Orphan Receptor GPR179 Forms Macromolecular Complexes With Components of Metabotropic Signaling Cascade in Retina ON-Bipolar Neurons

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Submitted: July 24, 2013
Accepted: September 27, 2013

Citation: Orlandi C, Cao Y, Martemyanov KA. Orphan receptor GPR179 forms macromolecular complexes with components of metabotropic signaling cascade in retina ON-bipolar neurons. Invest Ophthalmol Vis Sci. 2013;54:7153–7161. DOI:10.1167/iovs.13-12907

Purpose. In the mammalian retina, synaptic transmission between light-excited rod photoreceptors and downstream ON-bipolar neurons is indispensable for dim vision, and disruption of this process leads to congenital stationary night blindness in human patients. The ON-bipolar neurons use the metabotropic signaling cascade, initiated by the mGluR6 receptor, to generate depolarizing responses to light-induced changes in neurotransmitter glutamate release from the photoreceptor axonal terminals. Evidence for the identity of the components involved in transducing these signals is growing rapidly. Recently, the orphan receptor GPR179, a member of the G protein-coupled receptor (GPCR) superfamily, has been shown to be indispensable for the synaptic responses of ON-bipolar cells. In our study, we investigated the interaction of GPR179 with principle components of the signal transduction cascade.

Methods. We used immunoprecipitation and proximity ligation assays in transfected cells and native retinas to characterize the protein–protein interactions involving GPR179. The influence of cascade components on GPR179 localization was examined through immunohistochemical staining of the retinas from genetic mouse models.

Results. We demonstrated that, in mouse retinas, GPR179 forms physical complexes with the main components of the metabotropic cascade, recruiting mGluR6, TRPM1, and the RGS proteins. Elimination of mGluR6 or RGS proteins, but not TRPM1, detrimentally affects postsynaptic targeting or GPR179 expression.

Conclusions. These observations suggest that the mGluR6 signaling cascade is scaffolded as a macromolecular complex in which the interactions between the components ensure the optimal spatiotemporal characteristics of signal transduction.

Keywords: retina, ON-bipolar neurons, orphan GPCR, protein targeting, macromolecular complexes

The high sensitivity of vertebrate vision relies on the ability of light-stimulated rod photoreceptors to transmit signals to downstream ON-bipolar cells (ON-BC). The synaptic transmission between these cells is mediated by the neurotransmitter glutamate, which activates the postsynaptic mGluR6 receptor on the dendritic tips of ON-BC.1-4 Upon glutamate stimulation, mGluR6 activates the G protein, Go, which, in turn, keeps the effector channel, TRPM1, closed. Light hyperpolarizes photoreceptors and suppresses glutamate release. This leads to a reduction in Go activation by mGluR6 and the consequent opening of the TRPM1 channel, resulting in the depolarizing response of the ON-BC. Thus, Go deactivation is the key event that leads to the opening of the TRPM1 channel.

The G proteins exhibit slow rates of spontaneous deactivation, and the physiologically relevant timing of the process requires assistance from the regulator of G protein signaling (RGS) proteins, which greatly accelerate G protein inactivation.5,6 Recent studies have identified that two RGS proteins, RGS7 and RGS11, synergistically provide timely Go inactivation, which drives the light responses of ON-BC.7-10 The RGS7 and RGS11 proteins are targeted to the dendritic tips of ON-BC, where these proteins colocalize with components of the mGluR6 signaling cascade. The concurrent knockout of RGS7 and RGS11 in mice leads to a severe reduction in the light sensitivity of ON-BC and dramatically slows the onset of the depolarizing response.10

The GPR158 and GPR179 proteins are the two members of the orphan G protein-coupled receptor (GPCR) family that recently have been shown to bind to the RGS proteins.11 The GPR179 protein interacts with RGS7 and RGS11, and the disruption of GPR179 in mice leads to the failure of these RGS proteins to localize to the dendritic tips.11 Mutations in the GPR179 gene in humans or mice lead to defects in ON-BC responses and cause congenital stationary night blindness, characterized by an inability to see under dim light conditions.12,13 These observations suggest that GPR179 has an important role in regulating the synaptic signaling cascade of ON-BC neurons. However, virtually nothing is known about mechanistic aspects of GPR179 function. In our study, we demonstrated that, in addition to the previously reported interaction with RGS proteins, GPR179 forms macromolecular complexes with the mGluR6 receptor and TRPM1 channel, suggesting a role for this protein in the compartmentalization of the principal elements of the ON-BC cascade.
METHODS

Antibodies, Genetic Constructs, and Mouse Strains

The generation of sheep anti-RGS9-2 CT, sheep anti-RGS11, sheep anti-TRPM1, and sheep anti-mGluR6 antibodies has been described previously.\textsuperscript{14–16} Rabbit anti-RGS7 (7RC1) was a generous gift from William Simonds (National Institute of Diabetes, and Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD). Mouse anti-GPR179 (Ab877; Primm Biotech, Milan, Italy), mouse anti-\(\beta\) actin (A5441; Sigma-Aldrich, St. Louis, MO), rabbit anti-PKC\(\alpha\) (P4334; Sigma-Aldrich), rabbit anti-GTBP2 (C10751; Assay Biotech, Sunnyvale, CA), rabbit anti-myc (A00172; GenScript, Piscataway, NJ), and rabbit anti-GST (sc-459; Santa Cruz Biotechnology, Inc., Dallas, TX) were purchased. For the detection of GPR179 by Western blotting and immunoprecipitation studies, we used the sheep anti-RGS9-2 CT antibody\textsuperscript{15} (referred to in this study as sheep anti-GPR179 CT). This antibody was generated against a synthetic peptide with the following sequence: DSDDPRAGESGDQTEKEVCPWESLAEGKAG. From this sequence, 14 amino acids are repeated 21 times in the C-terminus of GPR179, with a homology ranging between 36\% and 72\% (see Supplementary Fig. S1A). We refer to this sequence as the conserved domain (CD). These antibodies recognize a single protein band upon Western blotting of HEK293 cells transfected with the GPR179 expression construct (see Supplementary Fig. S1B). No immunoreactivity was observed in nontransfected HEK293 cells or in cells transfected with a GPR179 construct lacking the C-terminal domain that harbors CD sequences (see Supplementary Fig. S1B). Furthermore, appending short CD sequences rendered an unrelated GST protein immunoreactive to sheep anti-GPR179 CT antibodies, confirming the antibody recognition epitope (see Supplementary Fig. S1C). Consistent with a previous report that the original target of these antibodies, RGS9-2, is absent completely from the retina,\textsuperscript{17} sheep anti-GPR179 CT antibodies recognized a single band on a Western blot with wild-type (WT) retinas (see Supplementary Fig. S1D). In contrast, this band was shifted to a lower molecular weight region in nob5 retinas (see Supplementary Fig. S1D), consistent with the predicted disruption of the N-terminal portion of GPR179 via transposon recombination.\textsuperscript{12}

The human mGluR6 plasmid was purchased from the Missouri cDNA Resource Center. Human myc-tagged-GPR179

\[\text{FIGURE 1.} \quad \text{The GPR179 coimmunoprecipitates with mGluR6 and TRPM1 in transfected cells and in vivo. (A) Coimmunoprecipitation of GPR179 with TRPM1 and mGluR6 in transfected cells. The HEK293T/17 cells were transfected with the indicated constructs. Immunoprecipitated proteins were detected by Western blotting using specific antibodies. Cells transfected with only TRPM1 or mGluR6 served as a control for nonspecific binding. The experiments were conducted three times involving independent transfections and yielded similar results. (B) Coimmunoprecipitation of GPR179, TRPM1, and mGluR6 in vivo. Total retina lysates prepared as described in Materials and Methods were subjected to immunoprecipitation with indicated antibodies (sheep GPR179 CT, sheep TRPM1, or sheep mGluR6). Sheep IgG were used as negative control for nonspecific binding for GPR179 immunoprecipitation. Retinas from TRPM1 knockout mice and nob3 mice were used as negative controls for nonspecific binding to sheep anti-TRPM1 and sheep anti-mGluR6 antibodies, respectively. The experiments were repeated at least three times, each time with retinas from separate animals.}\]
was purchased from OriGene (Rockville, MD). The cloning of full-length mouse myc-tagged–TRPM1 and RGS7 in pcDNA3.1/V5-His-TOPO has been described previously.15,18–21 A version of GPR179 lacking the C-terminus (nt 2922–7101, GPR179\textsuperscript{DCT\textit{myc}}) was generated by subcloning the corresponding portion of the GPR179 sequence into pcDNA3.1/V5-His-TOPO vector appended with the C-terminal c-myc tag. Two conserved sequences, each containing three repeats likely recognized by the sheep GPR179 CT antibodies, were fused in frame with GST and subcloned into the \textit{Escherichia coli} expression vector pGEX-2T. The first conserved domain (CD1) includes the sequence from 4237 to 4512 nt of human GPR179. The second domain (GPR179 CD2) includes the sequence from 5827 to 6141 nt. All cloned sequences were verified through DNA sequencing. Following expression in the BL21 \textit{E. coli} strain, the GST fusion proteins were purified through affinity chromatography on glutathione sepharose HP (GE Healthcare, Pittsburgh, PA) as described previously. 20 The \textit{nob3} mice carrying a frame-shifting mutation in the \textit{mGluR6} gene that leads to early termination at amino acid 264\textsuperscript{22} were obtained from The Jackson Laboratory (Bar Harbor, ME). The TRPM1 knockout mice (\textit{Trpm1}\textsuperscript{tm1Lex}) were obtained from European Mouse Mutant Archive (Munich, Germany). These mice contain a targeted deletion of exons 2 to 4, resulting in a null allele, as characterized in previous studies.23 The generation and characterization of \textit{nob5} mice carrying the transposon-mediated inactivation of \textit{GPR179} gene has been described.12 Male and female mice (1–3 months old) were used in the study. Mice were maintained on a diurnal 12-hour light/dark cycle. Procedures involving mice were approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute, and performed following the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

**Immunoprecipitation, Western Blotting, Cell Culture, and Transfection**

Retinas obtained from wild-type, TRPM1 knockout and \textit{nob3} mice were sonicated in ice-cold buffer (150 mM NaCl and complete protease inhibitor cocktail in PBS). The membrane
fraction was sedimented through centrifugation at 100,000 g for 30 minutes at 4°C. Each pellet was resuspended in ice-cold IP buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, and complete protease inhibitor cocktail) by sonication. For immunoprecipitation, the lysates were incubated with 20 lL of Dynabeads (Invitrogen, Carlsbad, CA) and 2 lL of specific antibodies on a rocker for 1 hour at 4°C. After three washes with IP buffer, the proteins were eluted with 50 lL of SDS sample buffer. The samples were analyzed by SDS-PAGE, followed by Western blotting using HRP-conjugated secondary antibodies and an ECL West Femto (Thermo Fisher Scientific, Waltham, MA) detection system. The HEK293T/17 cells were cultured at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), MEM nonessential amino acids, 1 mM sodium pyruvate, and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). The cells were transfected using Lipofectamine LTX and PLUS reagent (Invitrogen), harvested 24 hours later, lysed in ice-cold IP buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, and complete protease inhibitor cocktail) by sonication, and the lysates were clarified through centrifugation at 20,000 g for 15 minutes. For the immunoprecipitation experiments, the supernatant was incubated with 20 lL of Dynabeads and 2 lL of specific antibodies on a rocker for 1 hour at 4°C. After three washes with IP buffer, the proteins were eluted with 50 lL of SDS sample buffer and analyzed by SDS-PAGE.

**Immunohistochemistry**

The eyecups were dissected from mice, fixed for 15 minutes with 4% paraformaldehyde (PFA), cryoprotected with 30% sucrose in PBS for 2 hours at room temperature (RT), and embedded in OCT. The 12 µm frozen sections were obtained using a cryostat. The sections were permeabilized in 0.1% Triton X-100/PBS for 5 minutes, blocked with 0.1% Triton X-100/PBS and 10% donkey serum for 1 hour, and incubated with primary antibodies (mouse anti-GPR179 1:200; rabbit anti-PKCα 1:50000 as a marker of rod ON-BC or rabbit-CtBP2 1:2000 as a presynaptic marker) in 0.1% Triton X-100/PBS, and 2% donkey serum for 1 hour. After three washes, the sections were incubated with Alexa Fluor 488 or 546-conjugated...
secondary antibodies for 1 hour. The sections were stained for 5 minutes with 6-dimidino-2-phenylindole (DAPI) before mounting in Fluoromount (Sigma-Aldrich). The images used in this study were generated in The Light Microscopy Facility at the Max Planck Florida Institute (Jupiter, FL) using a Zeiss LSM 780 confocal microscope (LCI Plan-Neofluar 363/1.3 Imm Korr DIC M27 objective in water; Carl Zeis Meditec, Jena, Germany) at room temperature. Image acquisition and processing was accomplished using ZEN 2011 software (Carl Zeiss Meditec) with only minor manipulations of the images, using a fluorescence intensity setting under nonsaturating conditions and maintaining similar parameters for each acquired image.

Proximity Ligation Assay

The sections were prepared, permeabilized, and blocked for immunohistochemistry, and subsequently incubated with primary antibodies (mouse anti-GPR179 1:200 and sheep anti-TRPM1 1:200, or sheep anti-mGluR6 1:200 and rabbit anti-PKCα 1:5000, or rabbit anti-CtBP2 1:2000) in 0.1% Triton X-100/PBS and 2% donkey serum for 1 hour at RT, followed by 4 washes with 0.1% Triton X-100/PBS. The sections subsequently were incubated with Plus–anti-sheep and Minus–anti-

mouse PLA probes (Bethyl Laboratories, Montgomery, TX) together with Alexa Fluor 488-conjugated anti-rabbit antibody (Invitrogen) in PLA Probe Diluent and ×20 Assay Reagent for 1 hour at 37°C. The sections were washed 4 times for 5 minutes each in Wash Buffer A (0.01 M Tris, 0.15 M NaCl, and 0.05% Tween 20, pH 7.4) at RT and subsequently incubated with Ligation-Ligase mix (ligation stock ×5 with ligase in high purity water) for 30 minutes at 37°C. After washing 3 times with ×1 wash buffer A for 2 minutes at RT samples were incubated with amplification-polymerase mix (amplification stock ×5 and polymerase in high purity water) for 100 minutes at 37°C. The sections subsequently were washed 2 times with wash buffer B (0.2 M Tris and 0.1 M NaCl, pH 7.5) for 10 minutes at RT and once with ×0.01 wash buffer B for 1 minute at RT. The sections were stained with DAPI for 5 minutes before mounting in Fluoromount (Sigma-Aldrich). The quantification of PLA particles was performed using ImageJ software (available in the public domain at http://rsbweb.nih.gov/ij/). A constant area (3250 μm²) was drawn across either the outer (OPL) or inner (INL) plexiform layer, and all positive PLA particles within the area were counted. The total number of bipolar cells that contribute to the formation of these synapses was counted.
RESULTS
To probe the mechanisms of GPR179 action, we examined the possibility that GPR179 forms complexes with components of the synaptic signaling cascade in ON-BC. We first cotransfected HEK293 cells with GPR179 and either mGluR6 or TRPM1, and analyzed their interactions by coimmunoprecipitation (Fig. 1A). Considerable levels of TRPM1 and mGluR6 were detected in the eluates from beads conjugated to GPR179 antibodies, in the presence, but not the absence, of GPR179, indicating the specific interaction of GPR179 with TRPM1 and mGluR6 in transfected cells. Next, we immunoprecipitated GPR179 from native retinas (Fig. 1B) and found that it coelutes with TRPM1. The reciprocal immunoprecipitation of TRPM1 also pulled down GPR179. Importantly, no immunoprecipitation was observed in TRPM1 knockout retinas or when nonimmune IgGs were used as a control, further indicating that this interaction is specific. While we were able to detect mGluR6 band in GPR179 immunoprecipitates, no specific signal for GPR179 was observed when mGluR6 was precipitated (Fig. 1B), likely reflecting the nonspecific interaction of anti-mGluR6 antibodies with other mGluRs expressed in the retina or with components of the protein complex involving GPR179.

We next determined whether GPR179 forms complexes with TRPM1 and mGluR6 in the native environment of ON-BC using in situ proximity ligation assays. In this technique, protein–protein interactions are detected by the ability of closely associated proteins to support localized rolling-circle DNA amplification following the ligation of oligonucleotide probes attached to specific antibodies against proteins of interest. Individual interacting pairs of proteins subsequently are visualized through the hybridization of fluorescent oligonucleotide probes with the amplified DNA sequence. When using specific probes targeting the TRPM1/GPR179 complex, we detected a punctate pattern of fluorescence located in the OPL (Fig. 2). Double staining with the rod ON-BC marker PKCα revealed that the puncta were located at the dendritic tips, in immediate apposition to the sites of glutamate release, evidenced by counterstaining with the synaptic ribbon marker, CtBP2 (Fig. 2A). Importantly, no punctate signal was detected in the outer plexiform layer when the same probes were used.

FIGURE 5. Impact of TRPM1, mGluR6, and RGS proteins on localization of GPR179. Immunohistochemical detection of GPR179 localization in cross-sections of retinas from WT and knockout mice for TRPM1, nob3, and ROG7/11 double knockout. Sections were stained with antibodies recognizing GPR179 (green) together with a marker of ON-bipolar cells, PKCα (red, left panel), or a presynaptic terminal marker, CtBP2 (red, right panel). Immunofluorescence signal of GPR179 is localized at the dendritic tips of ON-bipolar cells in the OPL. Two independent immunostaining experiments were conducted with sections from a single animal.
on TRPM1 knockout retinas, indicating the specificity of TRPM1/GPR179 complex detection (Fig. 2B). Quantification revealed significant enrichment of puncta in the synaptic outer plexiform layer of wild-type, but not TRPM1 knockout, retinas. The extent of the positive punctate signal outside of the OPL, for example, in the INL, was negligible (Fig. 2D).

We used a similar proximity ligation approach to detect complexes of GPR179 with mGluR6 (Fig. 3). The WT retinas showed punctate signals at the dendritic tips of ON-BCs in close proximity to presynaptic markers (Fig. 3A), with no specific signal detected in nob3 retinas lacking mGluR6 (Fig. 3B). The number of the GPR179/mGluR6-positive synaptic puncta was lower relative to GPR179/TRPM1-positive signals (Fig. 3D), an observation consistent with less robust detection of the GPR179/mGluR6 interaction using immunoprecipitation. Altogether, these data suggested that GPR179 is in a complex with two major components of the metabotropic signaling cascade, mGluR6 and TRPM1, at the dendritic tips of ON-BC neurons.

Given the interaction and colocalization of GPR179 with major elements of the signal transduction cascade in ON-BC, we examined the consequences of deleting these interacting proteins (TRPM1, mGluR6, and RGSs) on the expression and localization of GPR179. Western blot analysis of total retina lysates revealed a severe reduction in GPR179 protein levels upon mGluR6 elimination (Fig. 4A). The concurrent knockout of the main GAP proteins RGS7 and RGS11 also resulted in a significant reduction in GPR179 expression, albeit to a lesser extent. We detected no significant changes in GPR179 levels in retinas lacking TRPM1, although there was a trend toward an increase in GPR179 protein content in these retinas (Fig. 4A).

The absence of detectable GPR179-positive postsynaptic puncta in nob3 mice (Fig. 5) suggested the requirement of mGluR6 for targeting GPR179 to the dendritic tips. However, severely reduced GPR179 levels in nob5 retinas might also hinder the immunohistochemical detection of GPR179 localization. The GPR179 targeting was normal in retinas lacking RGS proteins, despite a significant reduction in the overall expression levels. Consistent with a previous report, GPR179 colocalized with mGluR6 and TRPM1 at the dendritic tips (Fig. 6). The elimination of TRPM1 had no effect on the ability of GPR179 to colocalize with mGluR6 at the dendritic tips (Fig. 6A). Conversely, the elimination of mGluR6 prevented the dendritic accumulation of TRPM1 and GPR179 proteins (Fig. 6B). Thus, these results indicated that the expression and/or localization of GPR179 is sensitive to changes in the composition of the postsynaptic signaling complex in ON-BC neurons.

**Discussion**

Compartmentalization has an essential role in dictating the spatiotemporal characteristics and selectivity of cellular signaling. This process involves the localization of GPCR signaling pathways at discrete subcellular sites, where they often integrate into the web of protein–protein interactions that shape their function. These mechanisms are particularly important in transducing visual signals that often require exquisite sensitivity and high-temporal resolution. For instance, the ON-BCs, major interneurons in the retina, use a GPCR signaling cascade that relays changes in the activity of the mGluR6 receptor to the opening and closing of the TRPM1 effector channel. The mGluR6-TRPM1 pathway mounts a reliable response to changes in afferent inputs from the photoreceptor neurons produced by a single photon stimulation on a timescale of less than 100 ms. Clues about the organization of the pathway that enables the rapid and sensitive transmission of the signal comes from studies on fly phototransduction, where GPCR rhodopsin couples to the opening of the TRP via the interaction of multiple components of the signaling pathway with scaffolding proteins. However, mechanisms that facilitate signaling in mammalian ON-BC largely are unknown. The results obtained in our study implicated a recently discovered orphan GPCR, GPR179, in scaffolding several components of the metabotropic ON-BC pathway. In addition to the previously demonstrated association of GPR179 with RGS7 and RGS11 proteins, we reported the association of GPR179 with mGluR6 and TRPM1 in transfected cells and in vivo. The use of the emerging PLA approach further demonstrated the formation of...
these complexes directly at the tips of the ON-bipolar dendrites. However, despite the sensitivity and spatial resolution of PLA, the limitations of this approach do not allow us to determine whether the associated proteins form direct contacts.

While the potential mediators and binding determinants of this complex remain to be elucidated, it is clear that these interactions have an important role in regulating the expression levels and subcellular targeting of cascade components. For example, in a previous study, we reported that the elimination of GPR179 disrupts the targeting of RGS proteins, without affecting TRPM1 localization.11 In the present study, we demonstrated that the elimination of mGluR6 or RGS, but not TRPM1, abrogates the expression of GPR179. However, the postsynaptic accumulation of GPR179 is disrupted by the elimination of mGluR6, but not RGS or TRPM1. The localization of TRPM1, in turn, depends on its interaction with another partner, nyctalopin.27 Similar to GPR179, nyctalopin binds mGluR6 and TRPM1,16,27 and the disruption of nyctalopin in mouse models of congenital stationary night blindness prevents the postsynaptic targeting of TRPM1.27 Therefore, it appears that multiple components of the mGluR6 pathway exist as larger macromolecular complexes with scaffolding molecules, such as GPR179 and nyctalopin, where the coassembly of individual components is required for the proper targeting and stability of the complex. Determining the hierarchical relationship and determinants for this complex formation, and trafficking likely will yield important insights into the organization of the postsynaptic compartment of ON-BC neurons.

The heteromerization of GPCRs is an increasingly accepted phenomenon that shapes the properties of these receptors.28 Furthermore, class C GPCRs, such as mGluR6 and GPR179, are a particularly instructive example, as most of the members of this protein family form obligate oligomers.29 Therefore, we proposed that the recruitment of GPR179 to mGluR6 might alter the functionality of these receptors, making them suitable for the regulation of the signaling in ON-BC, for example, through the increased transiency of the response via the recruitment of the RGS proteins in close proximity to the G protein targets. Considering the interactions of GPR179 with multiple components of the mGluR6 pathway, investigating the functional impact of this receptor on individual elements will be an important future direction.

Acknowledgments

The authors thank Ignacio Sarria and Ikuo Masuho for the critical comments on the manuscript. Supported by National Institutes of Health Grant EY018139 (KAM) and a postdoctoral fellowship from Foundation BLANCEFLORE Boncompagni-Ludovisi (CO).

Disclosure: C. Orlandi, None; Y. Cao, None; K.A. Martemyanov, None

References


