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Differential Expression of Ionotropic Glutamate Receptor Subunits in the Outer Retina

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ABSTRACT

Ionotropic glutamate receptors (iGluRs) are extremely diverse in their subunit compositions. To understand the functional consequences of this diversity, it is necessary to know the subunits that are expressed by known cell types. By using immunocytochemistry with light and electron microscopy, we localized several subunits (GluR2/3, GluR4, and GluR6/7) in cat retinal neurons, postsynaptic to photoreceptors. Type A horizontal cells express all three subunits strongly, whereas type B horizontal cells express GluR2/3 strongly, GluR6/7 weakly, and do not express GluR4. When they are present, the subunits are expressed strongly throughout the cytoplasm of the somata and primary dendrites; however, in the terminals, they are concentrated at the postsynaptic region, just opposite the presumed site of photoreceptor glutamate release. Surprisingly, all bipolar cell classes (OFF cone bipolar cells, ON cone bipolar cells, and rod bipolar cells) express at least one iGluR subunit at their dendritic tips. Cone bipolar cells forming basal contacts with the cones (presumably OFF cells) express all three subunits in association with the electron-dense postsynaptic membrane. Invaginating dendrites of cone bipolar cells (presumably ON cells) express GluR2/3 and GluR4. Rod bipolar cells (ON cells) express GluR2/3 in their invaginating dendrites. The function of iGluRs in horizontal cells and OFF bipolar cells clearly is to mediate their light responses. GluR6/7 subunit in the receptor of these cells may be responsible for the dopamine-mediated enhancement of glutamate responses that have been observed previously in these cells. The function of iGluRs in ON bipolar cells remains an enigma. J. Comp. Neurol. 405:173–184, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: glutamate receptors 2/3, 4, and 6/7; bipolar cell; horizontal cell; rod bipolar cell

Photoreceptors in mammalian retina communicate with their postsynaptic neurons by continuously releasing glutamate in the dark and reducing this release with light increments (Massey and Maguire, 1995). There are about 12 types of postsynaptic neurons that are grouped into three functional classes: ON bipolar cells, OFF bipolar cells, and horizontal cells. ON and OFF bipolar cells carry parallel information regarding light increment and decrement and form the receptive field centers of ON and OFF ganglion cells. The horizontal cells, which, in most mammals, consist of two morphologically distinct types (Fisher and Boycott, 1974; Boycott et al., 1978; Gallego, 1986), provide inhibition to photoreceptors and bipolar cells, thereby contributing to the receptive field surround of ganglion cells (for review, see Sterling et al., 1995).

The segregation into parallel ON and OFF pathways is quite remarkable, in that the same glutamate molecules hyperpolarize ON bipolar cells (Nawy and Jahr, 1990, 1991; Shiells and Falk, 1990; Yamashita and Wässle, 1991; de la Villa et al., 1995) but depolarize OFF bipolar cells and horizontal cells (Attwell et al., 1987; Hensley et al., 1993; Euler et al., 1996; Sasaki and Kaneko,1996). The receptor type that hyperpolarizes ON cells is known to be a retina-specific metabotropic receptor, metabotropic glutamate receptor 6 (mGluR6; Nomura et al., 1994; Ueda et al., 1997; Vardi and Morigiwa, 1997). The receptor types that depolarize OFF cone bipolar cells and horizontal cells clearly belong to a family of ionotropic glutamate receptors (iGluRs) of the ionotropic α-amino-3-hydroxy-5-methyl-4-

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isoxazole propionic acid (AMPA) and/or kainate types (O'Dell and Christensen, 1989; Kim and miller, 1991; Zhou et al., 1993; Krizaj et al., 1994; Sasaki and Kaneko, 1996). However, there are four isoforms of AMPA subunits (GluR1–GluR4) and five isoforms of kainate subunits (GluR5–GluR7 and KA1 and KA2), and each receptor is an oligomer composed of four of these subunits (for reviews, see Hollman and Heinemann, 1994; Hollman, 1997; Rosenmund et al., 1998). The subunit composition in each bipolar and horizontal cell type is largely unknown. Because pharmacological tools to identify the subunit composition are unavailable, identification and localization of the iGluR subunits in these cells depend mainly on immunocytochemical methods.

Previous studies found that the transcripts of GluR1–GluR7 are present in the inner nuclear layer and the ganglion cell layer in rat and cat retina (Müller et al., 1992; Hamassaki-Britto et al., 1993). However, to understand retinal circuitry, it is important to localize the proteins themselves not only to cell types but also to specific regions of interest within the cell. In the outer retina, several AMPA subunits were localized to bipolar cells by using light microscopy (Peng et al., 1995; Qin and Pourcho, 1996), but only the kainate subunit KA2 has been localized specifically to OFF bipolar cells by using electron microscopy (Brandstätter et al., 1997). Also, one study has examined the expression of AMPA subunits in cat horizontal cells (Qin and Pourcho, 1996), but the horizontal cell types (type A or type B) were not identified. In the present study, by using immunocytochemistry, we examined the ultrastructural localization of the AMPA subunits GluR2/3 and GluR4 and the kainate subunits GluR6/7 in cat horizontal and bipolar cells. We show that the two types of horizontal cells express a different set of iGluRs. OFF bipolar cells, as expected, expressed iGluRs, but, surprisingly, both rod and ON cone bipolar cells also expressed some iGluR subunits. Some of these data have been summarized briefly as part of an overview (Vardi et al., 1998).

MATERIALS AND METHODS

Antibodies

Polyclonal antibodies against GluR2/3, GluR4 (Chemicon International Inc., Temecula, CA), GluR6/7 (gift of Dr. R.L. Huganir, Howard Hughes Medical Institute, John Hopkins School of Medicine, Baltimore, MD), and GluR6/7 (gift of Dr. R.J. Wenthold, Laboratory of Neurochemistry, NIDCD/NIH, Bethesda, MD) were raised against synthetic peptides corresponding to the C-termini sequences in the intracellular domain of the rat GluR clones and were affinity purified by using the corresponding peptides (Wenthold et al., 1992; Raymond et al., 1993). The difference between the Wenthold antibody and the Huganir antibody is that the first was directed against 14-amino-acid peptides and the second was directed against 16-amino-acid peptides. The additional epitopes in the Huganir antibody resulted in greater signal-to-noise ratio; therefore, it was used for most experiments. The subunit specificities of these antibodies have been well characterized (Wenthold et al., 1992; Puchalski et al., 1994). An antibody for calbindin-D was obtained from Sigma Chemical Company (St. Louis, MO).

Tissue preparation

Eyes of adult cats (n = 5) were enucleated under deep anesthesia (sodium pentobarbital, 45 mg/kg, i.p.), and the animals were killed with an overdose of the anesthetic drug. All experiments with animals were conducted in compliance with Federal regulations and the University of Pennsylvania policy. Eyes were hemisected along the ora serrata, the lens and vitreous were removed, and the posterior half was processed for either Western blots or immunocytochemistry.

Western blots

The neural retina was isolated and frozen immediately in liquid nitrogen. It was homogenized at 30 mg wet weight/ml in 1% sodium dodecyl sulfate (SDS) lysis buffer in 50 mM Tris-HCl containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.0. Tissue was further sonicated three times for 10 seconds each, and the protein concentration was determined (Bio-Rad, Richmond, CA). The retinal membrane proteins (20 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE; 12% acrylamide) and transferred by electroblotting onto PVDF membranes (Millipore, Bedford, MA). The blots were blocked overnight at 4°C with 5% nonfat dry milk and 0.5% (volume/volume) Tween-20 in Tris-buffered saline, pH 7.4. After blocking again at room temperature (RT) for 1 hour in 0.5% (volume/volume) normal goat serum (NGS), the blots were incubated at RT with primary antibodies for 2 hours and then with goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) for 1 hour. Blots were visualized with enhanced chemiluminescence (Renaissance; DuPont NEN, Boston, MA). Antibodies were used at the following dilutions: anti-GluR2/3 and anti-GluR4, 1:500 (1 µg/ml); anti-GluR6/7, 1:500; HRP-conjugated goat anti-rabbit IgG, 1:50. Control experiments were processed as described above omitting the primary antibody or, for GluR6/7, preabsorbing the antibody by overnight incubation with the antigenic peptide at 4°C (3 µg peptide to 1 µg antibody; in excess of × 150).

Immunocytochemistry

Eye cups were fixed by immersion for 1 hour at RT in a mixture of 4% paraformaldehyde and 0.01% glutaraldehyde in phosphate buffer (0.1 M), pH 7.4, containing 5% sucrose (SPB), rinsed, and cryoprotected in phosphate buffer (PB) containing 30% sucrose at 4°C overnight. In initial experiments, 0.1% glutaraldehyde was included in the fixative. This gave "clean," punctate staining, but careful observation revealed that staining was restricted to edges of the sections that were in contact with the antibody and that the punctate appearance may have been an artifact due to lack of antibody penetration. When such sections were processed for electron microscopy, stain was not visible. Presumably, stain was limited to small regions located mainly in tissue that was sectioned away. Therefore, in subsequent experiments, we fixed the retinas in fixative containing only 0.01% glutaraldehyde. Penetration (judging from electron photomicrographs) was improved but was still limited to a depth of about 1–6 µm.

For cryosections, pieces of central retina were embedded in tissue freezing medium (Electron Microscopy Sciences, Fort Washington, PA) and sectioned radially at 10 µm. For Vibratome sections, posterior eye cups were frozen in liquid nitrogen and thawed two or three times, and central pieces were embedded in agarose and sectioned radially at 50 µm.

The sections were preincubated in 5% SPB containing 10% NGS and 0.1–0.5% Triton X-100 for 30 minutes at RT and were incubated overnight at 4°C with the primary

antibodies in the same diluent. After rinsing, sections were incubated in secondary antibodies conjugated to either HRP or indocarbocyanine (Cy3) for 3 hours at RT. HRP was visualized as a diaminobenzidine (DAB; 0.05% DAB + 0.01% hydrogen peroxide in PB) reaction product. For double-labeling, in some experiments, frozen sections were incubated simultaneously in the two primary antibodies (anti-iGluRs raised in rabbit and anti-calbindin raised in mouse) followed by rinse and incubation with the two secondary antibodies. In other experiments, the sections were incubated first in antibodies for iGluRs and were developed with DAB as described above. They were then rinsed in glycine-HCl buffer, pH 2.2, for 5 minutes to elute the antibodies (DAB reaction product remains where it was deposited), incubated in the antibody for calbindin followed by anti-mouse IgG conjugated to Cy3, rinsed, and mounted. Primary antibodies were used at the following dilutions: anti-GluR2/3, anti-GluR4, and anti-GluR6/7, 1:60–1:100 (≈5 µg/ml); anti-calbindin, 1:200.

Immunoelectron microscopy

Radial Vibratome sections (50–100 µM) were stained as described above, except that Triton X-100 was omitted from the medium. After DAB reaction, sections were postfixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.2, for 2 hours at 4°C. After thorough rinsing in cacodylate buffer, sections were incubated in hexamethylene-tetramine (2.6%), silver nitrate (0.2%), and disodium-tetraborate (0.2%) for 5–15 minutes at 60°C. Subsequently, the sections were rinsed in distilled water (10 minutes × 3), incubated in gold chloride (0.05%) for 10 minutes at 4°C, rinsed again in distilled water, and incubated in sodium thiosulfate (2.5%) for 2 minutes. After a thorough rinse in distilled water followed by cacodylate buffer, sections were postfixed with 2% osmium tetroxide in cacodylate buffer for 1 hour, dehydrated in 50% and 70% methanol, then stained with 0.5% uranyl acetate in 70% methanol for 1 hour at 4°C in the dark. Sections were dehydrated further with propylene oxide and flat embedded in Epon 812. Epon blocks were sectioned either in parallel to the Vibratome sections for radial sections or, in certain cases, parallel to retinal layers for tangential sections.

For electronic processing of Figure 1, negatives of Western blots and light photomicrographs were scanned at about 1,300 dots per inch (dpi). Digital images were cropped, enlarged, and contrast enhanced by using Adobe Photoshop (Adobe Systems, Mountain View, CA). Final resolution was around 300 dpi. The figures were labeled with Adobe Illustrator and printed out at 300 dpi on a Phaser 450 (Tecktronix).

RESULTS

Antibodies recognize GluR protein in cat retina

To determine whether the antibodies that were prepared against rat GluR2/3, GluR4, and GluR6/7 cross-react with the corresponding cat glutamate receptor subunits, we subjected homogenates of cat retina to Western blot analysis. In three experiments, the antibodies for GluR2/3, GluR4, and GluR6/7 detected bands at about 110 kDa (Fig. 1A), consistent with their predicted molecular masses of 102 kDa, 108 kDa, and 116 kDa, respectively (Raymond et al., 1993; Puchalski et al., 1994; Wenthold et al., 1994; Peng et al., 1995). These bands were at the same positions

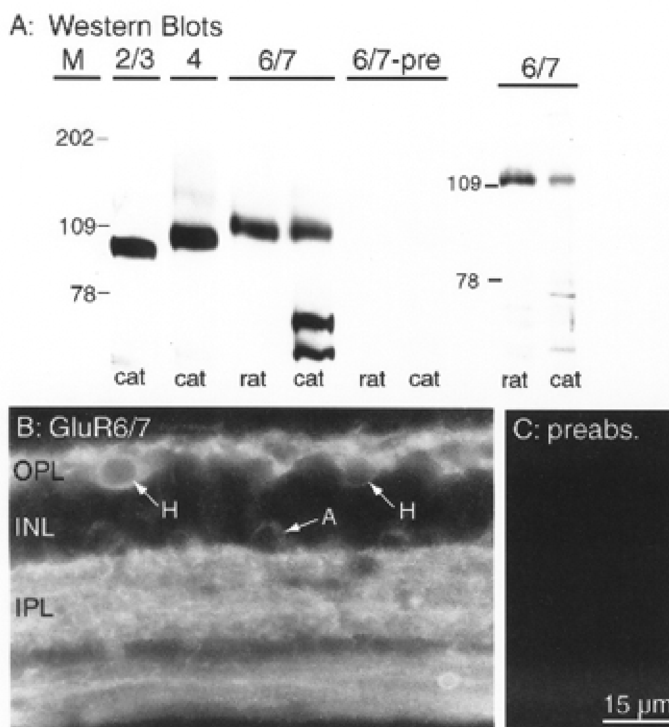

Fig. 1. Antibodies against glutamate receptors GluR2/3, GluR4, and GluR6/7 recognize the cat glutamate receptor subunits. **A:** Western Blots (20 µg/lane) show a single band at a molecular weight of about 110 kDa for GluR2/3 and GluR4. For GluR6/7, there is always a band at the expected 116-kDa position, but there often are additional bands with lower molecular masses. The strength of these bands relative to the prominent band is variable. All of the bands recognized by the antibody were eliminated by preabsorption with GluR6 peptide. **B:** Staining for GluR6/7 (10-µm frozen section; visualized with indocarbocyanine; Cy3). Stain is observed in both plexiform layers, in horizontal cells (H), and in certain amacrine cells (A). **C:** Staining was eliminated completely by preabsorbing the antibody with a 150-fold excess of GluR6 peptide/antibody. OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer.

as those in the rat retina (shown only for GluR6/7). Both antibodies for GluR6/7 detected additional bands. These bands probably are degraded GluR6, because 1) the relative strength of the 116-kD band and the other bands was variable (Fig. 1A), 2) they were eliminated by preabsorbing the antibody with GluR6 peptide (three experiments), and 3) this degradation has been reported in HEK-293 cells as well and was interpreted to be degradation products (Wenthold et al., 1994). To further test the specificity of the immunocytochemical staining for GluR6/7, we absorbed the antibody with the peptide prior to applying it to cat retinal tissues. Staining was eliminated totally (Fig. 1B,C). In addition, we stained for GluR6/7 with the Wenthold antibody. This gave higher background, but staining of the inner and outer plexiform layers and of horizontal cells was above background (not shown). These results indicate that the antibodies for rat GluR2/3, GluR4, and GluR6/7 subunits specifically recognize the homologous cat subunit proteins.

Staining is present in cell classes known to respond to AMPA and kainate

Staining patterns for GluR2/3, GluR4, and GluR6/7 in radial frozen sections of cat retina were similar by light

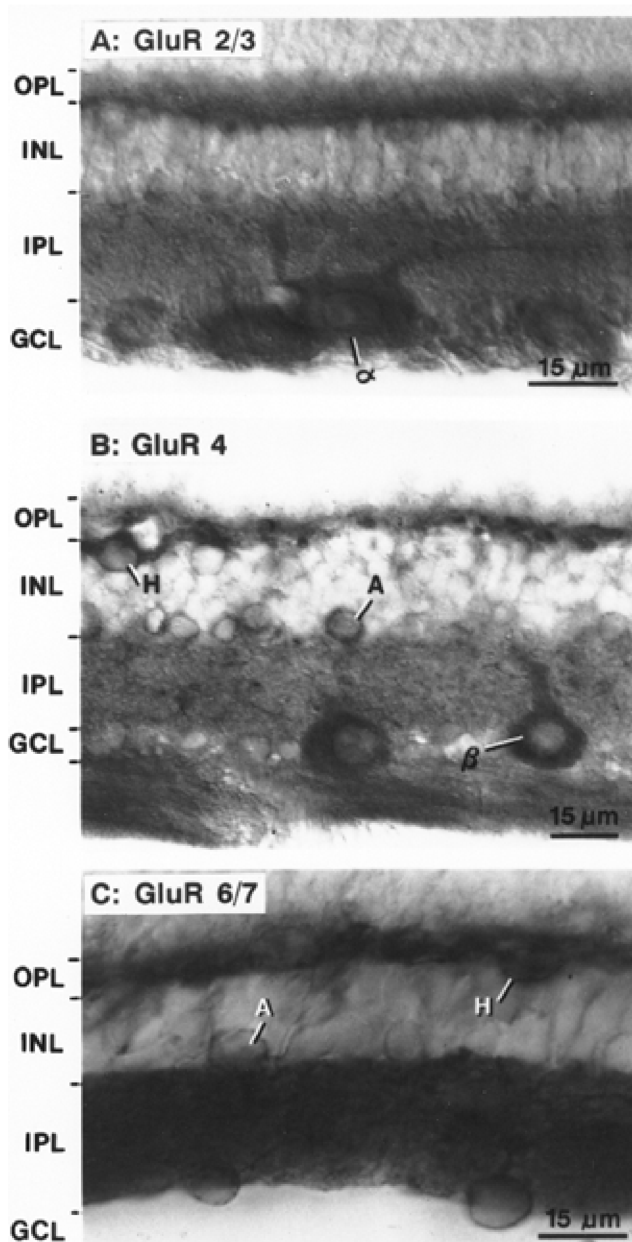

Fig. 2. Staining patterns for GluR2/3 (top), GluR4 (middle), and GluR6/7 (bottom) are similar [10-µm frozen sections; visualized with diaminobenzidine (DAB) reaction product]. Both outer and inner plexiform layers (OPL and IPL, respectively) stain strongly, as do the somata of horizontal cells (H), amacrine cells (A), and large and medium cells in the ganglion cell layer (GCL; presumable α and β ganglion cells). For GluR2/3 and GluR4, stain in somata appears stronger than in the plexiform layers; however, for GluR6/7, stain in somata appears as strong or weaker than in the plexiform layers. INL, inner nuclear layer.

microscopy (Fig. 2). The strongest staining appeared in horizontal cells and ganglion cells. Many amacrine cell somata and processes also were stained. The stained processes often appeared punctate, probably representing amacrine cell varicosities, which are the sites of synaptic communication. Notably, staining for GluR6/7 in processes of amacrine and ganglion cells was stronger than in their somata (Figs. 1B, 2C). Bipolar cell somata usually were below detection levels but became apparent after intensification with gold (not shown). Photoreceptors were unstained. Radial processes, which are characteristic of Müller cells, often were stained at the region where they crossed the outer nuclear layer. However, this staining was not consistent. Because Müller cells generally stain for many antibodies, including preimmune sera, we did not attempt to ascertain whether their staining was specific. The staining patterns described above were consistent for each of the three antibodies in six to seven experiments done on retinas taken from three to five cats. Similar results were observed also in monkey and rat retina (not shown). These results are in general agreement with previous work (Hughes et al., 1992; Müller et al., 1992; Hamassaki-Britto et al., 1993; Peng et al., 1995, Qin and Pourcho, 1996).

Type A and type B horizontal cell somata express different sets of iGluRs

In cat, the two horizontal cell types can be discriminated, because one type (type A; HA) is large and stains strongly for the calcium-binding protein, calbindin, and the other type (type B; HB) is smaller and stains weakly for calbindin (Röhrenbeck et al., 1989; Vardi et al., 1994). When we stained the same sections for GluR2/3 and calbindin, both horizontal cell types were stained (Fig. 3A). In contrast, when GluR4 and calbindin were used, only HA cells were stained (Fig. 3B). When GluR6/7 was used, both types stained, but staining of HA was consistently stronger than that of HB (Fig. 3C). These data were observed in three experiments and quantified in two (Table 1). The ratio of HB:HA somata in cat retina should be 2:1 (Wässle et al., 1978). Our counting revealed a ratio of about 1:1, probably because staining of HB for calbindin was weak, and many of these somata remained below threshold. The staining in HA cells for the iGluRs cannot be attributed to cross reactivity of anti-rabbit $F(ab)_2$ fragments with the mouse anticalbindin, because, in control experiments, the anti-rabbit secondary antibody did not react with the mouse primary antibodies. Potential cross talk between the fluorescent signals in the double-labeled tissue was avoided by visualizing iGluRs with a DAB reaction product and by visualizing calbindin with Cy3.

Horizontal cell terminals express iGluRs in a narrow strip along the synaptic ridge

To evaluate receptor expression in the terminals and to localize the synaptic regions between photoreceptor and horizontal cells, we examined the staining under the electron microscope. Horizontal cells contact the cone in a structure termed the "synaptic triad." In this triad, two horizontal cell processes (lateral elements) and one bipolar cell dendrite (central element) invaginate the cone and terminate in apposition to a synaptic ribbon in the cone. It was not possible to discriminate between lateral elements of HA and HB cells, but it was possible to localize the receptors within the lateral elements. For all subunits, staining was associated with the electron-dense region of the postsynaptic horizontal cell membrane just below the ribbon (Fig. 4). Regions that were located laterally within the terminal (farther from the center of the triad) usually were unstained. In several examples, we observed the staining in a tangential view of the triad. When the sectioning plane passed through the ribbon (Fig. 5A, top plane), there was little

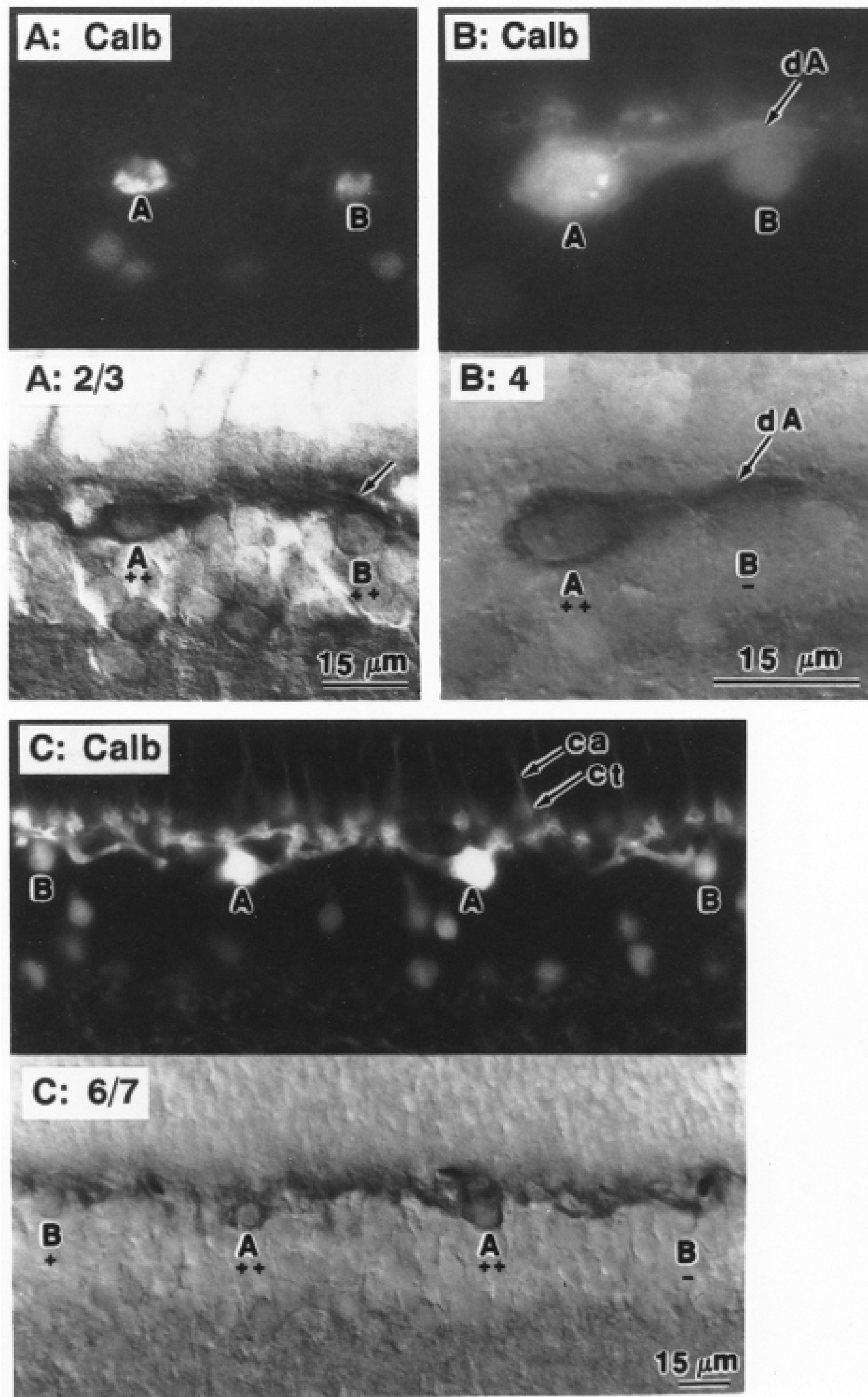

Fig. 3. Differential expression of ionotropic GluRs (iGluRs) by type A (A) and type B (B) horizontal cells. Frozen sections were double labeled for the iGluR subunits (as indicated in the figures) and calbindin (Calb). Calbindin is visualized with Cy3, and the iGluR subunits are visualized with DAB reaction product. **A:** Both types of horizontal cells stain equally for GluR2/3. The somata and primary dendrites of type A horizontal cells (HA) are stained darkly. The soma of the type B horizontal cell (HB) is pale, but the dendrite emanating from it (arrow) is stained darkly. **B:** HA but not HB cells stain for GluR4. Note that the darkly stained dendrite above the HB soma (dA) belongs to HA. The dendrite emanating from HB was verified to be unstained through different focal planes. For the purpose of the figure, we chose a region in which both HA and HB could be photographed simultaneously. When HA and HB were farther from one another, the lack of staining in HB was obvious. **C:** HA somata and primary dendrites are stained strongly for GluR6/7, but HB somata are stained weakly. In this section two HA somata are dark, one HB soma is pale (left), and one HB soma is unstained (right). Cone terminals (ct) and axons (ca) also were stained for calbindin. ++, Strongly stained; +, weakly stained; −, unstained.

TABLE 1. Differential Staining Between Type A and Type B Horizontal Cells[1]

Cell type	GluR2/3 A	GluR2/3 B	GluR4 A	GluR4 B	GluR6/7 A	GluR6/7 B
Stained strongly (%)	100	100	87.5	0	89	33
Stained weakly (%)	0	0	0	0	11	30
Unstained (%)	0	0	12.5	100	0	37
Number of examined somata	14	17	8	7	18	33

[1]Percentage of type A and type B horizontal cell somata stained for glutamate receptors (GluRs) GluR2/3, GluR4, and GluR6/7 (from two experiments in which ionotropic GluRs were visualized with 3,3′-diaminobenzidine tetrahydrochloride reaction product). Type A was distinguished from type B by its strong staining for calbindin.

staining (Fig. 5B). However, when the plane of section was below the ribbon, where the horizontal cell terminals apposed one another (Fig. 5A, bottom plane), staining was stronger and was distributed along this apposition line (Fig. 5C). These results demonstrate that the staining in horizontal cell terminals is concentrated at the electron-dense membrane along the synaptic ridge.

Next, we checked how often the two lateral elements of a triad stained for iGluRs. Only cones that were contacted by at least one stained process and only triads within these cones in which both lateral elements could be discerned clearly were considered. In these radial sections, the depth of a stained process from the edge could not be determined. A lateral element was determined to be stained if it contained an aggregate of at least two gold particles. This criterion was based on the level of background staining in photoreceptor terminals, which rarely contained an aggregate. In most triads, neither lateral element was stained; in about one-third, only one element was stained; and, in a small fraction (2–13%), both were stained (Table 2). It appears that the two lateral elements in a triad express different receptors, as shown by Brandstätter et al. (1997), but it is still possible that this staining pattern is an artifact of the low probability of staining.

In rod terminals, we initially examined radial sections and found very few stained lateral elements. To assess whether this weak staining was a false negative resulting from examination of regions that were inaccessible to the antibodies, we reexamined material that was processed for immunocytochemistry as radial Vibratome sections and then thin sectioned horizontally. In the horizontal plane, the distance from the edge could be determined easily. We traced rod and horizontal cell terminals from 7 to 12 serial sections located within 6 µm from the edge and counted the number of stained horizontal cell terminals. We looked at structures up to 6 µm from the edge, because horizontal cells terminating in cones could be observed at this depth. For GluR2/3, 12 lateral elements were stained in 22 traced rod terminals (Fig. 6). Because of the small number of stained processes, we did not determine the percentage of rods in which both lateral elements were stained. For GluR4, only three lateral elements were stained in 58 rod terminals, and, for GluR6/7, only five lateral elements were stained in 70 rod terminals. These results suggest that staining of lateral elements at the rod terminal is probably positive for GluR2/3 and negative for GluR4 and GluR6/7.

Bipolar dendritic tips forming basal contacts express GluR2/3, GluR4, and GluR6/7

Dendritic tips of OFF cone bipolar cells usually can be identified by the basal contacts they form with the cones (Dowling and Boycott, 1966; Kolb and Nelson, 1995). At these basal contacts, the electron-dense pre- and postsynaptic membranes are separated by a synaptic cleft, which is traversed by a striated, electron-dense material. Dendrites that formed basal contacts were stained for GluR2/3, GluR4, and GluR6/7 (Fig. 7). We noticed previously that some ON dendrites, identified by mGluR6 expression, form basal (or basal-like) contacts in one section and an invaginating contact in another (Vardi and Morigiwa, 1997; Vardi et al., 1998). To verify that the stained dendrites identified as "basal" did not invaginate, we created a short series of four to seven sections and traced the stained basal dendrites. In this material, none of the contacts identified as "basal" was invaginating, indicating that they probably were OFF dendrites. The numbers of observations are summarized in Table 3. A dendrite that was stained in consecutive sections was counted only once. Within the dendritic tips, staining often was distributed randomly (Fig. 7A), but we found many examples in which the largest and most numerous gold particles were present in association with the electron-dense dendritic membrane (Fig. 7B,C).

Invaginating cone bipolar dendritic tips express GluR2/3 and GluR4

Dendrites of ON cone bipolar cells commonly invaginate the pedicle and form the central element of the cone synaptic triad. However, not every invaginating dendrite is ON, because some OFF dendrites also invaginate (McGuire et al., 1984; Kolb and Nelson, 1995). The percentage of invaginating OFF bipolar cells is unknown due to an incomplete account of these cells in the cat. However, for one type of OFF bipolar cell that we stained with an antibody for glutamic acid decarboxylase 65, we found 14% invaginating processes (Vardi, unpublished). Invaginating bipolar dendritic tips were stained frequently for both GluR2/3 and GluR4 (Fig. 8B,C) and were stained infrequently for GluR6/7. This suggests that ON cells express iGluRs. However, it might be possible that the stained invaginating dendrites belong to the invaginating OFF cells and not to ON cells. To determine the likelihood of this possibility, we applied the following reasoning. If only OFF bipolar cells express iGluRs, then the number of stained invaginating dendrites that belong to OFF cells should be smaller than the number of stained basal dendrites, because 1) most OFF dendrites are basal, and 2) in general, there are more basal contacts than invaginating contacts (Chun et al., 1996). For GluR2/3 and GluR4, the number of stained invaginating dendrites was similar to that of stained basal dendrites (approximately one stained dendrite per cone; Table 3). We therefore conclude that, although some of the stained invaginating dendrites may be OFF, most of them are ON. For GluR6/7, the number of basal dendrites was greater by about fivefold than the number of stained invaginating dendrites; therefore, it is possible that only OFF cells express this isoform.

Rod bipolar dendritic tips express GluR2/3

Staining for GluR2/3 revealed a light stain (Fig. 2A) or a spray of fine dots (not shown) in the upper region of the outer plexiform layer. This could be interpreted as staining in horizontal cell processes that invaginate the rod terminals. Surprisingly, on electron microscopic examination,

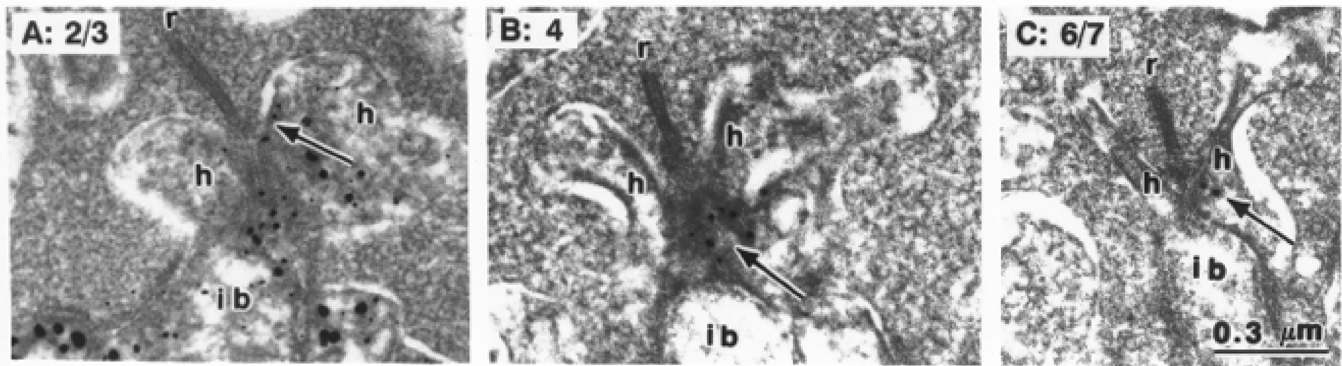

Fig. 4. Horizontal cell terminals (lateral elements) in cones express GluR2/3, GluR4, and GluR6/7 subunits (electron photomicrographs). Stain (arrows) is associated with the electron-dense membrane region of the lateral element (h) just below the ribbon (r). **A:** GluR2/3. Semioblique view of a cone triad. Staining is strong, but it is concentrated in the central region of the lateral element. **B,C:** Standard (radial) view of the cone triads. Stain is for GluR4 (B) and GluR6/7 (C). ib, Invaginating bipolar dendrite.

staining was more robust in rod bipolar dendritic tips (Fig. 8A; see also Fig. 6B). Two rod bipolar dendrites invaginate every rod terminal, each from a different rod bipolar cell (Sterling et al., 1988; Rao-Mirotznik et al., 1995). In many photomicrographs, staining was apparent only in one dendrite (Fig. 8A). However, in the tangential series, of 22 rod terminals that were traced six contained two stained invaginating dendrites (see Fig. 6B), and two more contained one stained dendrite. The ratio of stained:unstained dendrites, therefore was 14:44 (32%). Staining for GluR4 was weak: in the tangential series: Six dendrites in 58 rod terminals (5%) were stained. Staining for GluR6/7 was very weak: Only two dendrites in 70 rod terminals (1%) were stained. Therefore, rod bipolar dendrites appear to express GluR2/3 but probably do not express GluR4 or GluR6/7.

DISCUSSION

We localized several iGluRs (GluR2/3, GluR4, and GluR6/7) to specific cell types in the cat outer retina by double labeling and ultrastructural study. This is the first ultrastructural localization of the AMPA subunits GluR2/3 and GluR4 in retina. Staining patterns were specific: 1) The antibodies detected a band at the appropriate molecular weight, 2) preabsorption of the antibody for GluR6/7 eliminated both the bands in Western blots and the staining pattern in the immunoreacted sections, and 3) the subcellular distribution of iGluRs in horizontal cells and bipolar cells revealed here agrees with the distribution inferred from physiology (see below).

In all cases, only a small fraction of synaptic terminals of a given cell type was stained. This is a known problem with immunocytochemistry for iGluR subunits (Baude et al., 1995; Petralia, 1997), probably due to masking of the epitopes after insertion of the protein into the membrane. This is a particular problem with antibodies that are directed against the C-termini of the iGluR subunits. For GluR2, this domain is responsible for protein-protein interaction with the anchoring protein GRIP (glutamate receptor interacting protein) and could be masked by this interaction (Dong et al., 1997). Because of this limitation, negative staining should be undertaken very cautiously. However, when staining for a given subunit is observed in more than 10% of the terminals of a particular cell type, it is probably safe to conclude that this type expresses the subunit.

GluR2/3 staining is the most ubiquitous of the three subunits examined here. It is expressed in both types of horizontal cells and in OFF bipolar cells. It is also expressed in the dendritic tips of both rod bipolar and ON cone bipolar cells. GluR4 was expressed by one type of horizontal cell (HA), in OFF bipolar cells, and also, unexpectedly, in invaginating dendrites of ON cone bipolar cells. GluR6/7 was expressed in horizontal cells and in OFF cone bipolar cells.

Subcellular distribution of subunit proteins in horizontal cells and bipolar cells

In horizontal cells, staining for the three iGluR subunits in somata and primary dendrites is strong. Physiological recordings from isolated cat horizontal cells support this finding, because glutamate elicits a large response if it is applied focally to somata and primary dendrites (T. Sasaki, personal communication). Staining in the terminals is weak and is concentrated near the narrow strip of electron-dense postsynaptic membrane just below the ribbon (Raviola and Gilula, 1975). When glutamate was applied to dendritic tips, it elicited no response. This lack of response from dendritic tips is probably because the receptors that are localized in the region of the invaginating terminal tips are cut off in the process of isolation.

Unlike horizontal cells, staining in bipolar cell somata and primary dendrites is weak. Stain concentrates in the dendritic tips and in the basal dendrites (presumably of OFF bipolar cells), and it associates with the postsynaptic electron-dense membrane. This distribution is consistent with physiological recordings from isolated OFF cone bipolar cells, because glutamate elicits a large response if it is applied focally to dendritic endings, but it elicits no response if it is applied to somata (Sasaki and Kaneko, 1996).

Functional implication of iGluR expression in horizontal cells

We found that HA expresses both AMPA subunits (GluR2/3 and GluR4) and a kainate subunit (GluR6/7). In

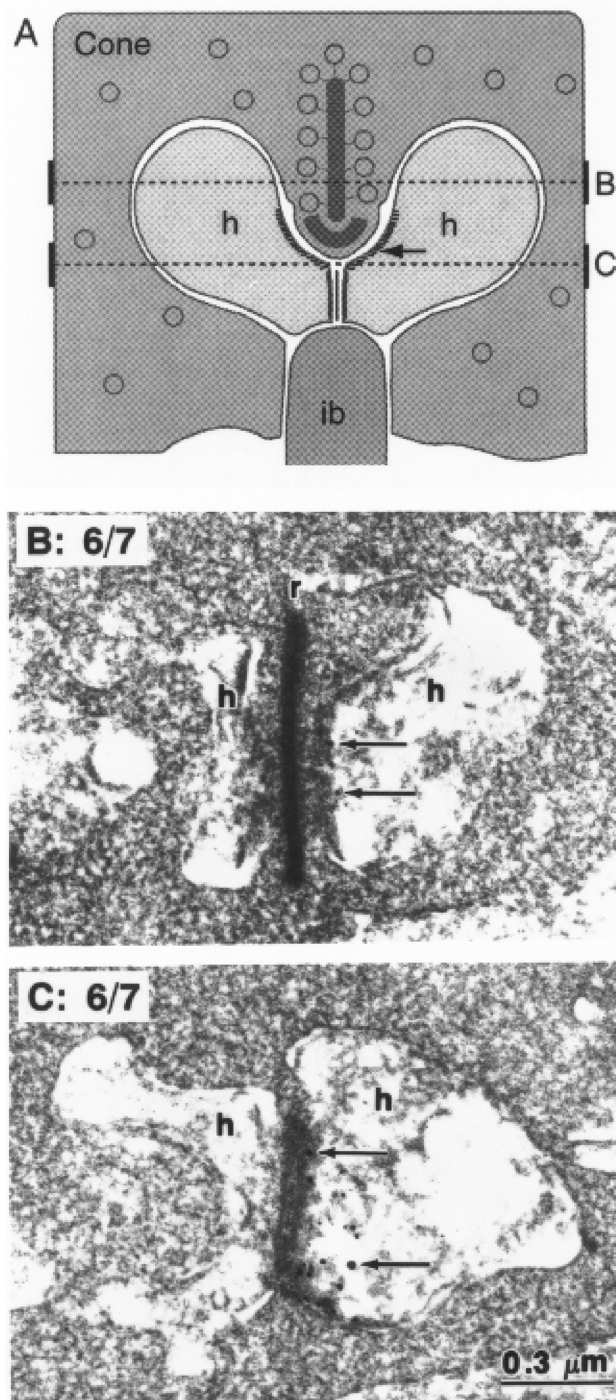

Fig. 5. Stain in cone horizontal cells is localized along the synaptic ridge (GluR6/7; electron photomicrographs). **A:** Schematic of the cone triad at a cross-sectional (standard) view. The dashed horizontal lines indicate the approximate sectioning planes in B and C, respectively. Arrow points to the electron-dense membrane region, which is thought to host the glutamate receptor. h, Horizontal cell terminal; ib, invaginating bipolar cell. **B:** Tangential view through a ribbon and two lateral elements. The ribbon is seen along its length. Stain (arrows) in this plane is weak, and it is associated with the electron-dense material of the right horizontal cell terminal. **C:** Tangential view of the same lateral elements about 80 nm below B. The lateral elements are in contact, and the ribbon is gone. Stain in this section (arrows) is stronger than in B. Note that staining is absent in the lateral region (farther from the ribbon) of the lateral element (C is reprinted from *Vision Research,* Vol. 38, Vardi et al., Neurochemistry of the Mammalian Cone 'Synaptic Complex', pp 1359–1369, Copyright 1998, with permission from Elsevier Science).

TABLE 2. Frequency of Stained Lateral Elements in Cone Synaptic Triads[1]

Stained elements	GluR2/3	GluR4	GluR6/7
None stained (%)	65	58	56
One stained (%)	33	29	35
Both stained (%)	2	13	9
Total examined triads	79	59	82

[1]Percentage of triads with zero, one, or both lateral elements stained for GluR2/3, GluR4, or GluR6/7.

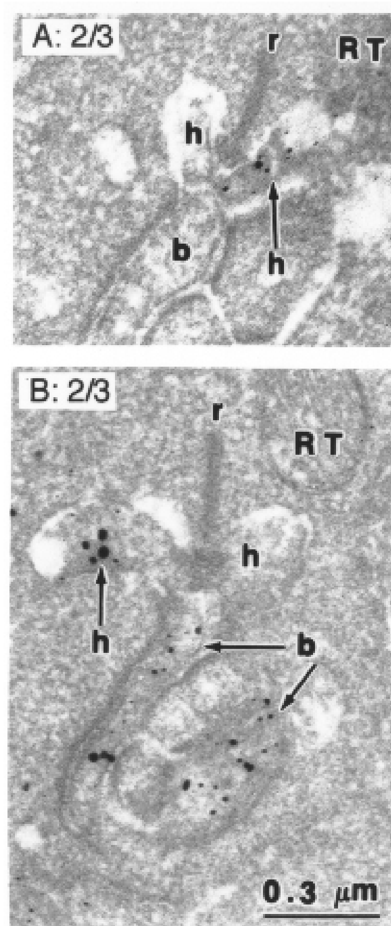

Fig. 6. Axon terminals of HB cells stain for GluR2/3 (electron photomicrographs). **A:** A rod terminal (RT) with one stained lateral element (arrow). Stain is strongest below the synaptic ribbon (r). **B:** Another rod terminal in which one of the lateral elements and both rod bipolar dendritic tips (b) are stained. h, Horizontal cell terminal.

brain, immunoprecipitation experiments suggest that most glutamate receptors combine AMPA (GluR1–GluR4) or kainate (GluR5–GluR7, KA1, and KA2) subunits but rarely combine both (Puchalski et al., 1994; for review, see Huettner, 1997). This may imply that HA has two different receptors, possibly contacting different cone types. In lower vertebrates, horizontal cells express only one type of AMPA/kainate receptor (Ishida and Neyton, 1985; O'Dell and Christensen, 1989; Krizaj et al., 1994). If HA also has only one receptor, then this receptor may be composed of both AMPA and kainate subunits and possibly has unique characteristics. Indeed, in carp horizontal cells, N-methyl D-aspartate (NMDA) acts as a weak antagonist at the non-NMDA glutamate receptors: quite an unusual pharmacology (Ariel et al., 1986).

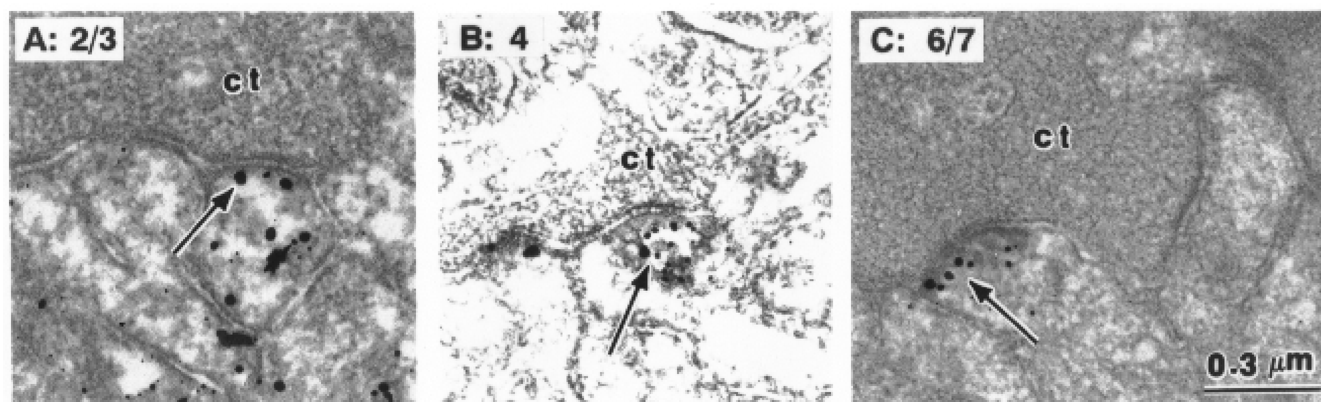

Fig. 7. Dendrites forming basal contacts express iGluR subunits (electron photomicrographs). **A:** GluR2/3. The stained dendrite (arrow) forms a basal contact. Gold particles are distributed randomly within the dendrite. Other nearby dendrites also are stained lightly. **B,C:** GluR4 and GluR6/7. Staining (arrows) is associated with the electron-dense membrane in the basal contacts (C is reprinted from *Vision Research,* Vol. 38, Vardi et al., Neurochemistry of the Mammalian Cone 'Synaptic Complex', pp 1359–1369, Copyright 1998, with permission from Elsevier Science). ct, Cone terminal.

TABLE 3. Comparison of Staining Frequency for Basal Bipolar Dendrites, Invaginating Bipolar Dendrites, and Horizontal Cell Terminals[1]

Elements	GluR2/3	GluR4	GluR6/7
No. of stained basal contacts	22 (6)	30	23 (10)
No. of stained invaginating dendrites	23 (6)	26	5 (3)
No. of stained horizontal cell terminals	30 (4)	44	28 (5)
No. of examined cone pedicles	26 (5)	33	22 (5)

[1]The number indicates the observed stained profiles for GluR2/3, GluR4, and GluR6/7. Only cone pedicles that had at least one stained postsynaptic element were counted. Numbers in parentheses are from a series of four to seven sections (0.3–0.7 μm).

Glutamate receptors in the horizontal cells have an additional interesting feature: Dopamine enhances their responses to glutamate through cyclic AMP-dependent protein kinase (PKA). This has been shown in both lower vertebrates (Knapp and Dowling, 1987; Krizaj et al., 1994; Maguire and Werblin, 1994) and mammals (P. de la Villa, personal communication). This enhancement probably is due to the GluR6 subunit in the receptor, because GluR6 phosphorylation by PKA was shown to enhance responses to kainate (Raymond et al., 1993, 1994; Wang et al., 1993). Therefore, the GluR6 subunit in horizontal cells appears to be responsible for modulation of horizontal cell responses by dopamine. This, in turn, would modulate the receptive field surround of ganglion cells (Smith, 1995). Because HA expresses GluR6/7 more strongly than HB, the effect of dopamine on the two types may differ. GluR1 also can be phosphorylated by PKA (Roche et al., 1996), but, to our knowledge, GluR1 is not expressed by horizontal cells (Peng et al., 1995; Vardi, unpublished observation).

It is noteworthy that dopamine has another differential effect on the two types. In the HA network, it closes gap junctions between cells through the D1 receptor, a G-protein, and PKA phosphorylation (Hampson et al., 1994). This increases the input resistance of the network and, thus, also increases the responses to glutamate. Therefore, in HA, dopamine acts synergistically to enhance the glutamate response both by increasing the current through the GluR channel and by reducing leaks to other cells due to the closing of gap junctions. Unlike the HA network, dopamine does not seem to play a modulatory role in the HB network (Mills and Massey, 1997). In support of this, the G-protein that is likely to couple the dopamine D1 receptor, G_{olf}, is expressed by HA but not HB (Vardi and Studer, 1994). GluR4, a Ca^{2+}-permeable receptor, also was expressed differentially, but how the GluR4 subunit affects the glutamate response in heteromeric native receptors is currently unknown.

Localization of iGluRs in OFF cone bipolar cells

We have demonstrated that cone bipolar dendritic tips that form basal contacts express AMPA (GluR2/3 and GluR4) and kainate (GluR6/7) subunits. The ratio of basal to invaginating dendrites that were stained for GluR6/7 was 23:5. Because most basal contacts belong to OFF cells, it appears that, within cone bipolar cells, GluR6/7 is expressed primarily (or possibly only) in OFF cone bipolar cells. The GluR6 subunit in these cells could explain the dopamine enhancement of glutamate response observed in bipolar cells of lower vertebrates (Maguire and Werblin, 1994). Combining knowledge accumulated from rat and cat, the OFF bipolar cell class expresses at least five iGluR subunits: the AMPA subunits, GluR1 (Qin and Pourcho, 1996), GluR2 or GluR3, and GluR4; and the kainate subunits, GluR6 or GluR7 and KA2 (Brandstätter et al., 1997). More experiments will be required to determine whether different types of OFF bipolar cells use receptors with different subunit combinations.

Localization of AMPA receptor subunits in ON bipolar cells

Previous reports have shown GluR2 expression in rod bipolar cells: GluR2 message in rat by in situ hybridization (Hughes et al., 1992) and GluR2/3 and GluR2/4 receptor proteins in rat and mouse by immunohistochemistry (Peng et al., 1995; Hughes, 1997). We add two significant contributions: 1) GluR2/3 (probably GluR2) is concentrated in the *dendritic tips* of *rod bipolar* cells, colocalized with mGluR6; and 2) *both* GluR2/3 and GluR4 are localized in invaginating dendritic tips that are probably *ON cone bipolar* cells.

The localization of the AMPA subunits in the ON bipolar cell dendritic tips suggests a synaptic function. The contribution of the AMPA receptor to synaptic function is difficult to predict, because, if glutamate activates the AMPA receptor in a conventional manner, then sodium channels will open and antagonize the principal effect of glutamate, which is to close nonselective cation channels (Dacheux and Raviola, 1986; Yamashita and Wässle, 1991; de la Villa

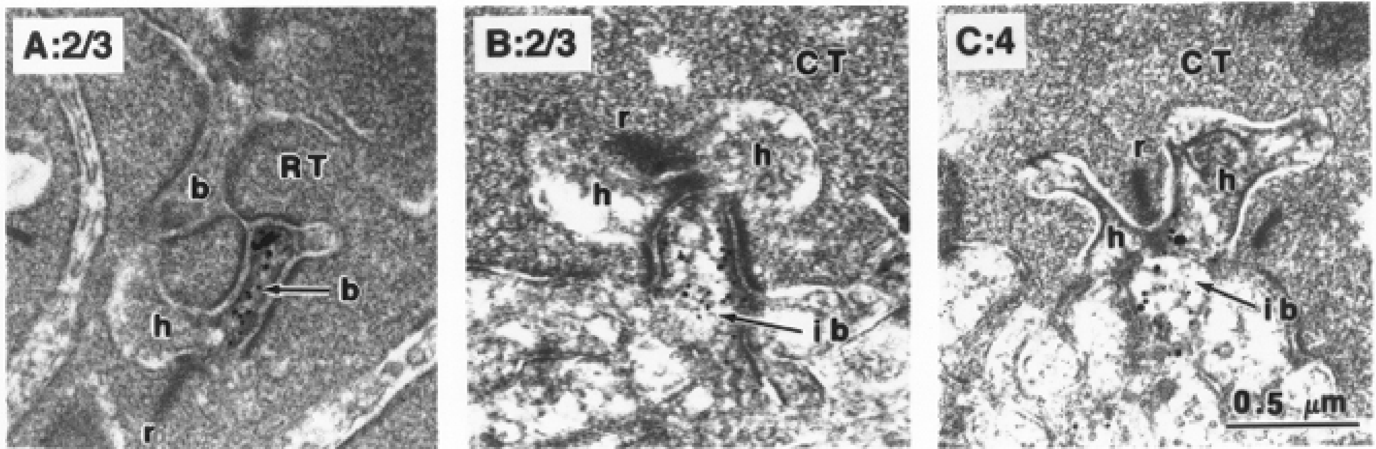

Fig. 8. Invaginating bipolar cells express the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunits GluR2/3 and GluR4 (electron photomicrographs). **A:** GluR2/3. Cross-section of a rod terminal (RT). Staining is localized to one invaginating rod bipolar dendritic tip (b, arrow), the other invaginating dendritic tip (b) is unstained. The membrane separating the horizontal cell terminal and the bipolar dendritic tip is invisible, as is often the case even after good fixation (for example, see Fig. 7A in Rao-Mirotznik et al., 1995). However, rod bipolar dendrites can be recognized based on the apposing electron-dense rod membrane. The horizontal cell terminals (h) are unstained in this section. **B:** GluR2/3. A cone triad with the central element (ib) of the triad stained (reprinted from *Vision Research,* Vol. 38, Vardi et al., Neurochemistry of the Mammalian Cone 'Synaptic Complex', pp 1359–1369, Copyright 1998, with permission from Elsevier Science). **C:** GluR4. A cone triad with the central element (ib) and one lateral element stained. CT, cone terminal; r, ribbon; h, horizontal cell terminal.

et al., 1995; Euler et al., 1996). In these studies, closure of channels is indirect, through a metabotropic action. Hartveit (1996, 1997) found a long latency conductance increase in response to AMPA and kainate, but these responses were not present after axotomy. This indicates that the conductance change was due to receptors that were present on the axon and not on the dendrite. However, in one study, when cyclic guanosine 5′-monophosphate (cGMP) was not added to the solution in the recording electrode, glutamate elicited a conductance increase that was blocked by the glutamate antagonist cis-2,3-piperidine dicarboxylic acid (Karschin and Wässle, 1990). This study did not determine the ions that contributed to the current. In lower vertebrates, ON bipolar cells receive input from rods and cones, and there, glutamate clearly gates ion channels in addition to activating mGluR6. The ions that contribute to the currents are K^+ or Cl^- (Saito et al., 1981; Nawy and Copenhagen, 1987; Lasater, 1988; Hirano and MacLeish, 1991; Grant and Dowling, 1995, 1996), so the receptors probably are not of the AMPA/kainate type. A recent study showed that GluR1 expressed in cortical neurons could activate a G-protein in a manner so far known only for the mGluRs (Wang et al., 1997). In support of this, cGMP-gated current that is known to be present in certain ganglion cells (Ahmad et al., 1994) can be suppressed by AMPA (Sterling, personal communication). Similar unconventional behavior may be found also in ON bipolar cells, in which case, the AMPA subunit would close the cGMP-gated channel in synergy with mGluR6. Therefore, it is possible that the AMPA subunits contribute a subtle modulation to the response of ON bipolar cells through an unknown chemical pathway.

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