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Isolation of ON Bipolar Cell Genes *via* hrGFP-coupled Cell Enrichment Using the mGluR6 Promoter

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mGluR6 expression is a characteristic property of retinal ON bipolar cells. mGluR6 is also the causal gene for a form of congenital night blindness. To elucidate physiological and pathological functions of ON bipolar cells and mGluR6, we thought it important to identify genes specifically expressed in them. We thus made transgenic mouse lines expressing humanized *Renilla reniformis* green fluorescent protein (hrGFP), under the control of the mGluR6 promoter. From their retina, we isolated hrGFP-positive cells by cell sorting, and analysed the gene-expression profile of these cells by using DNA microarray. Further analysis revealed that about half of the initially selected ON bipolar cell genes were expressed in the expected retinal layer. We confirmed previously ambiguous retinal localization of regulator of G-protein signalling 11 (RGS11) and transient receptor potential cation channel, subfamily M, member 1 (TRPM1). In addition, we showed the expression of calcium channel, voltage-dependent, alpha2/delta subunit 3 (Cacna2d3) in ON bipolar cells for the first time. Although we could not completely exclude the possibility that a small population of hrGFP-positive cells might not be ON bipolar cells, these mice as well as our strategy would be highly valuable for the further analysis of ON bipolar cells.

Key words: bipolar cells, Cacna2d3, mGluR6, RGS11, TRPM1.

Abbreviations: Cacna2d3, calcium channel, voltage-dependent, alpha2/delta subunit 3; GAP, GTPase-activating protein; Gβ5, the fifth member of the heterotrimeric G-protein β-subunit family; hrGFP, humanized *Renilla reniformis* green fluorescent protein; INL, inner nuclear layer; IPL, inner plexiform layer; mGluR6, metabotropic glutamate receptor subtype 6; PI, propidium iodide; RGS11, regulator of G-protein signalling 11; TRPM1, transient receptor potential cation channel, subfamily M, member 1.

When the retina is stimulated by light, ON bipolar cells depolarize and OFF bipolar cells hyperpolarize. Although all photoreceptor cells release glutamate in darkness, the bipolar cell types respond to glutamate differently (*1*, *2*). OFF bipolar cells have ionotropic glutamate receptors: glutamate opens a cation channel, and the cell depolarizes. ON bipolar cells have a sign-inverting receptor, mGluR6 (*3*); these bipolar cells hyperpolarize in response to glutamate *via* closure of a non-selective cation channel. Conversely, when synaptic glutamate release is decreased in the light, the transduction channel opens, and ON bipolar cells depolarize. This distinction, created at the first retinal synapse, is propagated throughout the visual system. This is a fundamental functional feature of the visual system, and elucidation of the function of bipolar cells that create this dichotomy is pivotal for understanding the system (*1*, *2*). Recently, mGluR6 has been shown to be the causal gene for a congenital form of night blindness, suggesting the importance of bipolar cells in visual diseases (*4*, *5*).

In our previous studies, we demonstrated that the 5′ upstream genomic sequence of mGluR6 is capable of directing a cell-specific and developmentally regulated expression of mGluR6 in ON bipolar cells (*6*). In this study, we used the same genomic region to express hrGFP specifically in ON bipolar cells and purified ON bipolar cells to homogeneity by cell sorting to investigate the genome-wide gene expression profile of ON bipolar cells. We found many genes expressed in ON bipolar cells in the retina. Several of them were well in line with ON bipolar cell function and are discussed in detail herein.

MATERIALS AND METHODS

The animals were treated as approved by the Animal Research Committee of Kyoto University for the ethical use of experimental animals.

Antibodies—Anti-hrGFP antibody was from Stratagene (La Jolla, CA, USA). Anti-PKCα was obtained from BD Biosciences (Franklin Lakes, NJ, USA); and anti-calretinin, from Milipore (Billerica, MA, USA). Anti-mGluR6 was described previously (*7*). Alexa 594-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA).

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Generation of Transgenic Animals—A transgenic vector was constructed by fusing hrGFP coding sequence of phrGFP1 (Stratagene) downstream to the mGluR6 promoter (*6*) to drive expression of hrGFP in ON bipolar cells. The vector was digested with a restriction enzyme, and the expression unit for hrGFP was gel-purified from agarose gels. The DNA fragment was microinjected into the pronuclei of C57BL/6J fertilized one-cell embryos. Injected embryos were transplanted into the oviducts of pseudopregnant female mice. Integration of the hrGFP gene was analysed by PCR of tail DNA.

Retinas and brains from each line were solubilized with 20 mM Tris–-HCl containing 2% SDS (pH7.5), and the proteins in the lysates (50 μg protein) were separated on SDS–PAGE gels and then transferred to nitrocellulose membranes. Expression of hrGFP was visualized by immunoblotting with anti-hrGFP antibody.

Immunohistochemistry—Eyes were enucleated, dissected at the limbus and immersed in 4% paraformaldehyde/PBS for 30 min. After fixation, the eyes were cryoprotected with 25% sucrose and embedded in O.C.T. compound (Miles, Elkhart, IN, USA). The samples were sectioned at 10 μm on a cryostat and examined for fluorescence of hrGFP. For immunofluorescence staining, mounted sections were rinsed, pre-incubated with 5% normal goat serum and 1% TX-100 in PBS, and incubated overnight with the primary antibody diluted in the same solution at 4°C. The sections were then reacted with the Alexa 594-conjugated secondary antibody at room temperature for 30 min. After final rinsing with PBS, the samples were coverslipped with Vectorshield (Vector Laboratories, Burlingame, CA, USA), and the edge of the coverslip was sealed with VALAP (mixture of Vaseline, lanolin and paraffin). Fluorescence immunoreactivity was viewed by confocal microscopy (LSM510, Carl Zeiss, Oberkochen, Germany).

Cell Dissociation and Cell Sorting—After enucleation of eyes from adult transgenic mice, the neural retinas were isolated from the retinal pigment epithelium in Earle's balanced salt solution at room temperature. Enzymatic digestion and dissociation of single cells were conducted by using the Papain Dissociation System (Worthington Biochemical Corporation, Lakewood, NJ, USA) according to the manufacturer's instructions. Dissociated cells were stained with 1 μg/ml of propidium iodide (PI), and two-colour cell sorting based on hrGFP and PI fluorescence was performed with a FACSVantage (BD Biosciences).

Microarray Analysis—Total RNA was isolated from the sorted cells with RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The integrity of the isolated RNA was analysed with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We used Sentrix Mouse-6 Expression BeadChips (Illumina, San Diego, CA, USA) containing > 47,000 probes to query expression profiles of the mouse genome. Preparation of cRNA, hybridization, and scanning of the microarray were performed according to the manufacturer's protocol. Data were analysed by using a software package provided by the manufacturer. Briefly, after subtraction of background signals, the remaining signals were normalized by the Rank-invert method. Significantly expressed genes were detected by calculating the Detection value, and the Diffscore was calculated to detect genes expressed differentially.

In Situ Hybridization—*In situ* hybridization with digoxigenin-labelled RNA probes was carried out as described earlier (*8*). PCR primers used for preparation of the probes are listed in Table 1. Templates for amplification were cDNA prepared from retinal RNA, mouse brain cDNA (Marathon-Ready, Clontech, Mountain View, CA, USA) or cloned cDNAs. All the amplified fragments were cloned into pCR-Blunt II-TOPO (Invitrogen) for *in vitro* transcription. For each probe, two to three sections were used for hybridization, and they gave an identical hybridization pattern. Representative data were presented. Images were obtained with Axiophot 2 (Carl Zeiss).

RESULTS

Generation and Characterization of Transgenic Animals—A fusion gene consisting of the mGluR6 promoter region and hrGFP was microinjected into fertilized mouse eggs, which then were transferred to foster mothers. Of the eight independent transgenic lines that passed their transgenes onto their offspring, two lines, mg6hrGFP11 and mg6hrGFP26, exhibited significant expression of hrGFP in the retina (Fig. 1A).

Sections of retinas from these two transgenic mouse lines are shown in Fig. 1B–E (for higher magnification of these pictures, see Supplementary Figs 1–4).

Table 1. **Forward and reverse primers used for the PCR amplification of genes.**

Gene	Forward primer	Reverse primer	Template	Expected PCR Product Size (bp)
Rhodopsin	GCCCCAATTTTTATGTGCCCTTC	ACTTCCTTCTCTGCCTTCTGAG	retina	733
PKCα	CCTGCGACATGAATGTTCACAAC	AAGGTTTTAATTTGAACGTGAAG	brain	300
TRPM1	GCGTGGTTCAATCAAGAGCTC	CATGAGGTGGAGCAGGGAATCTG	retina	367
Slc5a8	CATTCGTCTCTGTGGCACAATC	CAATCGCTGACACTTTCTTAGATG	Riken[a]	581
Cacna2d3	TGTAGGCATTCAGATGAAACTTG	TCGTCTGATCTTCTGAGCCTTTAAC	brain	700
Ddit4l	ATGGTTGCAACGGGCAGTTTGAG	TTAGCACTCTTCAATGACTGTC	brain	582
Prkcn	AACCTGAGAAAGAGACATTATTG	AAGCACCTCGTCCGCAAAGATC	brain	453
Smtn	ATGGCAGACGAGGCTTTAGCT	GGACTCTGTCTCTCACAGAG	IMAGE[b]	1000
RGS11	CGGGATCCATGGAGCGGGTAGTCGTGAGTATG	CGCGTCGACCTACTCCCCATCGGCACCTTC	retina	1332

[a]Riken cDNA clone, A430032A05. [b]IMAGE cDNA clone, #3709078.

Because more hrGFP was expressed in the retina of mg6hrGFP11 (Fig. 1A), sections from this transgenic line showed a stronger fluorescence (data not shown). To facilitate comparison of the two lines, we enhanced the fluorescent signals of hrGFP in mg6hrGFP26 in Fig. 1D and E. On immunofluorescence staining with the anti-PKCα antibody, which selectively labels rod bipolar cells (*9*), there were more hrGFP-positive/PKCα-negative bipolar cells than hrGFP-positive/PKCα-positive cells in both transgenic lines (Fig. 1B and D). The cell bodies of the hrGFP-positive/PKCα-negative bipolar cells were located at the position similar or close to the PKCα-positive rod bipolar cell bodies in the outer part of the inner nuclear layer (INL). As all the hrGFP-positive cells but not other cells had punctate mGluR6 immunoreactivities at their postsynaptic dendritic tips, we assumed that the hrGFP-positive cells are mostly, if not all, ON bipolar cells (Supplementary Fig. 5). Given that, these hrGFP-positive/PKCα-negative bipolar cells corresponded to cone ON bipolar cells. Immunostaining for calretinin revealed three dendritic strata within the inner plexiform layer (IPL; Fig. 1C and E): the middle stratum represents the border between sublamina a and b of the IPL (*1*, *10*). All the axon terminals of hrGFP-positive cells resided in sublamina b of the IPL. There were two distinct populations of axon terminals in sublamina b. hrGFP-positive/PKCα-negative axonal terminals in the outer part of sublamina b corresponded to those of cone ON bipolar cells (Fig. 1B and D). There was no apparent difference in the spatial distribution of fluorescent signals between the two transgenic lines. These morphological analyses were all in accord with our previous analysis of the mGluR6 promoter region (*6*) and quantitative anatomical view of retinal cell populations (*11*). Thus, our established two independent mouse lines apparently expressed hrGFP specifically in all ON bipolar cells (see DISCUSSION section).

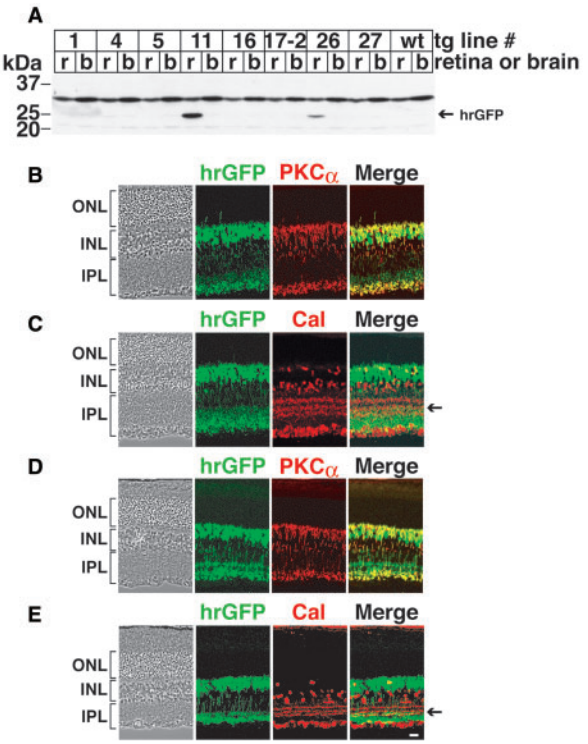

Fig. 1. **Characterization of transgenic mouse lines.** (A) Immunoblot analysis of the retina and brain from transgenic lines. The arrow indicates the position of hrGFP. The slower migrating band in each lane is a non-specific signal, which serves as a loading control. r, retina; b, brain. (B and D) Immunofluorescence staining of PKCα immunoreactivities in transverse retinal sections from the two transgenic mice. Note the presence of hrGFP-positive/PKCα-negative axon terminals in the outer layer of sublamina b in both sections. (C and E) Immunofluorescence staining of calretinin (Cal) immunoreactivities in transverse retinal sections from the two transgenic mice. The arrows indicate the position of the middle stratum of calretinin strata in the IPL. The outer (above the arrow in the figures) portion of IPL corresponds to sublamina a. Sections in (B) and (C) are from mg6hrGFP11; and those in (D) and (E), from mg6hrGFP26. In (D) and (E), the intensity of the hrGFP fluorescence was enhanced to facilitate comparison of expression patterns between the two transgenic lines. Scale represents 20 μm.

Purification of ON Bipolar Cells—Retinas were prepared from each adult transgenic mouse line, and the cells were dissociated into single cells by papain treatment (Fig. 2A). The typical yield of total cells was $\sim 1 \times 10^6$ from two retinas. The representative cells shown in Fig. 2B exhibited morphological profiles

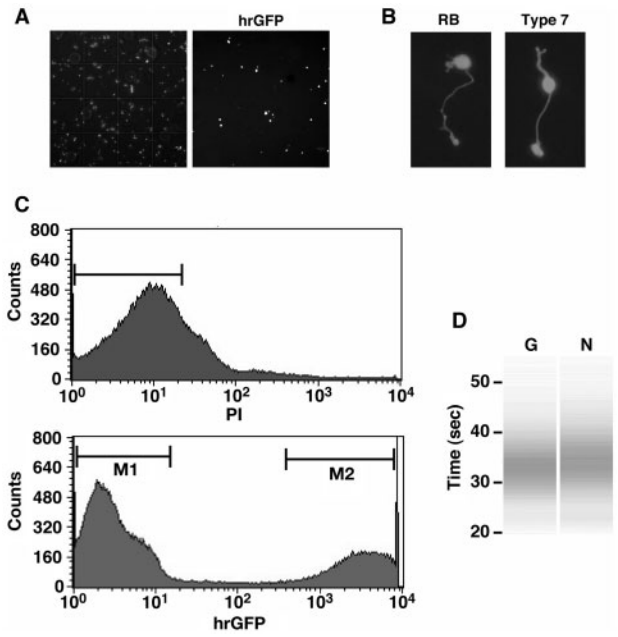

Fig. 2. **Purification of ON bipolar cells by cell sorting and preparation of cRNA probes for microarray analysis.** (A) Dissociated retinal cells. The right panel shows fluorescence of hrGFP. (B) Representative fluorescent bipolar cells. RB and Type 7 correspond to the types of ON bipolar cells defined by Ghosh *et al*. (*10*). (C) From PI-negative viable cells (PI-negative fraction in the upper panel), hrGFP-positive (fraction M2) and -negative (fraction M1) cells were sorted as ON bipolar cells and non-ON bipolar cells, respectively. (D) The labelled cRNAs have a comparable length distribution. G represents ON bipolar cells; and N, non-ON bipolar cells.

Table 2. **Genes selectively expressed in ON bipolar cells.**

Diff[a]	rank[b]	Accession	ISH[c]	Ref[d]	Symbol	Definition
371	26	AK044507	Yes		TRPM1	Transient receptor potential cation channel, subfamily M, member 1
364	19	NM_008790.1		Yes (*12*)	Pcp2	Purkinje cell protein 2
353	7	NM_145423.1	No	No (*13*)	Slc5a8	Solute carrier family 5, member 8
328	25	NM_022422.3		Yes (*14*)	Gng13	Guanine nucleotide binding protein 13, gamma
328	68	AK048773			Kcnma1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1
318	12	NM_009785.1	Yes		Cacna2d3	Calcium channel, voltage dependent, alpha2/delta subunit 3
315	22	NM_030143.2	No		Ddit4l	DNA-damage-inducible transcript 4-like
284	13	NM_021459.2		Yes (*15*)	Isl1	ISL1 transcription factor, LIM/homeodomain
282	62	NM_008759.1		Yes (*16*)	Og9x	OG9 homeobox gene
276	104	NM_029239.2	No		Prkcn	Protein kinase C, nu
275	34	NM_013870.1	No		Smtn	Smoothelin
275	116	NM_009367.1			Tgfb2	Transforming growth factor, beta 2
263	4	NM_011627.2		Yes (*17*)	Tpbg	Trophoblast glycoprotein
256	126	NM_013515.1			Epb7.2	Erythrocyte protein band 7.2
249	40	NM_173415		No (*18*)	Nyx	Nyctalopin
246	103	NM_183000.1			Accn3	Amiloride-sensitive cation channel 3
246	44	NM_008937.2		No (*19*)	Prox1	Prospero-related homeobox 1
245	74	NM_011101.1		Yes (*9*)	Prkca	Protein kinase C, alpha
236	72	NM_026653.1			Rpa1	Replication protein A1
235	112	XM_358362			Ptprz1	Protein tyrosine Phosphatase, receptor type, Z polypeptide 1
231	8	AK032866			Gpc6	Glypican 6
226	10	NM_013878.1			Cabp2	Calcium-binding protein 2
225	174	NM_019684.1			Stk23	Serine/threonine kinase 23
221	192	XM_358335.1			Cacna1s	Calcium channel, voltage-dependent, L type, alpha 1S
220	58	NM_183000.1			Accn3	Amiloride-sensitive cation channel 3
217	80	NM_011782.1			Adamts5	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5
214	30	NM_011261.1		? (*20*)	Reln	Reelin
209	145	NM_023380.1			Samsn1	SAM domain, SH3 domain and nuclear localisation signals, 1
209	42	XM_130497.2			Ryr3	Ryanodine receptor 3
207	11	XM_128488.3	Yes		Rgs11	Regulator of G-protein signalling 11
198	33	NM_010308.2		Yes (*21*)	Gnao	Guanine nucleotide binding protein, alpha o isoform A
197	101	NM_008075.1		Yes (*22*)	Gabrr1	GABA-C receptor, subunit rho 1
197	67	AK038695			A230055O04Rik	Carbonic anhydrase-related protein 10 homologue
196	63	NM_009367			Tgfb2	Transforming growth factor, beta 2
187	32	NM_025734.2			Kcng4	Potassium voltage-gated channel, subfamily G, member 4
185	27	NM_172869			Frmd3	FERM domain containing 3
183	87	NM_007701.2		Yes (*23*)	Chx10	*C. elegans* ceh-10 homeo domain containing homologue
178	155	XM_126517.4		Yes (*16*)	Car10	Carbonic anhydrase 10
177	6	NM_031161.1		No (*24*)	Cck	Cholecystokinin
177	111	NM_007904.2			Ednrb	Endothelin receptor type B
175	66	NM_019676.1			Plcd1	Phospholipase C, delta 1

[a]Calculated Diffscore from analysis of mg6hrGFP11. [b]The rank of the gene from analysis of mg6hrGFP26. [c]Bipolar cell expression revealed from *in situ* hybridization analysis in this study. [d]Possibility of getting each gene by microarray analysis deduced from reported expression analyses. Reelin is expressed in a population of cone bipolar cells but not in rod bipolar cells. As the type of these cone bipolar cells is not indicated, it is difficult to predict if Reelin can be isolated as an ON bipolar cell-specific gene.

corresponding to the rod bipolar cell and type-7 cone bipolar cell (*10*), reflecting that we had labelled various types of ON bipolar cells with hrGFP. After the dissociated cells had been stained with PI, a fluorescent marker of dead cells, the hrGFP-positive/PI-negative (*i.e.* viable) ON bipolar cells were purified by cell sorting (Fig. 2C). hrGFP-negative/PI-negative cells were also pooled and used as a cell population of non-ON bipolar cells. Although the purity of ON bipolar cells from mg6hrGFP11 was close to 100%, that from mg6hrGFP26 was about 85%. This tendency was observed in repeated experiments and possibly reflected the lower fluorescence of ON bipolar cells in the mg6hrGFP26 line. RNA was isolated from the two populations of cells without major degradation, and cRNA probes synthesized from the RNA showed a similar size distribution (Fig. 2D).

Gene-Expression Profile of ON Bipolar Cells—We used Sentrix Mouse-6 Expression BeadChips containing >47,000 probes to query expression profiles of the mouse genome. cRNA probes prepared from ON bipolar cells and the corresponding non-ON bipolar cells from both mg6hrGFP11 and mg6hrGFP26 mouse lines were

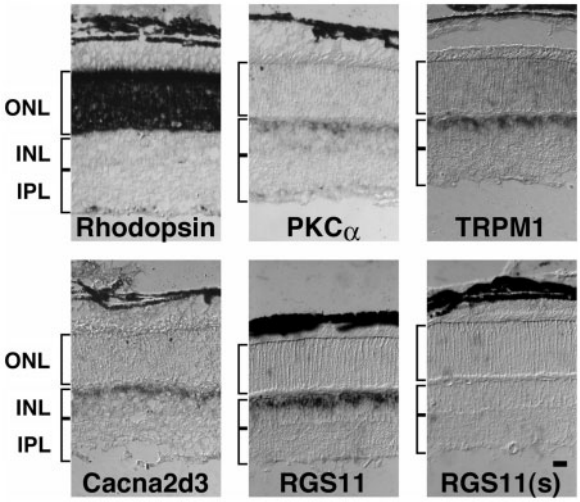

Fig. 3. ***In situ* hybridization analysis of ON bipolar cell genes in adult mouse retinal sections.** Note the positive signals of TRPM1, Cacna2d3 and RGS11 in the outer portion of the INL. In RGS11(s), the sense strand of RGS11 was used for hybridization as a negative control. The scale bar represents 20 μm.

subjected to microarray hybridization analysis. To select candidate genes specifically expressed in ON bipolar cells, we first focused on highly expressed genes in hrGFP-positive cells from the mg6hrGFP11 line (Detection value = 1), and then ranked the genes according to their DiffScore, which indicates the ratio of mRNA expression in ON bipolar cells to that in non-ON bipolar cells. Of 100 genes with the highest DiffScores from the mg6hrGFP11 line, 91 genes were ranked in the top 200 from the analysis of the mg6hrGFP26 line. Of these, according to the literature and database biological functions have been assigned to 41 of these genes (listed in Table 2); retinal expression patterns have been reported for 15 of these 41 genes; and 10 of these 15 genes have been shown to have bipolar cell-enriched expression patterns, coherent with the isolation by microarray analysis (for TRPM1, see DISCUSSION section).

To confirm the spatially restricted expression of the candidate genes, we performed *in situ* hybridization analysis on seven genes of interest (Table 1). Five of them (Slc5a8, Cacna2d3, Ddit4l, Prkcn and Smtn) had high Diffscores, but their localization in ON bipolar cells had not been reported previously. The molecular properties of TRPM1 and RGS11 suggest their close association with mGluR6 signal transduction (Table 2). Rhodopsin and PKCα were used as controls to locate photoreceptor cells and rod bipolar cells, respectively. As shown in Fig. 3, we found three genes, RGS11 (*25*, *26*), TRPM1 (*27*, *28*) and Cacna2d3 (*29–31*), that were expressed in the outer portion of the INL, where ON bipolar cells reside. Because there was no detectable signal for the other four genes (data not shown), their expression in the retina could not be determined. More importantly, however, we did succeed in localizing these three molecules implicated in the function of ON bipolar cells.

DISCUSSION

In the present study we established two mouse lines targetedly expressing hrGFP in ON bipolar cells by using the mGluR6 gene promoter. Although we could not completely exclude the possibility that a small population of hrGFP-positive cells might not be ON bipolar cells, our morphological analyses and published analyses (*6*, *11*) suggest that we have labelled selectively all ON bipolar cells with hrGFP. After purification of these cells, we searched for genes selectively expressed in them by conducting microarray analysis. Although the purity of hrGFP-positive cells was close to 100%, about half of the genes identified by our microarray analysis were previously reported as those not expressed specifically in ON bipolar cells. This may be caused by limitations associated with our analysis based on relative abundance of mRNA (*32*, *33*) and/or the limitation of DNA microarray measurements (*34*). Similar limitations of analysis using microarrays were also reported in the experiment conducted on bipolar cells using a different promoter to express GFP in ON bipolar cells (*16*). In spite of everything, our approach was powerful enough for screening, and we were able to identify many candidate genes that may be pivotal in the function of ON bipolar cells.

The synapse between photoreceptors and ON bipolar cells is the first one in the visual system. Glutamate, the photoreceptor transmitter, hyperpolarizes ON bipolar cells *via* activation of mGluR6 (*35*, *36*). Activated mGluR6 then modulates a cation channel. Although mGluR6 was previously thought to signal through a G-protein to a cGMP-preferring phosphodiesterase (*35*, *36*), reminiscent of the phototransduction cascade, it is currently believed that cGMP, rather than gating the transduction channel, potentiates ON bipolar cell responses by activating cGMP-dependent kinase (*37*) and that the G-protein, most likely Go (*21*, *38*, *39*), may inhibit the channel. Similar to the case of photoreceptor cells, the L-type Ca^{2+} channels are thought to be responsible for exocytosis of glutamate from bipolar cells (*40*). We confirmed the localization of three putative key molecules of ON bipolar cells, RGS11, TRPM1 and Cacna2d3, in the appropriate layer of the retina (Fig. 3).

RGS proteins are GTPase-activating proteins (GAPs) for Gα proteins, stimulating the hydrolysis of GTP by the G proteins to adjust to the physiological deactivation rate (*25*, *26*). Vision represents a particularly obvious example showing the importance of RGS proteins. The GAP activity of RGS9 is critical in setting the inactivation kinetics of phototransduction, and patients with mutations in RGS9 or R9AP9 (RGS9 anchor protein) exhibit bradyopsia, a visual defect with impaired light adaptation and contrast detection (*41*). It is likely that the rapid response of ON bipolar cells to light requires a similar GAP activity. Dhingra *et al*. (*42*) found a retina-specific RGS protein, Ret-RGS1, as an interacting molecule of GαoA. They found that Ret-RGS1 is expressed in the dendritic tips of all ON bipolar cells (but no OFF bipolar cells) and can serve as a GAP for GαoA. As Ret-RGS1 is expressed in many types of retinal cells (*42*), its function is not specific to ON bipolar cells. Gβ5 is the fifth member of the heterotrimeric G-protein β-subunit

family (*43*). Unlike the other four conventional G-protein β-subunits, Gβ5 interacts with the G-protein-γ-like domain found in the R7 members of the RGS proteins (RGS6, 7, 9 and 11) (*44*). The retinal phenotype of Gβ5 knockout mice (*45*) prompted analysis of R7 members of RGS proteins by 3 groups (*46–48*). One group showed that RGS11 is expressed in photoreceptor cells and neurons of the inner retina (*46*), although the other two groups agreed on the expression of RGS11 at the dendritic tip of ON bipolar cells with discrepant conclusions regarding RGS7 expression (*47*, *48*). Our *in situ* hybridization pattern of RGS11 is consistent with data from the latter two groups. Although involvement of multiple RGS proteins in regulation of the mGluR6 cascade is possible, further understanding of RGS11 function should reveal the ON bipolar cell-selective action of RGS proteins and may lead to the identification of mutations causing retinal disease as in the case of RGS9.

Mammalian TRP channel proteins form six-transmembrane cation-permeable channels (*27*, *28*). They are grouped into six subfamilies on the basis of amino acid sequence homology (TRPC, TRPV, TRPM, TRPA, TRPP and TRPML). The TRPM subfamily comprises eight mammalian members, which play a role in processes as diverse as taste detection, Mg^{2+} homoeostasis, and cold sensing. TRPM1 is a Ca^{2+}-permeable plasma membrane channel (*49*) and exhibits closest similarity to TRPM3, which has a low ability to discriminate between divalent and monovalent cations (*50*). Consistent with this, recent gene targeting of TRPM1 strongly suggests that it is the non-selective cation channel regulated by mGluR6 (*51*). On the contrary, however, Kim *et al*. (*16*) have reported that TRPM1 is expressed in OFF bipolar cells and in a small number of ON bipolar cells. Thus, further investigation is necessary for the localization of TRPM1 in the retina.

The L-type Ca^{2+} channels are thought to be responsible for exocytosis of glutamate from photoreceptor and bipolar cells (*40*). These channels are formed by hetero-oligomeric complexes consisting of various combinations of an α1 protein with auxiliary α2δ, β and γ subunits (*30*, *31*). The α1 subunit imparts most of the conductive properties of the channel, whereas the auxiliary subunits modulate calcium currents and channel activation/inactivation kinetics. The auxiliary subunits are also involved in proper assembly and membrane localization of the calcium-channel complexes (*30*). CACNA1F, an α1 subunit of the L-type Ca^{2+} channel, identified as a mutated gene in X-linked congenital stationary night blindness (*52*, *53*), is localized to rod photoreceptor and bipolar cell active zones (*54*). Mutations in the human CACNA2D4 gene, an α2δ subunit, define a gene defect that causes autosomal recessive cone dystrophy (*55*). Thus, CACNA2D3 (*29*), another α2δ subunit, may be a subunit of the L-type Ca^{2+} channel that plays an important role in information processing of ON bipolar cells and is a causal gene of a visual disease as in the case of CACNA2D4 in photoreceptors.

The genes identified in this study should reflect various aspects of ON bipolar cell function and provide clues to define the retina's coding of visual stimuli in the ON pathway. They may also be causal genes for congenital visual diseases. Further characterization of genes specifically active in ON bipolar cells is in progress, and the results obtained should provide a useful basis for elucidation of retinal function and treatment of visual diseases.

SUPPLEMENTARY DATA

Supplementary data are available at JB online.

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