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Probing Neurochemical Structure and Function of Retinal ON Bipolar Cells with a Transgenic Mouse

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Abstract

Retinal ON bipolar cells comprise about 70% of all bipolar cells. Glutamate hyperpolarizes these cells by binding to the metabotropic glutamate receptor mGluR6, activating the G-protein G_{o1}, and closing an unidentified cation channel. To facilitate investigation of ON bipolar cells, we here report on the production of a transgenic mouse (Grm6-GFP) in which EGFP, under control of mGluR6 promoter, was expressed in all and only ON bipolar cells. We used the mouse to determine density of ON bipolar cells, which in central retina was 29,600 cells/mm². We further sorted the fluorescent cells and created a pure ON bipolar cDNA library that was negative for photoreceptor unique genes. Using this library, we determined expression of 27 genes of interest. We obtained positive transcripts for: G_o interactors: regulators of G-protein signaling (RGS), Ret-RGS1 (a variant of RGS20), RGS16, RGS7, purkinje cell protein 2 (PCP2, also called L7 or GPSM4), synembryn (RIC-8), LGN (GPSM2), RAP1GAP, Gβ5; cGMP modulators: guanylyl cyclase (GC) 1α1, GC1β1, phosphodiesterase (PDE) 1C, PDE9A; and channels: inwardly rectifying potassium channel Kir2.4, transient receptor potential TRPC2, sperm-specific cation channels CatSper 2–4. The following transcripts were not found in our library: AGS3 (GPSM1), RGS10, RGS19 (GAIP), calbindin, GC1α2, GC1β2, PDE5, PDE2A, amiloride-sensitive sodium channel ACCN4, and CatSper1. We then localized Kir2.4 to several cell types, and showed that in ON bipolar cells, the channel concentrates in their dendritic tips. The channels and modulators found in ON bipolar cells likely shape their light response. Additional uses of the Grm6-GFP mouse are also discussed.

Keywords

mGluR6; CatSper; Kir2.4; RGS; G-protein; guanylyl cyclase; phosphodiesterase; EGFP

Introduction

About 70% of all retinal bipolar cells are ON bipolar cells; these include approximately 5 types of ON cone bipolar cells that mediate daylight vision, as well as rod bipolar cells that mediate night vision (Sterling and Demb, 2004). The high proportion of ON bipolar cells and their key position as linkers between photoreceptors and ganglion cells justifies the significant efforts that have been taken to understand their physiological responses and their

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underlying molecular mechanisms. ON bipolar cells receive their input from photoreceptors, which transmit their signal to both ON and OFF bipolar cells by modulating glutamate release (Ayoub et al., 1989). Glutamate has two modes of action: it depolarizes OFF bipolar cells via conventional ionotropic receptors, and hyperpolarizes ON bipolar cells via a metabotropic glutamate receptor, mGluR6 (Nomura et al., 1994; Vardi et al., 1998; Vardi et al., 2000; Haverkamp et al., 2000; Vardi et al., 2002). Under scotopic conditions when photoreceptors are continuously releasing glutamate, bound mGluR6 activates the heterotrimeric G-protein G_{o1} (Nawy, 1999; Dhingra et al., 2000; Dhingra et al., 2002), which eventually closes an unidentified non-specific cation channel (Nawy and Jahr, 1990; de la Villa et al., 1995). This maintains the cell in a hyperpolarized state. A light increment reduces glutamate release, unbinds mGluR6, inactivates G_{o1} , and the channels are permitted to open. This depolarizes the ON bipolar cells, whose summed responses give rise to the b-wave of the non-invasive electroretinogram (ERG) (Robson and Frishman, 1999).

Progress in solving the transduction cascade that mediates the light response in ON bipolar cells has been slow, because unlike photoreceptors which can be purified and easily studied biochemically, the ON bipolar cells cannot be purified. Moreover, since they are imbedded in an inner layer of the retina, recording from them is difficult. Thus, we still cannot identify the effector for G_{o1} , what forms the transduction channel, what modulates the response, and what is the function of cGMP. Such information would be difficult or impossible to achieve by physiological recordings if the key proteins were novel, or if the known drugs were useless. To alleviate some of these difficulties in studying ON bipolar cells, especially their mGluR6 transduction cascade, we produced several mouse lines in which EGFP expression was driven under the control of mGluR6 promoter. Here we use this transgenic mouse to create an ON bipolar cDNA library and to examine the expression profile of these cells.

Methods

Generation of the transgenic mouse

mGluR6 promoter sequence was extracted by digesting pN2N (a generous gift of Y. Nakajima and S Nakanishi) with NotI, blunt filling with Klenow fragment polymerase, and then digesting with XhoI. pEGFP1 (Clontech, Cambridge, UK) was digested with SmaI and XhoI, and ligated with mGluR6 promoter. Background was removed by digesting these plasmids with SacII. After transformation, clone sequence was confirmed by digestion and sequencing. This was then digested with XhoI and SphI, and run on a gel. The 10.5 kb band consisting of mGluR6 promoter fused to EGFP coding sequence was eluted for microinjection into fertilized eggs (performed by the University of Pennsylvania Transgenic and Chimeric Mouse facility). The founder mice (in B6SJL/F1/J background) were screened by genomic PCR for the presence of the transgene. The primers UEGFP1 (tcg agc tgg acg gcg acg ta) and LEGFP 817 (tta ctt gta cag ctc gtc cat) were used at the annealing temperature of 56°C. The positive lines were then examined for EGFP expression in retina, initially with an ophthalmoscope, and later with a low power objective on a fluorescent microscope. Mice with green fluorescence were bred with C57BL/6J for 6 generations to eliminate the rd (retinal degeneration) genes associated with the founder line strain. Animals were treated in compliance with federal regulations and University of Pennsylvania policy.

Cell count

Mice were deeply anesthetized by intraperitoneal injection (85 µg/g ketamine plus 13 µg/g xylazine) and the eye orientation was noted by an incision at the nasal pole. Eyes were enucleated and the mice were then sacrificed by anesthetic overdose (3X initial doses). Animals were treated in compliance with federal regulations and University of Pennsylvania policy. The eye was fixed and the retina was flat mounted, ganglion cell up. A filter paper

was placed around the retina to provide support for the cover slip and reduce compression. The inner nuclear layer was scanned with a confocal microscope with 1 μm steps in the Z-axis. Two retinas were scanned under 100x oil immersion lens (NA 1.4) (Leica microscope) where the scanned field was 100 $\mu\text{m} \times 100 \mu\text{m}$ and two retinas were scanned under 60x oil (NA1.42; Olympus) where the scanned field was 140 $\mu\text{m} \times 140 \mu\text{m}$. Cells were identified through a stack of focal planes taken at 1 μm intervals and counted manually using Openlab software (Improvision; Lexington, MA). For the first two retinas, three counts were made from each field and the counts were averaged, for the other two only one count per field was made since the variance between mice was greater than between counts.

Immunocytochemistry

After anesthesia, the eye was incised at the ora serrata and fixed by immersion in phosphate buffer (pH 7.4) containing 4% (or 2%) paraformaldehyde for 1 hour (or 10 min). It was then rinsed in buffer, soaked overnight in 30% buffered sucrose, and embedded in a mixture of two parts 20% sucrose in phosphate buffer and one part Tissue Freezing Medium. Retina was cryosectioned radially at 10–15 μm thickness and immunostained according to a standard protocol. Sections were soaked in diluent containing 10% normal goat serum, 5% sucrose, and 0.3% Triton X-100 in phosphate buffer. They were then incubated in primary antibodies overnight at 4°C, washed and incubated (3 hr) in anti-rabbit F(ab)₂ fragment conjugated to a fluorescent marker, and then rinsed and mounted in Vectashield.

HEK 293T cells were cultured in Minimal Essential Medium supplemented with Penstrep (Invitrogen, Carlsbad, CA) and 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO₂ incubator. Cells were transiently transfected with Kir2.4 (in pcDNA3.1) using Fugene transfection reagent (Invitrogen, Carlsbad, CA). Cells were harvested 48 hours later. Retinas and HEK cells were imaged either with a Leica or Olympus confocal microscope. All images were taken with the default pinhole (of one airy disk) calculated by the confocal microscopes. These give the following approximate z-resolutions: for 100x NA1.4 (Leica) and 60x NA1.42 (Olympus) objectives – 500–600 nm; and for 40x NA 1.25 (Leica) and 40x NA 1.3 (Olympus) – 650–750 nm. For display, images were contrast adjusted with Adobe Photoshop 7.0 (Adobe Systems, San Jose, Ca).

Western blottings

Fresh or frozen mouse retinas or HEK cells were homogenized in 0.2-1 ml ice cold homogenization buffer (5 ml per g wet weight) (in mM: 320 sucrose, 5 Tris-HCl, and 2 EDTA, 2.5 β -mercaptoethanol, pH adjusted to 7.4 at room temperature) containing protease inhibitor cocktails (P8340; Sigma Aldrich, St. Louis, MO), centrifuged at 6,000 X g for 10 min and the supernatant was collected. Protein assay was carried out using BCA protein reagent (Biorad). 20 μg of protein was mixed with NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA), incubated for 10 min at 95°C, then loaded onto 10% Tris-glycine gel (Bio-Rad). Electrophoresis was performed with a mini-protean II electrophoresis cell (Bio-Rad) under reduced denaturing conditions. The resolved proteins were transferred to a nitrocellulose membrane (Bio-Rad) in transfer buffer (192 mM glycine, 25 mM Tris-Cl, pH 8.3, and 20% methanol) for 40–50 min at 80V using a Bio-Rad Trans-Blot semi-dry transfer cell. The membrane was blocked with 7% skim milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, PH 7.4) for 1 hr, and incubated with the primary antibody (1:5000) overnight at 4°C in TBST-milk. After extensive washes in TBST, the membrane was incubated with the secondary antibody conjugated to peroxidase (Jackson Research laboratories, 1:3000) in TBST-milk for 2 hr at room temperature. After extensive washes in TBST, bound antibody was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

Antibodies

Three antibodies were used for marking cells. Rod bipolar cells were stained with a mouse monoclonal antibody against PKC α purified from bovine brain (clone MC5, Sigma-Aldrich, St. Louis MO, USA; cat. P 4334) and shown to specifically recognize a 79 kDa protein which can be blocked with peptides 296–317. They were also stained with a polyclonal antibody that was raised in rabbit against a synthetic peptide corresponding to rat PKC α amino acids 659–672 (Sigma-Aldrich; cat P5704) and gives identical staining pattern to that of the monoclonal. ON bipolar cells were marked with a polyclonal antibody that was raised in rabbit against peptide aa 47–59 of mouse G- γ 13 and whose specificity in retina was established by correlating transcript (RT-PCR) and immunostaining (Huang et al., 1999; Huang et al., 2003). This antibody was a gift of Robert Margolskee, Mount Sinai School of Medicine. Other antibodies were used for localizing proteins of interest. The only antibody that could be shown to be specific in retina was a polyclonal antibody against Kir2.4, which was raised in rabbit against the less conserved C-terminus amino acids 395–434 of rat Kir2.4, and used at 1:500 dilution. Its specificity in rat has been confirmed by transfecting COS cells and by correlating results with in situ hybridization (Pruss et al., 2003; Liu et al., 2001); its specificity in mouse retina is described in Results. Several other antibodies used in this study either did not reveal staining or their staining specificity could not be established in retina. We chose to report on their performance here in order to provide preliminary information that may benefit future studies. A polyclonal antibody against CatSper1 raised in rabbit against the first 150 amino acids of the amino terminus (a gift of Dejian Ren, University of Pennsylvania; Ren et al., 2001) gave similar Western blots and immunostaining in wild type and CatSper1-null retina. Two polyclonal antibodies against CatSper2, one against the N-terminus and the other against the C-terminus (gifts from Timothy Quill, University of Texas; Quill et al., 2001) gave similar Western blots and immunostaining in wild type and CatSper2-null mouse. A polyclonal antibody against TRPC2 raised in guinea pig against an N-terminal peptide (CSSDASGAGPGGPLRNNE) (gift from Richard Axel, Columbia University; Leybold et al., 2002) did not detect any band in Western blotting, and did not stain retina. Monoclonal antibody to RGS16 raised in mouse against a partial recombinant human protein aa90–180 (Abnova, Taipei City, Taiwan; cat H00006004-Ao1) revealed several bands by Western blotting, but the major bands were not at the correct molecular weight. Immunocytochemistry for RGS16 revealed strong staining in the outer plexiform layer. Similarly, a polyclonal antibody against RIC-8 (synembryn) raised in goat against a synthetic protein corresponding to a human sequence (Abcam, Cambridge MA, USA; cat ab 24383) gave several bands at unexpected molecular weights, and immunostaining revealed strongest staining in the photoreceptor outer segment. Polyclonal antibody to PDE9A raised in rabbit against a synthetic peptide corresponding to human C-terminus peptide CDISNEVRPMEVAEPWVDCLLEE (Genetex, San Antonio TX, USA; cat GTX14625) recognized PDE9A in transfected HEK cells, but gave 5 major bands in Western blotting. Immunostaining with this antibody gave strong punctate staining in the outer plexiform layer.

Cell isolation and FACS

Retinas were isolated and incubated in Ca²⁺- and Mg²⁺- free HBSS with 20 mM HEPES, 3 mg/ml BSA, 3 mg/ml glucose, 1.5 mM MgSO₄, and 0.0025% trypsin (Sigma-Aldrich) at 37°C for 25 min with gentle shaking. Enzymatic reaction was stopped by adding 0.25 mg/ml trypsin inhibitor and 40 μ g/ml DNaseI. Tissue was triturated with a one ml pipette tip followed by a 100 μ l tip until the suspension was homogenous. Cell viability and quality of dissociation was checked with EthD-1 (1 μ M; Invitrogen; Carlsbad, California). The dissociated cells were sorted by Fluorescent Assisted Cell Sorting (FACS) using FACSVantage (BD Biosciences, San Jose, CA, USA). Briefly, around 100,000 dissociated cells were resuspended in Ca²⁺ and Mg²⁺ free HBSS. Debris and aggregates were gated out

by forward and side scatter. The cells were then sorted based on size and fluorescence intensity. Only sizes between 6–8 μm and intensities higher than 100 units were selected. For RNA extraction, the sorted cells were collected in the lysis buffer. For viewing the cells, they were collected in phosphate buffer, spun down at 2500 rpm, and settled on a glass slide.

Library production

Total RNA from isolated cells was extracted with Nucleospin RNA II kit and reverse transcribed with the aid of “SMART™ cDNA library construction kit” (both from Clontech). About 1 μg RNA was primed to CDSIII primer containing the *Sfi* IB restriction site on the 5' end and a stretch of dT on the 3' end. The newly transcribed cDNA was extended with an oligonucleotide containing the *Sfi* IA sequence (SMART IV primer). The cDNA was then amplified with the CDSIII and a 5' primer, and the double stranded cDNA was fractionated. The first 4 fractions containing cDNA were pooled, digested with *Sfi* I, and ligated with the *Sfi* I-digested pEXP1. The ligation mixture was transformed in DH10B cells and the number of independent clones was calculated to be $>10^6$. The library was amplified and aliquots were stored at -80°C .

Polymerase chain reaction

PCR reaction on library DNA was carried out with gene specific or degenerate primers on a programmable thermocycler (Perkin Elmer, Branchburg, NJ). The PCR was performed for 35 cycles with the following conditions: denaturation at 94°C for 30 sec, annealing at 52 – 62°C (depending on the primers used) for 30 sec, followed by extension at 72°C for 30 sec. All primer sets were applied to the ON bipolar library and to reverse transcribed retinal cDNA. For most transcripts, amplification from retina was used as positive control, and unless otherwise noted, retina was indeed positive. For each test transcript, we designed three PCR primers: one lower primer and two upper primers (Table 1). At least one product with a predicted size from each primer set was sequenced to confirm that the product was not only the correct size, but also the correct sequence. When the correct product size was not obtained, we sequenced the remaining bands to test if they represented different splice variants.

Electroretinogram (ERG) recording

The experimental apparatus, methods of light stimulation and quantification, ERG recording, and cone signal isolation have been described in detail previously (Lyubarsky et al., 1999; Lyubarsky et al., 2000). Briefly, a mouse was dark-adapted overnight. Then, under dim red light, it was deeply anesthetized by intraperitoneal injection of ketamine (20 $\mu\text{g}/\text{gm}$), xylazine (8 $\mu\text{g}/\text{gm}$) and urethane (800 $\mu\text{g}/\text{gm}$) and placed onto a warmed (37°C) platform. The eyes were dilated with 1% Mydracyl (Alcon, Fort Worth, TX). A platinum recording electrode contacted both corneas, and the reference electrode lay in the animal's mouth. The animal was then placed inside a light-proof Faraday cage that also served as a Ganzfeld, with appropriate ports and baffles to ensure uniform illumination. The light stimuli were brief flashes (~ 10 ms) generated by xenon flash lamps, and delivered in a multiport, customized Ganzfeld through calibrated filters. Stimulus intensity and spectral composition were controlled with neutral density and bandpass interference filters. Light intensities were calibrated and converted to estimated number of photoisomerizations per photoreceptor (R^*) as described previously (Lyubarsky et al., 1999; Lyubarsky et al., 2000). ERGs were recorded from both eyes using differential amplifiers with a bandwidth of 0.1 Hz to 1 kHz. The filtered traces were digitized at 5 kHz. Data were analyzed with Clampfit software and the responses between wild type and CatSper-null mice or Grm6-GFP mice were compared with a student t-test.

Results

Description of the EGFP positive lines

Our initial screen revealed 6 lines of transgenic mice with EGFP expression in the retina. To determine which retinal cell types expressed EGFP, radial cryosections of mature retinas were performed and the location of the fluorescing cells was examined. In all lines, EGFP was expressed only in cells of the inner nuclear layer that were restricted to the upper tiers. None of the lines showed fluorescence in the ganglion cell layer or the photoreceptor layer.

To verify that the fluorescing cells were only ON bipolar cells, we immunostained the retina with an antibody against G- γ 13, the gamma subunit of gustducin. This subunit was shown to be expressed exclusively by ON bipolar cells (Huang et al., 2003). In two lines (lines 3 and 5) EGFP was expressed by all ON bipolar cells and nowhere else in the retina (Fig 1). Tangential views of retina from line 5 mice illustrate the dense array of ON bipolar somas in the INL with sparse “holes” that probably indicate OFF bipolar somas, and possibly Müller cells (Fig. 2A). The axons of these bipolar cells traversed sublamina *a* without branching or terminating (Fig. 2B). They then terminated in all strata of sublamina *b* with a dense network of large terminals in strata 5 (left region in Fig. 2C) and fine arborizations in strata 3 – 4 (right region in Fig. 2C).

The completeness of EGFP expression allowed us to study the density of ON bipolar cells in the mouse retina. Four retinas were analyzed: near the optic disk, the density was more variable than in periphery; in most retinas it was highest at about 100–300 μ m away from the disk edge, and it stayed relatively constant up to 2 mm away. Taking the highest density from each retina (regardless of eccentricity), the average was about 32,000 cells/mm² (Fig. 2D). The average at 200–600 μ m was $29,600 \pm 3700$ cells/mm², and the average across eccentricities was $25,250 \pm 1520$ cells/mm².

In another line of transgenic mice (line 1), staining for G- γ 13 revealed that EGFP was expressed only in ON bipolar cells, but not in all of them (Fig. 3A, B). To determine if the EGFP-expressing cells were rod bipolar cells, we stained for PKC. We found that certain PKC-stained (rod bipolar) cells did not express EGFP and certain EGFP-expressing cells were not rod bipolar cells (Fig. 3C). Thus, in this mouse line, the EGFP-expressing cells were mosaics of rod- and cone bipolar cells. In the rest of our mice lines, EGFP expression was weak or not restricted to ON bipolar cells so these lines and line 3 (which was redundant with line 5) were terminated.

Line 5 transgenic mouse has a normal ERG

We next tested the viability of the EGFP expressing ON bipolar cells and their usefulness in future studies. Thus we carried out ERG recordings under dark and light adapted conditions. Grm6-GFP and wild type mice were dark adapted overnight, and their ERG responses were tested first with dim stimuli, then with increasing flash intensities (Fig. 4A, B). Next, we tested response recovery with a pair of flashes separated by either 350 or 500 ms (Fig. 4D). Finally, we light adapted the mice and tested their cone ERGs (Fig. 4C). The wild type and Grm6-GFP mouse exhibited similar ERG wave shape and amplitude as well as similar adaptation effects (Fig. 4C, F). Also, the time to peak of the a- and b-waves were similar for both mouse groups (data not shown).

Expression profiling using an ON bipolar cDNA library

The fluorescence of dissociated ON bipolar cells was sufficiently strong to permit Fluorescent Assisted Cell Sorting (FACS) (Fig. 5 A, B). The ON bipolar cells were sorted according to fluorescence intensity and size. Only sizes between 6–8 μ m with intensities

higher than 100 units were selected. Combined Nomarsky and fluorescence optics showed that the purity of the collected cells was about 98% (Fig. 5C, D). The sorted cells were lysed, and their mRNA was used to create a mammalian expression library. For practice and comparison, we also made a library from the mouse rd/rd retina (in which all rods and most cones degenerate) and tested that as well.

We tested the purity of the ON bipolar cDNA library by testing if RNA unique to photoreceptors and not known to be expressed by ON bipolar cells is present in the library. This is a stringent test since photoreceptors are the most likely source of contamination. Consequently, we used primers specific for opsin, RGS9, GRK1, and cGMP-gated channels. Opsin, which always gave the expected band when amplified from cDNA obtained from whole mouse retina, never gave a band when amplified from the ON bipolar library (at least 4 amplifications) (Fig. 5G). In the rd library, these primers gave a correct band infrequently (2 out of 5 trials), probably due to the fact that some cones survived. RGS9, GRK1, cone CNG-gated channel, and rod CNG-gated channel amplified from retinal cDNA gave strong bands at the expected sizes, but only non-specific faint bands when amplified from either the rd (not shown) or ON bipolar library (Fig. 5F). In a few cases, the rod CNG-gated channel gave a very faint band at the correct size in the bipolar cell library. This may reflect the 2% contamination we estimated after FACS. In contrast, amplification of mGluR6, $G\alpha_{o1}$, Purkinje cell protein 2 (PCP2), or Ret-RGS1, a proteins known to be expressed by ON bipolar cells, gave bands for all samples (Fig. 5E, shown only for $G\alpha_{o1}$). Control samples, samples that did not include DNA in the PCR reaction mixture, never gave any bands. These results show that our experimental library is reasonably pure.

We next used our library to determine if, for 27 proteins of interest, their transcript is expressed by ON bipolar cells. Proteins of interest were divided into three categories: potential interactors of G_o , modulators of cGMP, and channels. To test the expression of each molecule, we used two sets of primers, a common lower primer and two upper primers (Table 1 and Fig. 6). For all transcripts we report here to be positive, the expected PCR products were sequenced at least once and found to be correct.

G_o interactors—Modulators of interest were based primarily on our yeast two hybrid screen of a retinal library, in which we used a constitutively active mutant of $G\alpha_{o1}$ as bait (Dhingra et al., 2004). Among 10 potential G_o interactors, the following transcripts were positive in ON bipolar cells: regulator of G-protein signaling (RGS), Ret-RGS1 (a variant of RGS20), purkinje cell protein 2 (PCP2, also called L7 or GPSM4), RGS16, synembryn (RIC-8), LGN (GPSM2), and RAP1GAP. Activator of G-protein signaling 3 (AGS3, also called GPSM1), RGS10, RGS19 (GAIP), and calbindin were positive when amplified from retina but negative when amplified from the ON bipolar library. Since new immunocytochemistry data suggests that RGS7 coupled to $G\beta_5$ is localized to ON bipolar dendritic tips and thus may contribute to the light response (Morgans et al., 2007; Rao et al., 2007), we also tested the expression of these transcripts and found both to be positive.

cGMP modulators—In early studies, cGMP was reported to be the second messenger of the mGluR6 transduction cascade (Nawy and Jahr, 1990; Shiells and Falk, 1990), and in later studies, it appeared to have a modulatory function (Snellman and Nawy, 2004). In either case, it is important to identify enzymes that synthesize or hydrolyze cGMP. Since physiological experiments suggest that the guanylyl cyclase in ON bipolar cells is soluble (Shiells and Falk, 1992), we tested for GC1 α 1, GC1 α 2, GC1 β 1, and GC1 β 2. We found that only GC1 α 1 and GC1 β 1 were expressed by ON bipolar cells. GC1 α 2 was positive in retina but negative in ON bipolar cells, and GC1 β 2 was negative in both retina and ON bipolar cells. For hydrolysis, we concentrated on phosphodiesterase PDE1C, PDE2A, PDE5, PDE9A – all known to hydrolyze cGMP. We found that only PDE1C and PDE9A were

expressed in ON bipolar cells. PDE5 was positive in retina and negative in bipolar cells; PDE2A was ambiguous.

Channels—Our yeast two hybrid screen also fished two channels: amiloride-sensitive cation channel 4 (ACCN4) and inwardly rectifying potassium channel J14 (KCNJ14 or Kir2.4). The amiloride-sensitive channel was positive in retina but negative in ON bipolar cells. PCR amplification of the library showed that Kir2.4 transcript was expressed by ON bipolar cells, and its full-length transcript was identical to that cloned in mouse; thus it represents a known potassium channel. Next we tested if the canonical TRP channels (TRPC) are expressed in ON bipolar cells because these channels are cation channels and TRPC2 is expressed together with G_o in the vomeronasal organ (Takami et al., 2001). Using degenerate primers for this subfamily, we amplified a single band that, upon sequencing, was found to be TRPC2. The expression of this transcript was confirmed with specific primers. However, when we tested TRPC2 protein expression using immunocytochemistry and Western blotting, we found that both methods failed to reveal protein expression in retina.

We also examined the four known CatSper isoforms thought to be specific to sperm. Similar to the ON bipolar transduction channels, CatSper channels are cation non-specific with high permeability for calcium. Interestingly, these channels can be activated by cyclic nucleotides, but they do not have cyclic nucleotide-binding domain, as might be the case with the ON bipolar transduction channel (Nawy, 1999; Quill et al., 2001; Carlson et al., 2003; Snellman and Nawy, 2004). Amplification of CatSper1 mRNA was ambiguous because the PCR product included one intron. Amplification of CatSper2 was always positive, but a detailed analysis of the amplified mRNA revealed that it lacked exons 2–5, which would yield a truncated protein. Amplification of CatSper 3 and 4 were positive in the ON bipolar library, and amplification from retinal cDNA showed them to be full-length. To test if any CatSper channels do indeed participate in the light response, we recorded the ERG responses of CatSper1-, CatSper3-, and CatSper4- null mice. These mice, donated by David Clapham and Dejian Ren, are sterile and their sperm is deficient in hyperactivated motility (Ren et al., 2001; Qi et al., 2007). We tested their ERG responses to light flashes whose intensities ranged from dim to saturating under dark-adapted conditions and to bright light under light-adapted conditions. We found all the responses to be similar to wild-type (Fig. 7A, B, shown only for CatSper 4-null). We then wondered if these channels are only responsible for the calcium flux, because then their deletion would not eliminate the flash response, but may affect adaptation and response recovery. We tested this with a double flash of different inter-flash intervals. Plotting the ratio of second b-wave peak amplitude to first against inter-flash interval showed that adaptation and response recovery were similar to wild type (Fig. 7C, D).

Kir 2.4 is expressed in dendritic tips of ON bipolar cells

For one protein, Kir2.4, we tested expression with a polyclonal antibody against the C-terminus of the rat channel (Liu et al., 2001; Pruss et al., 2003). Immunostaining of Kir2.4-transfected HEK293T cells with this antibody recognized the expressed protein and gave no background in untransfected cells (Fig. 8A, B). Western blots revealed two bands, one at ~52 kDa (corresponding to the monomer; unmodified protein is expected to be 48 kDa) and the second at ~110 kDa (corresponding to the dimer). Western blots of retinal protein revealed the same two bands and no other, showing that the antibody is specific (Fig. 8C). Immunostaining of retinal sections with this protein gave staining throughout the retina. Staining appeared strongest in ganglion cell somas, photoreceptor inner segments, and outer plexiform layer, where labeling appeared punctate (Fig. 8D). When fixation was weak (4% paraformaldehyde for 10 min), all somas in the inner nuclear layer were also lightly stained.

The ubiquitous expression of Kir2.4 shown here by immunostaining is consistent with in situ hybridization in which all nuclear layers were labeled (Hughes et al., 2000). To determine if the punctate labeling in the outer plexiform layer represents ON bipolar dendritic tips, we stained sections of the transgenic mouse and found that indeed most (but not all) of these puncta colocalized with EGFP (Fig. 8E–G). Puncta in the INL mainly localized to the edge of the somas, suggesting the channel clustered at several membrane regions. Thus ON bipolar cells express Kir2.4 in their dendritic tips, but possibly other structures in the outer plexiform layers also express this channel.

Discussion

We produced several transgenic mouse lines, and maintained two lines (1 and 5) in which EGFP was expressed under the control of mGluR6 promoter. In line 5, EGFP was expressed in all ON bipolar cells and only in these cells. Using this line, we estimated the density of ON bipolar cells near the optic disk on the horizontal meridian to be about 30,000 cells/mm². The density of all bipolar cells in a similar eccentricity can be estimated to be 42,000 cells/mm² (Jeon et al., 1998), so ON bipolar cells comprise about 71% of all bipolar cells. At this eccentricity, the density of rod bipolar cells is about 13,000 cells/mm² (Strettoi and Pignatelli, 2000), thus comprising only 43% of ON bipolar cells. Rod bipolar cells peak at about 1 mm (15,000 cells/mm²), where the average ON bipolar cell density is 22,800, so at this eccentricity rod bipolars comprise 66% of ON bipolar cells. From line 5, we also created an ON bipolar cell library that we then used to determine expression profile. We found that ON bipolar cells transcribe a plethora of G_o modulators, including a number of RGS molecules, channels, and enzymes that might contribute to the mGluR6 cascade and to maintain the membrane's resting potential.

Potential uses for the Grm6-GFP transgenic mouse

The ON bipolar cells in transgenic mouse line 5 are highly fluorescent, and their physiological properties remain unchanged. Thus this mouse line can be used for multiple purposes, several of which have already been exploited. In a previous study, we followed the developmental course of ON bipolar cells in these mice. We found that fluorescent cells could already be detected at postnatal day 3, and they had an unusual mode of axogenesis and dendritogenesis (Morgan et al., 2006). In the current study, we used the transgenic mouse to determine the ON bipolar cell density across different eccentricities and to generate an ON bipolar cDNA library and quest for their gene expression profile.

In the future, this mouse could be used to facilitate several more tasks such as to (a) produce cDNA libraries from different developmental stages and examine differential expression patterns; (b) facilitate targeting of ON bipolar cells in physiological experiments either in a slice preparation, or after cell dissociation; (c) facilitate targeting of OFF bipolar cells by choosing EGFP-negative cells located in the middle of the inner nuclear layer (a procedure that is especially important since OFF bipolar cell density is relatively low); and (d) determine the connectivity pattern of ON bipolar cells with ganglion cells. In addition, scanning of other organs of the mouse showed EGFP-positive structures throughout the body including the brain (Vardi, unpublished data), and this will no doubt add to potential uses. The high density of fluorescent cells in line 5 makes it difficult to reconstruct single cells, but since in line 1 EGFP expression is patchy, it is better suited for this purpose.

G_o interactors in ON bipolar cells

Light hyperpolarizes a photoreceptor but depolarizes the ON bipolar cells. This response reversal is mediated by the metabotropic glutamate receptor mGluR6, which couples to the heterotrimeric G-protein G_o, whose activation causes closure of non-specific cation

channels. Although the effector for G_o and the channel remains unknown, it is clear that a major site for modulation is the activation and inactivation of G_o . Using a constitutively active mutant of $G\alpha_o$ as bait in a yeast two-hybrid system, we previously fished out a number of potential interactors for G_o . We then localized the protein that was hit most often, Ret-RGS1, to bovine ON bipolar cells and found that it can facilitate the rising phase of the light response (Dhingra et al., 2004). Here we probed for the other potential modulators to determine if they too are expressed by ON bipolar cells.

Following Ret-RGS1, the molecule that was hit most times by the yeast-two hybrid screen was PCP2. PCP2 is known to be expressed by ON bipolar cells (Berrebi et al., 1991), so PCR amplification of its transcript was expected to be positive. Nevertheless, the library provided new information since RACE PCR showed that retinal PCP2 represents a novel splice variant (Ret-PCP2; deposited in GenBank, accession EU164759). Further studies of this protein showed that Ret-PCP2 accelerates the ON bipolar light response (Xu et al., submitted). Among the other “fished” molecules, four more, RGS16, synembryn, LGN, and RAP1GAP, were found to be positive in ON bipolar cells. In addition, RGS7, which was not fished but may be a potential modulator of G_o , was also positive in these cells, consistent with immunocytochemistry results (Morgans et al., 2007; Rao et al., 2007). Thus it appears that ON bipolar cells express many modulators. Several of these may shape different phases of the light response while others may modulate cascades related to other receptors such as mGluR7 located on the axonal arbor (Brandstätter et al., 1996). One potential interactor, calbindin (that was hit only once), was not found in ON bipolar cells. This is consistent with mouse immunostaining results that localize this protein exclusively to horizontal and amacrine cells (Dhingra et al., 2000; Haverkamp and Wässle, 2000). Lack of amplification of calbindin from the ON bipolar cDNA library shows that the library is not contaminated by amacrine cells.

Modulators of cGMP concentration

An important modulator of cGMP concentration is guanylyl cyclase. ON bipolar cells are thought to express a soluble guanylyl cyclase because NO donors (the known activators of this cyclase) open the cation channels, presumably by increasing cGMP concentration (Shiells and Falk, 1992). However, functional localization of soluble guanylyl cyclase, by stimulating with NO donors and immunostaining for cGMP, gave staining in ON cone bipolar cells, but not in rod bipolar cells (Koistinaho et al., 1993; Blute et al., 1998; Gotzes et al., 1998; reviewed by Sitaramayya, 2002). There are four isoforms of soluble guanylyl cyclase: two $GC1\alpha$'s and two $GC1\beta$'s. $GC1\beta2$ appears to be non-functional (Koesling et al., 2004), and indeed our PCR from retinal cDNA did not amplify its mRNA. Among the other three isoforms, only $GC1\alpha1$ and $GC\beta1$ were expressed, suggesting they form the functional heterodimer in ON bipolar cells (Koesling and Friebe, 1999).

Another modulator of cGMP is PDE. The ON bipolar transduction cascade was thought to be similar to phototransduction, where transducin activates PDE6, which then hydrolyzes cGMP and leads to the closing of cGMP-gated channels (Nawy and Jahr, 1990; Shiells and Falk, 1990). Consequently, we tested antibodies against photoreceptor PDEs (PDE6A–C) and against PDE γ . All gave nice staining of photoreceptor outer segments, but no staining in bipolar cells (Dhingra et al., 2004 and unpublished data). We then examined other PDEs that hydrolyze cGMP and found two to be positive: PDE1C and PDE9A. The positive transcript for PDE1C is consistent with immunolocalization of this isoform to cells located high in the inner nuclear layer (Santone et al., 2006). PDE1C is activated by Ca^{2+} /calmodulin and hydrolyzes both cGMP and cAMP (Bender and Beavo, 2006). As such it can be involved in multiple signaling cascades, such as regulation of GABA response by dopamine via cAMP (Feigenspan and Bormann, 1994). Unlike PDE1C1, PDE9A hydrolyzes only cGMP. This protein is highly expressed in the brain, especially in the olfactory bulb and Purkinje cells

(Andreeva et al., 2001; van Staveren et al., 2002; van Staveren and Markerink-van Ittersum, 2005). Among the 20 known PDE isoforms, PDE9A has the highest affinity for cGMP (Fisher et al., 1998; Soderling et al., 1998). Thus cGMP levels in ON bipolar cells might be low, a situation that could explain why cGMP in rod bipolar cells remained undetected after NO stimulation of the cyclase.

Channels

Very little is known about the molecular identity and physiological properties of the transduction cation channel in ON bipolar cells. We do know that this channel is non-selective for cations, and is thought to permeate calcium. Moreover, the channel is modulated by cGMP, but it does not contain a cGMP-binding domain (Nawy, 1999; Snellman and Nawy, 2004). Guided by this knowledge, we examined the expression of members of the CatSper family which permeate calcium and other cations, are modulated by cyclic nucleotides, and do not have a cyclic nucleotide binding domain (Ren et al., 2001; Carlson et al., 2003). Four isoforms of this channel are known, and here we found that at least 3 (CatSper 2–4) are transcribed by ON bipolar cells (Qi et al., 2007; Jin et al., 2007). CatSper 2 transcript skips exons 2–5, and thus cannot be translated correctly. However, both CatSper 3 and 4 are positive and are transcribed in full length. Nonetheless, light responses recorded by ERG and whole cell mode from CatSper3- and CatSper4- null mice showed that responses were normal, suggesting that, if these channels do contribute to the light response, the contribution is minor.

We also examined Kir2.4 because it was fished out by our yeast two-hybrid screen. This inwardly rectifying potassium channel (Topert et al., 1998; Topert et al., 2000) is expressed by all retinal cell types including ON bipolar cells, consistent with in situ hybridization results (Hughes et al., 2000). Interestingly, in ON bipolar cells, Kir2.4 is expressed in dendritic tips more strongly than in somas. Somatic expression is limited to a few clusters in 3–4 regions of the membrane. Since Kir2.4 is a potassium channel, and since it interacts with $G\alpha_1$ (Sulaiman, unpublished data) it probably contributes to regulation of the resting potential, possibly regulated by G_o .

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Fig. 1. EGFP is expressed exclusively in all ON bipolar cells (Grm6-GFP, mouse line 5)
 Images are radial sections of line 5 retina stained for G- γ 13 (red). Upper row, low magnification; projection of 3 consecutive confocal planes. Lower row, a single confocal plane of INL at higher magnification. All cells stained for G- γ 13 were EGFP-positive and vice versa. In case staining was not evident in a particular section, it became evident in sequential sections. A–C were photographed under 40x oil immersion objective NA 1.25. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; a, the OFF sublamina; b, the ON sublamina.

Fig. 2. Density of ON bipolar cells varies little across eccentricity

A–C. Tangential view of line 5 retina at the level of the INL (A), sublamina a of the IPL (B), and sublamina b of the IPL (C). Because the fields are slightly slanted, structures on the left are deeper in the retina. For example in C, the left field images sublamina 5 where large structures belonging to rod bipolar terminals are seen, while the right field images sublaminae 3 and 4 with smaller terminals of ON cone bipolar cells.

D. ON bipolar cell density *vs.* distance from the optic disk (OD); distance of 0 defined as the edge of the disk. All points are temporal to the disc except the X at 100 μm, which is nasal. Different symbols are from different mice.

Fig. 3. Grm6-GFP transgenic mouse (line 1) shows a mosaic expression of EGFP in ON bipolar cells

A. A low magnification radial section (projection of 2 confocal planes) of line 1 retina showing that EGFP is restricted to cells whose somas lie at the upper tiers of the INL and whose axons arborize in sublamina b.

B. A radial section stained for G- γ 13 (red). All EGFP-positive cells are stained for G- γ 13 (double arrowheads), indicating that they are all ON bipolar cells. However, many G- γ 13-positive cells are EGFP-negative (arrows) indicating that not all ON bipolar cells are marked by EGFP in this mouse line.

C. A radial section stained for PKC (red). Many PKC-positive cells are EGFP-negative (arrows). Double arrowheads point to EGFP-positive rod bipolar cells, and asterisks indicate EGFP-positive ON cone bipolar cells.

Fig. 4. ERG of Grm6-GFP transgenic mouse (line 5) is similar to that of wild type

A–D. ERG responses of wild type mouse (wt, black traces) and Grm6-GFP mouse (gray traces). Scale bar for time is 100 ms and for voltage is 100 μ V.

A. Responses to dim flashes (30 R*/rod) under dark-adapted conditions.

B. Responses to an intense flash (10⁵ R*/rod, isomerizing ~0.1% of the rhodopsin) under dark-adapted conditions.

C. Responses to an intense flash (10⁵ R*/rod) under light-adapted background (white light, 5.3×10^4 R*/rod/s) that completely suppressed the rod driven current.

D. Test of ERG response recovery: a dim flash (30 R*/rod) under dark-adapted background was followed by a second dim flash at an interval of 350 ms. The amplitude of the second response was smaller than that of the first and recovered to its original size after about 500 ms.

E and F. Comparing the ERG parameters of wild-type and Grm6-GFP mice. Error bar shows standard error. **E.** Comparison of a- and b-wave amplitudes under dark- and light-adapted conditions. The small differences were insignificant; n=3 mice, 6 eyes, for each group.

F. Ratio of the peak amplitude of the 2nd b-wave to that of the 1st. The difference was not significant (n=3).

Fig. 5. ON bipolar cDNA library is pure

A, B. Two examples of dissociated ON bipolar cells.

C, D. Two examples of cells that were sorted by FACS: top images are DIC and bottom images are fluorescent. In C examples, all four FACS-sorted cells express EGFP. In D, there are 8 cells (bright white speckles are DIC artifacts); one is EGFP-negative (*) and one is very faint. Scale bars are 10 μm.

E–G. PCR products used to test the library. **E.** Gα_{o1} was amplified as positive control for purity testing. **F, G.** Photoreceptor genes were amplified to test purity. Con, control used water instead of DNA; bp, ON bipolar cDNA library; ret, retinal cDNA; rd, rd retina cDNA library; CNGA1, rod cGMP-gated channel; CNGA3, cone cGMP-gated channel. E and G come from the same gel.

Fig. 6. Representative PCR products

PCR products were amplified from the bipolar cDNA library (bp) using transcript-specific primers. Transcripts are specified above the lanes in A, and on the right for β -E. For all panels except A, the left three lanes show product amplified with primer set 1 (the top rows for each transcript in Table 1) and the right three show product amplified with primer set 2 (the lower rows in Table 1). Negative control (con) used water instead of DNA, and positive control used retinal cDNA (ret). Left lanes are 100 bp markers (M) with top bright band at 1000 bp and bottom bright band at 500 bp. In B (GC1 α 2), the PCR product that was amplified from retina with primer set 1 was sequenced and found to be correct, while that amplified from bipolar library was sequenced and found to be incorrect.

Fig. 7. CatSper-null mice show normal ERG responses to light stimuli

A. Response of CatSper 4-null mouse to an intense flash (10^5 R*/rod, isomerizing $\sim 0.1\%$ of the rhodopsin) under dark-adapted conditions.

B. Response of the same animal to an intense flash (10^5 R*/rod) under light-adapted conditions (background intensity = 5.3×10^4 R*/rod/s).

C. Response recovery of another CatSper4-null mouse under dark-adapted conditions. The stimulus consisted of two consecutive flashes (30 R*/rod) with an inter-flash interval of 300 ms.

D. Ratio of the amplitude of the 2nd b-wave to that of the 1st as a function of inter-stimulus interval. The recovery of the 2nd response was similar for wild type (n=2), CatSper3-null (n=1), and CatSper4-null (n=2) mice, with an average recovery time of 500 ms. Time scale bar is 100 ms.

Fig. 8. Kir2.4 is expressed throughout the retina and in ON bipolar dendritic tips

A, B. HEK cells were transfected with Kir2.4 cloned from retina and immunostained with anti-Kir2.4. The antibody recognizes Kir2.4 and gives no background in untransfected cells. A, fluorescent image, immunostaining visualized with FITC; B, DIC image with merged fluorescence.

C. Western blots of HEK cells transfected with Kir2.4 (Kir2.4), HEK cells transfected with two different empty vectors (Vec1 and Vec2), and retinal homogenate (Ret). The same two bands appear in HEK cells and retina. Faint non-specific bands appear only in HEK cells.

D. Wild type mouse retina immunostained for Kir2.4. All cell types are stained; this picture represents a collapse of 3 confocal optical planes (taken with a 40x oil immersion objective NA 1.3).

E–G. Grm6-GFP retina (with EGFP in ON bipolar cells; green) stained for Kir 2.4 (magenta) showing higher magnification of OPL and INL: punctate staining in OPL is present in dendritic tips of ON bipolar cells; puncta in INL mainly localized to the edge of the somas. These pictures are from a single confocal optical plane taken with a 60x oil immersion objective (NA 1.42).

Table 1
Primers used for PCR amplification

Each sequence of an upper primer is preceded by the number of the first nucleotide at the 5' end, while each lower primer is preceded by the number of the last nucleotide at the 3' end (reverse primer). Lower primers were used with either of the two upper primers. The expected PCR product size (in bp) for each primer pair is written in parentheses following the upper primer sequence.

Transcript Accession no.	R	bp number 5'/upper primer/(size)	bp number 3'/lower primer
G_o interactors			
Ret-RGS1 NT* BI734645	+	19 gcattagagagccaaagaa (283)	301 gcatttcgctccgcttcagat
Ret-RGS1 BC116929	+	574 cttaaagaggtcagcttgact (197)	770 ctatgctcaactgtttct
PCP2 EU164759	+	99 gcttctcaacctgctgacc (358)	456 gccagatggctagaactc
	+	22cacaatggaccaggaagaaga (435)	
RGS16 NM_011267	+	193 gctccgatactgggggtatt (363)	555 ccacatcgaagcaactgga
	+	382 cctgcgaggagttcaagaag (174)	
AGS3 NM_134116	–	167 gcaggatgatcaggatgg (408)	574 gatctctgctctccattcg
	–	410 ccgctcttgaagacaaag (165)	
RIC-8 NM_053194	+	1388 ggacacggacacagaggagt (313)	1700 cagttctgaaggggagcaag
	+	1545 aggcacagatcatccaacc (156)	
RGS10 NM_026418	–	150 gccttaagagcacagccaag (470)	619 ggtgaagggctcagcttatg
	–	420 agccacacctctgatgttc (200)	
LGN NM_029522	+	1783 cgctcccagaagaaatcag (662)	2444 ggggaccagtcctttcttc
	+	2031 ccagagggccagtttcagta (414)	
RAP1GAP NM_001081155	+	1817 ctccagagctcccagagatg (1017)	2833 ggactcaggttgggtgattg
	+	2509 gggaacagggttaggaaaagg (325)	
RGS19 NM_026446	–	260 ctgtagctgctcctggaacc (381)	640 gatggctcttgcatcttct
	–	441 ttctgcgcacagaatacag (200)	
RGS7 NM_011880	+	1311 tcgagagtgcaggaaatatgg (359)	1669 gtccttgctccagtaaca
	+	1459 agctgatgaagagcgactcc (211)	
Calbindin NM_007586	–	30 gcctgagagagaggctta (389)	418 gccagcctccataaactca
	–	215 agaaggcaaggagggttct (204)	
Gβ5 NM_138719	+	723 cgtgcaagcctttgaaactc (328)	1050 gaatgctgcccacaggag
	+	723 cgtgcaagcctttgaaactc (469)	1191 gtgaggtgctccataagc
cGMP modulators			
GC1 α1 ** NM_021896	+	1910 agcagtggtggagagctgat (550)	2459 taagagcatgtggctcactaat
	+	2191 tctgcagtggtgctcggaag (269)	
GC1 α2 NM_001033322	–	3857 tccagattccacaacacca (850)	4706 gctgtttcccctgtttctca
	–	4477 tgcctgtgatcttttagc (230)	
GC β1 ** NM_017469	+	2235 gctacaagcattgcctagacg (758)	2992 accaacagggaattcagcca
	+	2792 caggacaaggctgggaata (201)	

Transcript Accession no.	R	bp number 5'/upper primer/(size)	bp number 3'/lower primer
GC β 2 NM_172810	–	1174 cgaagcactaagggtcaagc (757)	1930 atggctttcaacgggtactg
	–	1780 ggctgtgaacctatccaaa (151)	
PDE 1C1 NM_011054	+	2118 gaaggaagctgaagagaagg (673)	2790 catgcctcagttacctgaagt
	+	2620 ggtgagttttgctctccta (171)	
PDE 2A NM_001008548	–	2526 caccacctccgcatttcaa (482)	3007 acaggagctctgggtccgtcca
	–	2740 gaccgatggagatgatggac (268)	
PDE 5 NM_153422	–	2693 cccgtgtacaaactgccaca (618)	3310 gaatcatctggcctgggggt
	–	3154 cgtgtacctaattgctctgc (157)	
PDE 9A NM_008804	+	1038 gacgtgagtgcaacatttc (582)	1619 cactgtcttttgcattctgt
	+	1440 ccaagctcttcccgtgtt (180)	
Channels			
Kir 2.4 NM_145963	+	1192 gtggctcccagatgatgtt (369)	1560 catcagaggctggaagggaag
	+	1327 gctccctcactgcatttgt (234)	
ACCN4 NM_183022	–	1238 gagctcagggtactcagc (722)	1959 cccacacagcactgttcta
	–	1718 tatgaggtctcctgggatcg (242)	
CatSper 1 NM_139301	?	1515 ggttctccgagcactgttc (217)	1731 taccagacagcaatcacca
	?	1720 gctgtcctggtagataattc (321)	2040 tccagcctcaaatgccatgt
CatSper 2 NM_153075	+	279 ccaaatgtcagctgttgc (1919)	2197 cttcattagcttttgcctggg
	+	1015 gctgaggtctctcaactg (1183)	
CatSper 3 NM_029772	–	3 agaattcaggtccaaggac (1202)	1204 ctctccttgggcatgtcttc
	+	461 tgcatactccactttgctg (744)	
	+	905 ccttgcgattatggaggaga (300)	
CatSper 4 NM_177866	–	11 agagaaggggacacagaaa (1600)	1610 tgatgcagacagaggtcagg
	+	911 gagggaagaggacacctgaa (700)	
	+	1361 gaagcgagtatgggaactgg (250)	
TRPC ***	+	ga(a/g)acitcica(a/g)tc(c/t)ctt(c/t)tg	(c/t)ttgtic(g/t)igc(a/g)aa(c/t)ttcca(c/t)tc
TRPC2	+	3065 gaaacgttcagtttctcttttgg (225)	3289 cttggagcgagcaaaactccactc
Purity testing			
Opsin AF011389	–	574 atctttggttgagcaggtact (320)	893 tgggcagtagcaaagcatgcaa
Rod CNGC NM_007723	– /+	2052 tgaagatggctgctggat (270)	2321 ttcatcctgtagagactccgt
Cone CNGC NM_009918	–	1951 ttctgatgaaggacaaccta (225)	2175 tgcaggtgagaagccacaa
GRK1 NM_011881	–	1287 agaagttcagccaggccagca (310)	1596 tcctccagggaatggagcag
RGS9 NM_011268	–	1695 cctttgcttccccgagtcgt (310)	2004 accttccctgtgacacaga
G_{α1} M36777	+	544 atcctccgaaccagagtcac (427)	970 tgttgggtgagcggtttt

* For Ret-RGS N-terminus, the mouse sequence is not available in the GenBank; the given accession number is for an EST (Dhingra et al., 2004).

** Names of isoforms as they appear in the NCBI data bank is incorrect; we adapted the names according to Zabel et al. and Koesling et al. (Zabel et al., 1998; Koesling et al., 2004).

*** TRPC degenerate primers are based on Liman et al. (Liman et al., 1999). NT, N-terminus; CNGC, cyclic nucleotide-gated channel; GRK, G-protein receptor kinase; R, results of amplification from ON bipolar library; −, no amplification; +, positive amplification; −/+, mostly negative; ?, results were ambiguous; for CatSper2, the obtained PCR products were shorter than expected; for CatSper3 and CatSper4, first primer indicates “-” because the full length transcript could not be amplified from the library, but was amplified from retinal cDNA; Proteins that are positive in ON bipolar cells are bolded and underlined.