ5, RGS7, RGS11 and R9AP. ***B.*** Retina sections from R9AP +/+ and −/− littermates were labeled by immunofluorescence for Gβ5, RGS7, and RGS11. ***C.*** Transmission electron micrograph showing normal ultrastructure of the OPL in the R9AP deficient mouse. The examples show a rod spherule (***left***) and a cone pedicle (***right***) showing normal invagination of postsynaptic horizontal cell processes (H) and bipolar cell dendrites (B) at the ribbon synapses (arrowheads). Scale bar = 0.2 μm. ***D.*** RGS11 western blot of: Lane 1: extract from cells transfected with RGS11, Gβ5, and R9AP precipitated with Protein G sepharose beads alone; Lane 2: extract immunoprecipitated with an antibody to R9AP and Protein G sepharose beads; Lane 3: total extract.

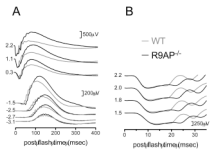

Figure 3. R9AP deficiency slows the ERG b-wave

A. Mean ERGs from WT (gray traces) and R9AP$^{-/-}$ (black traces) mice for a representative range of flash intensities. Numbers to the left of traces indicate flash intensities in log sc cd-s/m^2. Note change of scale for flash intensities greater than 0 log sc cd-s/m^2. ***B.*** Mean ERGs to four high flash intensities shown over a shorter time base, highlight the similarity in the ERG a-waves between WT (gray traces) and R9AP$^{-/-}$ (black traces) mice.

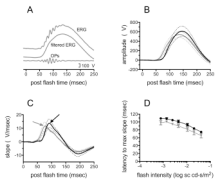

Figure 4. R9AP deficiency delays the onset of the rising phase of the ERG b-wave

A. ERG to a −2.5 log sc cd-s/m^2 flash (top). Filtered ERG b-wave (middle) was obtained following digital subtraction of the OP's (bottom) from the ERG. ***B.*** Mean filtered ERG b-waves from WT (gray traces) and R9AP$^{-/-}$ (black traces) mice to a −2.5 log sc cd-s/m^2 flash. Dotted lines show traces 1 S.E. either side of the mean. ***C.*** The means of the derivatives of the filtered ERGs to the −2.5 log sc cd-s/m^2 flash. The peak of the derivative (arrows) corresponds to the maximal slope of the rising phase of the filtered ERG b-wave. ***D.*** Latencies to reach maximum slope as a function of flash intensity. Error bars, ±SE

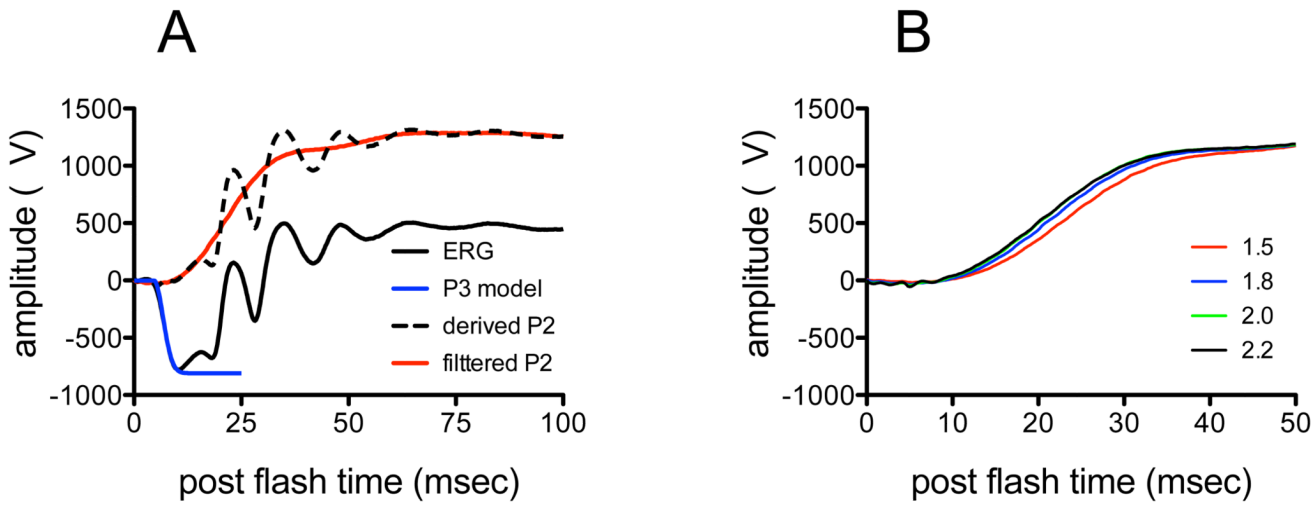

Figure 5. Isolation of the P2 response

A. ERG to a 1.8 log sc cd-s/m^2 flash (solid black line); Phototransduction P3 model fit to the leading edge of the ERG a-wave (blue line); Post-photoreceptoral ERG after subtraction of P3 model fit (dashed black line); P2 response following digital subtraction of OPs (red line) ***B.*** P2 responses from a WT mouse generated by flash intensities of 1.5, 1.8, 2.0 and 2.2 log sc cd-s/m^2 (responses to highest 3 flash intensities overlap).

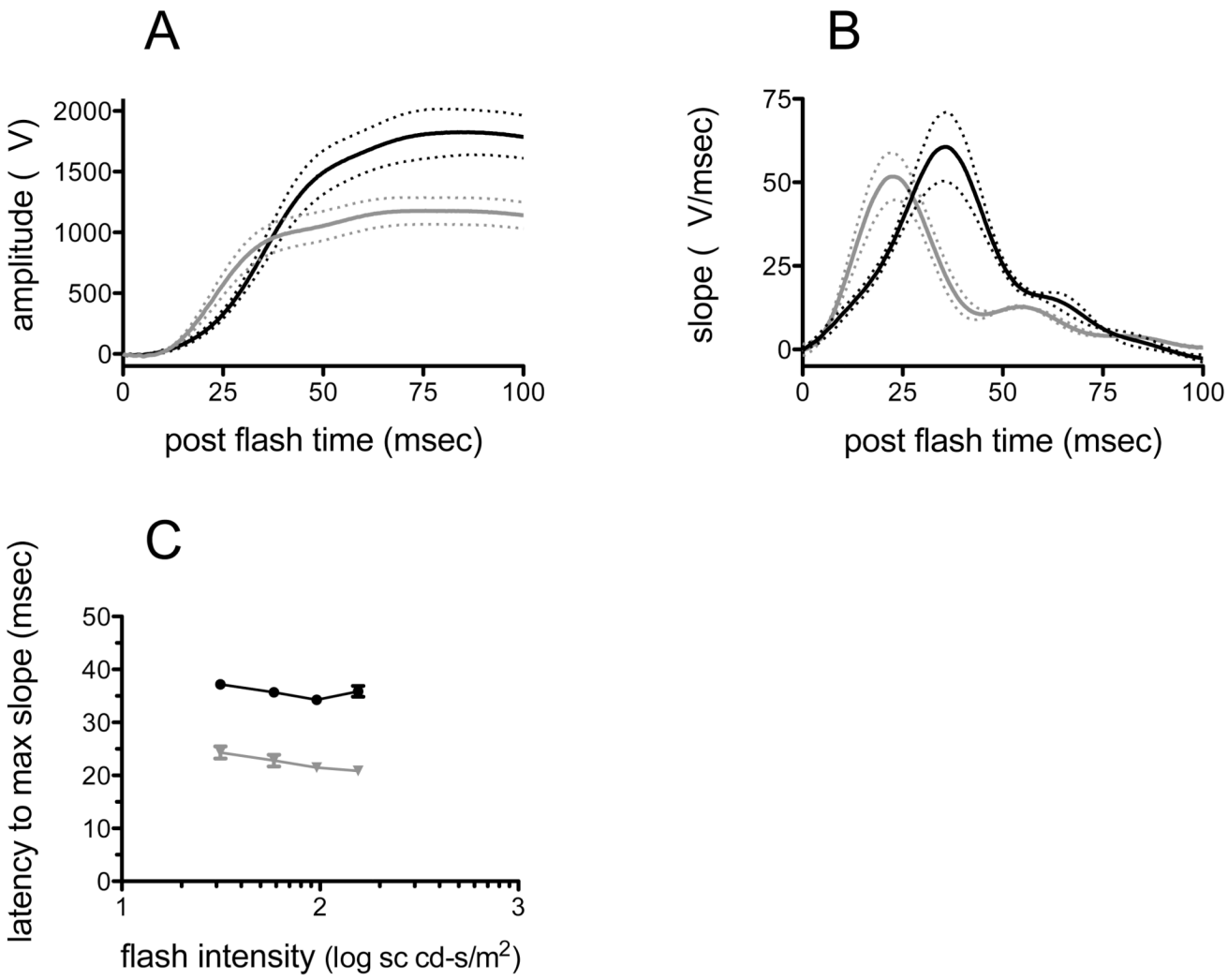

Figure 6. R9AP deficiency delays the onset of the rising phase of the bright flash P2 response

For all graphs, gray traces are from WT mice and black traces are from R9AP$^{-/-}$ mice. Dotted lines show traces 1 S.E. either side of the mean. ***A.*** Mean P2 responses to a 1.8 log sc cd-s/m^2 flash ***B.*** The means of the derivatives of the P2 response for a 1.8 log sc cd-s/m^2 flash. ***C.*** Latencies to reach maximum slope on the rising phase of the P2 response as a function of flash intensity. Error bars, ±SE