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Expression of genes encoding glutamate receptors and transporters in rod and cone bipolar cells of the primate retina determined by single-cell polymerase chain reaction

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Purpose: Light signals from rod and cone photoreceptors traverse distinct types of second-order, bipolar neurons that carry these signals from the outer to inner retina. Anatomical and physiological studies suggest that the specialization of rod and cone bipolar cells involves the differential expression of proteins involved in glutamatergic signaling. In a previous study, we compared the expression of genes for the AMPA- (GluR1-4) and kainate-sensitive (GluR5-7, KA1-2) ionotropic glutamate receptors, the metabotropic glutamate receptors (mGluR1-8), and five non-vesicular glutamate transporters (EAAT1-5) in full-complement cDNA constructed from fresh and aldehyde-fixed macaque retina using a technique suitable for amplification of a variety of differentially expressed transcripts. Here we apply the same protocol to compare expression of these genes in cDNA constructed from single rod and cone bipolar cells previously-labeled for morphological identification in fixed slices of macaque retina.

Methods: We used immunocytochemical labeling and unique morphological features in lightly fixed slices of macaque retina to target the rod bipolar or the DB3 cone OFF bipolar cell. Under visual control, we used a micropipette to target and extract labeled cells, and we isolated mRNA from each through enzymatic digestion. Full-length cDNA was synthesized using 3'-end amplification (TPEA) PCR, in which the highly diverse 3' regions were amplified indiscriminately to ensure detection of both high and low abundance genes. We used gene-specific RT-PCR to probe the cDNA of each bipolar cell both for expression of known genes to confirm cell identification as well as expression of genes encoding glutamate receptors GluR1-7, KA1-2, and mGluR1-8 and for transporters EAAT1-5.

Results: Of 27 rod bipolar cells confirmed to express the genes for the a subunit of protein kinase C, mGluR6, and its G protein $G\alpha_o$, 26 expressed at least one AMPA GluR subunit gene, 16 expressed at least two, and nine expressed three or more. Nearly every cell expressed the GluR4 gene (23/27), followed by GluR2 (16/27) and GluR1 (11/27). In addition to mGluR6, 20/27 cells also expressed the mGluR3 gene. Nearly every rod bipolar cell also expressed the genes for the EAAT2 (23/27) and EAAT4 (21/27) transporters. Of 26 DB3 cells confirmed by expression of calbindin D-28 and absence of GAD-65/67, each expressed the gene for the AMPA subunit GluR4, followed by GluR2 (22/26), and GluR1 (15/26), the only kainate subunit gene expressed was GluR6 (18/26). Nearly every DB3 cell also expressed the gene for the EAAT2 transporter (25/26), but no others.

Conclusions: Rod bipolar cells in the *Macaca* monkey retina expressed not only the mGluR6 gene, a subunit necessary for transmission of light-ON signals, but also nearly always GluR4 in combination with the glutamate transporter EAAT4 (21/27 cells). The DB3 cell involved in processing light-OFF signals from cones expressed most highly the combination of GluR4 and the transporter EAAT2 (25/26). These results suggest that glutamatergic signaling in rod and cone circuits in the primate retina depends upon complex molecular interactions, involving not only multiple glutamate receptor subunits, but also glutamate transporters. Our data demonstrate a consistent primary pattern for each cell type with subtle variability involving other genes. Thus, like neuronal cell types in other brain regions, morphological and physiological homogeneity among retinal bipolar cell types does not exclude variations in expression that could serve to adjust the stimulus-response profile of each cell.

A tremendous degree of functional diversity in the early visual system is rendered at the first synapse, where glutamate released from rod and cone photoreceptors excites specialized circuits tuned to different aspects of the visual scene [1-5]. Most of this diversity arises at the cone axon terminal. There, some 20-50 pre-synaptic active zones parcel the cone signal to about 10 morphological types of second-order bipolar neurons [6,7]. Roughly half of these (OFF cells) depolarize in response to the increased glutamate caused by a decrement in light, while the other half (ON cells) depolarize with the drop in glutamate caused by a light increment. Functional specialization among the OFF cells is rendered through specific combinations of ionotropic glutamate receptor (GluR) subunits, distinguished by their affinities for the glutamate agonists AMPA (GluR1-4) or kainate (GluR5-7, KA1 and

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KA2; [8-16]). While physiological specialization is also likely among the cone ON bipolar cells, a certain degree of uniformity exists among these cells through their common expression of the metabotropic glutamate receptor mGluR6 [16-19]. In darkness, when glutamate release is highest, mGluR6 closes nonspecific cation channels through activation of the heterotrimeric G-protein $G\alpha_o$; when glutamate decreases with light, the channels open [20,21].

The rod photoreceptor, though outnumbering cones by 19:1, mediates a far smaller portion of the visual dynamic range. Each rod reliably signals a single photon of light, thus establishing the lower limit of visual sensitivity [22-24]. The rod axon terminal, unlike that of the cone, generally contains only a single, large active zone [12,25,26]. And unlike the cone, the rod signal is parceled to just a single type of bipolar cell that collects from 20-60 rods to maximize its sensitivity in the dark [27,28]. Like the cone ON bipolar cells, the rod bipolar cell also depolarizes to light through mGluR6 and $G\alpha_o$ [15,16,20,21,29-32].

That mGluR6 is omnipresent among the cone and rod ON bipolar cells and is unquestionably necessary for generating the ON response could naturally lead to the tacit assumption that it is the only receptor involved in transferring the photoreceptor signal to these cells. However, recent studies using both immunocytochemical and genetic markers suggest that both cone and rod ON bipolar cells in the rodent retina express multiple GluR subunits (AMPA) as well as mGluR6 [33-35]. In contrast, based on physiological measurements in non-primate mammalian retina [13-16], cone OFF bipolar cells are expected to express multiple GluR subunits, but not mGluRs. The problem of assigning particular subunits to specific bipolar cell types is confounded not only by the difficulty in physiologically resolving currents from specific subunits, but also by the tremendous ambiguity inherent to anatomically resolving subunit localization in the dauntingly dense medley of neuronal processes in the outer retina [6,35].

In a recent study, we applied a modified version of 3'-end amplification (TPEA) RT-PCR to construct full-length cDNA libraries from retina of *Macaca fascicularis* [36]. We tested broad patterns of expression of genes for GluR and mGluR subunits as well as the glutamate transporters EAAT1-5 in RNA harvested from both fresh retina and from lightly fixed retinal slices. In the present study, we used TPEA to study gene expression, but in cDNA libraries constructed from morphologically identified single bipolar cells pre-labeled in fixed slices using immunocytochemical markers. We focused on two anatomically and physiologically disparate cell types: the rod bipolar cell and the diffuse bipolar cell DB3. In primate retina, the DB3 cell is known to transmit OFF signals from cones to multiple types of ganglion cell in the inner retina, including the small bistratified and the parasol ganglion cell [37,38]. By probing these libraries for specific genes, we demonstrate not only that the rod bipolar cell and DB3 have a unique neurochemical signature in terms of glutamate receptor and transporter expression, but also that TPEA is a useful, precise technique for comparing gene expression between individual neurons [39].

METHODS

Animals and tissue preparation: We obtained three adult (6-8 years) macaque retinas, one each from a male *Macaca fascicularis* used in our previous TPEA study [36], a female *Macaca nemestrina*, and male *Macaca nemestrina*. Each animal was procured from unrelated behavioral studies under a normal diurnal cycle, in adherence with federal guidelines and institutional policy. Prior to euthanasia in the light, eyes were removed from anesthetized monkeys and submerged in cold Ames media continuously bubbled to saturation with 95% O_2-5% CO_2 to minimize RNA degradation. Isolated eyecups were cleaned of vitreous and the retinas dissected free in the same media within 30 min of removal. Each retina was then immersion-fixed in 4% paraformaldehyde for 2-4 h at room temperature. The retinas were washed extensively in 0.01 M PBS, embedded in 5% agarose and cut into 60-80 μm sections on a vibratome. A number of sections were placed in 0.01 M PBS containing 0.01% sodium azide and stored at 4 °C for later experiments.

Morphological identification of cells: For immunocytochemical identification of rod bipolar cells, we used a rabbit polyclonal antibody against the PKCα subunit (1:500; Upstate Biotechnology, Billerica, MA; [27]). For identification of DB3 cells, we used a mouse monoclonal antibody against calbindin-28 (1:10,000; Sigma, St. Louis, MO) [40]. Sections were first placed in blocking solution consisting of 0.8% bovine serum albumin (BSA), 0.1% gelatin, and 5% normal goat serum (NGS) and 0.1% Triton-X at room temperature for 1-2 h, before being transferred into a primary antibody solution containing 0.8% BSA, 0.1% gelatin, 3% NGS, and 0.1% Triton-X for 24-72 h at 4 °C. To reduce background noise, we rinsed the sections thoroughly in 0.01 M PBS at room temperature for 2-3 h, before placing them in a secondary antibody solution containing Alexa-red 594 (Molecular Probes, Carlsbad, CA) at a dilution of 1:200 in 0.8% BSA, 0.1% gelatin, 1% NGS, and 0.1% Triton-X for 3-4 h at room temperature. Sections were washed with 0.01 M PBS repeatedly and kept at 4 °C in 0.01 M PBS.

In macaque retina, PKCα also faintly labels the DB4 ON bipolar cell [28]. This cell is discerniable from the rod bipolar cell through its shallower axonal termination near the ON/OFF border of the inner plexiform layer and by the morphology of its dendritic tree, which because of its contacts from cones, ramifies closer to the outer plexiform layer below the layer of rod terminals [28]. We restricted our harvesting of PKCα-labeled bipolar cells to rod bipolar cells therefore by targeting cells with (1) an axon terminating deep in the inner plexiform layer, just above the ganglion cell layer, and (2) a dendritic tree reaching above the shallower cone terminal layer to the higher placed rod terminals [27,28]. Using Nomarski/differential interference contrast (DIC) optics (next section), we were able to visualize both the ganglion cell layer and rod axon terminals in each slice for this confirmation. Similarly, for DB3 cells, since calbindin also labels horizontal cells in the inner nuclear layer [40], we restricted our harvesting to only those cells with a clearly labeled axon descending to the OFF sublamina of the inner plexiform layer.

Harvesting single cells for construction of cDNA libraries: Labeled sections were immersed in a shallow holding chamber containing a distilled water solution of proteinase K (20 μg/ml) to facilitate the removal of cells from the sections. The chamber was warmed to 37 °C with a temperature controller (TC-324B; Warner Instruments, Inc., Hamden, CT.) on a fixed stage coupled to an Olympus BX50 upright microscope equipped for fluorescent imaging with a 40x-water-immersion DIC objective (Olympus LUMPlanFI/IR) and a real-time digital camera (Q-Imaging Retiga 1300; Quantitative Imaging Corp., Burnaby, British Columbia, Canada). We pulled micropipettes from borosilicate glass capillaries (1.0/0.75 mm OD/ID with filament; World Precision Instruments) on a horizontal electrode puller (Model P-97, Sutter Instruments Co., Novato, CA) to a resistance following heat polishing of 5-8 MW. Under visual control, the tip of the glass pipette was lowered onto fluorescently labeled cells using a micromanipulator (PSC-5000; Burleigh Instruments, McHenry, IL.). Negative pressure was applied using a 5 cc syringe connected to a head stage (HS-2A; Axon Instruments, Inc., Sunnyvale, CA) with 1.5 mm O.D. polyethylene tubing (TC-324B; Warner Instruments, Inc.) to gently dislodge the targeted cell from the section. Each pipette tip was confirmed visually to contain a single cell, and the labeled cell was separated into a 0.5 ml microfuge tube and set aside at -20 °C for RNA extraction.

Reverse transcription and amplification of RNA from single cells: Once we had harvested a large number of both rod bipolar and DB3 cells, we placed them in storage, keeping them separate. The cells were then lysed in 100 μl of RNA extraction buffer containing 10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), 2% sodium dodecyl sulfate (SDS; pH 7.3), 5U of RNAseOUT (Invitrogen, Carlsbad, CA.), and 500 mg/ml Proteinase K (Ambion Inc, Austin, TX). We used a relatively high volume of extraction buffer to ensure that most (about 95%) of mRNA was recovered following the phenol chloroform extraction. This is common for single-cell studies. The solution was incubated at 60 °C for 16 h to allow dissolution. After Proteinase K treatment, the cellular components were digested with DNase I (Invitrogen, 10 mg/ μl) at 37 °C for 30 min to avoid DNA contamination. The RNA from each cell was purified by phenol chloroform extraction (equal volume) followed by precipitation with an equal volume of isopropanol in the presence of 0.1 volume of 3 M sodium acetate (pH 4.0) and 1 ml of 20 mg/ml glycogen, which acts as an RNA carrier, at -20 °C. The resulting RNA pellet was washed in 70% ethanol and centrifuged for 5 min at 4 °C. Following a 70% ethanol wash, the RNA was re-suspended in 10 ml of DEPC-treated water. The entire 10 ml was used for reverse transcription and cDNA amplification.

Reverse transcription and cDNA amplification from single cells was the same procedure as described for fixed tissue in our previous paper [36]. We again followed a modification of the 3'-end amplification technique of Dixon et al. [39]. In this method, the 3' region of extracted mRNA is amplified arbitrarily by PCR using a combination of primers, so the amplified cDNA represents as diverse and complete sample of gene sequences as possible. Specific sequences are then amplified with a second round of PCR using gene-specific primers. Here, we used only 50 U of reverse transcriptase during the reverse transcription reaction, as suggested when minimal amounts of RNA starting material are used. DNase was used following reverse transcription in order to remove genomic DNA. Approximately 30 μg of amplified cDNA in 200 μl total volume can be generated using this method from a single cell. To avoid DNA contamination of the extracted RNA, we harvested cells in a room that was separate from the laboratory in which PCR reactions and gel electrophoresis were performed. RNA extraction and cDNA generation were executed on separate bench tops from where PCR was completed, and the bench tops were cleaned with hydrogen peroxide.

Gene-specific polymerase chain reaction: We performed gene-specific PCR on 0.5 μg of the amplified cDNA samples generated from RNA extracted from single cells in 50 μl reactions containing: 1x PCR buffer, 0.5 μM $MgCl_2$, 0.2 mM dNTPs, 2.5 U *Taq* DNA polymerase (Gibco-BRL, Carlsbad, CA), and gene specific primers at 250 ng/reaction. Following a hot-start addition of Taq and an initial 2-min denaturing step (94 °C), each of 35 PCR cycles consisted of 45 s denaturing (94 °C), 90 s annealing (60 °C), and 60 s elongation (72 °C) steps. After the final cycle, elongation was allowed to occur at 72 °C for another 10 min. All PCR reactions were run for 35 cycles, which we determined to be optimal in our previous study [36]. Five ml of the PCR products were then separated on a 2% agarose gel (Invitrogen) stained with ethidium bromide (0.5 μg/ml). Gel images were captured using a Polaroid camera on Polaroid 667 film. Photographs were scanned (UMAX Astra 6450, UMAX Technologies, Inc, Dallas, TX) into a computer and adjusted uniformly with Adobe Photoshop (Version 7, Adobe Systems, Inc., San Jose, CA). Amplification during gene-specific PCR was performed at 35, a relatively low PCR cycle number, and 60 °C a moderately high annealing temperature, in an attempt to reduce false positives and nonspecific signals. For gene-specific PCR to detect glutamate receptors and non-vesicular transporters, we designed primers to target unique 180-220 base-pair regions of known human sequences accessed from GenBank using the sequence alignment feature of Vector NTI Suite 7.1 (InforMax, Inc., Carlsbad, CA; Table 1). These regions are highly homologous to the primate sequences we determined previously for these genes [36]. All PCR reactions were confirmed at least in duplicate. When possible, gene-specific primers were designed to span at least one exon/intron boundary, to avoid amplification of any residual genomic DNA.

Controls: In a recently published paper [36], we detected every gene of interest that we probe here. All PCR products for each cell were confirmed as the expected target genes via purification and product sequencing as described previously [36]. Briefly, we precipitated representative PCR products for each subunit and sequenced the DNA using the original amplification primers in conjunction with the BigDye terminator kit (Perkin Elmer, Inc., Boston, MA). Samples of the precipitated reaction were run on a 3100 Automated Sequencer (Applied Biosystems, Inc., Foster City, CA) and identity confirmed. No PCR product greater than the directed base-pair

region was detected in any reaction, nor did we detect more than a single specific product. To support positive identification of rod bipolar cells, we used primers designed against PKCα and $G\alpha_o$. Any cell lacking these genes (along with mGluR6) was excluded. For DB3 bipolar cells, we used primers against calbindin-28. To check against horizontal cell contamination for all bipolar cells, we used primers against the GABA precursors, the 65 and 67 kDa glutamate decarboxylase enzymes GAD-65 and GAD-67 [3,4]. To check against Müller glial cell contamination, we used primers against glutamine synthetase, a Müller-specific enzyme involved in glutamate clearance [3,4]. We also confirmed positive expression of the housekeeping marker β-actin, to ensure that absence of any gene-specific PCR product was not due to RNA degradation. These primers are also listed in Table 1.

RESULTS

Expression of glutamate receptor and transporter subunits in rod bipolar cells: In our previous work [36], we used the TPEA protocol, detailed in the previous section [39], to construct cDNA from a paraformaldehyde-fixed slice from the same *Macaca fascicularis* retina employed in this study. With this library, we demonstrated the expression of genes encoding all AMPA- (GluR1-4) and kainate-sensitive (GluR5-7, KA1-2) ionotropic glutamate receptors, all metabotropic glutamate receptors (mGluR1-8), and five non-vesicular glutamate transporters (EAAT1-5). These results served as a positive control for the single cell studies, to be described as follows, using the *fascicularis* retina, since they confirmed that each gene can be readily detected in cDNA generated from the limited amount of RNA extracted from an aldehyde-fixed retinal slice. In Figure 1, we present similar results using a single, fixed slice from each of two *Macaca nemestrina* retinas harvested for this study. The expression of each GluR, mGluR and transporter subunit was qualitatively similar to the published results for *fascicularis*, with demonstrated expression of each subunit in the slice (Figure 1A,B). The reaction products for the two *nemestrina* retinas are qualitatively similar. Thus, these reactions served as a positive control for the single cell studies to follow using fixed slices. In Figure 1C, we demonstrated the same PCR reactions from the *nemestrina* cDNA, but with reverse transcriptase omitted. The absence of any reaction produce served as an effective negative control. Similar results were obtained from the *fascicularis* retina [36] and for the second *nemestrina* retina (not shown).

Next we used our microelectrode to target the rod bipolar cell. These were identified by (1) positive label with PKCα, (2) the presence of a clear axon terminating at the border of the ganglion cell layer and inner plexiform layer, and (3) a clearly labeled dendritic tree reaching above the cone terminal layer to rod terminals (Figure 2A,B). These morphological features were confirmed using DIC optics in conjunction with fluorescence to identify the appropriate retinal layers. Once a single cell was targeted and secured, we removed it from the slice using the electrode (Figure 2C,D). From the RNA extracted from each harvested cell, we then created a cDNA library using the same protocol applied to RNA from the fixed slice. With this method, 35 individual cells were isolated and cDNAs generated.

We first tested each rod bipolar cDNA for positive expression of the housekeeping gene β-actin to ensure that absence of any subsequent PCR product was not due to degradation of RNA. The results for 11 representative cells are shown in Figure 3A. Levels of β-actin were qualitatively similar across all single cells tested. Next, to ensure that we did not harvest either neighboring horizontal cells or Müller glia, we tested for GAD-65/67 (Figure 3B,C) and glutamine synthetase (Figure 3D), respectively. No cell showed contamination from these two sources.

Table 1. Primers for gene-specific polymerase chain reaction in single bipolar neurons

Gene	Accession number	Location	Human sequence (5'-3')	Size (bps)
GluR1	NM_000827	2071F	TGGTGTCTCCCATTGAGAGTGC	188
		2258R	AATCATCCCCTCCTCTGTGGTC	
GluR2	NM_000826	1559F	TTTTTGCCTACATTGGGGTCAG	184
		1742R	TAGTCTCTGGTTTTCCTTGGGTGC	
GluR3	NM_000828	2072F	CAGACTGAAATTGCATATGGGACC	191
		2262R	TCCAGCAGGAAGGCAAACTTTC	
GluR4	NM_000829	2068F	TGAAAAGATGTGGACCTACATGCG	186
		2253R	GAATCCAGATTTCCTCCCACTTTC	
GluR5	NM_000830	2428F	TCTGGCTGCCTTCTTGACAGTAG	204
		2631R	TCACTGTTTCTTACCAGGGCGG	
GluR6	NM_021956	1977F	GACAGTGGAACGCATGGAATCC	206
		2182R	CTCGCTGGATTCCTTCTTCATTAC	
GluR7	NM_000831	2153F	CTGATGACCTGGCCAAGCAAAC	232
		2384R	TTCCTCTGCGTGACGTACTCGATG	
KA1	NM_014619	2218F	GGGAATCGCCAGGGTGTTGAATCC	216
		2433R	TTCAGGATCTCCAGGCGGTTGTTC	
KA2	NM_002088	2194F	CTCAACTGCAACCTCACCCAGATC	205
		2398R	CCAAACCTTTAGCTCGATGGTCC	
mGluR1	NM_000838	2900F	AATGGCAAGTCTGTGTCATGGTC	186
		3085R	GCTGGTATCTGAAAAGGTCAGGC	
mGluR2	NM_000839	2043F	CCATCTGCCTGGCACTTATCTC	199
		2241R	AAGGCATAAAGCGTGCAGAGC	
mGluR3	NM_000840	2047F	ATCAAGCACAACAACACACCCTTG	186
		2232R	CAGCAGGGCTGAGTAACAGATAGC	
mGluR4	NM_000841	2683F	ACATGCCCAAAGTCTACATCATCC	199
		2881R	TAAGTCTGTTTGGTGGCCAGCG	
mGluR5	NM_000842	2019F	TGGCATCTGCCTGGGCTACTTATG	205
		2223R	ACAGGCACTCATGCCTCTGGGC	
mGluR6	NM_000843	2302F	AGGTGGTGGGGATGATAGCATG	190
		2491R	TTGATGGCGTACACTGTGCACG	
mGluR7	NM_000844	2005F	CTGGGCTGTGATTCCTGTCTTC	225
		2229R	AAGCCCAAGAAAACTCGCCG	
mGluR8	NM_000845	2001F	CTCCTTCCGACGGGTCTTCCTA	181
		2181R	AAGGAGCTGGACGGAGATGA	
EAAT1 (GLAST)	NM_004172	819F	TAAACAATGTGTCTGAGGCCATGG	207
		1025R	CTACCAGTCTCATGATGGCTTCG	
EAAT2 (GLT-1)	NM_004171	1256F	TTCATTGCTTTTGGCATCGC	191
		1446R	TGCCTAGCAACCACTTCTAAGTCC	
EAAT3 (EAAC-1)	NM_004170	808F	ATTCAGATGGCATAAACGTCCTGG	188
		995R	CCGAGCAATCAGGAACAAAATACC	
EAAT4	NM_005071	706F	GAACCAGCTTCCTGGAAAATGTC	202
		907R	TTGAGGCTGTCGAAGAAGTCCCTG	
EAAT5	NM_006671	877F	TCAGCTTCTGCCAGTGCCTCAATG	208
		1084R	TAGAGCAGGGGCAGGATAAAGAGC	
PKCα	XM_034737	1045F	TCCTCATGGTGTTGGGAAAGG	212
		1256R	ATCCACTGTCTGGAAGCAGGAGTG	
Gao	NM_020988	522F	CATCCTCCGAACCAGGGTCAAAAC	192
		713R	TCTTCGTGGAGCACCTGGTCATAG	
Calbindin	NM_004929	667F	TGGAATTAACTGAGATGGCCAGG	175
		841R	TCGCACAGATCCTTCAGTAAAGC	
GAD-65	XM_038553	949F	TGCTCTTCCCAGGCTCATTG	194
		1142R	TCACGAGGAAAGGAACAAACCC	
GAD-67	NM_000817	1585F	TGGCACGACTGTTTATGGAGC	163
		1747R	GACTGAGTTGGCCCTTTCTATGC	
β-actin	NM_001101	411F	5'-AGGCCAACCGCGAGAAGATG	123
		533R	5'-CCATCACGATGCCAGTGGTA	
Glutamine Synth.	NM_002065	1239F	5'-GGGAACTGGAATGGTGCAGG	110
		1348R	5'-TGCCGCTTGCTTAGTTTCTC	

The primers were used to detect genes from mRNA extracted from single bipolar cells previously labeled using immunocytochemistry in fixed retinal slices. “Accession number” is the GenBank accession number for the corresponding human gene. “Location” is the position of initial nucleotide for forward and reverse primer pairs. “Human sequence” represents the sequence for design of forward and reverse primers. “Size” is the number of base pairs in expected PCR product.

To further support positive identification of rod bipolar cells, we probed for PKCα and for two particular genes coding proteins previously localized in mammalian rod bipolar cells, mGluR6 and $G\alpha_o$ [17-21,29,30]. Figure 4A,F show representative results for these genes from the cDNA of two bipolar cells. Of the 35 putative rod bipolar cells, 27 showed clear expression of mGluR6, PKCα and $G\alpha_o$ without GAD-65/67 or glutamine synthetase contamination. Only these cells were included in subsequent analysis. Relative amounts of these genes varied slightly from cell to cell, although we did not detect secondary or other non-specific bands with our set of primers. For negative control, gene-specific PCR omitting reverse transcriptase always resulted in the absence of a product, as shown in Figure 1B and Figure 3A.

Though mGluR6 is the predominant mediator of the light response of retinal ON bipolar cells, including the rod bipolar cell [20,29], recent evidence suggests these cells may also express one or more ionotropic glutamate receptors [33-35]. Gene-specific PCR from the 27 confirmed rod bipolar cDNA libraries demonstrated expression of multiple additional receptors. Figure 4B,G show that two exemplary rod bipolar cells expressed GluR4; the first cell also expressed GluR2 (Figure 4B). Interestingly, neither cell expressed any kainate-sensitive subunits (Figure 4C,H). Figure 4D,I demonstrate expression of mGluR3 in addition to mGluR6. Both cells also expressed the EAAT2 and EAAT4 glutamate transporters (Figure 4E,J).

The overall expression pattern for all 27 rod bipolar cells is illustrated in Figure 5. Beside mGluR6, which all 27 cells expressed, the most prevalent subunits were GluR4 and EAAT2 (85% each), EAAT4 (78%), mGluR3 (74%), and GluR2 (59%). Often the relative expression level of mGluR3 was greater than for mGluR6. Because this was somewhat surprising to us, we conducted mGluR3 gene-specific PCR in triplicate with comparable results throughout. Only four of the 27 rod bipolar cells expressed a kainate-sensitive subunit (GluR6). The most prevalent combination of subunits expressed was GluR4 with EAAT4 (78%), followed by GluR4 with EAAT2 (74%), GluR4 with EAAT2, and EAAT4 (67%), mGluR3 with EAAT2 (67%), GluR4 with mGluR3 (63%), GluR2 with EAAT2 (56%), and mGluR3 with EAAT4 (56%). In 26/27 (96%), either EAAT2 or EAAT4 was expressed in conjunction with at least one of the GluR2/GluR4 subunits.

Expression of glutamate receptor and transporter sub-

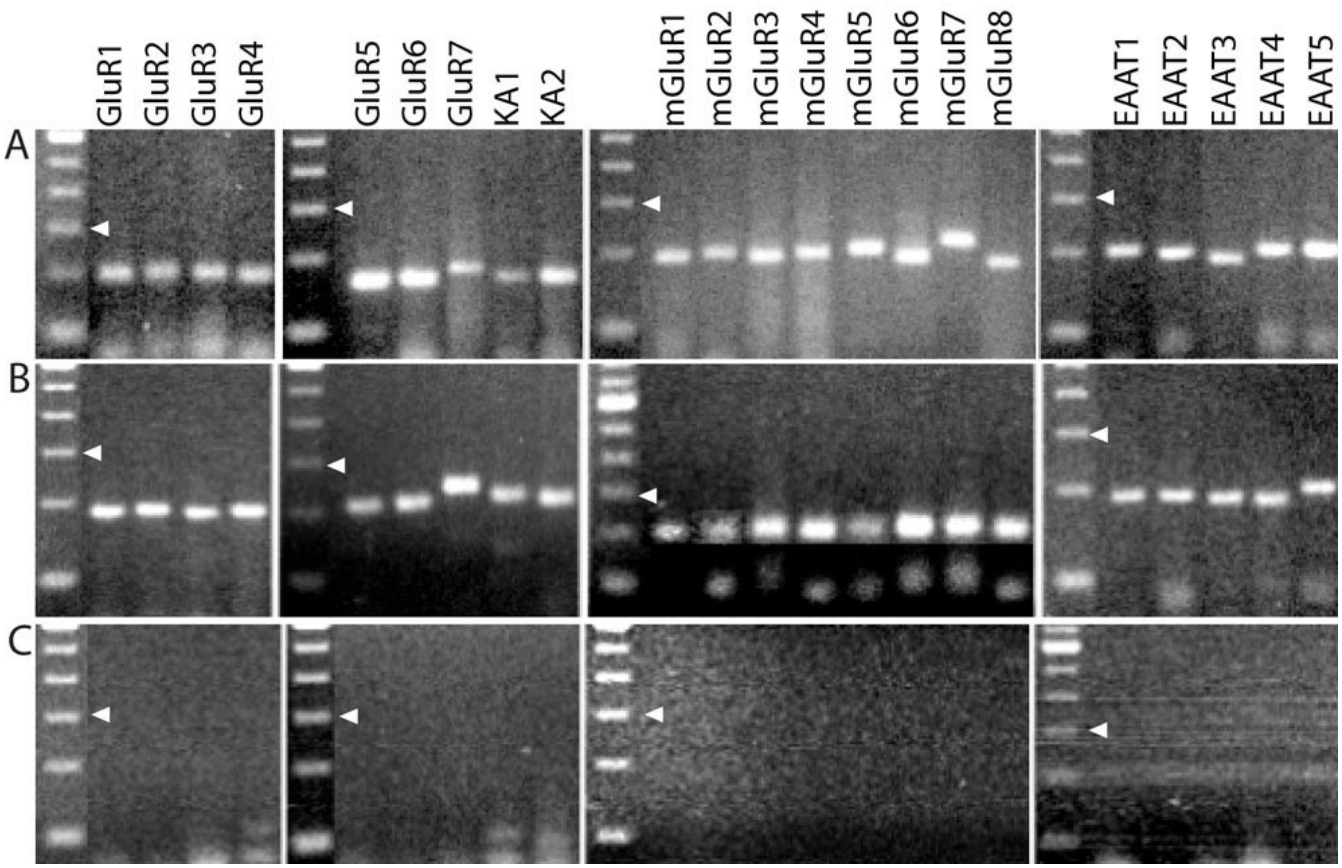

Figure 1. Positive and negative controls using cDNA from fixed retinal slices. **A**: Ethidium bromide-stained 2% agarose gel shows gene-specific PCR from a cDNA library created using 3'-end amplification (TPEA) RT-PCR for RNA extracted from a fixed slice from *Macaca nemestrina* retina. Results demonstrate positive expression of each GluR, mGluR, and transporter subunit tested using the primers listed in Table 1 **B**: The same PCR reactions are shown, but for a fixed slice from a second *Macaca nemestrina* retina. The results are similar to the first retina. **C**: The same reactions are shown, but with reverse transcriptase omitted as a negative control. A single PCR product was detected in each reaction, so gels are truncated. Arrowheads indicate 300 bp.

units in DB3 cells: Next we set out to investigate the genetic profiles of the OFF diffuse bipolar cell, DB3, known to provide synaptic input to the small bistratified and the parasol ganglion cell in the macaque retina [37,38,41]. In macaque retina, the DB3 cell is labeled selectively among bipolar cells by antibodies against calbindin [40], and we exploited this property to isolate single cells and measure their glutamate receptor and transporter gene expression profiles as we did for rod bipolar cells.

While DB3 cells are the only bipolar cell type to express calbindin, the H2 horizontal cell and cone photoreceptors in macaque retina also express calbindin [42,43]. Fortunately, anatomical and morphological differences between these three cell types enabled exclusive selection of DB3 cells (Figure 6A). Cones were relatively easy to avoid because of their location in the outer retina and unique morphology. Similarly, horizontal cell bodies lie just below the axon terminals of photoreceptors and lack a descending axon to the inner retina. On the other hand, the somas of DB3 cells are positioned deeper in the middle of the inner nuclear layer and give rise to a clear, descending axon (Figure 6A). Using these criteria, we were able to target and extract 34 calbindin-positive putative DB3 cells from our fixed retinal slices; two samples are demonstrated in Figure 6B,C. As before, we first tested the quality of the cDNAs created from harvested DB3 cells by confirming the expression of β-actin. The results for 11 representative DB3 cells are shown in Figure 6D. Like the rod bipolar cell, levels of β-actin were similar across all cells. Next, to ensure that we did not harvest neighboring Müller glia, we tested for expression of glutamine synthetase (Figure 6E).

Since only DB3 cells and H2 horizontal cells in the inner retina express calbindin, we used gene-specific PCR against GAD-65 and GAD-67 to exclude contamination from horizontal cells [3,4,44]. The cDNA created from the two extracted cells in Figure 6B,C demonstrated expression of calbindin, but not GAD-65 or GAD-67 (Figure 7A,F). Using this method, we were able to confirm positive identification of 26 of the 34 putative DB3 cells we had extracted. These 26 cells were used for subsequent gene-specific PCR.

Both DB3 cells in Figure 6B,C expressed genes for the

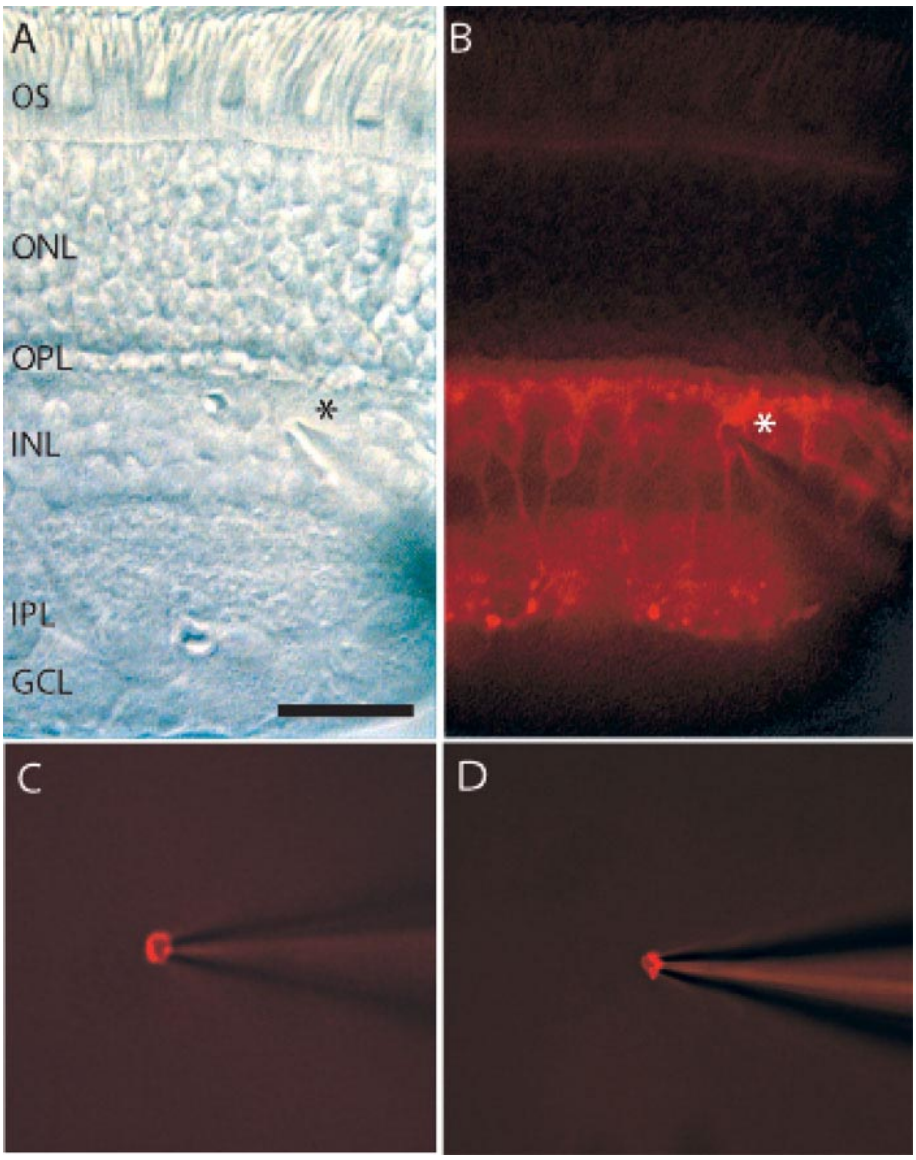

Figure 2. Labeling and extraction of rod bipolar cells. **A**: Nomarski-DIC image shows a 60-80 μm thick vertical section through macaque retina. A patch pipette electrode was positioned near the inner nuclear layer (INL), indicated by asterisk. **B**: The same retinal slice under fluorescence shows immuno-label of rod bipolar cells with antibodies against PKCα; their axons terminate just at the border of the inner plexiform layer (IPL) with the ganglion cell layer (GCL). **C**, **D**: Two representative single cells labeled for PKCα are shown following removal with patch pipette electrode. The retinal slice is no longer visible beyond the plane of focus. The following abbreviations are in effect: outer segments (OS), outer nuclear layer (ONL), outer plexiform layer (OPL). Scale bar equals 20 μm for both **A** and **B**.

AMPA subunits GluR2 and GluR4, while the second cell also expressed the GluR1 gene (Figure 7B,G). In terms of kainate subunits, both cells expressed the GluR6 gene, but no others (Figure 7C,H). Figure 7D,I demonstrate that neither cell expressed any mGluR gene, but both cells did express the EAAT2 transporter gene (Figure 7E,J). Gene-specific PCR omitting reverse transcriptase resulted in the absence of all PCR products, as shown in Figure 1B and Figure 6D (negative controls). Figure 8 demonstrates the general expression pattern for the 26 DB3 cells. Every cell expressed the GluR4 gene and all but one expressed the EAAT2 gene. The next most prevalent subunit genes were GluR2 (85%), GluR6 (69%), GluR1 (58%), and GluR3 (38%). No cell expressed any other receptor or transporter gene. The most prevalent gene combination with GluR4 was EAAT2 (96%), followed by GluR2 (81%), GluR2 with EAAT2 (80%), and GluR6 and EAAT2 (69%).

DISCUSSION

Single-cell polymerase chain reaction for retinal neurons: The retina is a highly heterogeneous tissue, containing a rich variety of both neuronal and glial cell types. Physiological and morphological investigation of these various cell types is greatly facilitated by anatomical separation by retinal layer [5]. Studies of retinal circuitry often exploit the unique neurochemical signature of individual cell types to map connectivity and spatial distribution [45]. Most studies of glutamate receptor and transporter expression or localization in the retina have therefore focused on correlating patterns with distinct retinal layers [7-12,46-49]. To link the physiological and morphological properties of retinal cells with gene expression, purification or isolation of individual cell types is essential. Due to the relatively small size and close proximity of individual cells in the retina, a comparison of expression profiles between populations of cells is problematic. Thus, many recent studies were performed using cells isolated from disassociated retina [50-52].

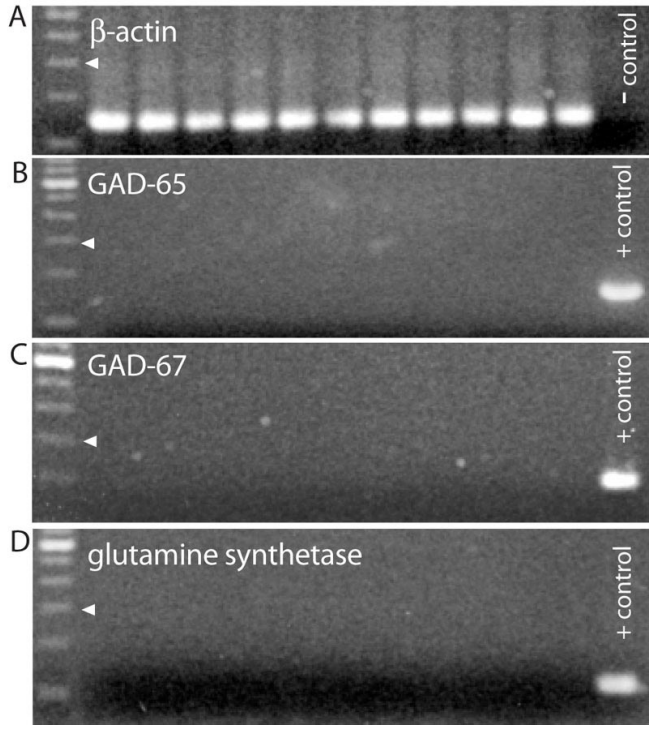

Figure 3. Positive and negative controls for rod bipolar cell cDNA. Gene-specific PCR from cDNA libraries was created using 3'-end amplification (TPEA) RT-PCR on RNA extracted from 11 representative rod bipolar cells, harvested as shown in Figure 2. **A**: Ethidium bromide-stained 2% agarose gel demonstrated expected PCR product for β-actin in each cell. Negative control run without reverse transcriptase from a bipolar cell was lacking product, as expected. **B**, **C**, **D**: Same rod bipolar cells tested negative for expression of genes expressed by horizontal cells (GAD-65 and GAD-67) and Müller glia (glutamine synthetase). Positive control was obtained using cDNA from fixed slice, as shown in Figure 1. Gels are truncated since only a single PCR product was detected for either β-actin (**A**) or the positive control (**B**, **C**, **D**). Arrowheads indicate 300 bp.

Physiological and morphological variability among cells maintained in vitro complicates genetic analysis, since expression in live tissue is susceptible to changes in real-time [53]. Variability between cells, can be limited by preserving tissue sections in fixative, creating a "snap-shot" of cell function at a particular point in its physiological history. In order to link morphology with gene expression while eliminating erroneous variability, we sought to collect a homogenous population of cells in a consistent physiological state by harvesting them from fixed retinal slices immuno-labeled with cell-type specific markers. Previous studies have demonstrated that nucleic acids may be extracted reliably from fixed material [54,55]. Nevertheless, the reliable analysis of gene expression in fixed tissue is subject to certain limitations. Fixation causes cross-linkage between nucleic acids and proteins and covalently modifies RNA by the addition of mono-methylol groups to the bases, making subsequent RNA extraction, reverse transcription, and amplification problematic [56].

While extensive degradation of RNA can occur before completion of the fixation process, we have shown that RNA of quality comparable to that isolated from fresh tissue can be obtained from fixed retina provided the time between sacrifice and fixation is short [36]. Our results demonstrate that the pattern of expression of glutamate receptors and transporters in cDNA created from RNA extracted from a fixed retinal slice (Figure 1) is comparable to that in cDNA created from fresh retinal RNA using TPEA [36]. Here we applied the same protocol to amplify and probe the small amount of RNA extracted from single retinal bipolar cells. We were able to reliably detect β-actin in both harvested rod bipolar and DB3 cells (Figure 3 and Figure 6), indicating that the absence of any subsequent gene-specific PCR products was not due to degradation of RNA during the fixation step. Conversely, that we did detect all genes of interest listed in Table 1 in the fixed slice (positive controls in Figure 1 and Figure 3) indicates that the absence of particular gene in a single cell was not due to poor choice of primers or other artifact.

Glutamate receptor expression in rod bipolar cells: We verified the identity of rod bipolar cells by detection of three genes coding proteins previously localized to these cells. The first and most obvious gene is PKCα, the protein marker most

closely correlated with rod bipolar cells in a variety of mammalian species [27,35]. However, in four harvested cells, the PKCα gene was not detected, and we excluded these cells from further analysis. Rod bipolar cells depolarize to light through coupling of mGluR6 to $G\alpha_o$, specifically the $G\alpha_o$ splice variant [18-21,29]. We used gene-specific primers against mGluR6 and primers that amplify both splice variants of $G\alpha_o$ also to confirm identity. While PKCα also faintly labels the DB4 ON cone bipolar cell [28], we were careful to harvest only those labeled cells whose descending axon terminated at the border of the ON sublamina with the ganglion cell layer and whose dendritic tree extended beyond the layer of cone axon terminals to rod spherules [27,28].

More than one-third (11/27) of our rod bipolar cell cDNA libraries showed GluR1 expression. Numerous studies in monkey, mouse, and rat demonstrated protein localization primarily in the ganglion cell and inner plexiform layers [57-59]. However, GluR1 localizes just below the rod spherule in mouse [60], and RT-PCR analysis of dissociated rod bipolar cells reveals strong GluR1 gene expression [35]. We found that the gene encoding GluR2 was detected in a majority of rod bipolar cells. In 1992, Hughes and colleagues first detected GluR2 in dissociated rod bipolar cells [61]. Genetic analysis in rat and cat support this pattern of GluR2 expression [34,48], as does protein localization in rat and monkey retina [6,34,62]. While GluR3 is detectable in fixed macaque retinal slice (see Figure 1 and [36]), we detected it in the cDNAs generated from only three rod bipolar cells (Figure 5). We detected the GluR4 gene in more cells than any other GluR (23/27). While RT-PCR analysis of dissociated rod bipolar cells does not show GluR4 expression, the use of multiplex PCR in that study could have caused amplification masking [35]. Furthermore, specific antibodies against GluR4 indicated localization to rod bipolar dendrites in rodent retina [63]. While we readily detected the mRNAs for kainate receptors in our fixed retinal slice [36], only 4/27 rod bipolar cells expressed any kainate subunit (GluR6). Even so, we performed gene-specific PCR

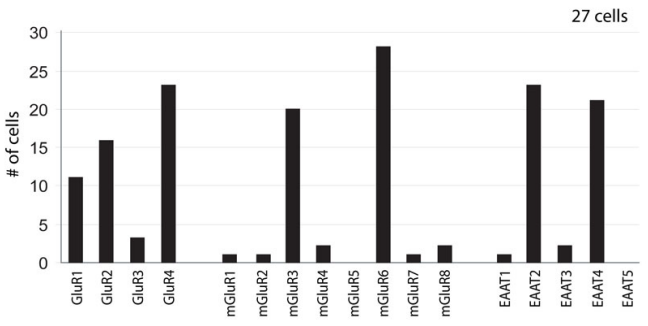

Figure 5. Summary of glutamate receptor and transporter gene expression in rod bipolar cells. For AMPA-sensitive subunits, nearly every cell expressed the GluR4 gene (23/27), followed by GluR2 (16/27), and GluR1 (11/27). In addition to mGluR6, 24 cells also expressed an additional mGluR gene, most often mGluR3 (20/27). Nearly every rod bipolar cell also expressed the gene for the EAAT2 (23/27) and EAAT4 (21/27) transporter. Expression of kainate subunit genes (GluR5-7, KA1-2) was limited to GluR6 in 4/27 cells, so these are not represented.

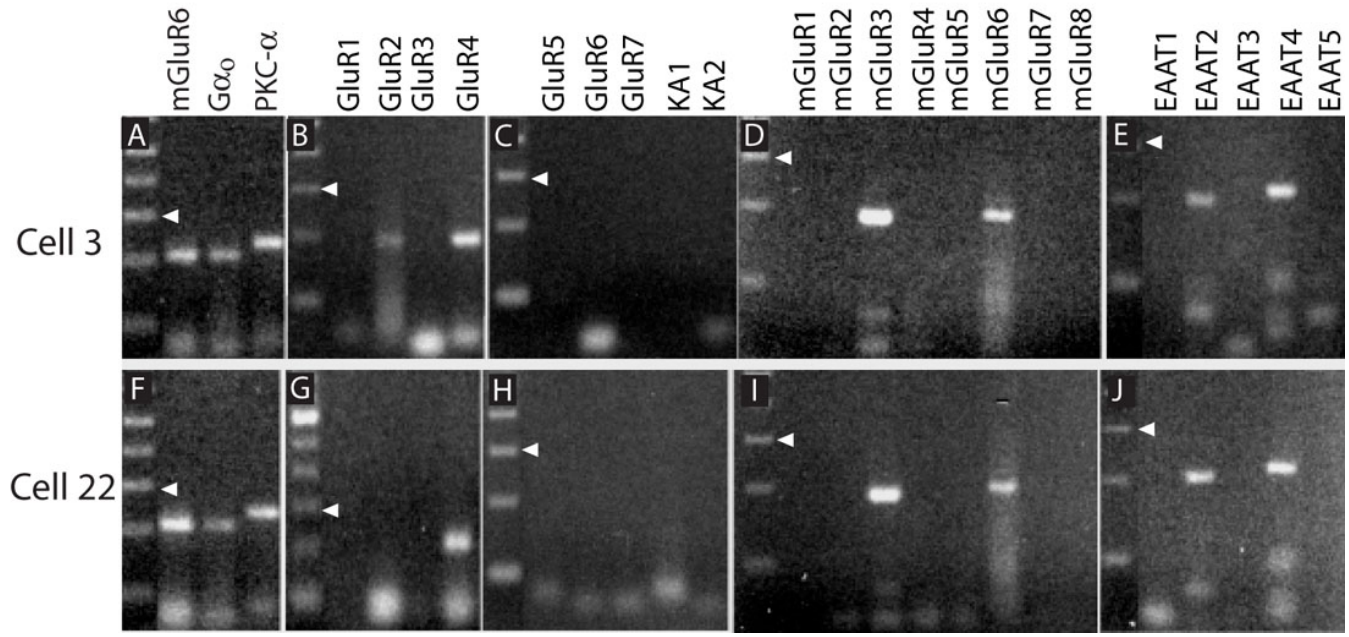

Figure 4. Gene expression in two rod bipolar cells. Gene-specific PCR from cDNA libraries were created using 3'-end amplification (TPEA) RT-PCR on RNA extracted from two representative rod bipolar cells. **A-F**: Shown are gels for two cells demonstrating expected PCR products for mGluR6, $G\alpha_o$ and PKCα. **G**: We confirmed a similar pattern of expression for 28 of 35 putative rod bipolar cells and used gene-specific PCR from the same cDNA libraries to test expression of AMPA (**B**, **G**), kainate (**C**, **H**), and mGluR subunits (**D**, **I**). We also tested the expression of the five non-vesicular glutamate transporter (EAAT) subunits (**E**, **J**). A single PCR product was detected in each reaction, so gels are truncated. Arrowheads indicate 300 bp.

reactions for kainate receptors in triplicate; each reaction confirmed the general pattern. This result supports the idea that any localization of kainate subunits beneath the rod spherule that is not in horizontal cells is likely within cytoplasmic "fingers" of the rod terminal itself [10].

We also investigated whether primate rod bipolar cells expressed other mGluR genes (besides that encoding mGluR6), as is found in other mammalian retina [64]. We found that 20/27 rod bipolar cDNA libraries contained the gene for mGluR3, a group II mGluR, and the genes for other mGluRs were expressed in 1-2 cells each (Figure 5). Although its localization on rod bipolar cells is unknown, mGluR3 may function as a pre-synaptic autoreceptor at the rod bipolar axon terminal, like other mGluR subunits. The group III mGluRs mGluR7 and mGluR8 have both been localized to pre-synaptic terminals in rat retina: mGluR7 to cone bipolar cells and mGluR8 to photoreceptors [65,66].

Glutamate receptor expression in DB3 cells: In macaque, calbindin labels the diffuse bipolar cell type DB3 [40]. While DB3 cells are the only bipolar cell in macaque monkey retina to express calbindin, two other cell types contain calbindin as well: HII horizontal cells and cones [42,43]. Fortunately, ana-

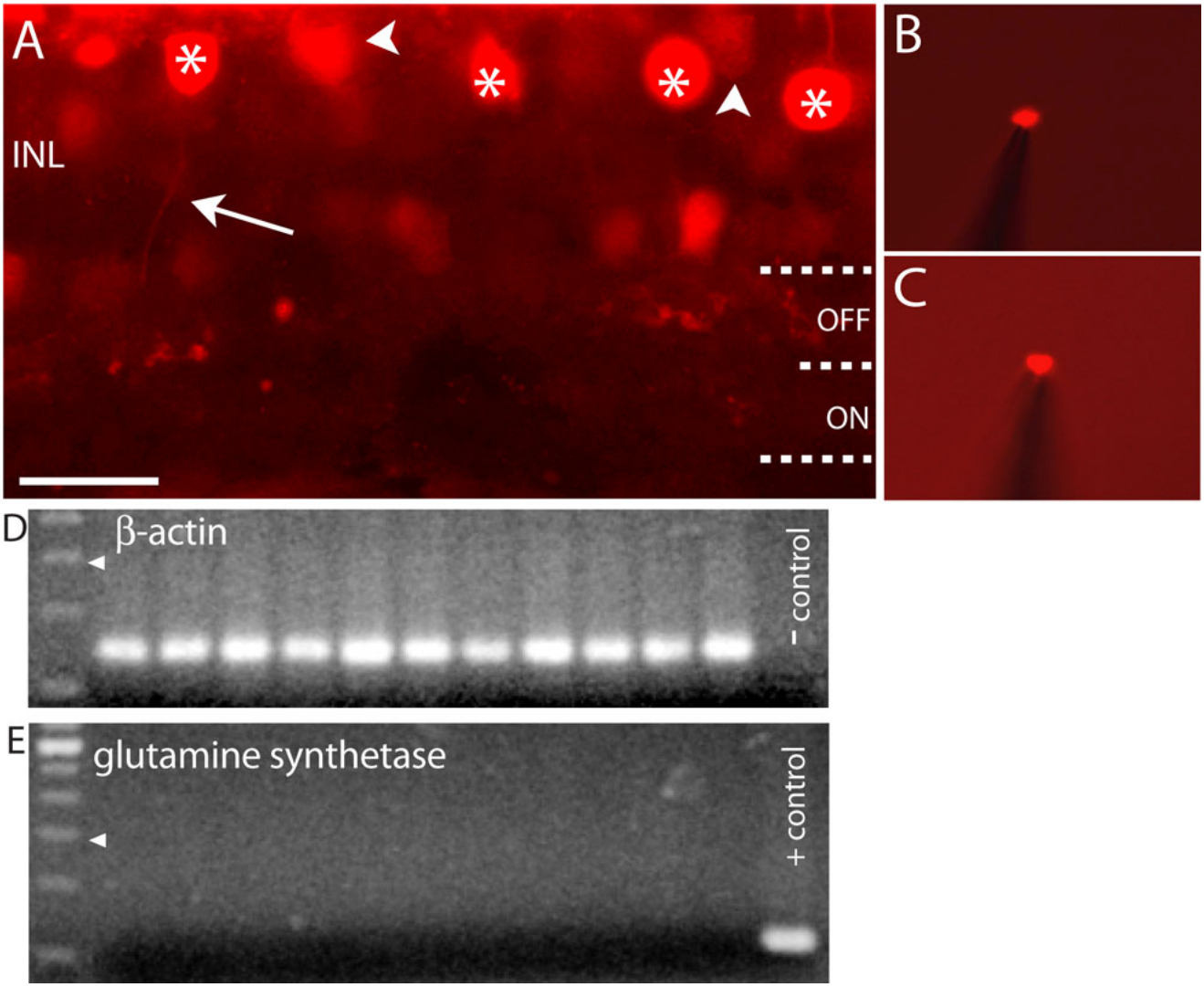

Figure 6. Labeling and extraction of DB3 cells and controls. **A**: High-magnification fluorescent image shows a vertical slice of macaque retina immuno-labeled with anti-calbindin-28 kDa. In this focal plane, labeled horizontal cells (arrowheads) appear in the background above the more prominent DB3 bipolar cells (asterisks). A DB3 axon (arrow) is shown descending from the inner nuclear layer (INL) deep into the OFF sublamina just above the border with the ON sublamina of the inner plexiform layer (IPL) [40]. The ganglion cell and photoreceptor layers are out of the image, while the shadow of the patch pipette electrode is apparent in the bottom middle of the image. Some amacrine cells deep in the INL may be weakly labeled. These we avoided by targeting only cells just below the horizontal cell layer. **B**, **C**: Two representative single cells labeled for calbindin were harvested from the slice with a patch electrode. Identification was based on the clear presence of a descending axon. The retinal slice is no longer visible as it lies out of the plane of focus. Scale bar represents 10 μm. **D**: Expected PCR product for β-actin in cDNA from each of 11 representative DB3 cells is shown negative control without reverse transcriptase for DB3 cell is lacking product, as expected. **E**: The same DB3 cells tested negative for expression of glutamine synthetase, which is expressed by Müller glia. Positive control was obtained using cDNA from fixed slice, as shown in Figure 1.

tomical segregation and morphological differences among these three cell types enabled our exclusive selection of DB3 cells. Since horizontal cells produce GABA as a primary neurotransmitter via the glutamate decarboxylase 65 and 67 kDa enzymes (GAD-65/67, [44]), we used primers against these to remove any errant horizontal cells from our sample. We cannot rule out the possibility that by removing the eight cells expressing either GAD gene from the sample, we also removed possible bipolar cells, some of which in macaque retina are labeled with GAD antibodies [44].

GluRs in OFF bipolar cells close in response to a light stimulus, thereby hyperpolarizing the cell. Our results indicate consistent expression in DB3 cells of transcripts for the AMPA subunits GluR1-4 and the low-affinity kainate subunit GluR6. Since DB3 cells form basal contacts at the cone axon terminal [37,38,67], our results are consistent with the localization of GluR2/3, GluR4, and GluR6/7 at basal dendrites in the primate retina [7,12,68]. The joint expression of AMPA receptors with GluR6 could aid in the recovery from glutamate excitation and desensitization, with rapid recovery mediated by AMPA subunits and a slower phase by the kainate subunit. By limiting the postsynaptic current, receptor desensitization and slow recovery would prevent saturation of the OFF voltage response and allow the synapse to operate over the cone's entire physiological voltage range.

GluR6 is one of three kainate receptors that can form functional homomeric ligand-gated channels when expressed in isolation [69]. In darkness, when glutamate release from cones is highest, both AMPA and kainate receptors may play a role in the depolarizing properties of DB3 cells. Both recombinant AMPA and kainate receptors are rapidly activated by high concentrations of glutamate with a high probability of opening [70]. While both AMPA and kainate receptors are largely permeable to cations and exclude anions from the pore, an exception is homomeric GluR6 receptors, where the positively charged arginine within the GluR6 pore causes a substantial increase in Cl^- permeability [71].

Expression of glutamate transporters in rod bipolar and DB3 cells: The concentration of glutamate encountered by postsynaptic receptors as it diffuses from the photoreceptor active zone must differ for distinct sets of postsynaptic processes. The different distances from release for dendrites of rod bipolar (invaginating; [10]) and DB3 cells (basal, [68]) yield a dark glutamate concentration that differs for the two sets of synapses. At the rod axon terminal, the concentration for the dendritic tips of the rod bipolar cell is approximately 100 μM, which decreases with diffusion to 1-2 μM for distances corresponding to the DB3 synapse at the cone terminal [72]. Though the true concentration is unknown, because of the inevitable differences in glutamate concentration at the two synapses, different mechanisms may be in place for glutamate removal and recycling. One potential method is the differential expression of non-vesicular glutamate transporters. Our work demonstrated expression of genes for all five non-vesicular glutamate transporters, EAAT1-5, in fixed slices of primate retina (see Figure 1 and [36]). While transporters localized to horizontal and Müller cells undoubtedly contribute to rapid glutamate turnover, our single-cell PCR results

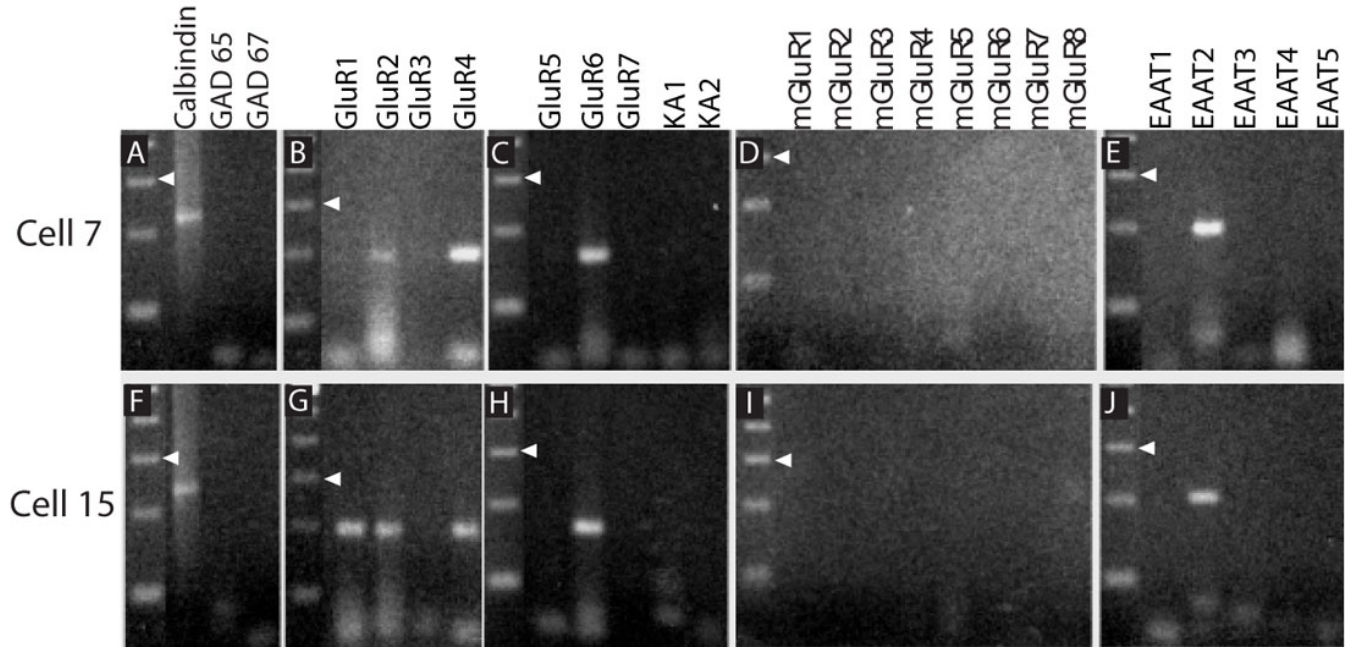

Figure 7. Gene expression in two DB3 cells. Gene-specific PCR was performed using cDNA libraries created using TPEA RT-PCR on RNA extracted from two representative cells, as shown in Figure 4. **A**, **F**: Ethidium bromide-stained 2% agarose gels for two cells harvested based on immuno-label for calbindin demonstrate expression of the calbindin gene, but not GAD-65 or GAD-67, which would be expressed in horizontal cells. We confirmed this pattern for 26 of 34 harvested DB3 cells and used gene-specific PCR from the same cDNA libraries to test expression of AMPA (**B**, **G**), kainate (**C**, **H**), and mGluR subunits (**D**, **I**). We also tested the expression of the five EAAT subunits (**E**, **J**). Arrowhead in each gel indicates 300 bp.

suggest the rod bipolar cell plays an active role as well. By expressing both EAAT2 and EAAT4, rod bipolar cells would be able to aid in the fast removal of glutamate from the extrasynaptic space at the rod terminal. This could help in the reduction of noise at the first synapse. We cannot rule out the possibility that these transporters also localize in the inner retina near the rod bipolar axon terminal, where they could play a similar role.

In contrast to the rod bipolar cell, we found that DB3 cells expressed only one transporter gene, EAAT2 (Figure 7 and Figure 8). Since DB3 cells should encounter a much lower level of glutamate at their dendrites, it is conceivable that these cells only need one transporter to aid in glutamate removal. However, further characterization of transporter localization is necessary to determine whether these transporters occur in the outer or inner retina. Additionally EAATs mediate two distinct processes: the stoichiometrically coupled transport of glutamate, Na+, K^+, and H^+, and a pore-mediated anion conductance. Whole-cell patch clamp recordings have demonstrated that EAAT4 but not EEAT2, channels display voltage-dependent gating of the anion conductance and that this gating can be modified by external glutamate [73]. These findings suggest EAAT4 may contribute to a different resting membrane potential in rod bipolar cells and that this difference may ultimately affect glutamate release from the axon terminal. No cell in either sample expressed EAAT5.

Intrinsic variability in expression of receptors and transporters: How reliable is our technique for separating out distinct cell types based on gene expression? Consider our sample of rod bipolar cells, harvested using their unique morphology and excluded if any lacked expression of the mGluR6, PKCα and $G\alpha_o$ genes. Each of the genes for GluR3, mGluR1-2, mGluR4, mGluR7-8, EAAT1, and EAAT3 were expressed by only 1-3 cells (Figure 5), and these were expressed almost entirely by different cells. Since we detected these genes readily using the same PCR protocol in retinal slices (Figure 1), their low abundance in rod bipolar cells cannot be attributed to poor primer design. Also, we confirmed the identity of each PCR product by sequencing (as in [36]), so our detection of these genes in these few cells is not due to spurious primer annealing. In contrast, the genes for GluR4 and EAAT2 were expressed in nearly every cell (23/27 each), with the incidence of each extremely close to that of cells expressing both (20/27; see Table 2). Similarly, if a rod bipolar expressed the EAAT4 gene, it also expressed the GluR4 gene (21/27). Importantly, these trends held across all three macaque retinas we used: two *nemestrina* and a single *fascicularis* (Table 2). That the fraction of rod bipolar cells expressing all three genes (67%) approached the fraction expressing either GluR4+EAAT2 (74%) or GluR4+EAAT4 (78%) indicates that these were generally the same population of cells (Table 2). This trend, too, held across all three macaque retina, with each of the top three gene combinations represented in each retina. Along these lines, we detected no clear pattern distinguishing the rod bipolar cells from any particular retina.

If the sample of rod bipolar cells actually contained additional cell types, the data would show distinct clusters of cells separated by their expression of different combinations of genes. By definition, the data would also show at least some degree of mutual exclusivity between genes. To test this possibility, we calculated the frequencies of other common patterns of co-expression. The next tier of frequently encountered genes included mGluR3 (20/27), GluR2 (16/27), and GluR1 (11/27). For the 18/27 cells demonstrating the GluR4+EAAT2+EAAT4 pattern, six of the possible eight permutations with these second tier genes were represented nearly equally (Table 2), with no two genes being mutually exclu-

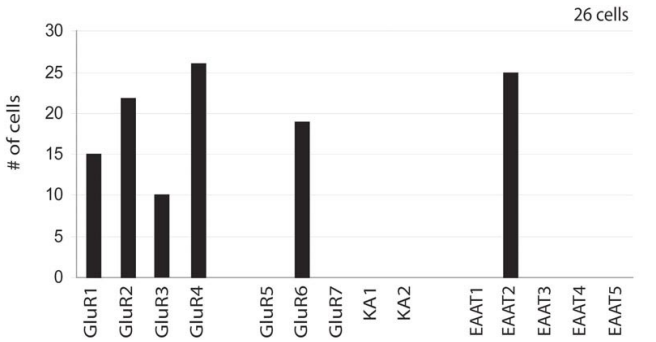

Figure 8. Summary of glutamate receptor and transporter gene expression in DB3 cells. For AMPA-sensitive subunits, of the 26 confirmed DB3 cells, each expressed the GluR4 gene, followed by GluR2 (22/26), and GluR1 (15/26). The only expressed kainate subunit gene was GluR6 (18/26). Nearly every DB3 cell also expressed the EAA2 transporter gene (25/26), but no others. Since no cell expressed an mGluR subunit, these are not represented in the graph.

Table 2. Gene expression in rod bipolar cells

Retina	Cell number	GluR4	EAAT2	EAAT4	mGluR3	GluR2	GluR1
M. nem. 2	9	+	+	+	+	+	+
M. nem. 2	10	+	+	+	+	+	+
M. nem. 2	14	+	+	+	+	+	+
M. nem. 2	17	+	+	+	+	+	+
M. nem. 2	18	+	+	+	+	+	+
M. nem. 2	21	+	+	+	+	+	+
M. nem. 1	4	+	+	+	+	+	-
M. nem. 2	25	+	+	+	+	+	-
M. nem. 1	5	+	+	+	-	+	+
M. nem. 2	8	+	+	+	+	-	-
M. nem. 2	22	+	+	+	+	-	-
M. fasc.	29	+	+	+	+	-	-
M. fasc.	32	+	+	+	+	-	-
M. fasc.	33	+	+	+	+	-	-
M. nem. 1	1	+	+	+	-	+	-
M. nem. 2	12	+	+	+	-	+	-
M. fasc.	34	+	+	+	-	-	-
M. fasc.	35	+	+	+	-	-	-
M. nem. 2	19	+	+	-	+	+	+
M. nem. 2	15	+	+	-	+	+	-
M. nem. 1	2	-	+	-	+	+	+
M. nem. 2	11	-	+	-	+	+	+
M. nem. 1	3	-	+	-	-	+	-
M. nem. 2	20	+	-	+	+	-	+
M. nem. 2	16	+	-	+	+	-	-
M. nem. 1	7	+	-	+	-	-	-
M. nem. 1	6	-	-	-	+	-	-

The table shows expression of the six most encountered genes in 27 rod bipolar cells identified morphologically in fixed slices from three retinas as described and with confirmed expression of the genes for mGluR6, $G\alpha_o$ and PKCα. Each of the three retinas used is indicated by name.

sive in expression (Table 3). Similarly, for the 9/27 cells *not* expressing GluR4+EAAT2+EAAT4, there were eight combinations of these three genes with mGluR3, GluR2, and GluR1 (Table 2), with no two genes being mutually exclusive across combinations (Table 3). Conversely, most of the cells expressing mGluR3, GluR2, and GluR1 also expressed the GluR4+EAAT2+EAAT4 combination: 13/20 for mGluR3, 11/16 for GluR2, and 7/11 for GluR1.

The sample of 26 DB3 cells also showed great homogeneity. For the kainate subunits, no cell expressed any gene but GluR6. Expression of mGluRs, EAAT1 and EAAT3-5 was also completely absent. In contrast, each cell expressed GluR4, while 25/26 cells expressed the EAAT2 gene and 22/26 cells expressed the gene for GluR2. The incidence of cells expressing both EAAT2 and GluR2 was 21/26 (Table 4). With regard to the top three genes, the DB3 cells therefore demonstrated even greater homogeneity than the rod bipolar cells. Concerning other possible distinguishing patterns, the next tier of frequently encountered genes included GluR6 (18/26), GluR1 (15/26), and GluR3 (10/26). Of the 21/26 cells expressing the GluR4+EAAT2+GluR2 pattern, six of the possible eight permutations with these second tier genes were represented about equally (Table 4), with no two genes being mutually exclusive (Table 5). For the 5/26 cells not expressing all three top genes, there were three combinations of these with GluR6, GluR1, and GluR3, again with no two genes mutually exclusive across combinations (Table 5). Thus, like the rod bipolar cell, there is great consistency in the primary pattern of expression for the DB3 cell with no clear division into sub-patterns defined by expression of genes of lesser incidence. As with the rod bipolar cells, no pattern emerged distinguishing the two *nemestrina* and a single *fascicularis* retina from one another (Table 4). Based on our analysis, the possibility that each of the various combinations we documented in Table 2 and Table 4 represents a distinct cell type seems untenable. It is possible that our sample of rod bipolar cells contained a small number of errant DB4 ON bipolar cells, therefore, we suggest that the variability for both the rod bipolar and DB3 cell most likely indicates subtle, stochastic differences in transcription.

Is there recent precedence for such variability in the retina? A new study of gene expression in cells dissociated from the mouse retina found a rather limited array of GluR expression in a small number of rod bipolar cells [74]. In that study, gene expression was examined using a two-stage PCR paradigm similar to ours. However, that study used a first-round amplification step incorporating sets of primers designed to recognize classes of receptor subunits (i.e., AMPA, kainate, NMDA) simultaneously, followed by nested PCR to distinguish specific subunits. In contrast, our first-round PCR reaction amplified all mRNAs universally (see [1]), followed by gene-specific PCR. The use of nested PCR reduced the cycles needed for amplification, but limited the number of genes probed. Those investigators found that 4/4 rod bipolar cells expressed the GluR1 subunit, and 3/4 expressed the KA2 subunit; no other AMPA or kainate subunit was expressed, and mGluRs and transporters were not tested [74]. This is in stark contrast

Table 3. Contingency table for gene expression in rod bipolar cells

	Gene B: Cells Co-expressing with Gene A				
Gene A (number cells)	GluR2	mGluR3	EAAT4	GluR4	EAAT2
GluR1 (11)	10/11	10/11	8/11	7/11	10/11
GluR2 (16)	-	12/16	11/16	13/16	16/16
mGluR3 (20)	-	-	15/20	17/20	17/20
EAAT4 (21)	-	-	-	21/21	18/21
GluR4 (23)	-	-	-	-	20/23

The left-most column lists in ascending order the five most frequently encountered genes ("Gene A") among our 27 rod bipolar cells and provides, in parentheses, the number of cells expressing each. The right columns represent the fraction of those cells co-expressing the next most frequently encountered gene ("Gene B").

Table 4. Gene expression in DB3 cells

Retina	Cell number	EAAT2	GluR2	GluR6	GluR1	GluR3
M. nem. 1	1	+	+	+	+	+
M. nem. 1	13	+	+	+	+	+
M. nem. 2	17	+	+	+	+	+
M. nem. 2	19	+	+	+	+	+
M. nem. 1	2	+	+	+	+	-
M. nem. 1	4	+	+	+	+	-
M. nem. 1	8	+	+	+	+	-
M. nem. 2	28	+	+	+	+	-
M. nem. 1	15	+	+	+	-	+
M. nem. 2	18	+	+	+	-	+
M. nem. 2	27	+	+	+	-	+
M. fasc.	31	+	+	+	-	+
M. nem. 1	10	+	+	+	-	-
M. nem. 2	24	+	+	+	-	-
M. nem. 2	30	+	+	+	-	-
M. nem. 1	9	+	+	-	+	+
M. nem. 1	11	+	+	-	+	-
M. nem. 2	21	+	+	-	+	-
M. nem. 2	23	+	+	-	+	-
M. nem. 2	25	+	+	-	+	-
M. nem. 2	26	+	+	-	+	-
M. nem. 1	6	+	-	+	-	-
M. nem. 1	12	+	-	+	-	-
M. nem. 2	22	+	-	+	-	-
M. fasc.	33	+	-	-	+	-
M. nem. 1	3	-	+	-	-	+

Expression of the five most often encountered genes in 26 individual DB3 cells, broken down by retina. GluR4 is excluded since every cell expressed this gene. Each of the three retinas used is indicated by name and shading. Blocks of different shading mark the separate patterns of expression of the six genes.

Table 5. Contingency table for gene expression in DB3 cells

	Gene B: Cells co-expressing with gene A			
Gene A (number cells)	GluR1	GluR6	GluR2	EAAT2
GluR3 (10)	5/10	8/10	10/10	9/10
GluR1 (15)	-	8/15	14/15	15/15
GluR6 (18)	-	-	15/18	18/18
GluR2 (22)	-	-	-	21/22

The left-most column lists in increasing frequency the four most frequently encountered genes ("Gene A") among our 26 DB3 cells and provides, in parentheses, the number of cells expressing each. The right columns represent the fraction of those cells co-expressing the next most frequently encountered gene ("Gene B").

to our results from macaque retina, in which GluR4 was expressed by nearly every rod bipolar cell and expression of kainate subunits was limited to GluR6 in only 4/27 cells (Figure 5). Among 13 rod and cone bipolar cells sampled in [74], only two cells expressed a kainate subunit other than KA2 (GluR5); no cell expressed GluR3 or GluR4. The sample size for any single bipolar cell type was limited. However, it is worth noting that within the set of four rod bipolar cells, there were three distinct expression patterns. Similarly, within a cluster of five ganglion cells of the same morphological type, no pattern of AMPA or kainate subunit expression was repeated; each cell had a unique signature (see Figure 8 of [74]). In a second cluster of four ganglion cells of a different type, each cell also demonstrated its own unique pattern of expression. So while species differences apparently may affect which subunits are expressed, variability within a cell type seems to be an unavoidable occurrence.

In this sense, retinal neurons are similar to other neurons in the brain, where there is documented precedence for intrinsic variability within a morphological type. In the hippocampus, single-cell profiling and comparison of CA1 and CA3 cells using microarray technology indicates substantial variability within a type, though far less than that separating out the different types [75,76]. In CA1 pyramidal cells, differences in cation channel expression between cells as assessed by single-cell RT-PCR translates to subtle physiological differences in their gating properties [77]. In dorsal root ganglion neurons, heterogeneous expression of early immediate genes determined by single-cell comparisons appears to provide a mechanism by which a variety of stimulus-response cascades can arise within a homogenous class of neurons [78]. It has been argued that stochastic fluctuations in gene expression contribute to intrinsic noise that is essential for normal, physiological tuning [79]. If so, our results suggest that fundamental physiological properties in bipolar cells arise from a primary expression pattern, but that a certain degree of stimulus-response modulation is possible by introducing subtle changes in the expression profile.

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REFERENCES

1. Thoreson WB, Witkovsky P. Glutamate receptors and circuits in the vertebrate retina. Prog Retin Eye Res 1999; 18:765-810.
2. Kalloniatis M, Tomisich G. Amino acid neurochemistry of the vertebrate retina. Prog Retin Eye Res 1999; 18:811-66.
3. Wassle H. Parallel processing in the mammalian retina. Nat Rev Neurosci 2004; 5:747-57.
4. Calkins DJ, Tsukamoto Y, Sterling P. Foveal cones form basal as well as invaginating junctions with diffuse ON bipolar cells. Vision Res 1996; 36:3373-81.
5. Haverkamp S, Grunert U, Wassle H. The synaptic architecture of AMPA receptors at the cone pedicle of the primate retina. J Neurosci 2001; 21:2488-500.
6. Morigiwa K, Vardi N. Differential expression of ionotropic glutamate receptor subunits in the outer retina. J Comp Neurol 1999; 405:173-84.
7. Qin P, Pourcho RG. Localization of AMPA-selective glutamate receptor subunits in the cat retina: a light- and electron-microscopic study. Vis Neurosci 1999 Jan-Feb; 16:169-77.
8. Qin P, Pourcho RG. Immunocytochemical localization of kainate-selective glutamate receptor subunits GluR5, GluR6, and GluR7 in the cat retina. Brain Res 2001; 890:211-21.
9. Haverkamp S, Grunert U, Wassle H. Localization of kainate receptors at the cone pedicles of the primate retina. J Comp Neurol 2001; 436:471-86.
10. Harvey DM, Calkins DJ. Localization of kainate receptors to the presynaptic active zone of the rod photoreceptor in primate retina. Vis Neurosci 2002 Sep-Oct; 19:681-92.
11. DeVries SH. Bipolar cells use kainate and AMPA receptors to filter visual information into separate channels. Neuron 2000; 28:847-56.
12. Euler T, Masland RH. Light-evoked responses of bipolar cells in a mammalian retina. J Neurophysiol 2000; 83:1817-29.
13. Euler T, Schneider H, Wassle H. Glutamate responses of bipolar cells in a slice preparation of the rat retina. J Neurosci 1996; 16:2934-44.
14. Hartveit E. Functional organization of cone bipolar cells in the rat retina. J Neurophysiol 1997; 77:1716-30.
15. Vardi N, Morigiwa K. ON cone bipolar cells in rat express the metabotropic receptor mGluR6. Vis Neurosci 1997 Jul-Aug; 14:789-94.
16. Vardi N, Duvoisin R, Wu G, Sterling P. Localization of mGluR6 to dendrites of ON bipolar cells in primate retina. J Comp Neurol 2000; 423:402-12.
17. Nawy S. Regulation of the on bipolar cell mGluR6 pathway by Ca2+. J Neurosci 2000; 20:4471-9.
18. Dhingra A, Jiang M, Wang TL, Lyubarsky A, Savchenko A, Bar-Yehuda T, Sterling P, Birnbaumer L, Vardi N. Light response of retinal ON bipolar cells requires a specific splice variant of $G\alpha_o$. J Neurosci 2002; 22:4878-84.
19. Dhingra A, Faurobert E, Dascal N, Sterling P, Vardi N. A retinal-specific regulator of G-protein signaling interacts with $G\alpha_o$ and accelerates an expressed metabotropic glutamate receptor 6 cascade. J Neurosci 2004; 24:5684-93.
20. Schneeweis DM, Schnapf JL. The photovoltage of macaque cone photoreceptors: adaptation, noise, and kinetics. J Neurosci 1999; 19:1203-16.
21. Schneeweis DM, Schnapf JL. Noise and light adaptation in rods of the macaque monkey. Vis Neurosci 2000 Sep-Oct; 17:659-66.
22. Rao-Mirotznik R, Harkins AB, Buchsbaum G, Sterling P. Mammalian rod terminal: architecture of a binary synapse. Neuron 1995; 14:561-9.
23. Rao-Mirotznik R, Buchsbaum G, Sterling P. Transmitter concentration at a three-dimensional synapse. J Neurophysiol 1998; 80:3163-72.
24. Grunert U, Martin PR. Rod bipolar cells in the macaque monkey retina: immunoreactivity and connectivity. J Neurosci 1991; 11:2742-58.
25. Mills SL, Massey SC. AII amacrine cells limit scotopic acuity in central macaque retina: A confocal analysis of calretinin labeling. J Comp Neurol 1999; 411:19-34.
26. Dhingra A, Lyubarsky A, Jiang M, Pugh EN Jr, Birnbaumer L, Sterling P, Vardi N. The light response of ON bipolar neurons requires $G\alpha_o$. J Neurosci 2000; 20:9053-8.
27. Vardi N. Alpha subunit of Go localizes in the dendritic tips of ON

bipolar cells. J Comp Neurol 1998; 395:43-52.
28. de la Villa P, Kurahashi T, Kaneko A. L-glutamate-induced responses and cGMP-activated channels in three subtypes of retinal bipolar cells dissociated from the cat. J Neurosci 1995; 15:3571-82.
29. Baylor DA, Nunn BJ, Schnapf JL. The photocurrent, noise and spectral sensitivity of rods of the monkey Macaca fascicularis. J Physiol 1984; 357:575-607.
30. Tian L, Kammermeier PJ. G protein coupling profile of mGluR6 and expression of G alpha proteins in retinal ON bipolar cells. Vis Neurosci 2006 Nov-Dec; 23:909-16.
31. Hughes TE. Are there ionotropic glutamate receptors on the rod bipolar cell of the mouse retina? Vis Neurosci 1997 Jan-Feb; 14:103-9.
32. Kamphuis W, Klooster J, Dijk F. Expression of AMPA-type glutamate receptor subunit (GluR2) in ON-bipolar neurons in the rat retina. J Comp Neurol 2003; 455:172-86.
33. Kamphuis W, Dijk F, O'Brien BJ. Gene expression of AMPA-type glutamate receptor subunits in rod-type ON bipolar cells of rat retina. Eur J Neurosci 2003; 18:1085-92.
34. Huang H, Luo DG, Shen Y, Zhang AJ, Yang R, Yang XL. AMPA receptor is involved in transmission of cone signal to ON bipolar cells in carp retina. Brain Res 2004; 1002:86-93.
35. Brandstatter JH. Glutamate receptors in the retina: the molecular substrate for visual signal processing. Curr Eye Res 2002; 25:327-31.
36. Hanna MC, Calkins DJ. Expression and sequences of genes encoding glutamate receptors and transporters in primate retina determined using 3'-end amplification polymerase chain reaction. Mol Vis 2006; 12:961-76.
37. Bordt AS, Hoshi H, Yamada ES, Perryman-Stout WC, Marshak DW. Synaptic input to OFF parasol ganglion cells in macaque retina. J Comp Neurol 2006; 498:46-57.
38. Calkins DJ, Sterling P. Microcircuitry for two types of achromatic ganglion cell in primate fovea. J Neurosci 2007; 27:2646-53.
39. Dixon AK, Richardson PJ, Lee K, Carter NP, Freeman TC. Expression profiling of single cells using 3 prime end amplification (TPEA) PCR. Nucleic Acids Res 1998; 26:4426-31.
40. Grunert U, Martin PR, Wassle H. Immunocytochemical analysis of bipolar cells in the macaque monkey retina. J Comp Neurol 1994; 348:607-27.
41. Calkins DJ, Tsukamoto Y, Sterling P. Microcircuitry and mosaic of a blue-yellow ganglion cell in the primate retina. J Neurosci 1998; 18:3373-85.
42. Wassle H, Dacey DM, Haun T, Haverkamp S, Grunert U, Boycott BB. The mosaic of horizontal cells in the macaque monkey retina: with a comment on biplexiform ganglion cells. Vis Neurosci 2000 Jul-Aug; 17:591-608.
43. Chiquet C, Dkhissi-Benyahya O, Chounlamountri N, Szel A, Degrip WJ, Cooper HM. Characterization of calbindin-positive cones in primates. Neuroscience 2002; 115:1323-33.
44. Vardi N, Kaufman DL, Sterling P. Horizontal cells in cat and monkey retina express different isoforms of glutamic acid decarboxylase. Vis Neurosci 1994 Jan-Feb; 11:135-42.
45. Hendry SH, Calkins DJ. Neuronal chemistry and functional organization in the primate visual system. Trends Neurosci 1998; 21:344-9.
46. Peng YW, Blackstone CD, Huganir RL, Yau KW. Distribution of glutamate receptor subtypes in the vertebrate retina. Neuroscience 1995; 66:483-97.
47. Eliasof S, Arriza JL, Leighton BH, Amara SG, Kavanaugh MP. Localization and function of five glutamate transporters cloned from the salamander retina. Vision Res 1998; 38:1443-54.
48. Hamassaki-Britto DE, Hermans-Borgmeyer I, Heinemann S, Hughes TE. Expression of glutamate receptor genes in the mammalian retina: the localization of GluR1 through GluR7 mRNAs. J Neurosci 1993; 13:1888-98.
49. Hartveit E, Brandstatter JH, Enz R, Wassle H. Expression of the mRNA of seven metabotropic glutamate receptors (mGluR1 to 7) in the rat retina. An in situ hybridization study on tissue sections and isolated cells. Eur J Neurosci 1995; 7:1472-83.
50. Logiudice L, Henry D, Matthews G. Identification of calcium channel alpha1 subunit mRNA expressed in retinal bipolar neurons. Mol Vis 2006; 12:184-9.
51. Ruan GX, Zhang DQ, Zhou T, Yamazaki S, McMahon DG. Circadian organization of the mammalian retina. Proc Natl Acad Sci U S A 2006; 103:9703-8.
52. Fries JE, Goczalik IM, Wheeler-Schilling TH, Kohler K, Guenther E, Wolf S, Wiedemann P, Bringmann A, Reichenbach A, Francke M, Pannicke T. Identification of P2Y receptor subtypes in human muller glial cells by physiology, single cell RT-PCR, and immunohistochemistry. Invest Ophthalmol Vis Sci 2005; 46:3000-7.
53. Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 2000; 46:69-81.
54. Rupp GM, Locker J. Purification and analysis of RNA from paraffin-embedded tissues. Biotechniques 1988; 6:56-60.
55. Shibata DK, Arnheim N, Martin WJ. Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. J Exp Med 1988; 167:225-30.
56. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 1999; 27:4436-43.
57. Lin B, Martin PR, Grunert U. Expression and distribution of ionotropic glutamate receptor subunits on parasol ganglion cells in the primate retina. Vis Neurosci 2002 Jul-Aug; 19:453-65.
58. Grunert U, Haverkamp S, Fletcher EL, Wassle H. Synaptic distribution of ionotropic glutamate receptors in the inner plexiform layer of the primate retina. J Comp Neurol 2002; 447:138-51.
59. Hack I, Peichl L, Brandstatter JH. An alternative pathway for rod signals in the rodent retina: rod photoreceptors, cone bipolar cells, and the localization of glutamate receptors. Proc Natl Acad Sci U S A 1999; 96:14130-5.
60. Haverkamp S, Wassle H. Immunocytochemical analysis of the mouse retina. J Comp Neurol 2000; 424:1-23.
61. Hughes TE, Hermans-Borgmeyer I, Heinemann S. Differential expression of glutamate receptor genes (GluR1-5) in the rat retina. Vis Neurosci 1992; 8:49-55.
62. Brandstatter JH, Hack I. Localization of glutamate receptors at a complex synapse. The mammalian photoreceptor synapse. Cell Tissue Res 2001; 303:1-14.
63. Hack I, Frech M, Dick O, Peichl L, Brandstatter JH. Heterogeneous distribution of AMPA glutamate receptor subunits at the photoreceptor synapses of rodent retina. Eur J Neurosci 2001; 13:15-24.
64. Euler T, Wassle H. Different contributions of GABAA and GABAC receptors to rod and cone bipolar cells in a rat retinal slice preparation. J Neurophysiol 1998; 79:1384-95.
65. Brandstatter JH, Koulen P, Kuhn R, van der Putten H, Wassle H. Compartmental localization of a metabotropic glutamate receptor (mGluR7): two different active sites at a retinal synapse. J Neurosci 1996; 16:4749-56.

66. Koulen P, Brandstatter JH. Pre- and Postsynaptic Sites of Action of mGluR8a in the mammalian retina. Invest Ophthalmol Vis Sci 2002; 43:1933-40.
67. Hopkins JM, Boycott BB. The cone synapses of cone bipolar cells of primate retina. J Neurocytol 1997; 26:313-25.
68. Calkins DJ. Localization of ionotropic glutamate receptors to invaginating dendrites at the cone synapse in primate retina. Vis Neurosci 2005 Jul-Aug; 22:469-77.
69. Swanson GT, Green T, Sakai R, Contractor A, Che W, Kamiya H, Heinemann SF. Differential activation of individual subunits in heteromeric kainate receptors. Neuron 2002; 34:589-98.
70. Heckmann M, Bufler J, Franke C, Dudel J. Kinetics of homomeric GluR6 glutamate receptor channels. Biophys J 1996; 71:1743-50.
71. Burnashev N, Zhou Z, Neher E, Sakmann B. Fractional calcium currents through recombinant GluR channels of the NMDA, AMPA and kainate receptor subtypes. J Physiol 1995; 485 (Pt 2):403-18.
72. Calkins DJ. Representation of cone signals in the primate retina. J Opt Soc Am A Opt Image Sci Vis 2000; 17:597-606.
73. Melzer N, Biela A, Fahlke C. Glutamate modifies ion conduction and voltage-dependent gating of excitatory amino acid transporter-associated anion channels. J Biol Chem 2003; 278:50112-9.
74. Jakobs TC, Ben Y, Masland RH. Expression of mRNA for glutamate receptor subunits distinguishes the major classes of retinal neurons, but is less specific for individual cell types. Mol Vis 2007; 13:933-48.
75. Kamme F, Salunga R, Yu J, Tran DT, Zhu J, Luo L, Bittner A, Guo HQ, Miller N, Wan J, Erlander M. Single-cell microarray analysis in hippocampus CA1: demonstration and validation of cellular heterogeneity. J Neurosci 2003; 23:3607-15.
76. Ginsberg SD, Che S. Expression profile analysis within the human hippocampus: comparison of CA1 and CA3 pyramidal neurons. J Comp Neurol 2005; 487:107-18.
77. Zawar C, Plant TD, Schirra C, Konnerth A, Neumcke B. Cell-type specific expression of ATP-sensitive potassium channels in the rat hippocampus. J Physiol 1999; 514 (Pt 2):327-41.
78. Sheng HZ, Lin PX, Nelson PG. Combinatorial expression of immediate early genes in single neurons. Brain Res Mol Brain Res 1995; 30:196-202.
79. Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. Science 2002; 297:1183-6.

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