Coordinated control of sensitivity by two splice variants of $G\alpha_o$ in retinal ON bipolar cells

Haruhisa Okawa,[1] Johan Pahlberg,[2] Fred Rieke,[4] Lutz Birnbaumer,[5] and Alapakkam P. Sampath[1,2,3]

[1]Neurosciences Graduate Program, [2]Zilkha Neurogenetic Institute, and [3]Department of Physiology and Biophysics, University of Southern California, Los Angeles, CA 90089
[4]Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, WA 98195
[5]National Institutes of Environment and Health Sciences, Research Triangle Park, NC 27709

The high sensitivity of scotopic vision depends on the efficient retinal processing of single photon responses generated by individual rod photoreceptors. At the first synapse in the mammalian retina, rod outputs are pooled by a rod "ON" bipolar cell, which uses a G-protein signaling cascade to enhance the fidelity of the single photon response under conditions where few rods absorb light. Here we show in mouse rod bipolar cells that both splice variants of the G_o α subunit, $G\alpha_{o1}$ and $G\alpha_{o2}$, mediate light responses under the control of mGluR6 receptors, and their coordinated action is critical for maximizing sensitivity. We found that the light response of rod bipolar cells was primarily mediated by $G\alpha_{o1}$, but the loss of $G\alpha_{o2}$ caused a reduction in the light sensitivity. This reduced sensitivity was not attributable to the reduction in the total number of G_o α subunits, or the altered balance of expression levels between the two splice variants. These results indicate that $G\alpha_{o1}$ and $G\alpha_{o2}$ both mediate a depolarizing light response in rod bipolar cells without occluding each other's actions, suggesting they might act independently on a common effector. Thus, $G\alpha_{o2}$ plays a role in improving the sensitivity of rod bipolar cells through its action with $G\alpha_{o1}$. The coordinated action of two splice variants of a single Gα may represent a novel mechanism for the fine control of G-protein activity.

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INTRODUCTION

At the first synapse of the visual system, the output of the photoreceptor cells is segregated into ON and OFF pathways, which respond to increments and decrements of light intensity, respectively. ON bipolar cells use a G protein–coupled receptor-signaling pathway to signal light-evoked reductions in glutamate release from the rod photoreceptor spherule. However, unlike the phototransduction cascade, many of the components of the bipolar signaling cascade have yet to be identified. What is known is that a metabotropic glutamate receptor, mGluR6 (Nakajima et al., 1993; Nomura et al., 1994; Masu et al., 1995), senses glutamate release from photoreceptors and conveys this activity through a heterotrimeric G protein, $G\alpha_o$ (Nawy, 1999; Dhingra et al., 2000), to close nonselective cation channels, recently identified to be TRPM1 (Bellone et al., 2008; Koike et al., 2009; Morgans et al., 2009; Shen et al., 2009). However the target of the G protein and the gating particle controlling the TRPM1 current remain unidentified.

Despite the lack of identity of key signaling components in the mGluR6 pathway, work on mammalian rod ON bipolar cells has led to several insights about the pathway's functional properties. For instance, rod bipolar cells generate responses to light that are briefer than the response of rods (Field and Rieke, 2002; see also Sampath et al., 2005). In addition, a nonlinear threshold for signal transmission between rods and rod bipolar cells (van Rossum and Smith, 1998; Field and Rieke, 2002; Berntson et al., 2004a) produced by saturation of the mGluR6 signaling cascade (Sampath and Rieke, 2004) improves the signal-to-noise ratio of the single photon response by preserving responses in rods absorbing photons while eliminating noise from the majority of rods that do not. These properties are ultimately dependent on the speed and sensitivity of G-protein signaling in the rod bipolar dendrites.

Here we investigated the role played by the $G\alpha_o$ splice variants in setting the properties of the light response in mouse rod bipolar cells. The expression of $G\alpha_o$ in the mouse retina is mainly restricted to ON bipolar cells, with little or no expression in the photoreceptors (Vardi et al., 1993; Vardi, 1998; Dhingra et al., 2000;

Correspondence to Alapakkam P. Sampath: asampath@usc.edu
H. Okawa's present address is Department of Biological Structure, University of Washington, Seattle, WA 98195.
Abbreviation used in this paper: APB; L-2-aminophosphonobutyric acid.

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Dhingra et al., 2002). Two splice variants of the G_o α subunit ($G\alpha_{o1}$ and $G\alpha_{o2}$) are found in mouse ON bipolar cells (Dhingra et al., 2002). However, the expression of $G\alpha_{o2}$ is much lower than $G\alpha_{o1}$, and electroretinography from knockout mice for each splice variant suggests that rod bipolar responses appeared to require $G\alpha_{o1}$, but not $G\alpha_{o2}$ (Dhingra et al., 2002). We find surprisingly that both $G\alpha_{o2}$ and $G\alpha_{o1}$ contribute to dark-adapted responses of rod bipolar cells. Rod bipolar cells in mice lacking $G\alpha_{o2}$ exhibited reduced light sensitivity. The reduction in sensitivity was not attributable to the reduction in the retinal expression level of $G\alpha_o$ protein, as ~50% reduction in total $G\alpha_o$ expression for $G\alpha_o^{+/-}$ mice did not alter light sensitivity. Furthermore light sensitivity was not affected by the altered balance of retinal expression levels between two splice variants in $G\alpha_{o1}^{+/-}$ mice. These data indicate that the saturation within the mGluR6 signaling cascade that separates the rod single photon response from rod noise is not set by $G\alpha_o$ concentration, and that $G\alpha_{o2}$ works in a coordinated manner with $G\alpha_{o1}$ to improve the light sensitivity of rod bipolar cells.

MATERIALS AND METHODS

Animals and preparation

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Southern California (Protocol 10890) and followed guidelines set by the National Institutes of Health on the care and use of animals. Several lines of mice were crossed and used in these experiments, including mice lacking $G\alpha_o$ (Jiang et al., 1998), lacking either $G\alpha_o$ splice variants $G\alpha_{o1}$ or $G\alpha_{o2}$ (Dhingra et al., 2002), or lacking the gap junction subunit connexin 36 (Deans et al., 2002). Wild-type (WT), $Cx36^{-/-}$, $G\alpha_o^{+/-}$, $G\alpha_{o1}^{+/-}$, and $G\alpha_{o2}^{-/-}$ mice were used between 6 wk and 3 mo of age. $G\alpha_o^{-/-}$, $G\alpha_{o1}^{-/-}$, and $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ mice rarely survived more than 4 wk and were used at the age of 3–4 wk when their retina reached maturity as assessed by morphology and electroretinography (see Dhingra et al., 2000). Given the mixed 129Sv/C57BL-6J background of these mice (Jiang et al., 1998), comparisons in cellular responses were always made between littermates. The preparation of retinal slices was performed under infrared illumination as described previously (Sampath et al., 2005; Okawa et al., 2010). In brief, mice were dark adapted overnight and sacrificed, and the lens and cornea were removed. Retinas were isolated and kept in Ames' media equilibrated with 5% CO_2/95% O_2 at 32°C. A small piece of retina was embedded in agar, and slices were cut with a vibrating microtome, transferred into a recording chamber, and superfused with Ames' media heated to 35–37°C for recordings.

Electrophysiology and light stimulation

Light-evoked currents in rod bipolar cells and AII amacrine cells were recorded by whole-cell voltage clamp ($V_m = -60$ mV). The intracellular solution for bipolar cells consisted of (in mM): 125 potassium-aspartate, 10 KCl, 10 HEPES, 5 NMG-HEDTA, 0.5 $CaCl_2$, 1 ATP-Mg, 0.2 GTP-Mg; pH was adjusted to 7.2 with NMG-OH. The intracellular solution for AII amacrine cells consisted of (in mM): 110 cesium-methanesulfonate, 20 TEA-Cl, 10 HEPES, 10 EGTA, 2 QX-314, 1 ATP-Mg, 0.2 GTP-Mg; pH was adjusted to 7.2 with Cs-OH. Both rod bipolar cells and AII amacrine cells types were identified both by the location of cell somas within the inner nuclear layer and their distinct response properties. However when the cell types were difficult to distinguish by these criteria, such as for cells in $G\alpha_o^{-/-}$ and $G\alpha_{o1}^{-/-}$ mice, they were confirmed by visualizing the axonal stratification within the inner plexiform layer with 100–200 µM Alexa 750 (Invitrogen) added to the internal solution. Full-field 10-ms flashes were delivered from a blue LED ($\lambda_{max} \sim 470$ nm, FWHM ~ 30 nm) and focused onto the retinal slice with 20X 0.75NA objective (Nikon). Light-evoked currents were low-pass filtered at 300 Hz with an 8-pole Bessel filter and digitized at 1 kHz. The series resistance in these recordings was 10−25 MΩ and was uncompensated. Light intensity was calibrated daily and converted to an effective photon flux at the peak of spectral sensitivity for mouse rhodopsin ($\lambda_{max} \sim 501$ nm) by convolving the power-scaled LED output spectrum with the normalized spectral sensitivity curve for mouse rhodopsin. The number of activated rhodopsins per rod for a given flash was calculated by multiplying this effective photon flux with the estimated collecting area of mouse rods in retinal slices, which we calculated in the experimental setup to be 0.18 μm^2 (Cao et al., 2008; Okawa et al., 2010).

Western blotting

Isolated retinas were homogenized in lysis buffer containing protease inhibitor (Roche), 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. The homogenate was treated with 100 U/ml DNase for 30 min at room temperature. The protein concentration was checked using a BCA Protein Quantification Assay (Thermo Fisher Scientific). The extracted protein was run on a 10% NuPAGE Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane using a Transphor Electrophoresis Unit (Hoefer). The membrane was blocked in 10% milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature and incubated in a $G\alpha_o$ rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) in TBST (1:200), or in a $G\alpha_{o2}$ mouse monoclonal antibody (clone#101.4, provided by R. Jahn [Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany] and G. Ahnert-Hilger [Medical University of Berlin, Berlin, Germany]; see Winter et al., 2005) in TBST (1:5,000) at 4°C overnight. The membrane was washed with TBST and incubated with IRDye 800 CW anti-rabbit antibody or anti-mouse antibody (LI-COR) in TBST (1:20,000) for 1 h at room temperature and then washed with TBST. The positive bands were detected and expression quantified using an Odyssey Infrared Image System (LI-COR), with the expression of β-actin used as a loading control for total protein.

Online supplemental material

The supplemental material (Fig. S1) is available online at http://www.jgp.org/cgi/content/full/jgp.201010477/DC1. Fig. S1 A displays the average response to the dimmest flash tested in WT and $G\alpha_{o2}^{-/-}$ rod bipolar cells. Fig. S1 B documents the relationship between the maximal response to light and the flash strength that evokes a half-maximal response across all WT rod bipolar cells in this study.

RESULTS

Residual responses in $G\alpha_{o1}^{-/-}$ rod bipolar cells are mediated by $G\alpha_{o2}$

Experimental evidence suggests strongly that $G\alpha_o$ is responsible for transduction channel closure (Nawy, 1999; Dhingra et al., 2000, 2002; Koike et al., 2009), with a splice variant of $G\alpha_o$, $G\alpha_{o1}$, mediating the ON bipolar

response (Dhingra et al., 2002). We recorded from rod bipolar cells (Fig. 1 A) in $G\alpha_{o1}^{-/-}$ mice in an attempt to characterize the influence of $G\alpha_{o1}$ on transduction channel gating. Fig. 1 B shows the average response to the first flash for nine rod bipolar cells from the $G\alpha_{o1}^{-/-}$ retina after achieving the whole-cell voltage-clamp recording (one such cell is visualized). Surprisingly, we found that ON responses persisted in the absence of $G\alpha_{o1}$. In $G\alpha_{o1}^{-/-}$ retinas that showed light responses, rod bipolar cell responses were typically small in amplitude (5.3 ± 0.8 pA; n = 9) and decayed quickly after establishing the whole-cell configuration (Fig. 1 B). For comparison, the maximal amplitude of WT rod bipolar responses routinely exceeds several hundred picoamperes (see Table I). Thus, the electroretinography appears to have failed to detect this small remaining ON response (see Dhingra et al., 2002).

Previous work indicated that ON bipolar cells also express at a lower level the splice variant $G\alpha_{o2}$ in addition to $G\alpha_{o1}$ (Dhingra et al., 2002). To determine if $G\alpha_{o2}$ generated the small residual response in $G\alpha_{o1}^{-/-}$ mice, we recorded from rod bipolar cells in the full $G\alpha_{o}$ knockout

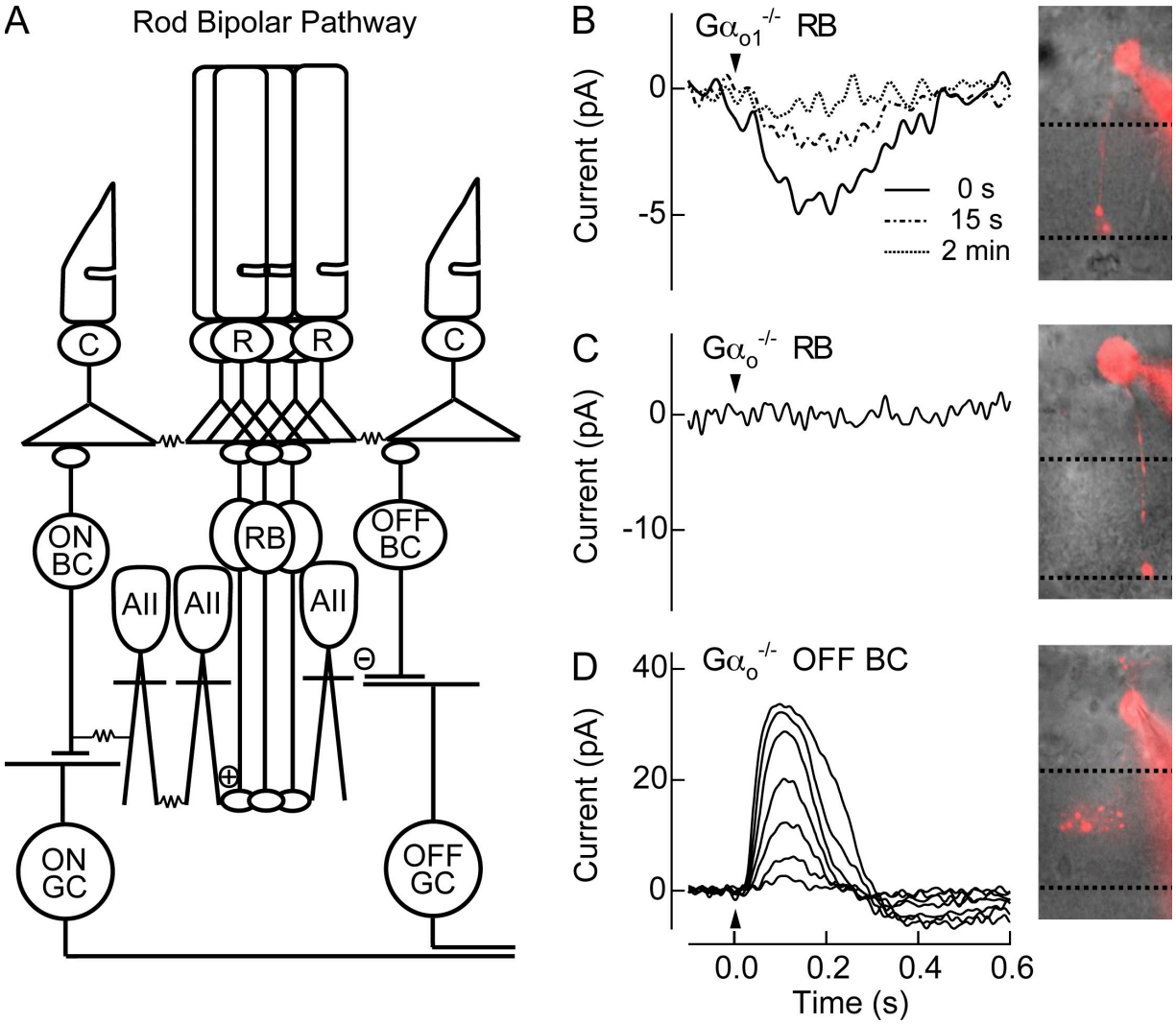

Figure 1. Rod bipolar responses are partially mediated by $G\alpha_{o2}$. (A) Schematic of the mammalian rod bipolar pathway. Rod photoreceptors (R) synapse onto rod bipolar cells (RB), which in turn synapse onto AIIACs (AII). Signals from AIIACs, which are coupled to one another by Cx36 gap junctions (Deans et al., 2002), send light-driven signals to ON cone bipolar cells (ON BC) through gap junctions composed of Cx36 on the AII side, and make glycinergic (−) synapses with OFF cone bipolar cells (OFF BC). Each bipolar cells synapses with its respective ganglion cell (GC). Cone photoreceptors (C) are also depicted. (B) A representative $G\alpha_{o1}^{-/-}$ rod bipolar cell visualized with Alexa 750 and the average flash response of 9 $G\alpha_{o1}^{-/-}$ rod bipolar cells immediately after whole-cell break in (0 s), and 15 s and 2 min later. The flash strength was 15 Rh*/rod, a strength that saturates WT rod bipolar cells. (C) A representative $G\alpha_{o}^{-/-}$ rod bipolar cell visualized with Alexa 750 did not generate light responses to flashes producing 32 Rh*/rod. In every rod bipolar cell tested from $G\alpha_{o}^{-/-}$ mice, rod bipolar light responses were never observed. (D) To confirm viability within the retinal slice, a $G\alpha_{o}^{-/-}$ Off-bipolar cell located near rod bipolar cell was visualized with Alexa 750, and displayed normal response families, indicating that the lack of rod bipolar responses was not due to the conditions of the retinal slice. Flash strengths were 0.5, 1.0, 2.0, 4.0, 8.0, 16, and 32 Rh*/rod.

TABLE I

Response properties of rod bipolar cells and AIIACs in different mouse lines

	Rod bipolar					
	$G\alpha_o^{+/+}$	$G\alpha_o^{+/-}$	$G\alpha_o^{-/-}$	$G\alpha_{o1}^{+/+}$	$G\alpha_{o1}^{+/-}$	$G\alpha_{o1}^{-/-}$
I_{max} (pA)	420 ± 38 (14)[b]	250 ± 22(15)[b,d]	−0.3 ± 0.3 (10)[d]	490 ± 36 (16)[b]	350 ± 28(17)[b,d]	5.3 ± 0.8 (9)[d]
I_{dark} (pA)	−33 ± 4.2 (14)	−28 ± 3.3 (15)	−27 ± 2.2 (10)	−25 ± 3.0 (16)	−27 ± 2.4 (17)	−33 ± 5.0 (9)
σ^2 (pA^2)	14 ± 3.1 (13)[a]	6.4 ± 1.6 (15)[a,c]	2.2 ± 0.3 (10)[c]	9.6 ± 1.3 (14)	12 ± 2.0 (16)[c]	4.7 ± 0.6 (9)[c]
$I_{1/2}$ (Rh*/rod)	2.5 ± 0.13 (14)	2.5 ± 0.17 (15)		2.8 ± 0.12 (16)	2.5 ± 0.15 (17)	
n	1.5 ± 0.04 (14)	1.6 ± 0.06 (15)		1.4 ± 0.05 (16)	1.5 ± 0.07 (17)	
τ_{int} (ms)	120 ± 10 (9.1)	100 ± 8 (10.0)		110 ± 6 (11.7)	120 ± 6 (10.3)	
T_{peak} (ms)	120 ± 4 (9.1)[a]	130 ± 5 (10.0)[a]		120 ± 3 (11.7)	120 ± 4 (10.3)	
% I_{sat}	16 ± 1.2 (9.1)	15 ± 2.0 (10.0)		16 ± 1.4 (11.7)	16 ± 1.7 (10.3)	

	Rod bipolar		AIIAC	
	$G\alpha_{o2}^{+/+}$	$G\alpha_{o2}^{-/-}$	$G\alpha_{o1}^{+/+}$ $Cx36^{-/-}$	$G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$
I_{max} (pA)	430 ± 44 (15)	370 ± 37 (16)	210 ± 44 (10)[a]	100 ± 22 (9)[a]
I_{dark} (pA)	−30 ± 4.3 (16)	−29 ± 2.7 (16)		
σ^2 (pA^2)	12 ± 2.0 (14)	13 ± 1.8 (16)		
$I_{1/2}$ (Rh*/rod)	2.2 ± 0.15 (15)[a]	2.6 ± 0.19 (16)[a]	0.17 ± 0.01(10)[b]	2.6 ± 0.13 (9)[b]
n	1.5 ± 0.02 (15)	1.6 ± 0.05 (16)	1.6 ± 0.09 (10)	1.5 ± 0.09 (9)
τ_{int} (ms)	120 ± 7 (9.2)	130 ± 11 (8.0)		
T_{peak} (ms)	120 ± 4 (9.2)	130 ± 8 (8.0)		
% I_{sat}	19 ± 1.8 (9.2)[a]	14 ± 1.6 (8.0)[a]		

All the values are given as mean ± SEM (*n*). The effective number of cells was used to calculate the SEM of τ_{int}, T_{peak}, and % I_{sat} (see Sampath et al., 2005). I_{max} is the maximal response amplitude. I_{dark} and σ^2 are the mean and the variance of holding current measured in the first 5 s after the establishment of whole-cell configuration. $I_{1/2}$ is the half-maximal flash strength. *n* is the exponent in the Hill Equation fit to flash strength vs. normalized response amplitude curves. τ_{int} and T_{peak} are the integration time and the time-to-peak of dim flash responses. % I_{sat} is the fractional amplitude of a dim flash response to an average flash strength of 1 Rh*/rod.

[a]$P < 0.05$, significant difference between littermates.

[b]$P < 0.01$, significant difference between littermates.

[c]$P < 0.05$, significant difference between $G\alpha_o^{-/-}$ or $G\alpha_{o1}^{-/-}$ rod bipolar cells compared to their heterozygote.

[d]$P < 0.01$, significant difference between $G\alpha_o^{-/-}$ or $G\alpha_{o1}^{-/-}$ rod bipolar cells compared to their heterozygote.

($G\alpha_o^{-/-}$). Voltage-clamp recordings ($V_m = -60$ mV) from rod bipolar cells in $G\alpha_o^{-/-}$ mice are shown in Fig. 1 C, and indicate that the ON response was completely lost from all ON bipolar cells tested ($n = 23$), including rod bipolar cells (10 of 23). Neighboring OFF bipolar cells in the same retinal slices demonstrated normal responses ($n = 6$; Fig. 1 D). Thus, $G\alpha_{o2}$ appears to mediate the remaining response in $G\alpha_{o1}^{-/-}$ rod bipolar cells. Interestingly, the initial holding current in voltage-clamp recordings from $G\alpha_o^{-/-}$ rod bipolar cells was not statistically different from that in WT cells (Table I), indicating that transduction channels remained closed despite the loss of $G\alpha_o$.

Characterization of $G\alpha_{o2}$-mediated rod bipolar responses in AII amacrine cells

The $G\alpha_{o2}$-mediated ON response in $G\alpha_{o1}^{-/-}$ rod bipolar cells was small and decayed too quickly to be characterized. To assess the sensitivity of the $G\alpha_{o2}$-mediated response in rod bipolar cells we instead recorded their output in the postsynaptic AII amacrine cells (AIIAC; see Fig. 1 A). Because AIIACs are more sensitive than rod bipolar cells and operate at light levels where few of the rod bipolar cell inputs are active (Pang et al., 2004; Dunn et al., 2006), their light responses will reflect subtle changes in the rod bipolar response. In addition, AIIACs are not subject to washout because their response is mediated by ionotropic glutamate receptors (Boos et al., 1993; Hartveit and Veruki, 1997). To isolate the direct output of rod bipolar cells, we eliminated input to the recorded AIIACs from neighboring AIIACs and ON cone bipolar cells by crossing $G\alpha_{o1}$ mice with $Cx36^{-/-}$ mice (Deans et al., 2002; see Fig. 1 A).

Fig. 2 (A and B) shows voltage-clamped ($V_m = -60$ mV) response families to flashes of increasing strength from $G\alpha_{o1}^{+/+}$ $Cx36^{-/-}$ and $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs. The maximum response amplitude among all the $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs tested was ~200 pA ($n = 9$), indicating that even small rod bipolar responses mediated by $G\alpha_{o2}$ can produce more substantial changes in downstream signals. In Fig. 2 C, the normalized response amplitude is plotted versus the flash strength and reveals that response families in $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs are shifted to ~10-fold brighter flash strengths compared with $G\alpha_{o1}^{+/+}$ $Cx36^{-/-}$ AIIACs. Furthermore, the maximal response amplitude of $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs was, on

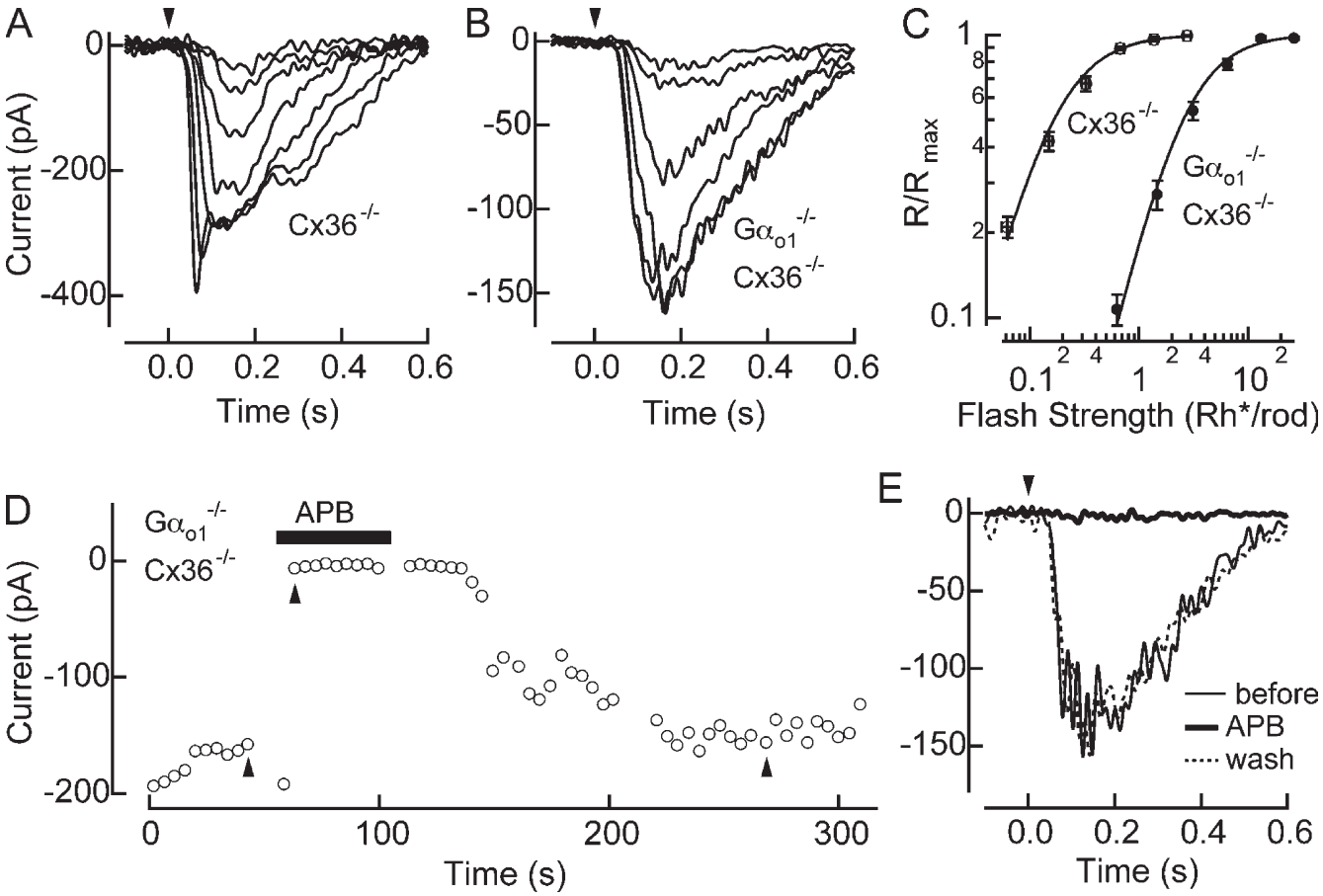

Figure 2. $G\alpha_{o2}$-mediated light responses measured in $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs. (A and B) Flash response families were recorded in a $Cx36^{-/-}$ (i.e., $G\alpha_{o1}^{+/+}$ $Cx36^{-/-}$ littermate) AIIAC (A) and a $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIAC (B). Flash strengths in the $Cx36^{-/-}$ AIIAC were 0.04, 0.1, 0.22, 0.46, 0.94, 1.9, and 3.8 Rh*/rod, and in the $G\alpha_{o1}^{+/+}$ $Cx36^{-/-}$ AIIAC were 0.63, 1.5, 3.1, 6.5, 13, and 27 Rh*/rod. (C) Normalized response amplitudes from individual families were averaged across cells for $Cx36^{-/-}$ AIIACs ($n = 10$) and $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs ($n = 9$), and plotted as a function of the flash strength. Half-maximal flash strengths estimated from the Hill curve fits were 0.17 ± 0.01 and 2.56 ± 0.13 Rh*/rod (mean ± SEM) for $Cx36^{-/-}$ and $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs, respectively. (D) Changes in the amplitude of the maximal flash response as a function of time before, during, and after application of APB are plotted. (E) Maximal flash responses (27 Rh*/rod) in a $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIAC before, during, and after the bath application of 8 µM APB, as marked by upward arrows in D.

average, approximately twofold smaller than $G\alpha_{o1}^{+/+}$ $Cx36^{-/-}$ AIIACs (Table I). Provided that AIIACs provide an accurate measure of rod bipolar sensitivity, this suggests that the rod bipolar response mediated by $G\alpha_{o2}$ alone is ~20-fold less sensitive than the response mediated by both $G\alpha_{o1}$ and $G\alpha_{o2}$. Interestingly we find that dark-adapted light responses to the strongest flashes in the $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs lacked the initial nose seen under normal circumstances (Nelson, 1982), suggesting that rod bipolar responses mediated by $G\alpha_{o2}$ alone are not able to fully drive glutamate release from the rod bipolar synaptic terminal.

$G\alpha_{o2}$-mediated responses were also controlled by mGluR6. Fig. 2 D plots the maximal inward response amplitude during the application of the mGluR6 agonist, L-2-aminophosphonobutyric acid (APB), for $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs. APB (8 µM) completely suppressed the response in $G\alpha_{o1}^{-/-}$ $Cx36^{-/-}$ AIIACs, an effect that was reversible after washout (Fig. 2 E). Thus, both the $G\alpha_{o1}$ and the $G\alpha_{o2}$ mediate a depolarizing light response in rod bipolar cells through the activity of mGluR6.

Reduced amplitude and sensitivity of light responses in $G\alpha_{o2}^{-/-}$ rod bipolar cells

We assessed the functional role played by $G\alpha_{o2}$ on the dark-adapted response of rod bipolar cells in $G\alpha_{o2}^{-/-}$ mice (Fig. 3 A). Response families in $G\alpha_{o2}^{-/-}$ rod bipolar cells appeared similar to WT, with statistically indistinguishable maximal amplitudes (Table I). The time-to-peak and integration time (defined as the integral of the dim flash response divided by its peak amplitude) of the dim flash response was also statistically indistinguishable from WT rod bipolar cells (Fig. 3 B; see Table I). However, the loss of $G\alpha_{o2}$ caused a reduction in the amplitude of the $G\alpha_{o2}^{-/-}$ dim flash responses (Fig. 3 B; see also Fig. S1 A), which led to an overall reduction of light sensitivity of rod bipolar cells, as seen by the shift to higher flash strengths in the plot of normalized response amplitude

versus flash strength (Fig. 3 C). The half-maximal flash strength provides a robust measure of the sensitivity of rod bipolar cells that is independent of the maximal response amplitude (Fig. S1 B). Thus the presence of $G\alpha_{o2}$ increases the sensitivity of the average response to a dim flash in rod bipolar cells of WT mice.

To determine how the decreased amplitude of the dim flash response influenced its detection, we characterized how the absence of $G\alpha_{o2}$ impacted the dark noise. We calculated the total variance (0–300 Hz) of the noise in darkness for $G\alpha_{o2}^{-/-}$ and WT rod bipolar cells in the 5 s immediately after establishing the whole-cell recording for the cells shown in Fig. 3. The total variance of the dark noise in WT rod bipolar cells was 11.5 ± 2.0 pA^2 (n = 14) and in $G\alpha_{o2}^{-/-}$ rod bipolar cells was 12.7 ± 1.8 pA^2 (n = 16) (mean ± SEM; P = 0.67), values that are indistinguishable statistically. The loss of $G\alpha_{o2}$ appears then to cause a reduction in the amplitude of the light response with the magnitude of the dark noise remaining unchanged, resulting in an overall reduced signal-to-noise ratio in $G\alpha_{o2}^{-/-}$ rod bipolar cells.

Reducing the total expression of $G\alpha_o$ does not alter rod bipolar responses

Reduced sensitivity in $G\alpha_{o2}^{-/-}$ rod bipolar cells may be simply due to the decrease in the total amount of $G\alpha_o$ protein rather than any specific role played by $G\alpha_{o2}$. To test whether the concentration of $G\alpha_o$ influenced response sensitivity, we recorded rod bipolar responses from heterozygous mice for $G\alpha_o$ ($G\alpha_o^{+/-}$). As shown in

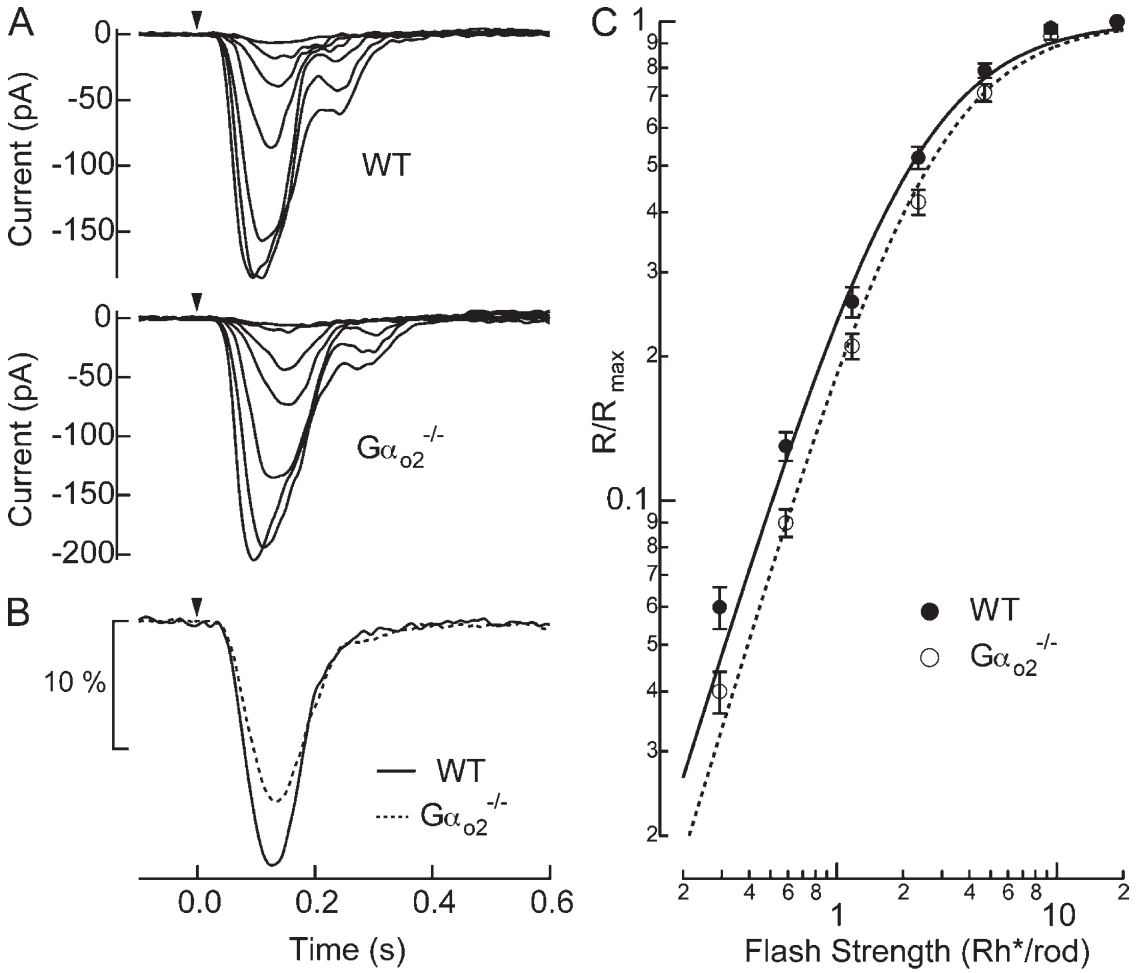

Figure 3. $G\alpha_{o2}^{-/-}$ rod bipolar cells exhibited reduced light sensitivity. (A) Responses to a family of flashes producing 0.29, 0.59, 1.2, 2.3, 4.7, 9.4, and 19 Rh*/rod were recorded in a WT (i.e., $G\alpha_{o2}^{+/+}$ littermate) and a $G\alpha_{o2}^{-/-}$ rod bipolar cell. (B) Normalized rod bipolar response to dim flashes producing 1 Rh*/rod was estimated by averaging normalized responses to dim flashes casing 5–25% of maximal responses and dividing those by the average flash strength, which was 0.60 Rh*/rod for WT and 0.72 Rh*/rod for $G\alpha_{o2}^{-/-}$ rod bipolar cells. The WT response is the average of 332 dim flash responses across 15 cells from 8 mice, and $G\alpha_{o2}^{-/-}$ response is the average of 321 dim flash responses across 16 cells from 6 mice. (C) Normalized response amplitudes from individual families were averaged across cells for WT (n = 15) and $G\alpha_{o2}^{-/-}$ rod bipolar cells (n = 16), and plotted as a function of flash strength. Half-maximal flash strengths estimated from the Hill curve fits were 2.2 ± 0.15 vs. 2.6 ± 0.19 Rh*/rod, and the Hill exponents were 1.51 ± 0.02 vs. 1.55 ± 0.05 for WT vs. $G\alpha_{o2}^{-/-}$ rod bipolar cells, respectively (mean ± SEM). While differences in the Hill exponent were not statistically significant (P = 0.13), the shift in half-maximal flash strengths was significant (P = 0.047).

Fig. 4 C, Western blot analysis for the whole retina using an antibody raised against $G\alpha_o$ confirmed that $G\alpha_o^{+/-}$ retinas had reduced $G\alpha_o$ expression by ~50% compared with WT. Since $G\alpha_o$ expression in the mouse retina is primarily in ON bipolar cells (Vardi et al., 1993; Vardi, 1998; Dhingra et al., 2000, 2002), and rod bipolar cells are approximately one third of all bipolar cells (Dhingra et al., 2008; Wassle et al., 2009), we expect the $G\alpha_o$ expression in rod bipolar cells is also approximately halved. Despite the loss of half of $G\alpha_{o1}$ and $G\alpha_{o2}$ (Fig. 4 C), the overall response kinetics and the sensitivity of $G\alpha_o^{+/-}$ rod bipolar cells remained similar to those of the littermate WT rod bipolar cells (Fig. 4, A and D; Table I). While the average time-to-peak was delayed slightly in $G\alpha_o^{+/-}$ rod bipolar cells (from 118 to 133 ms; see Table I), the integration time of the dim flash response was statistically indistinguishable from WT rod bipolar cells (Fig. 4 B; Table I). Thus, the reduced sensitivity in $G\alpha_{o2}^{-/-}$ rod bipolar cells appears instead to result from a specific effect of $G\alpha_{o2}$, and not from a reduction in the overall $G\alpha_o$ level. Furthermore, Fig. 4 D shows the Hill exponent between WT and $G\alpha_o^{+/-}$ rod bipolar cells are statistically identical, indicating that saturation within the mGluR6 cascade is not set by the $G\alpha_o$ concentration (see Discussion).

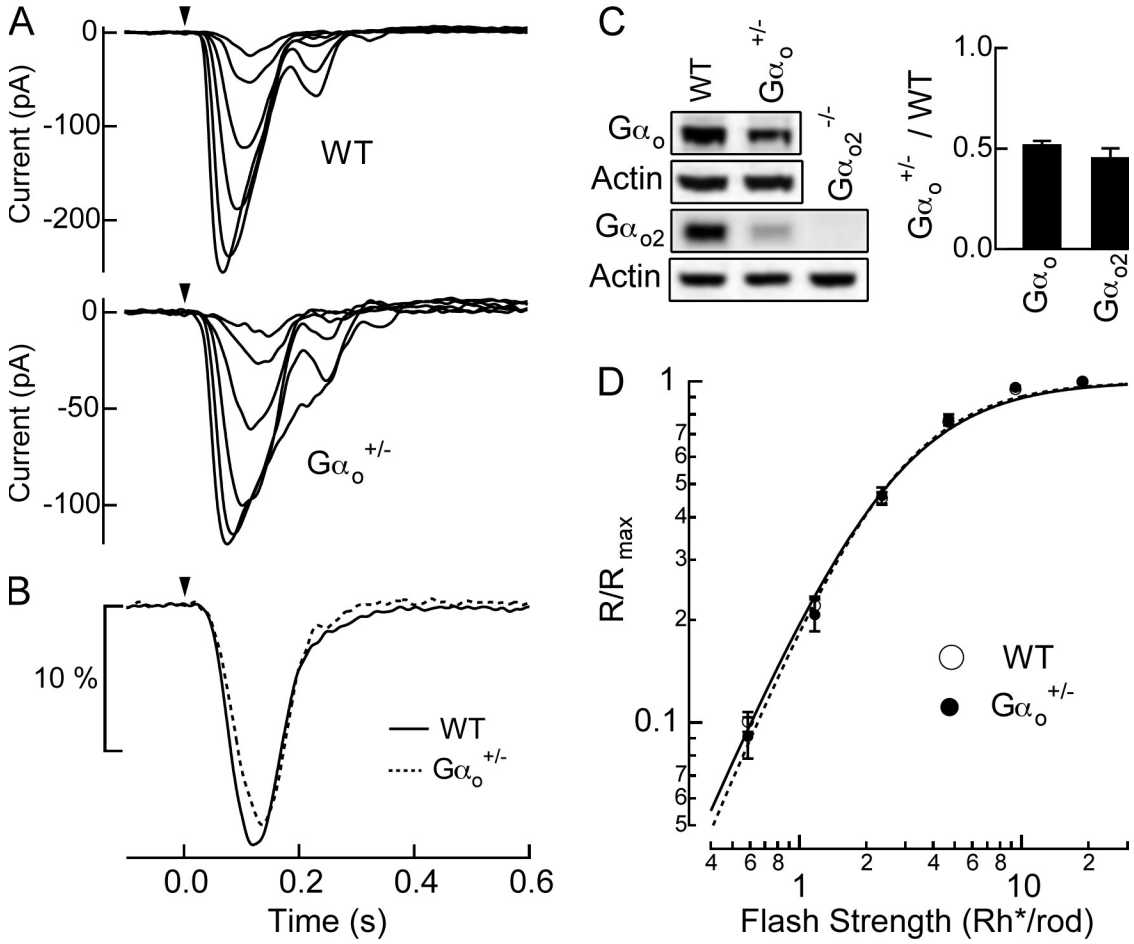

Figure 4. Reduced $G\alpha_o$ expression in $G\alpha_o^{+/-}$ mice does not alter rod bipolar responses (A) Responses to a family of flashes producing 0.59, 1.2, 2.4, 4.7, 9.4, and 19 Rh*/rod were recorded in a WT ($G\alpha_o^{+/+}$ littermate), and a $G\alpha_o^{+/-}$ rod bipolar cell. (B) Normalized rod bipolar response to dim flashes producing 1 Rh*/rod was estimated by averaging normalized responses to dim flashes casing 5–25% of maximal responses and dividing it by the average dim flash strength, which was 0.73 Rh*/rod for WT and 0.79 Rh*/rod for $G\alpha_o^{+/-}$. The WT response is the average of 437 dim flash responses across 14 cells from 3 mice, and the $G\alpha_o^{+/-}$ response is the average of 271 dim flash responses across 15 cells from 3 mice. (C) The total amount of $G\alpha_o$ and $G\alpha_{o2}$ proteins in WT and $G\alpha_o^{+/-}$ retinas were compared using Western blot analysis. The amount of $G\alpha_{o2}$ proteins in $G\alpha_{o2}^{-/-}$ retinas was also examined to check the specificity of the antibody. The protein level of $G\alpha_o^{+/-}$ retina was normalized to that of WT retina for a pair of WT and $G\alpha_o^{+/-}$ mice used in one experiment, and the collected results are shown in the bar graph. The error bars are the SEM. The $G\alpha_o$ protein levels were 1 vs. 0.52 ± 0.02 (n = 4) and the $G\alpha_{o2}$ protein levels were 1 vs. 0.46 ± 0.04 (n = 3) (mean ± SEM, WT vs. $G\alpha_o^{+/-}$). (D) Normalized response amplitudes from individual families were averaged across cells for WT rod bipolar cells (n = 14) and $G\alpha_o^{+/-}$ rod bipolar cells (n = 15) and plotted as a function of flash strength. Half-maximal flash strengths estimated from the Hill curve fits were 2.5 ± 0.13 vs. 2.5 ± 0.17 Rh*/rod, and the Hill exponents were 1.54 ± 0.04 vs. 1.62 ± 0.06 (mean ± SEM, WT vs. $G\alpha_o^{+/-}$).

Altering the balance of expression between $G\alpha_{o1}$ and $G\alpha_{o2}$ does not alter rod bipolar responses

Splice variants of G proteins typically display alterations in cellular functions, and frequently act on different effectors in the same cell. $G\alpha_{o1}$ and $G\alpha_{o2}$ both mediate depolarizing light responses in rod bipolar cells (Figs. 1 and 2), suggesting in the simplest scheme that they act on a common effector in the mGluR6-signaling cascade, although actions on different effectors cannot be ruled out. Regulation of the effector might then be dependent on the relative ratios of each of these splice variants. We tested how the ratio of $G\alpha_{o1}$ to $G\alpha_{o2}$ influences the properties of rod bipolar light responses in $G\alpha_{o1}^{+/-}$ mice. Fig. 5 C shows that the total $G\alpha_o$ expression was decreased by ~60% in these mice, whereas $G\alpha_{o2}$ expression was increased by ~25% compared with WT retinas. Overall, the ratio of $G\alpha_{o2}$ expression over $G\alpha_{o1}$ increased approximately threefold in $G\alpha_{o1}^{+/-}$ retinas compared with WT. Since the presence of $G\alpha_{o2}$ increased the sensitivity of the light response (Fig. 3), increasing the relative ratio of $G\alpha_{o2}$ to $G\alpha_{o1}$ might further increase the sensitivity of rod bipolar cells. However, neither the

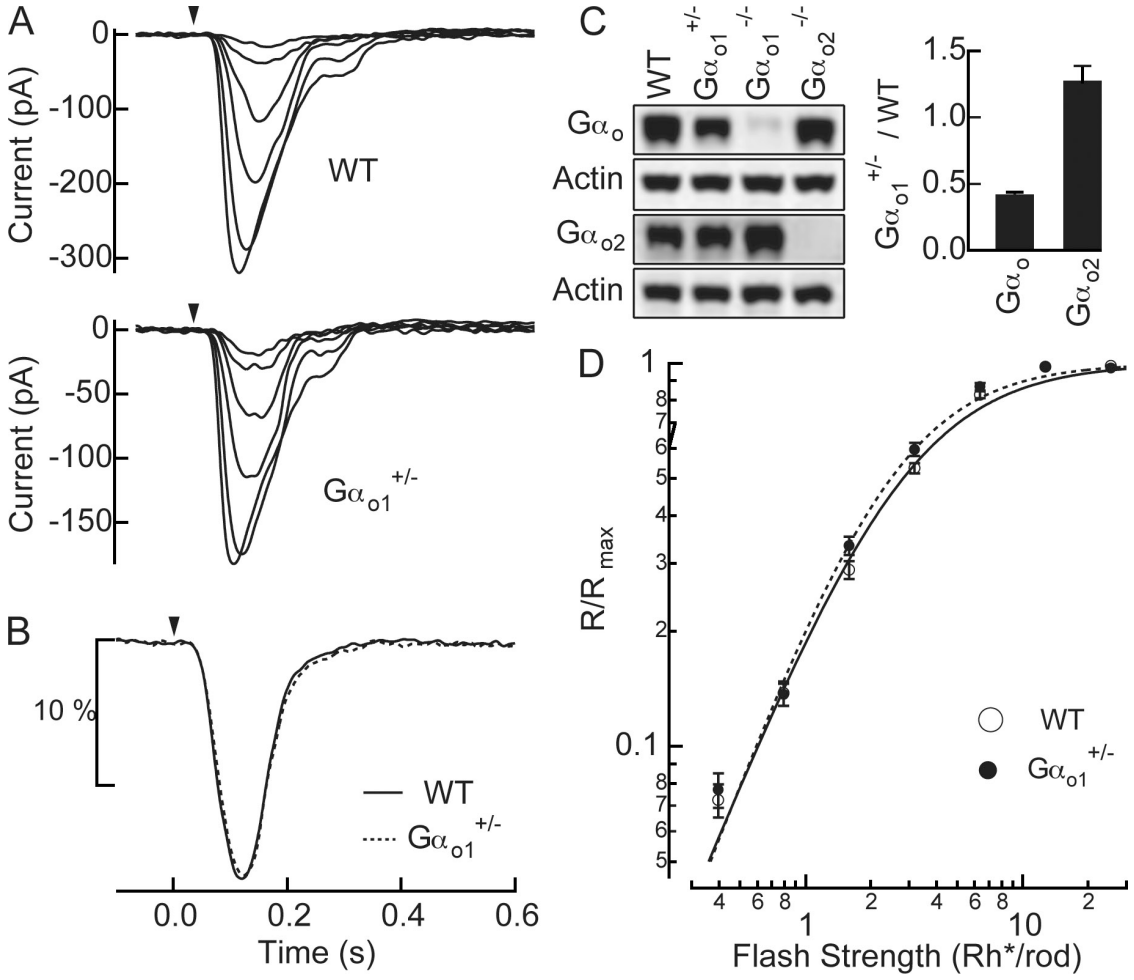

Figure 5. Altered $G\alpha_{o1}$ vs. $G\alpha_{o2}$ expression in $G\alpha_{o1}^{+/-}$ mice does not alter rod bipolar responses (A) Responses to a family of flashes producing 0.4, 0.8, 1.6, 3.2, 6.4, and 13 Rh*/rod were recorded in a WT ($G\alpha_{o1}^{+/+}$ littermate) and a $G\alpha_{o1}^{+/-}$ rod bipolar cell. (B) Normalized rod bipolar response to dim flashes producing 1 Rh*/rod was estimated by averaging normalized responses to dim flashes casing 5–25% of maximal responses and dividing it by the average dim flash strength, which was 0.70 Rh*/rod for WT and 0.72 Rh*/rod for $G\alpha_{o1}^{+/-}$. The WT response is the average of 351 dim flash responses across 16 cells from 4 mice and the $G\alpha_{o1}^{+/-}$ response is the average of 331 dim flash responses across 17 cells from 4 mice. (C) The total amount of $G\alpha_o$ and $G\alpha_{o2}$ proteins in WT, $G\alpha_{o1}^{+/-}$, $G\alpha_{o1}^{-/-}$, and $G\alpha_{o2}^{-/-}$ retinas were compared with Western blot analysis. The protein levels were normalized to those of WT retinas for a group of mice used in one experiment, and the results of repeated experiments are shown in the bar graph. The error bars show SEM. The $G\alpha_o$ protein levels were 1 vs. 0.42 ± 0.02 vs. 0.05 ± 0.01 vs. 0.95 ± 0.22 (n = 3), and the $G\alpha_{o2}$ protein levels were 1 vs. 1.27 ± 0.11 vs. 1.84 ± 0.16 vs. 0.01 ± 0.01 (n = 3) (mean ± SEM, WT vs. $G\alpha_{o1}^{+/-}$ vs. $G\alpha_{o1}^{-/-}$ vs. $G\alpha_{o2}^{-/-}$). (D) Normalized response amplitudes from individual families were averaged across cells for WT rod bipolar cells (n = 16) and $G\alpha_{o1}^{+/-}$ rod bipolar cells (n = 17) and plotted as a function of flash strengths. Half-maximal flash strengths estimated from the Hill curve fits were 2.81 ± 0.12 vs. 2.47 ± 0.14 Rh*/rod, and the Hill exponents were 1.43 ± 0.05 vs. 1.54 ± 0.07 (mean ± SEM, WT vs. $G\alpha_{o1}^{+/-}$), and are statistically indistinguishable (P = 0.12 for half-maximal flash intensity values, and P = 0.19 for Hill exponents between WT and $G\alpha_{o1}^{+/-}$ rod bipolar cells).

half-maximal flash strength nor the nonlinearity of flash response family in $G\alpha_{o1}^{+/-}$ rod bipolar cells differed from those in WT rod bipolar cells (Fig. 5, A and D; Table I). The time-to-peak and the integration time of the dim flash response were also statistically indistinguishable from WT (Table I). Thus, the balance of expression levels between two splice variants cannot explain the coordinated action of these splice variants in increasing the sensitivity of the rod bipolar cell response.

DISCUSSION

G proteins are essential signaling molecules that connect hundreds of G protein–coupled receptors with a relatively limited number of downstream effectors. In particular, G-protein signaling cascades play fundamental roles in early visual processing where they generate the response to light exposure in both rod and cone photoreceptor cells, and in ON bipolar cells. In ON bipolar cells, relatively little is know about the intermediate components of the signaling cascade that allow mGluR6 receptors through the action of $G\alpha_o$ to close TRPM1 transduction channels (for reviews see Okawa and Sampath, 2007; Snellman et al., 2008). Here we have studied how $G\alpha_o$ activity influences the dark-adapted light response in mouse rod bipolar cells and found the following: (a) the coordinated action of two splice variants of $G\alpha_o$ ($G\alpha_{o1}$ and $G\alpha_{o2}$) maximizes light sensitivity, (b) reductions in the concentration of $G\alpha_o$ do not influence the open probability of transduction channels, and (c) the nonlinear threshold due to the saturation of the transduction cascade does not depend on the $G\alpha_o$ concentration.

Coordinated actions of $G\alpha_{o1}$ and $G\alpha_{o2}$ improve rod bipolar sensitivity

The α subunit of G_o is expressed as two splice variants ($G\alpha_{o1}$ and $G\alpha_{o2}$) that differ by 26 amino acids in the GTPase domain near the C-terminal end (Hsu et al., 1990; Strathmann et al., 1990; Tsukamoto et al., 1991),

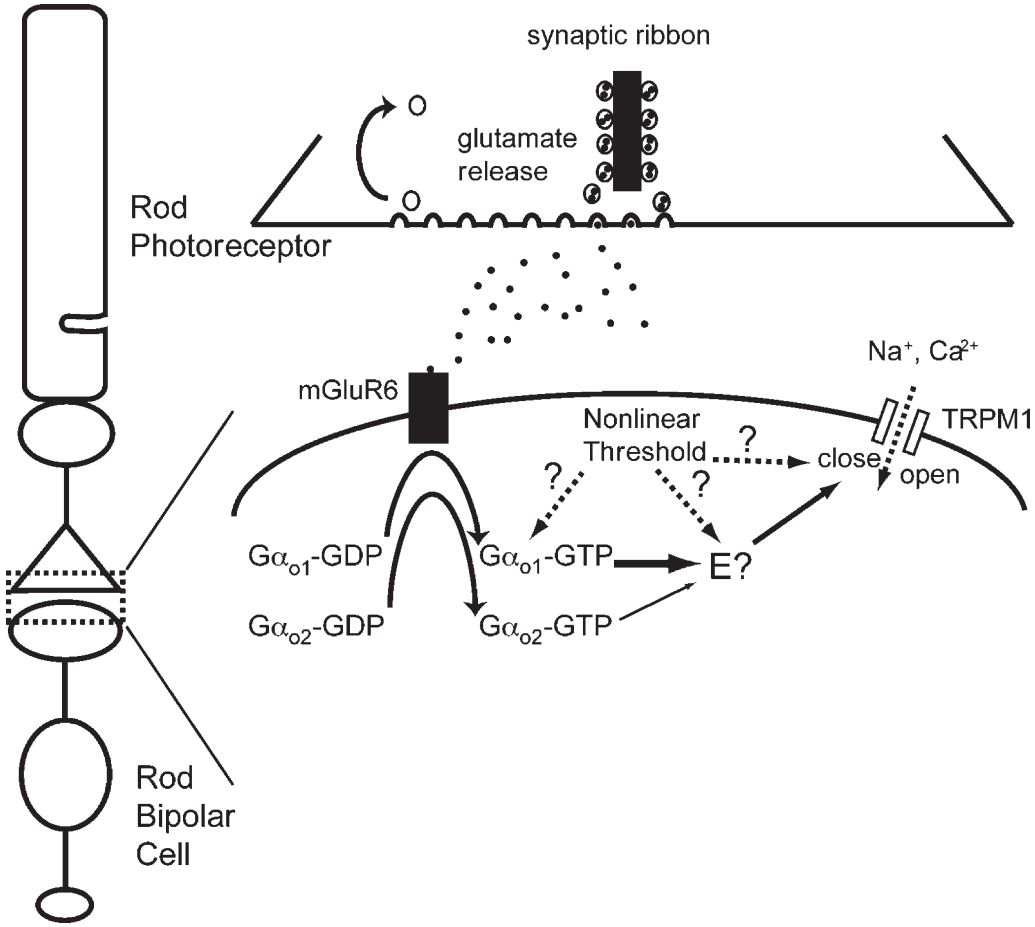

Figure 6. Proposed mGluR6-signaling cascade in rod bipolar cells. mGluR6 receptors activated upon binding glutamate released from rods exchange GTP for GDP on both $G\alpha_{o1}$ and $G\alpha_{o2}$, which leads to the closure of nonselective cation channels (TRPM1) through an unknown downstream cascade. The efficiency of the $G\alpha_{o2}$ pathway is lower than that of the $G\alpha_{o1}$ pathway, as represented by the thin arrow leading to the putative effector (E?). While a single effector is shown, this work does not exclude the possibility that $G\alpha_{o1}$ and $G\alpha_{o2}$ act on separate effectors that lead to the coordinated closure of TRPM1 gating. Arrows show that nonlinearity in the signaling cascade might reside at several locations.

a region known to link α_o subunits to their receptors and effectors (for review see Clapham and Neer, 1997). $G\alpha_o$ splice variants have typically been assigned with different or redundant functions. For instance, in the rat pituitary GH_3 cells, $G\alpha_{o1}$ and $G\alpha_{o2}$ mediate Ca^{2+} channel inhibition through muscarinic and somatostatin receptors, respectively (Kleuss et al., 1991, 1993; Degtiar et al., 1997). In rod bipolar cells, both $G\alpha_{o1}$ and $G\alpha_{o2}$ are controlled by the mGluR6 receptor and mediate the depolarizing light response (Figs. 1 and 2) without occluding each other's actions (Fig. 5). The most parsimonious explanation for these two facts are that both splice variants act independently on a common effector, as diagrammed in Fig. 6, however, we cannot rule out actions on different effectors.

Although $G\alpha_{o2}$-mediated signals are much less efficient than $G\alpha_{o1}$-mediated signals, a feature that may result from differing affinities of each splice variant for mGluR6 or the effector, the reduced efficiency likely reflects the relatively low expression of $G\alpha_{o2}$ compared with $G\alpha_{o1}$ (Dhingra et al., 2002). However, given that AIIACs are highly sensitive to rod bipolar cell input (Dunn and Rieke, 2008; Tian et al., 2010), any subtle variation in the rod bipolar response should result in detectable changes in AIIAC activity. We find that the amplitude of dim flash responses per photon in rod bipolar cells of $G\alpha_{o2}^{-/-}$ mice is ~25% smaller on average than in WT rod bipolar cells (Fig. 3 B; Fig. S1 A; Table I). This reduced sensitivity is attributable to a $G\alpha_{o2}$-specific effect because it cannot be explained either by the total $G\alpha_o$ concentration or the balance of expression between $G\alpha_{o1}$ and $G\alpha_{o2}$. Thus, the light response in rod bipolar cells is primarily mediated by $G\alpha_{o1}$, but $G\alpha_{o2}$ is necessary to increase the magnitude of the response without increasing the dark noise, thereby increasing the signal-to-noise ratio. Such coordination between two splice variants of a single $G\alpha$ may represent a novel mechanism that fine tunes the functional properties of signaling cascades.

TRPM1 channels remain closed in the absence of $G\alpha_o$ activity

A surprising finding of this work is that reductions of $G\alpha_o$ concentration (Figs. 4 and 5), or even the elimination of $G\alpha_o$ entirely (Fig. 1), does not appear to influence the amplifier holding current for voltage-clamped ($V_m = -60$ mV) rod bipolar cells (see Table I). The interpretation of this result is that reductions in $G\alpha_o$ concentration do not correspond to increases in the nonselective cation current of TRPM1 channels. Previous studies for TRPM1 channels expressed in CHO cells (Koike et al., 2009) suggest that these channels are constitutively open, with the presumed role of $G\alpha_o$ to close them (Nawy, 1999; Dhingra et al., 2000, 2002; Koike et al., 2009). The lack of influence of $G\alpha_o$ on the open probability of TRPM1 channels argues that this scheme is more complicated in situ, and may require additional factors for TRPM1 opening (Fig. 6). Alternatively, strong Ca^{2+}-dependent reductions in TRPM1 open probability (Nawy 2000, 2004; Berntson et al., 2004b) may relegate these channels closed even in the absence of $G\alpha_o$.

$G\alpha_o$ concentration does not set the nonlinear thresholding of rod signals

Our most sensitive vision is encoded in a specialized retinal circuit that pools rod signals, known as the rod bipolar pathway (see Fig. 1 A; Dacheux and Raviola, 1986; Smith et al., 1986). Under conditions where few rod photoreceptors receive photons, downstream cells must discriminate between rods that absorb light from those that do not. The optimization of signal transfer in this pathway requires a nonlinear threshold in rod bipolar cells that separates the single photon response from noise (van Rossum and Smith, 1998; Field and Rieke, 2002), which is generated by saturation of the postsynaptic signaling cascade in the rod bipolar cell dendrites and not by mGluR6 receptor saturation (Sampath and Rieke, 2004). The molecular mechanism that underlies the nonlinear threshold is not well defined, largely due to the uncertain identity of components of this signaling cascade downstream of $G\alpha_o$.

Here we show that the nonlinear threshold is not influenced by an ~50% reduction in concentration of retinal $G\alpha_o$ (Fig. 4), providing insight into where saturation may occur in the mGluR6 signaling cascade. If the rate of G-protein activation is saturated, such that the reduced $G\alpha_o$ expression does not cause an equivalent reduction in G-protein activity, these results indicate that the binding of $G\alpha_o$ to mGluR6 does not cause this saturation. However, we cannot eliminate the possibility that the exchange of GTP for GDP on $G\alpha_o$, or the dissociation of $G\alpha_o$ from mGluR6, places a bottleneck on the dark steady-state G-protein activity. Experimental evidence from rod outer segment preparations indicate that transducin ($G\alpha_t$) activation can occur very quickly (>1000 s^{-1} at physiological temperatures; Bruckert et al., 1992; Heck and Hofmann, 2001), perhaps not totally surprising given the high concentration of transduction elements. However, relatively little is known about G-protein activation rates in other intact systems. It remains to be seen whether GTP exchange and $G\alpha$ dissociation limit $G\alpha_o$ activation on the ~120 ms integration time of dark-adapted rod bipolar light responses.

If the rate of G-protein activation by mGluR6 is not saturated in darkness, then these results would indicate that the position of the nonlinear threshold must reside downstream of $G\alpha_o$ activation (see Fig. 6), or alternatively that G-protein activity is sufficient to saturate a downstream component of the signaling cascade even under conditions where this activity is reduced (i.e., $G\alpha_o^{+/-}$). Saturation could potentially be in the activity of the effector molecule that controls the gating particle of

TRPM1, or in the open probability of TRPM1 itself (compare Sampath and Rieke, 2004). For the level of saturation to be optimized with respect to the rod signal and noise, it must be set high enough to eliminate most of the continuous noise produced by spontaneous PDE activation, but not to eliminate too many single photon responses (Field and Rieke, 2002). Thus a delicate tradeoff between noise and sensitivity must exist, giving great importance to identifying the component of the signaling cascade mediating this nonlinear step.

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