Published in final edited form as:

Eur J Neurosci. 2016 June ; 43(11): 1509–1522. doi:10.1111/ejn.13243.

Lack of mGluR6-Related Cascade Elements leads to Retrograde Trans-synaptic Effects on Rod Photoreceptor Synapses via Matrix-associated Proteins

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Abstract

Heterotrimeric G-proteins couple metabotropic receptors to downstream effectors. In retinal ON bipolar cells, G_o couples the metabotropic receptor mGluR6 to the TRPM1 channel and closes it in the dark, thus hyperpolarizing the cell. Light, via GTPase activating proteins, deactivates G_o , opens TRPM1, and depolarizes the cell. G_o comprises $G\alpha_{o1}$, $G\beta_3$ and $G\gamma_{13}$; all are necessary for efficient coupling. In addition, $G\beta_3$ contributes to trafficking of certain cascade proteins and to maintaining the synaptic structure. The goal of this study was to determine the role of $G\alpha_{o1}$ in maintaining the cascade and synaptic integrity. Using mice lacking $G\alpha_{o1}$, we quantified the immunostaining of certain mGluR6-related components. Deleting $G\alpha_{o1}$ greatly reduced staining for $G\beta_3$, $G\gamma_{13}$, $G\beta_5$, RGS11, RGS7, and R9AP. $G\alpha_{o1}$'s deletion did not affect mGluR6, TRPM1, or PCP2. In addition, deleting $G\alpha_{o1}$ reduced the number of rod bipolar dendrites that invaginate the rod terminal, similar to the effect seen in the absence of mGluR6, $G\beta_3$, or the matrix-associated proteins, pikachurin, dystroglycan, and dystrophin, which are localized presynaptically to the rod bipolar cell. We therefore tested mice lacking mGluR6, $G\alpha_{o1}$, and $G\beta_3$ for expression of the matrix-associated proteins. In all three genotypes, staining intensity for these proteins was lower than in wild type, suggesting a retrograde trans-synaptic effect. We propose that the mGluR6 macromolecular complex is connected to the presynaptic rod terminal via a protein chain that includes the matrix-associated proteins. When a component of the macromolecular chain is missing, the chain may fall apart and loosen the dendritic tip adherence within the invagination.

Keywords

G-protein; Mouse; G-proteins; dystrophin; β -dystroglycan; pikachurin

Introduction

The key function of heterotrimeric G-proteins (comprising $G\alpha$ and $G\beta\gamma$ subunits) is to couple metabotropic receptors to their downstream effectors. These downstream effectors (enzymes or channels) modulate processes as diverse as growth and differentiation to physiological signal processing. In the retina, an important G-protein-mediated signaling cascade occurs in a class of second order neurons, the ON bipolar cells, where it is essential for night vision and for detecting light increments in daylight vision. This cascade inverts the photoreceptor's hyperpolarizing signal to a depolarizing response (Nawy and Jahr, 1990; Shiells and Falk, 1990). In the dark, glutamate released from photoreceptors binds the metabotropic glutamate receptor mGluR6, this activates a G-protein cascade that closes the non-selective cation channel TRPM1 and thereby hyperpolarizes ON bipolar cells (Koike *et al.*, 2010; Masu *et al.*, 1995; Morgans *et al.*, 2010). Light hyperpolarizes the photoreceptors, reducing glutamate release, opening the TRPM1 channels, and thus depolarizing ON bipolar cells.

ON bipolar cells express $G\alpha_o$, $G\beta_3$ and $G\gamma_{13}$ (Huang *et al.*, 2003; Ritchey *et al.*, 2010; Vardi *et al.*, 1993). While the three subunits in the heterotrimeric G-protein are thought to form a single functional unit that couples the receptor to an effector, it is becoming increasingly clear that each subunit also has a different function (Dhingra *et al.*, 2012; Ramakrishnan *et al.*, 2015). In ON bipolar cells, while deleting $G\alpha_o$ ($G\alpha_{o1}$ and $G\alpha_{o2}$) eliminates the light ON response (Dhingra *et al.*, 2002; Dhingra *et al.*, 2000; Okawa *et al.*, 2010), deleting $G\beta_3$ greatly reduces it but does not eliminate it, and deleting $G\gamma_{13}$ has a much milder effect (Dhingra *et al.*, 2012; Ramakrishnan *et al.*, 2015). In addition, deleting $G\beta_3$ mislocalizes many of the cascade components and compromises the synapse while deleting $G\gamma_{13}$ has a milder effect on the expression and/or localization of these components and no effect on the synapse. The effect of deleting $G\alpha_{o1}$, the predominant splice variant in ON bipolar cells, on the localization of mGluR6 cascade components has not yet been determined. In order to better understand how the G-protein subunits contribute to the maintenance of synaptic structure, we first tested the effect of deleting $G\alpha_{o1}$ on the expression of mGluR6-related cascade components within the ON bipolar cells. We found that the most vulnerable cascade components are those that comprise the GTPase activating protein (GAP) complex. We also found that lack of $G\alpha_{o1}$ compromises the synaptic structure. Testing the effects of deleting mGluR6, $G\alpha_{o1}$ or $G\beta_3$ on the expression of matrix-associated proteins in the rod to rod bipolar synapse, we found that their absence causes a retrograde trans-synaptic deficit in these proteins, and this likely leads to the compromised synaptic structure.

Methods

Ethical Approval

Procedures involving animals were performed in accordance with the guidelines of the National Institute of Health and the protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The total number of animals used for this study was 29 wild type (WT), 22 *Gnao1* (gene encoding $G\alpha_o$) knockout (KO), 6 *Grm6* (gene encoding mGluR6)-KO, and 6 *Gnb3* (gene encoding $G\beta_3$)-KO. For most experiments, mice were 4–7 weeks old, but we also used one 11 week old

mouse for electron microscopy; both male and female mice were used. Mice were deeply anaesthetized with an intraperitoneal injection of ketamine and xylazine (100 and 10 µg/gm bodyweight). Eyes were enucleated and incised just below the lens.

Immunostaining of retinal cryosections

Eyeballs were fixed in 4% paraformaldehyde for 10 or 60 minutes (for Gβ3 staining), rinsed in phosphate buffer, soaked overnight at 4°C in 0.1M phosphate buffer containing 30% sucrose for cryoprotection and embedded in a mixture of two parts 20% sucrose in phosphate buffer and one part optimal cutting temperature (OCT) compound (Tissue Tek, Electron Microscopy Sciences, Hatfield, PA, USA). Radial sections (15–18 µm) were cut on a cryostat (Leica Biosystems, Buffalo Grove, IL, USA) at –20°C, and sections were collected on a superfrost plus slide (Fisher scientific, Pittsburgh, PA, USA). Sections were permeabilized and blocked with 10% normal goat serum, 5% sucrose and 0.5% Triton X-100 in phosphate buffer for 1 hour at 20°C. Sections were then incubated in primary antibodies (diluted in blocking solution) at 4°C overnight or 3 days for mGluR6. Retinas were washed 3 times in 0.1M phosphate buffer containing 5% sucrose at room temperature. Sections were then incubated in secondary antibodies at 20°C for 3 hours, washed, mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and cover slipped. For double labeling, sections were simultaneously incubated in both primary antibodies (raised in different hosts) followed by both secondary antibodies.

Imaging and quantification

Sections were photographed with a confocal laser scanning microscope (Olympus Fluoview 1000, Center Valley, PA, USA) under a 60x oil-immersion objective (NA=1.42). Immunostaining intensities were compared between littermate (or age-matched) WT and KO retinas that had been processed in parallel and imaged under the same settings (a KO and its littermate WT are referred to as a 'set'). For images with single staining, regions of interest (ROI) were drawn around the different layers (outer plexiform, OPL; inner nuclear, INL; inner plexiform, IPL) and the background-subtracted average intensity per pixel for each ROI was measured using Volocity software (Improvision, PerkinElmer, Waltham, MA). For Gβ3 staining in cones, we drew ROIs around the outer segments of individual cones and determined the background-subtracted average intensity per pixel. For images with double stainings (ribbon and a matrix-associated protein), a user-defined MATLAB script was used to quantify the average intensity per pixel per punctum as described previously (Tummala *et al.*, 2014). Briefly, the program identifies ribbons and isolates puncta in a single focal plane using a user-specified threshold for intensity. It then fits a 2-D Gaussian to each isolated punctum and determines the size (full width at half maximum, FWHM) as the number of pixels with intensities above the half maximum intensity of the punctum and the intensity of each punctum as the average intensity per pixel of these pixels. The average intensity of staining or punctum size for each retina is the average of values from at least three confocal images. For each metric, the values obtained for KOs were normalized with the average values obtained from all the WT mice.

Antibodies

The following antibodies and concentrations were used: rabbit anti-G γ 13 (1:500, a gift from R. Margolskee); rabbit anti-G β 3 (1:300, HPA005645; Sigma); guinea pig anti-mGluR6 (1:100, Neuromics Edina, MN Oregon); mouse anti-G α_o (1:100, MAB 3073; Millipore); rabbit anti-TRPM1 (1:100, a gift from T. Furukawa, Osaka Bioscience Institute, Osaka, Japan) (Koike *et al.*, 2010; Kondo *et al.*, 2011); rabbit anti-G β 5 (1:500) and rabbit anti-RGS11 (1:1000, gifts from T. Wensel, Baylor College of Medicine, Houston, TX); rabbit anti-RGS7 (1:200, gift from K. Martemyanov, Scripps Research Institute, Jupiter, FL); rabbit anti-Ret-PCP2 (1:1,000, gift of Dr. Rod Feddersen; see Xu *et al.* 2008); rabbit anti-pikachurin (1:2,000, Wako 011-22631, Richmond, VA); mouse anti-dystroglycan (1:100, Abcam, ab49515, Cambridge, MA); mouse anti-dystrophin (1:100, Millipore MAB 1694, Billerica, MA); rabbit anti-RGS9 anchoring protein (R9AP) (1:100–500, a gift from V. Arshavsky, Duke University, Durham, NC); mouse anti-kinesin [1:100–200, Covance (currently Bio-legends)]; rabbit anti-ribeye (1:500, a gift from T. Sudhof, Stanford University, CA); sheep anti-TRPM1 (for Western blotting, 1:1,000, Gift of Dr. Kirill Martemyanov, The Scripps Research Institute, Jupiter, Florida); and mouse anti-beta-actin (1:3,000, Clone AC-74 Sigma, St. Louis, MO).

Electron microscopy

Eyes were fixed in phosphate buffer (0.1 M, pH 7.4) containing 2% paraformaldehyde and 2% glutaraldehyde for 6–12 hours. Eyes were then rinsed in phosphate buffer, and small pieces of retina were taken (with the optic disk at one end). Tissues were incubated in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 hours, rinsed with phosphate buffer, dehydrated in series of ethanol rinses and incubated sequentially with Embed 812:propylene oxide mixtures (1:1 and 2:1) and then in pure Embed 812 (Electron Microscopy Sciences, Hatfield, PA) overnight. Tissues were then embedded in fresh Embed 812 mixture at 60°C for 48 hours. Semi-thin sections (1 μ m) were stained with toluidine blue. Ultrathin sections (90 nm) were counterstained with uranyl acetate and lead citrate, and were examined with a JEM 1011 transmission electron microscope (10K magnification). Images were captured by ORIUS 835.10W CCD camera (Gatan, Pleasanton, CA) and analyzed using ImageJ. For analysis, we quantified the number of rod terminal profiles at which we see a ribbon, a horizontal cell process, or a bipolar dendrite. Thus, we first numbered all the identifiable profiles of rod terminals in an image, and then determined the presence or absence of the relevant structures in each profile. While it is possible to analyze the structure in reference to ribbons or dyads, a study on *Grm6*-KO that compared results obtained from reconstruction to those obtained from the two different methods of sampling showed that when sampling, referencing the data to number of profiles rather than number of ribbons produces more comparable results (Tsukamoto and Omi, 2014).

Western Blotting

Retinas from *Gnao1*^{-/-} and WT littermate mice were detached and frozen in liquid nitrogen. Tissue was homogenized using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in a lysis buffer containing 5 mM Tris-HCl, pH 7.5, 320 mM sucrose, 2 mM EDTA, 2.5 mM β -mercaptoethanol, and protease inhibitor mixture (P 8340, Sigma, St. Louis, MO). The

homogenate was centrifuged for 10 min at $6000 \times g$. Proteins from the supernatant were assayed using BSA protein reagent (Bio-Rad, Hercules, CA). The proteins were run on 4–15% SDS-PAGE gels and transferred to a nitrocellulose membrane using a wet transfer apparatus (Bio-Rad, Hercules, CA). After a quick rinse in PBS, the blots were incubated sequentially in the following: Odyssey blocking buffer diluted with PBS (1:1, blocking buffer) at room temperature for 1 h; primary antibody diluted in blocking buffer containing 0.1% Tween 20 at 4°C overnight; washes in PBS+0.1% Tween 20 (PBST); IRDye-conjugated secondary antibodies (from LI-COR Biosciences, Lincoln, NE, 1:15,000 dilution in blocking buffer containing 0.1% Tween 20) at room temperature for 1 h; washes in PBST; and a final rinse in PBS. The blots were then scanned using the Odyssey Infrared Imaging system (LI-COR Biosciences) as per the manufacturer's instructions. To control for equal loading, antibody against β -actin (as a loading control protein) was also included along with test antibody. The two antibodies used together were chosen such that they differed in the host species to enable their discrimination by different secondary antibodies. The band intensities were quantified using Odyssey software, adjusted for background and normalized against β -actin levels. Student's *t*-test was used to compare a quantity in WT and null KOs.

Results

Deleting $G\alpha_{o1}$ reduces $G\beta 3$ and $G\gamma 13$ expression in ON bipolar cell dendritic tips

Expression of G-protein subunits in a heterotrimeric complex often depends on each other. We have previously shown that in ON bipolar cells, deleting $G\beta 3$ dramatically reduces $G\alpha_o$ and $G\gamma 13$ (Dhingra *et al.*, 2012). Surprisingly, deleting $G\gamma 13$ greatly reduces $G\beta 3$, but hardly affects the expression of $G\alpha_o$ (Ramakrishnan *et al.*, 2015). To test whether these relationships are reciprocal, we tested how the deletion of $G\alpha_{o1}$, the primary splice variant in ON bipolar cells, affects its partners. Immunostaining of WT and *Gnao1*-KO retinas (4 sets) for $G\beta 3$ was dramatically reduced in all compartments of ON bipolar cells lacking $G\alpha_{o1}$ (Fig. 1A–B). In the OPL, the staining was reduced to about 35% of WT and the difference was highly significant ($p=0.003$) (Fig. 1E). Significant reduction was also seen in the IPL ($p=0.008$). In contrast, staining for $G\beta 3$ in cones was unaffected (Fig. 1A, E). Staining for $G\gamma 13$ (6 WT and 7 KO mice) was also reduced substantially in all layers; in the OPL staining was reduced to 43% of WT ($p=0.044$; Fig. 1C, D, F). Thus, $G\alpha_{o1}$ is required for normal expression of its partners.

Deleting $G\alpha_{o1}$ does not affect the expression of mGluR6 and TRPM1

Next we tested the effect of $G\alpha_{o1}$ deletion on the localization of the receptor mGluR6 and the channel TRPM1. For mGluR6, in both WT and KO (6 sets), we observed the characteristic punctate staining where each dot represents 2 rod bipolar dendritic tips. However, staining was variable between animals, and the puncta in some KOs appeared less organized (Fig. 2A). For TRPM1 (5 WT and 6 KO), staining was observed both in the dendritic tips in a punctate pattern and in the ON bipolar cells somas. Here too, staining in KOs was variable and appeared less organized than in WT (Fig. 2B). Quantitatively, the average intensity in the OPL of KO was lower than in WT, but the variability was higher and the difference did not reach statistical significance (Fig. 2C). Because mGluR6 and TRPM1 are restricted to ON bipolar cells, we could also assess expression by Western blotting; this

showed that these two cascade components were expressed at similar amounts in WT and KO retinas (4 sets of WT and KO for mGluR6, $p=0.6$; 3 sets for TRPM1, $p=0.7$; Fig. 2D, E and Supplementary Fig. 1A, B).

Deleting $G\alpha_{o1}$ dramatically reduces expression of the GTPase activating proteins, but not other $G\alpha_{o1}$ modulators

In *Gnb3*- and in *Gng13* (gene encoding $G\gamma13$)-KO mice, the most affected cascade components are those of the GTPase activating protein (GAP) complex, which are necessary for producing the rising phase of the light response (Cao *et al.*, 2012; Dhingra *et al.*, 2004; Orlandi *et al.*, 2012; Shim *et al.*, 2012; Zhang *et al.*, 2010). To determine if the absence of $G\alpha_{o1}$ results in a similar deficiency, we immunostained retinas for $G\beta5$, RGS11, RGS7, and R9AP. Indeed, for all these components the staining in the OPL was drastically reduced (Fig. 3). For $G\beta5$, staining intensity in KO OPL was 47% of that in WT ($p=0.043$; 5 WT and 6 KO); for RGS11, it was 26% ($p=0.002$; 5 WT and 6 KO); for RGS7 it was 69% ($p=0.022$; 4 sets); and for R9AP it was 47% ($p=0.021$; 5 WT and 6 KO). To test whether the reduced localization is due to reduced expression level in ON bipolar cells, we used Western blotting to determine total expression of RGS11, the only protein out of these 4 that, in retina, is restricted to ON bipolar cells. We found that RGS11 in KO is significantly reduced to 54% of WT ($p=0.013$) (Fig. 3B3, B4 and supplementary Fig. 1C). Reduced expression in OPL suggests that $G\alpha_{o1}$ is needed for correct localization at the tips and reduced total expression suggests that $G\alpha_{o1}$ is also needed to stabilize the protein.

We also tested the expression of PCP2, a known interactor of $G\alpha_o$ that in the retina is expressed only in ON bipolar cells and serves to accelerate the light response (Xu *et al.*, 2008). Expression and localization of this protein was only mildly changed with a small but not significant increase in INL (30%; $p=0.167$; 5 WT and 6 KO mice) (Fig. 4A, B). Expression of PCP2 as measured by Western blotting (Fig. 4C), showed about 31% increase in KO (4 sets) but measurements were highly variable, perhaps due to the small size of the protein (not shown). Thus, while the localization and perhaps expression level of the G-protein subunit partners of $G\alpha_{o1}$ and its GAPs are affected by $G\alpha_{o1}$'s expression, some cascade components are unaffected.

In *Gnao1*-KO, the ultrastructure of the synapse between rods and rod bipolar dendrites is slightly altered

Rods contact rod bipolar cells in a synaptic structure called tetrad. In this synaptic complex, the rod terminal contains a synaptic ribbon, on which the synaptic vesicles are tethered. The ribbon is presynaptic to four postsynaptic processes that invaginate the rod terminal: two lateral processes that belong to horizontal cells, and two central dendrites that belong to bipolar cells. We have previously qualitatively examined the synapse between the rod terminal and ON bipolar dendrites in *Gnao1*-KO and found them to be similar, but because the evaluation was not quantitative, in this study, we re-examined the ultrastructure in *Gnao1*-KO. We analyzed 1370 profiles of rod terminal from 4 WT retinas and 1656 profiles from 4 littermate KO retinas of 4–7 week old mice and one 11 week old KO in a total retinal length of 1.5 mm for WT and 2.2 mm for KO. For all profiles, we counted the number that contained: a synaptic ribbon, at least one horizontal cell process, and at least

one bipolar dendrite. We found that in KO, both horizontal cell processes and bipolar dendrites were represented in a lower fraction of profiles compared to WT (Fig. 5A, B). Horizontal cells in KO were reduced to 88% of WT (58% in KO vs 66% in WT, $p=0.010$) and bipolar dendrites were reduced to 71% of WT (25% in KO vs 34% in WT; $p=0.026$). The percentage of profiles in which ribbons were observed was also smaller in KO (47% vs 52%), but this difference was not significant ($p=0.450$). In comparison, analyzing the effect of deleting G β 3 on the ultrastructure showed no effect on percent of ribbons or horizontal cells and a greater effect on bipolar cells whose percentage was reduced to 55% of WT (3 sets of retinas from littermate mice, $p=0.01$; Fig. 5C).

Absence of mGluR6, G α_{o1} or G β 3 affects expression of presynaptic proteins

The finding that the synaptic structure is compromised in several mice lines in which the ON bipolar cascade components are deleted or mutated (such as mGluR6, G β 3, RGS7+RGS11) (Cao *et al.*, 2009; Dhingra *et al.*, 2012; Ishii *et al.*, 2009; Shim *et al.*, 2012; Tsukamoto and Omi, 2014) suggests that the deletion of these cascade components affects not only proteins directly involved in the cascade, but also proteins that are responsible for maintaining a normal synaptic structure. Three extracellular matrix-associated proteins are known to localize at the rod-to-rod bipolar synapse. These are pikachurin, dystroglycan, and dystrophin (reviewed by Constantin, 2014; Pillers, 1999 and). Deleting either pikachurin or dystroglycan impedes complete invagination of the rod bipolar dendrites, similar to that seen after deleting the mGluR6 cascade components (Omori *et al.*, 2012; Sato *et al.*, 2008). This raises the possibility that the observed deficiency in synaptic structure after deleting a cascade component is secondary to its effect on the expression or localization of any of these matrix-associated proteins.

To determine the effect of deleting a cascade component on the localization of the matrix-associated proteins, we double stained retinas of WT and three different KO genotypes (Gnao1-KO, Gnb3-KO, and Grm6-KO) for each of these proteins and also for a ribbon marker (ribeye or kinesin). First we stained for pikachurin; this protein is similar to agrin and perlecan with three laminin G domains (Sato *et al.*, 2008). It is produced by photoreceptors where it is cleaved and released into the extracellular space between the photoreceptor and the ON bipolar dendrite (Han and Townes-Anderson, 2012). In wild type mice, immunostaining for pikachurin showed the expected punctate appearance in the outer plexiform layer (Fig. 6A). In Gnao1-KO, staining appeared reduced, but the variability in punctate staining was higher than in WT (Fig. 6B). Using the staining for a presynaptic ribbon to localize the expected location of its postsynaptic bipolar dendritic tip, for each punctum, we quantified the average staining intensity per pixel. This method is more sensitive than that in which we quantified the average intensity per pixel in the OPL because when considering only the puncta, the average intensity is not diluted by empty spaces in the OPL. We also estimated the size of each punctum (see methods). Note however that a change in punctum size does not necessarily mean smaller apposition between the rod membrane and the dendritic tip because the computed size depends on the staining intensity of the punctum. Using these metrics we found that the average intensity for pikachurin in Gnao1-KO was slightly lower than in WT (Fig. 6C1), but the difference was not statistically significant ($p=0.73$; 7 sets). The average punctum size, however, was slightly smaller in KO,

and the difference was significant ($p = 0.047$), probably because of the lower variability in this metric. Given the large variability in the staining intensities of the puncta, we plotted the distribution of the intensities. We binned intensity values from all the 7 retinas into 41 bins and plotted the proportion of puncta in each bin (the intensity values ranged from 0-4095; each bin included 100 values). We found that in WT, the puncta intensities distributed normally and peaked at 1200 units, but in KO most puncta had low intensities, and the distribution peaked at 400 units (Fig. 6C2). The distribution in KO also showed that the proportion of puncta with high intensity was slightly higher than in WT, thus explaining why the average intensity was similar, despite appearing lower in the KO.

Evaluating pikachurin expression in *Gnb3*-null retinas, we found similar results: the average staining intensity was similar in WT and KO (3 mice each) with higher variability in KO (Fig. 6D–F), the puncta size in KO was slightly (88%) but significantly ($p=0.020$) lower than in WT, and the puncta distribution in *Gnb3*-KO was shifted to the left, similar to that in *Gnao1*-KO (Fig. 6F). Finally, in *Grm6*-KO (Fig. 6G–I), the average intensity was significantly lower than in WT (74% of WT; $p=0.01$; 4 WT and 6 KO mice), and the average size was highly significantly lower (73%; $p<0.0001$). The KO distribution was greatly shifted to the left of WT, and very few puncta showed high intensity.

Next we tested localization of β -dystroglycan, a glycoprotein with one transmembrane domain that is cleaved into α and β dystroglycan, and localizes with pikachurin at the electron-dense region of the photoreceptor membrane that apposes the ON bipolar dendrite (Blank *et al.*, 1999; Drenckhahn *et al.*, 1996; Hu *et al.*, 2011; Jastrow *et al.*, 2006; Kanagawa *et al.*, 2010; Montanaro *et al.*, 1995; Omori *et al.*, 2012). In this case, we used Ctbp2 (Ribeye) as the pre-synaptic ribbon marker. The staining pattern for β -dystroglycan was also punctate, and the intensity and the punctum average size were significantly reduced in all three genotypes. In *Gnao1*-KO, the intensity decreased to 43% of WT ($p=0.003$; 6 mice each) and the size decreased to 83% of WT ($p=0.033$) (Fig. 7A–C). In *Gnb3*-KO, these ratios were 68% for intensity ($p=0.013$; 3 sets) and 85% for size ($p=0.025$) (Fig. 7D–F). In *Grm6*-KO, the intensity in KO was reduced to 51% of WT ($p=0.025$; 4 WT and 5 KO mice) and the size was reduced to 72% ($p<0.001$) (Fig. 7G–I). The distribution curves in all three KO genotypes were shifted to the left.

Finally we tested immunoreactivity for dystrophin, a dystroglycan ligand that is expressed in the photoreceptor terminals and whose deficiency causes a slow and reduced ERG b-wave in both humans and mice (Blank *et al.*, 1999; Dalloz *et al.*, 2001; Drenckhahn *et al.*, 1996; Green *et al.*, 2004; Haenggi and Fritschy, 2006; Kameya *et al.*, 1997; Lenk *et al.*, 1996; Omori *et al.*, 2012; Pillers *et al.*, 1995; Satz *et al.*, 2009; Wersinger *et al.*, 2011). Similar to pikachurin and dystroglycan, immunostaining for dystrophin was punctate. In *Gnao1*-KO, staining intensity per punctum was reduced to 67% of wild type ($p=0.085$; 5 WT and 6 KO mice) and the size was reduced to 86% ($p=0.022$) (Fig. 8A–C). In *Gnb3*-KO these ratios were 58% ($p=0.007$; 3 sets) and 81% ($p=0.007$) correspondingly (Fig. 8D–F) and in *Grm6*-KO, these were 43% ($p<0.001$; 4 WT and 6 KO mice) and 73% ($p<0.001$) (Fig. 8G–I). Thus all the three matrix-associated proteins were reduced by removing mGluR6-related cascade components.

Discussion

We present here four key findings. **(1)** The absence of $G\alpha_{o1}$, the predominant subunit of G_o , greatly reduces the staining of its partners ($G\beta 3$ and $G\gamma 13$) throughout the cell and the staining of its GAPs in rod bipolar dendritic tips. **(2)** The absence of $G\alpha_{o1}$ leads to slight reduction and disorganization of stainings for mGluR6 and TRPM1. **(3)** In the absence of $G\alpha_{o1}$, fewer rod bipolar dendrites invaginate the rod terminal. **(4)** In retinas lacking $G\alpha_{o1}$, $G\beta 3$, or mGluR6, genotypes with a phenotype of reduced invaginating rod bipolar dendrites, stainings for the presynaptic extracellular matrix-associated proteins are reduced.

Differential function of G_o 's subunits

The primary function of the heterotrimeric G-protein is to couple the receptor to its effector; however, our previous work showed that the heterotrimer is not obligatory. Thus, while deleting $G\alpha_o$ eliminates the light ON response (Dhingra *et al.*, 2002; Dhingra *et al.*, 2000; Okawa *et al.*, 2010), deleting $G\beta 3$ greatly reduces it but does not eliminate it (Dhingra *et al.*, 2012). Furthermore, deleting $G\gamma 13$ has a much smaller effect (Ramakrishnan *et al.*, 2015). The ability to generate a light response in the absence of $G\beta 3$ suggests that $G\alpha_o$ can cycle between its inactive GDP-bound form to its active GTP-bound form without a $G\beta\gamma$ dimer. This interpretation is also supported by the finding that in cones lacking $G\beta 3$, response sensitivity is reduced, but the maximal light response is as in WT (Nikonov *et al.*, 2013). Similar results were also obtained in rods lacking $G\gamma 1$ (Kolesnikov *et al.*, 2011; Lobanova *et al.*, 2008). These data suggest that the function of $G\beta$ and $G\gamma$ is not necessarily to permit coupling, but to increase its efficiency. An increase in efficiency may be achieved by locally facilitating conformational changes in the macromolecular complex comprising the receptor, the G-protein, and the effector. But an increase in efficiency can also be achieved by ensuring that the molecules comprising the macromolecule are trafficked to the same intracellular locus and maintained there. We previously postulated that $G\beta 3$ is crucial for either trafficking or maintaining the integrity of the mGluR6 macromolecular complex because in its absence, both mGluR6 and TRPM1 do not concentrate in the dendritic tips as they do in WT. Here we surmise that this trafficking function may not be shared by $G\alpha_o$ since the absence of $G\alpha_{o1}$ only slightly affects the localization of the receptor and channel, and this could happen indirectly by the reduction of $G\beta 3$. Further support for $G\beta$'s role in trafficking is provided by the observation that surface expression of Kir2.4 and Kir3.2 increases after exogenously expressing $G\beta\gamma$ (Rubinstein *et al.*, 2009; Sulaiman *et al.*, 2013). Additionally, in the absence of $G\beta 3$, Gata2, which typically concentrates in the cone outer segments, is localized diffusely throughout the cone compartments, suggesting that its trafficking to the outer segment also requires a $G\beta$ (Nikonov *et al.*, 2013). As for $G\gamma 13$, while the absence of $G\alpha_{o1}$ greatly reduces expression of $G\beta 3$ and $G\gamma 13$, and the absence of $G\beta 3$ reduces expression of $G\alpha_o$ and $G\gamma 13$, the absence of $G\gamma 13$ reduces $G\beta 3$, but not $G\alpha_{o1}$. This suggests a minor function for $G\gamma 13$ in stabilizing the heterotrimer.

Susceptibility of the GAP proteins in ON bipolar cells

We showed here that deleting $G\alpha_{o1}$ dramatically reduces the GAP proteins (RGS11, RGS7, $G\beta 5$, and R9AP) in the dendritic tips of ON bipolar cells; similar results were obtained when $G\beta 3$ or mGluR6 were deleted. This is different from cone photoreceptors, in which the

absence of G β 3 does not affect the expression of R9AP (Nikonov *et al.*, 2013). These observations raise the question as to why the localization of the GAPs in the dendritic tips of ON bipolar cells is so susceptible to the absence of the receptor or the key coupling subunits. We speculate that in ON bipolar cells, active G α_o recruits the GAPs to the dendritic tips. Thus, in the absence of G α_{o1} , which occurs both when *Gnao1* or *Gnb3* are deleted, there is no active subunit and GAPs are diffused throughout the cell. In case of *Grm6*-KO, although G α_o is still expressed (Xu *et al.*, 2012), the absence of mGluR6 renders G α_o inactive at all times, once again preventing the GAPs from being recruited to the tips. It is currently unknown whether the GAPs are also affected in the absence of TRPM1; however, our hypothesis predicts that their localization in the tips would not be affected.

Retrograde trans-synaptic communication from rod bipolar cells to rod photoreceptors

We showed that without G α_{o1} , the number of rod bipolar dendrites that invaginate the rod terminal is reduced. Qualitatively similar results were obtained when the following genes were mutated or deleted: *Grm6*, *Gnb3*, *Gnb5* (gene encoding G β 5), and *RGS7+RGS11* (Rao *et al.*, 2007; Cao *et al.*, 2009; Ishii *et al.*, 2009; Dhingra *et al.*, 2012; Shim *et al.*, 2012; Tsukamoto and Omi, 2014). These findings raise an important question: what is the mechanism that causes the deficit? In principle, the deficit can arise during development, in which case, one would propose that the postsynaptic cascade elements affect developmental processes. Or, it is possible that development proceeds normally, but the structure cannot be maintained. In one of these mutants, *Gnb5*-KO, the loss of invaginating bipolar dendrites was attributed to deficits in the developmental process (Rao *et al.*, 2007); however because G β 5 is also expressed in photoreceptors, the deficits may not be due to alterations in the mGluR6 cascade. In the *Gnb3*-KO, the loss of invaginating bipolar dendrites was more severe in the adult than in early development, indicating that the structural deficit is due to the failure of synaptic maintenance (Dhingra *et al.*, 2012).

To start understanding why the synaptic structure is affected by deleting postsynaptic cascade components, we stained for three matrix-associated proteins known to play a role in maintaining the structure of the rod to rod bipolar synapse. Our findings that these proteins are greatly reduced in three genotypes that show deficits in invaginating rod bipolar dendrites suggest that the lack of proper expression of these proteins causes withdrawal of the invaginating dendrites. Thus, we envision that certain proteins of the mGluR6-related cascade interact directly and others interact indirectly with the matrix-associated proteins. Together, they form a protein chain that connects the postsynaptic rod bipolar membrane and the cascade components to the presynaptic rod membrane (Fig. 9). When a protein in the chain is missing, the chain breaks apart, leading to reduced adherence between the rod and the rod bipolar cell, and then to retraction of the dendrite from the invagination. Thus, although the rod bipolar dendrites can develop normally by a genetic program and invaginate the rod terminal using for example the interaction between the cell adhesion protein ELFN1 and mGluR6 (Cao *et al.*, 2015), a stable adhesion requires the full complement of pre- and postsynaptic complexes that interact with the matrix proteins. A large number of extracellular matrix proteins have been localized to the rod synapse (reviewed by Mercer and Thoreson, 2011); these likely form several microdomains (such as the apposition between rod and horizontal cells or apposition between rod and bipolar cells). The identity of the

protein on the rod bipolar that directly interacts with the extracellular matrix-associated proteins is unknown, but is likely one of the proteins that contain an extracellular domain. Nyctalopin could be a candidate, but staining for dystrophin in a mouse lacking nyctalopin appears normal (Ball et al., 2003). Other candidates are GPR179, LRIT3, mGluR6, TRPM1, and Cacna1S (Fig. 9). Although the function of such a chain appears to be primarily to create a stable morphological connection between the pre and the post synaptic cells, reminiscent of what is seen in the neuromuscular junction (Nishimune et al., 2004), it inherently contributes to the size and proper timing of the rod bipolar response to light as seen when pikachurin, dystroglycan, or dystrophin are deleted or mutated (Pillers et al., 1999; Sato et al., 2008; Omori et al., 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health Grants EY11105 (Noga Vardi), R21 EY021308 (Noga Vardi and Robert G Smith), EY 013333 (Michael A Freed) and NEI P30 EY01583 (Vision Research Core of the University of Pennsylvania). We thank Drs. Takahisa Furukawa, Robert Margolskee, Theodore G. Wensel, Vadim Y Arshavsky, Rod Feddersen, Thomas Sudhof, and Kirill Martemyanov for donating the antibodies used in this study. We also thank, Robert Smith, Michael Freed, Mikhail Y. Lipin, and Sergei Nikonov for multiple discussions and help over the course of this study.

Abbreviations

WT	wild type
KO	knockout
GAP	GTPase activating proteins
ONL	outer nuclear layer
OPL	outer plexiform layer
INL	inner nuclear layer
IPL	inner plexiform layer
ROI	region of interest
FWHM	full width at half maximum

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Fig. 1. Deletion of $G\alpha_{o1}$ greatly reduces expression of $G\beta 3$ and $G\gamma 13$ in ON bipolar cells
A. Immunostaining for $G\beta 3$ in wild type (A1) and *Gnao1*-KO (A2) retinas shows that localization is unaffected in cones and is greatly reduced in ON bipolar cells. **B.** Higher magnification of ON bipolar cells in WT (B1) and KO (B2). **C and D.** Lower and higher magnification images showing staining for $G\gamma 13$ in WT (C1, D1) and *Gnao1*-KO retinas (D1, D2). **E, F.** Quantification of staining intensity (average intensity per pixel) in OPL, INL, IPL, and in cones (for $G\beta 3$). * indicates p value < 0.05 , and ** indicates $p < 0.01$. For all figures: OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; WT wild type mice, KO, *Gnao1* knockout.

Fig. 2. Localization and expression of mGluR6 and TRPM1 may be only mildly affected
A. Immunostaining for mGluR6 in WT and *Gnaol*-KO as indicated. **B.** Immunostaining for TRPM1 in WT and KO as indicated. **C.** Average staining for TRPM1 in OPL and INL was not significantly different between WT and KO. **D.** Western blots for mGluR6 and actin (top) and TRPM1 and actin (bottom) for WT and KO as indicated. **E.** Quantification of Western blots for mGluR6 and TRPM1. Total band intensity for mGluR6 (or TRPM1) was divided by that for actin in each sample (three replicates per sample, sample was taken from 1–2 retinas from each mouse) and then averaged across samples to yield relative intensity (n=4 mice for mGluR6 and n=3 mice for TRPM1).

Fig. 3. Localization of GAP proteins in dendritic tips of ON bipolar cells is greatly reduced
A1, 2. Immunostaining for Gβ5 in WT (A1) and in *Gnao1*-KO (A2) shows greatly reduced staining in KO. **A3.** Quantification of staining intensities for Gβ5 in different retinal layers; staining in IPL is mainly in amacrine cells. **B.** Immunostaining for RGS11 in WT (B1) and KO (B2). **B4.** An example of Western blots for RGS11 and actin. **B3.** Quantification of immunostaining (left axis) and of Western blots (right axis). **C, D.** Immunostaining and quantification for RGS7 (C) and R9AP (D) organized as in A and as indicated.

Fig. 4. PCP2 is not affected by deleting $G\alpha_{01}$

A. Immunostaining for PCP2 in WT (A1) and *Gnao1*-KO (A2); staining is present in all compartments of the ON bipolar cell. **B.** Quantification of staining intensity in the different layers. **C.** Western blots for PCP2 in WT and KO.

Fig. 5. The structure of the rod terminal is slightly compromised

A. Electron micrographs of rod terminal profiles in WT and *Gnao1*-KO. r- ribbon, h- horizontal cell process found lateral to the ribbon and b- bipolar dendrite. **B.** A bar graph showing the percentage of profiles in which we identified a ribbon, at least one horizontal cell process, or at least one bipolar dendrite in *Gnao1* KO and in littermate WT. **C.** Similar to B, but evaluation was made from *Gnb3*-KO and littermate WT.

Fig. 6. In mutants missing mGluR6-related cascade elements, pikachurin in the synaptic cleft between photoreceptor terminals and ON bipolar cells is greatly reduced
 Comparison of staining in WT and littermate *Gnao1*-KO (A–C), in WT and littermate *Gnb3*-KO (D–F), and in WT and aged-matched *Grm6*-KO (G–I). On the left (X1) are images of pikachurin staining (red) in WT and KO as indicated; in the middle (X2), same images merged with staining for kinesin to show synaptic ribbons; and on the right (C, F, I) are the quantitative data for each genotype. **C1, F1, I1.** Bar graphs shows average normalized values for intensity per pixel in a punctum and punctum size (values were normalized to average WT; gray bars are WT and red bars are KOs). **C2, F2, I2.** Curves show the intensity

distribution of all quantified puncta for WT (black) and KOs (red); the values on the y-axis are % of puncta for each intensity bin, where the total puncta are 100%; the actual numbers of puncta analyzed (n) for each curve are color code next to each graph.

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Fig. 7. In mutants missing mGluR6-related cascade elements, β -dystroglycan associated with the photoreceptor terminals to ON bipolar dendrite synapse is greatly reduced

The organization of this figure is as for Fig. 6, but showing staining for dystroglycan. Here, ribeye (green) was used to localize ribbons. Gray bars and black curves are for WT, red bars and red curves for KOs.

Fig. 8. In mutants missing mGluR6-related cascade elements, dystrophin present at the photoreceptor terminals in apposition to ON bipolar dendrites is greatly reduced
 The organization of this figure is as for Fig. 7 with staining for dystrophin. Gray bars and black curves are for WT, red bars and curves for KOs.

Fig. 9. Schematic diagram illustrates hypothetical protein chain that holds presynaptic rod membrane close to postsynaptic rod bipolar dendrite

Matrix-associated proteins on the rod side are known: dystrophin binds actin and β -dystroglycan, β -dystroglycan binds α -dystroglycan which in turn binds pikachurin. We hypothesize that these elements link to proteins that reside on the rod bipolar membrane. On the rod bipolar side, the possible players (those which contain an extracellular domain) are: LRIT3, GPR179, Nyx, mGluR6, Cacna1s, and TRPM1. It is currently unknown which is the connecting protein, but clearly all are part of a large macromolecular complex that contains also the heterotrimeric G-protein G_o (in red). β -DG, α -DG are beta and alpha dystroglycan respectively; Nyx, nyctalopin. Dashed lines show some possible connections.