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Localization of mGluR6 to Dendrites of ON Bipolar Cells in Primate Retina

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ABSTRACT

We prepared antibodies selective for the C-terminus of the human mGluR6 receptor and used confocal and electron microscopy to study the patterns of immunostaining in retina of monkey, cat, and rabbit. In all three species punctate stain was restricted to the outer plexiform layer. In monkey, stain was always observed in the central element of the postsynaptic "triad" of rod and cone terminals. In monkey peripheral retina, stain was seen only in central elements, but in the fovea, stain was also observed in some dendrites contacting the base of the cone terminal. S-cone terminals, identified by staining for S opsin, showed staining of postsynaptic dendrites. These were identified as dendrites of the ON S-cone bipolar cell by immunostaining for the marker cholecystokinin precursor. The staining pattern suggests that all types of ON bipolar cells, despite their marked differences in function, express a single isoform of mGluR6. Ultrastructurally, mGluR6 was located not on the tip of the central element, near the site of vesicle release, but on its base at the mouth of the invagination, 400–800 nm from the release site. Thus, the mGluR6 receptors of ON bipolar cells lie at about the same distance from sites of vesicle release as the iGluR receptors of OFF bipolar cells at the basal contacts. J. Comp. Neurol. 423: 402–412, 2000.

Indexing terms: G-protein; metabotropic glutamate receptor; blue-sensitive bipolar cell; photoreceptor synaptic complex

Glutamate, released as a neurotransmitter by cones and rods, closes cation channels on the dendritic tips of ON bipolar cells (Nawy and Jahr, 1990; Shiells and Falk, 1990; Yamashita and Wässle, 1991; de la Villa et al., 1995; Euler et al., 1996). The glutamate receptor that triggers this response was identified in rodents as mGluR6 (Nakajima et al., 1993; Nomura et al., 1994), but the homologous receptor had not been identified in other mammals. To identify this receptor in primate retina seemed particularly important because there are at least five types of ON bipolar cell with clearly different functions. These include a "midget" bipolar cell that collects from a single cone and serves high spatial acuity; a bipolar cell that collects from cones sensitive to short wavelengths (S) and serves blue-yellow color vision; several types of "diffuse" bipolar cell that each collect from about 10 cones and serve sensitivity to motion and contrast; and a "rod" bipolar cell that collects from 20–60 rods and serves a high gain circuit for starlight vision (reviewed by Sterling, 1998; Calkins and Sterling, 1999; Boycott and Wässle, 1999). We asked whether mGluR6 is employed by all these bipolar types.

Another question concerned the fine localization of mGluR6. The dendrites of ON bipolar cells invaginate photoreceptor terminals to form the central elements of the postsynaptic "triad." At the mouth of the invagination, the dendrite lies about 500 nm from the active zone (where vesicles are released), and at the apex of the invagination, the dendrite ends some 100–200 nm below the active zone (Dowling and Boycott, 1966; Lasansky, 1972; Rao-Mirotznik et al., 1995; Calkins et al., 1996). Nowhere along this invaginating twig of the ON bipolar dendrite is there any postsynaptic specialization that might indicate where the mGluR6 receptors are clustered (Raviola and Gilula, 1975). To localize the receptor precisely is important because spatial and temporal concentration of gluta-

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mate caused by release of one vesicle decays exponentially. Therefore, depending on where mGluR6 is expressed along the dendrite—at the apex of the invagination or the base—its EC_{50} and binding kinetics might be quite different (Rao-Mirotznik et al., 1998).

To investigate these issues we used the sequence of rat mGluR6 (Nakajima et al., 1993; Nomura et al., 1994) to clone the carboxy-terminal fragment of human mGluR6 and then prepared antibodies against a peptide comprising the last 19 amino acids. These antibodies applied to macaque retina stained all types of ON bipolar cell; surprisingly, they stained the proximal but not the distal region of the dendritic tip.

MATERIALS AND METHODS

Cloning the carboxy-terminal end of human mGluR6

The fragment of cDNA (1279 bp) that encodes amino acids 8–413 of rat mGluR6 (Nakajima et al., 1993; generously provided by Prof. Nakanishi) was amplified by polymerase chain reaction (PCR) and labeled with ^{32}P by random priming. This probe was used to screen a cDNA library of human retina in λgt10 (generously provided by Jeremy Nathans). From approximately 10^6 phages, six positive clones were identified, and λ DNA was prepared, cut with *Eco*RI, and subcloned into pBluescript. A preliminary nucleotide sequence was determined for each clone using T3 and T7 primers, plasmid miniprep DNA, and the Sequenase kit (USB, Cleveland, OH). Several internal primers derived from the rat sequence were also used for sequencing. This allowed the design of primers with human sequence, which were used to determine the nucleotide sequence encoding the carboxy-terminus of human mGluR6.

Antibody preparation

Four antibodies were prepared against the C-terminal peptide (KATSTVAAPPKGEDAEAHK) whose N-terminus was extended with cysteine and conjugated to keyhole limpet hemocyanin either by glutaraldehyde or by N-succinimidyl-3-(2-pyridyldithio) propionate. Peptide synthesis and conjugation were performed by the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine (supported by Diabetes and Cancer Center Core Grants, DK-19525 and CA-16520). Antibodies were prepared by Cocalico Biologicals (Reamstown, PA). Rabbits were immunized initially with 1 mg of peptide, followed by three 0.5-mg boosters. Mice were immunized with 50 μg of peptide followed by three 25-μg boosters. All antisera gave the same staining pattern in monkey, but antisera against the glutaraldehyde-conjugated peptide stained stronger, with less background than the N-succinimidyl-3-(2-pyridyldithio) propionate conjugate. The rabbit antiserum used here (Ab717) was purified on a protein A column. The mouse antiserum (Ab38) was affinity purified on an agarose SulfoLink column (Pierce, Rockford, IL) to which the immunizing peptide was coupled according to the manufacturer's protocol.

Immunoblots

For HEK cells, protein samples (10 μg/lane) were dissolved in sodium dodecyl sulfate (SDS) loading buffer (pH 9.5) and separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a PVDF-plus membrane (MSI, Westboro, MA), incubated with primary antibody against mGluR6 (1:200–1000 for AB717 or 1:100 for AB38; 2 hours, room temperature), washed, incubated with alkaline phosphatase (AP)-conjugated anti-mouse or anti-rabbit IgG (1:1000; 2 hours; Promega, Madison, WI), washed, and detected by colorimetric reaction using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro-blue tetrazolium (NBT; Boehringer Mannheim, Indianapolis, IN). For retina the procedure was similar: 60–100 μg protein was loaded, proteins were separated on a 4–20% gradient gel, secondary antibody was conjugated to horseradish peroxidase (HRP), and the bands were detected with chemiluminescence (Amersham, Arlington Heights, IL). For some blots we prepared membrane fractions as follows: retina was homogenized in 20 ml homogenization buffer (250 mM sucrose, 10 mM Tris, 10 mM HEPES, 1 mM EDTA; pH 7.2 at 24°C) containing protease inhibitors. The homogenate was centrifuged at 6,000*g* for 10 minutes at 4°C, the supernatant was centrifuged at 40,000*g* for 30 minutes at 4°C, and the final pellet was resuspended in ~70 μl of homogenization buffer with protease inhibitors and stored at −80°C.

Immunocytochemistry

Fixed eye cups of adult rhesus monkey were obtained from Covance Research Products (Alice, TX). Fixation was for 1 hour in various concentrations of aldehydes diluted in 0.1 M phosphate buffer at pH 7.4. Eyes from adult cat or rabbit were enucleated under deep anesthesia (sodium pentobarbital: 45 mg/kg, i.p.). The animals were then sacrificed by overdose of the anesthetic drug (200 mg/kg). All experiments with animals were conducted in compliance with Federal regulations and University of Pennsylvania policy. Eyes were hemisected along the ora serrata, the lens and vitreous were removed, and the retina was detached from pigment epithelium and processed for immunocytochemistry.

Light microscopy. Retina was cryoprotected with 30% sucrose in phosphate buffer (overnight), frozen in a mixture of Tissue Freezing Medium (Electron Microscopy Sciences, Ft. Washington, PA) and 20% sucrose (1:2), and cryosectioned vertically at 10 μm. Sections were then stained according to a standard protocol: they were soaked in diluent containing 0.1 M phosphate buffer, 10% normal goat serum, 5% sucrose, and 0.3% Triton X-100; incubated in anti-mGluR6 overnight at 4°C; washed and incubated for 3 hours in anti-rabbit F(ab) fragment conjugated to HRP; and reacted with H_2O_2 + 3, 3′-diaminobenzidine tetrahydrochloride (DAB). Section were visualized with a Polyvar II microscope (Leica). In monkey and cat, best results were obtained with 3% paraformaldehyde or 4% paraformaldehyde + 0.01% glutaraldehyde for 1 hour. Stronger glutaraldehyde decreased specific staining and increased unspecific staining. In rabbit, best results were obtained with 2% paraformaldehyde for 1 hour.

Double labeling. Sections were incubated simultaneously in anti-human mGluR6 raised in mouse and anti-S opsin [or anti-cholecystokinin precursor (CCK)] raised in rabbit. In other experiments, sections were incubated in anti-human mGluR6 raised in rabbit and anti-calbindin raised in mouse. For the mouse anti-mGluR6, the best staining was obtained with an anti-mouse that was conjugated to Alexa 488 (Molecular Probes, Eugene,

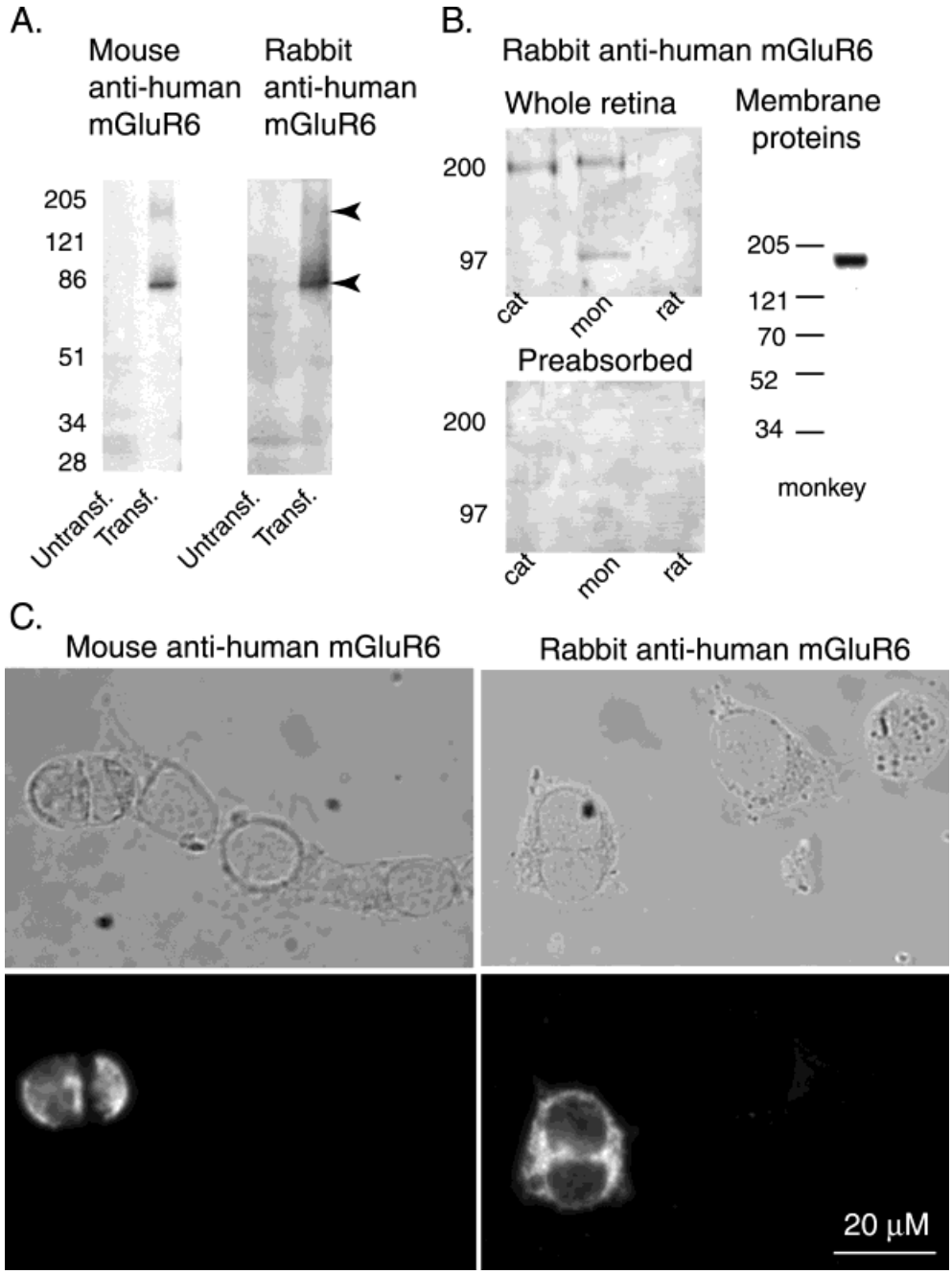


Fig. 1. Antibodies against human mGluR6 are specific. **A:** Antibodies (raised in rabbit and in mouse) used in Western blots of mGluR6 transfected HEK cells (transf.) recognized a prominent band at about 85 kDa and a weak band at about 170 kDa (arrowheads), corresponding to the monomer and the dimer forms of the receptor. The bands were absent in untransfected cells. **B:** In whole monkey retina (mon), the antibody (raised in rabbit) recognized a band at about 190 kDa and a fainter band at about 95 kDa. In cat, only the dimer was labeled. The antibody did not recognize the rat mGluR6. When membrane proteins were prepared, the 190-kDa band was prominent. **C:** HEK 293 cells transfected with mGluR6 stained with anti-mGluR6 made in rabbit and mouse. Top: HEK cells in phase contrast; bottom: same cells in fluorescence. Only transfected cells were stained.

OR). The second antigen was visualized with Lisamine Rhodamine. Sections were mounted in Vectashield (Vector, Burlingame, CA) and visualized with a confocal microscope (Leica). Optical sections were taken with a different pinhole size depending on the illustrated point; the approximate resulting resolution for each figure is indicated in figure legends. Images were contrast enhanced in Adobe Photoshop and placed into Adobe Illustrator. Anti-S opsin was a generous gift of Prof. Jeremy Nathans (John Hopkins University), and anti-CCK was a generous gift of Prof. John DelValle (University of Michigan). Anti-calbindin was from Sigma (St. Louis, MO).

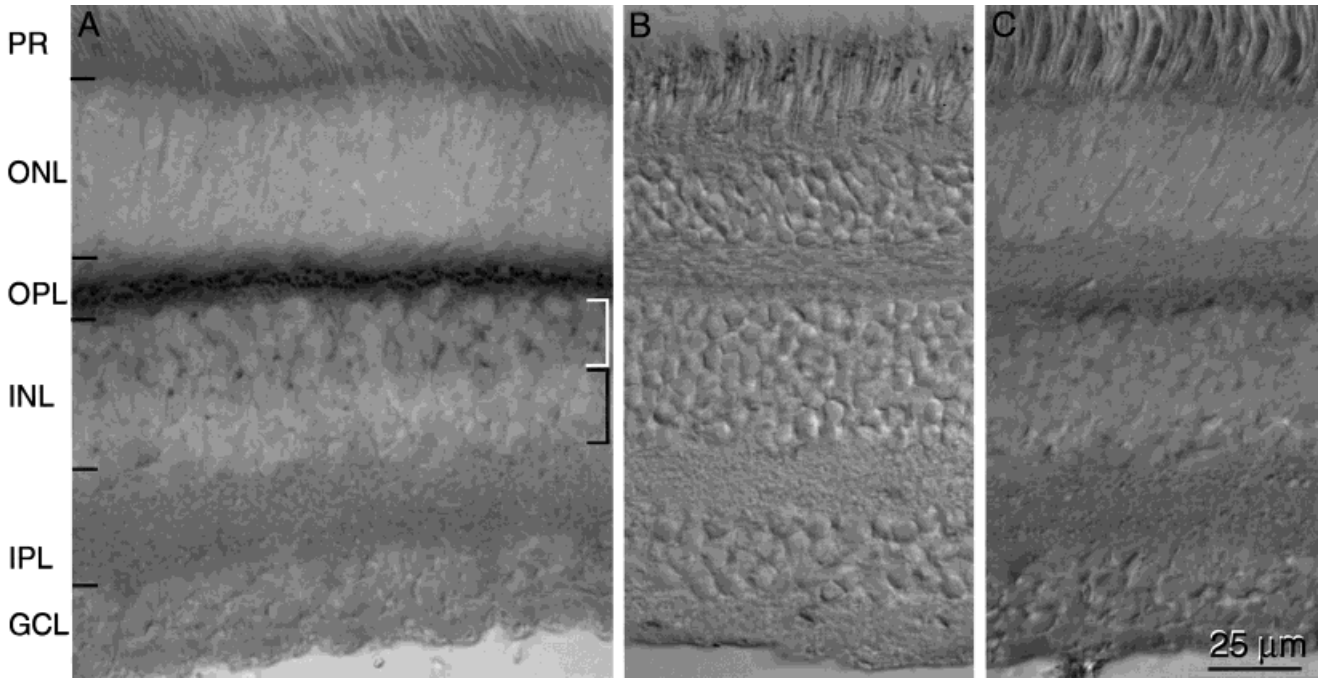


Fig. 2. mGluR6 is strongly expressed only in the outer plexiform layer (monkey, radial cryostat section; DAB reaction product). **A:** Antibody against mGluR6 (Ab717; diluted 1:5,000) showed strong "punctate" staining in the OPL and weaker staining in the outer half of the INL (white bracket). The inner half of the INL was unstained (black bracket). **B:** Preimmune serum gave no consistent staining. There was occasional staining of the photoreceptor outer segment (as in this section) or of cone inner segments. **C:** Same antibody (Ab717) preabsorbed with 0.03 mg/ml peptide failed to stain. PR, photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Electron microscopy. Fixative was 4% paraformaldehyde, 0.01% glutaraldehyde, and 0.2% tannic acid, or 4% paraformaldehyde and 0.02% glutaraldehyde. After cryoprotection, eyecups were freeze-thawed three times, embedded in 4% agarose, and Vibratome sectioned at 100 μm. Sections were processed as for light microscopy, but Triton X-100 was omitted, and incubation in primary antibody was extended to 3 days. DAB reaction product was intensified by the method of silver intensification followed by gold substitution (modified from van den Pol, 1988; Johnson and Vardi, 1998). The tissue was then osmicated (2% osmium tetroxide, 60 minutes), stained with 1% uranyl acetate in 70% ethanol (60 minutes), dehydrated in ethanol, soaked in propylene oxide, and embedded in Epon 812. Ultrathin sections were mounted on formvar-coated slot grids and stained with uranyl acetate.

Reconstruction and cell identification

The synaptic terminals of cones and the dendritic tips of cone bipolar cells were reconstructed from 40 serial sections and digitized (Stevens et al., 1980; Smith, 1987). Except for contrast enhancement, no alterations were made to the figures. Illustrations were prepared with Adobe Photoshop version 5 and Adobe Illustrator version 8 on the Mac.

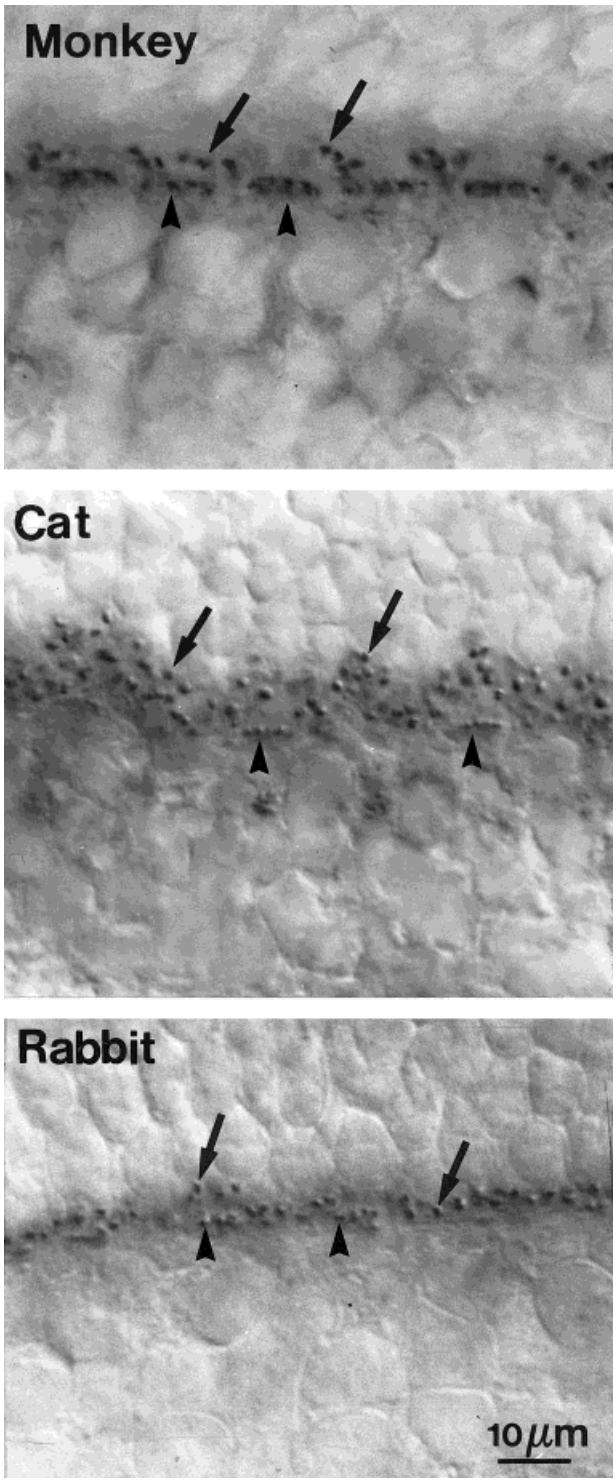
RESULTS

Antibodies for human mGluR6 are specific

Antibodies raised against a rat mGluR6 C-terminal peptide (Nomura et al., 1994) did not cross react with either monkey or cat retinas, suggesting that the C-terminal is not conserved between rodent and other species. Therefore, we isolated cDNA clones encoding mGluR6 from a human retinal library and determined the nucleotide sequence of the region encoding the C-terminus. The predicted amino acid sequence for the human C-terminal differs in 7 of the 19 amino acids of the rat peptide. Antibodies made to the human C-terminus (whether raised in rabbit or mouse) recognized two bands in Western blots of HEK cells transfected with the human mGluR6 cDNA (Fig. 1A). One band was about 85 kDa, running slightly faster than the predicted molecular weight of mature human mGluR6 (95 kDa) (Laurie et al., 1997); the other band was about twice as heavy, indicating a tendency for dimerization, as previously found for mGluR5 (Romano et al., 1996). Similar results were obtained in rat using anti-rat mGluR6 (Nomura et al., 1994). Our antibodies prepared against the human C-terminus also recognize mGluR6 in other mammalian retina since Western blots of monkey retina showed a weak band at 95 kDa and a stronger band at about 190 kDa (Fig. 1B). Blots of cat retinas showed only the 190-kDa band (dimer). Both bands were eliminated by preabsorbing the antibody with the mGluR6 C-terminus peptide. An additional band often observed at about 60 kDa was not eliminated by preabsorption. Antibody against human mGluR6 never detected rat mGluR6. When membrane fractions were prepared, only the dimer was observed.

To test whether the antibodies would recognize mGluR6 in *fixed* tissue, we immunostained fixed HEK cells that had been transiently transfected with the human mGluR6

cDNA. The antibodies strongly reacted with transfected cells but not with untransfected cells (Fig. 1C). In monkey retina, all four antibodies for mGluR6 gave strong and punctate staining in the outer plexiform layer (OPL). Each antibody gave a variable degree of background staining in cones and/or ganglion cells, especially when the antibody was concentrated (>1:2,000). The antibody raised in rabbit against the glutaraldehyde-conjugated peptide gave the strongest staining with the least background. This antibody, in addition to its staining of puncta in OPL, stained somas in the upper half of the inner nuclear layer (Fig. 2A). This region is typically occupied by ON bipolar cells (Vardi, 1998). There, stain was concentrated in the Golgi apparatus (as observed at the electron microscope level). Retinas incubated in preimmune sera were devoid of the punctate staining in the OPL (Fig. 2B). The punctate stain could be blocked by preincubating the antibody with 0.03 mg/ml peptide (Fig. 2C). Occasional staining in cones and ganglion cells was not blocked, suggesting it was nonspecific.



Stained puncta are the tips of rod bipolar and cone bipolar dendrites

In the upper OPL, individual stained puncta were relative large (~0.7 μm) and isolated (Fig. 3). These structures were absent in sections through the fovea but became more numerous with eccentricity. In the middle OPL, puncta were smaller (~0.4 μm) and neatly organized in rows. A similar staining pattern (isolated, large puncta above and rows of small puncta below) were also seen in cat and rabbit (Fig. 3). The large puncta correspond to the site where two to five rod bipolar dendritic twigs invaginate a rod terminal; the rows of small puncta correspond to sites where multiple twigs of ON cone bipolar cells invaginate a cone terminal.

All types of ON cone bipolar cells express mGluR6

Immunostain viewed by electron microscopy was concentrated in the central element of the rod and cone triad (Fig. 4). This structure in the primate always corresponds to the invaginating tip of an ON bipolar cell (Kolb, 1970; Calkins et al., 1996). However, a primate cone terminal harbors 20–50 triads (Chun et al., 1996; Calkins et al., 1996), and we could not tell from viewing single sections whether *all* central elements had been stained. Therefore, we prepared serial sections and partially reconstructed three foveal cone terminals. Fifty-five central elements were identified, and 51 of these were stained (Table 1).The few unstained central elements probably represent incomplete penetration of the antibody.

In this material immunostain was also observed at 36 dendritic tips that contact the base of the cone terminal (Figs. 5, 8E). Although most basal contacts are made by OFF bipolar dendrites (Kolb, 1994), in the fovea some basal contacts are made by diffuse ON bipolar dendrites (Chun et al., 1996; Calkins et al., 1996; Hopkins and Boycott, 1997). Thus staining of basal elements suggested that diffuse ON bipolar

Fig. 3. mGluR6 localizes to the tips of rod bipolar and cone bipolar dendrites (radial cryostat section; DAB reaction product). Monkey: Outermost scattered puncta (arrows) represent staining of multiple rod bipolar dendritic tips that invaginate a rod spherule. Innermost puncta organized in rows (arrowheads) represent staining of cone bipolar dendritic tips that invaginate the base of a cone pedicle. Cat (central area) and rabbit (visual streak): Staining pattern is similar to that of monkey; it is localized to distal rod bipolar dendritic tips in rod spherules (arrows) and cone bipolar dendritic tips at the base of cone pedicles (arrowheads). Puncta are more numerous in cat because rods and rod bipolar cells in this species are two- to fivefold denser than in rabbit.

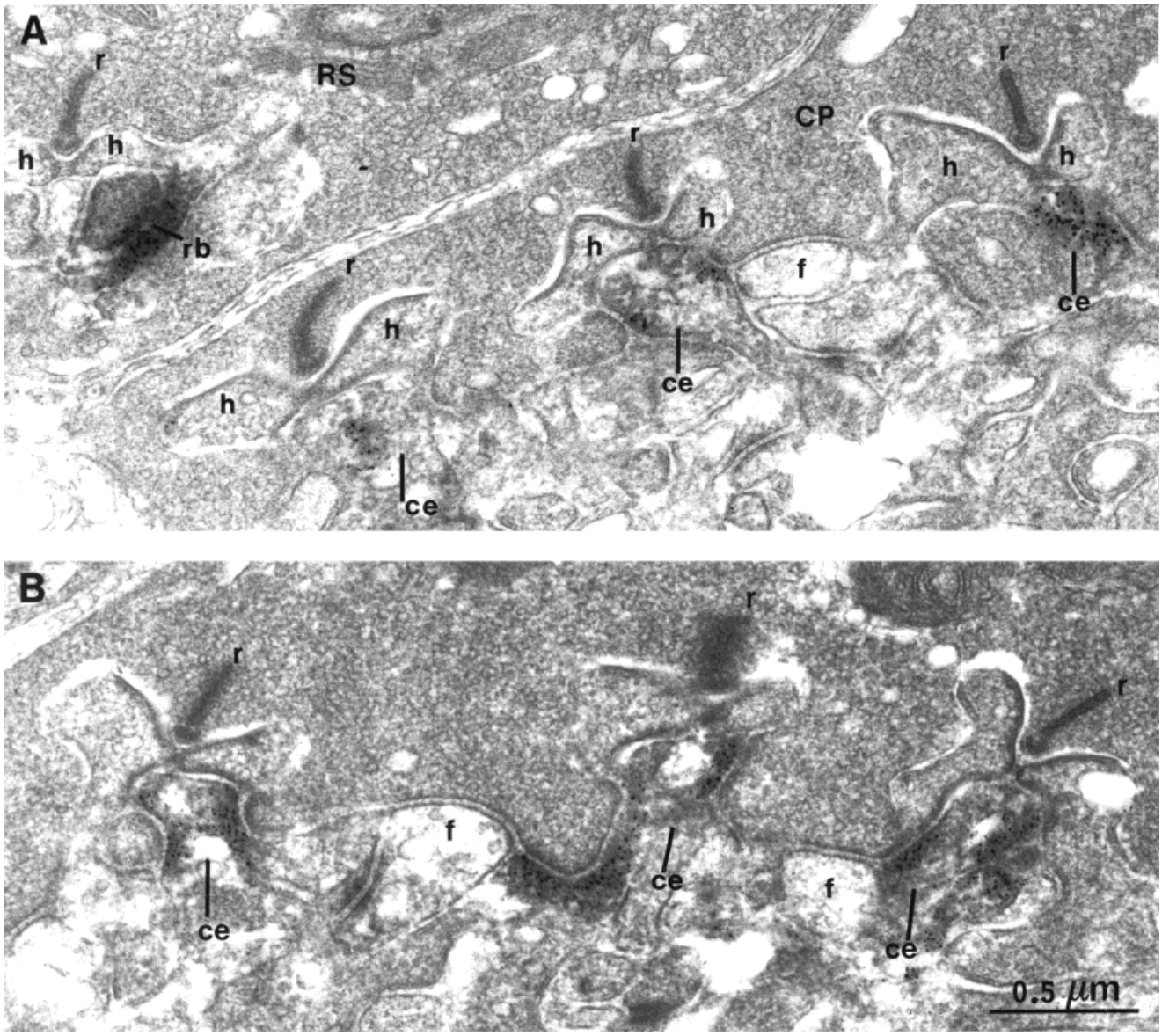


Fig. 4. In peripheral retina of monkey, mGluR6 localizes to "central elements" of the cone triad. **A:** Cone pedicle (CP) with three triads, each marked by a presynaptic ribbon (r). Below each ribbon are paired horizontal cell processes (h) and a central element (ce). Fine deposits of silver-gold-intensified immunostain are present in each central element. A rod spherule (RS) in upper left also shows two horizontal cell processes and a stained invaginating rod bipolar dendritic tip (rb). **B:** Different cone pedicle with three triads. Central elements are stained, but dendritic tips *between* the central elements at the cone base, which form typical "flat contacts," are unstained (f).

cells also express mGluR6. To test this, we examined serial sections from peripheral retina where all basal contacts elements are OFF bipolar dendrites (Chun et al., 1996). Partial reconstructions of cone terminals showed that all central elements and no basal contacts were stained (Table 1), suggesting that OFF dendrites do not express mGluR6. To test this further in a specific type of OFF bipolar cell, we stained the diffuse bipolar cell DB3 with mouse anti-calbindin (Grünert et al., 1994) and observed staining for mGluR6 with rabbit anti-mGluR6. Somas stained for calbindin were unstained for mGluR6 (Fig. 6). This showed that whereas ON midget, ON diffuse cone bipolar cells (DB5, DB6), and the rod bipolar cell all express mGluR6, OFF bipolar cells do not.

The cone terminals reconstructed from electron micrographs might not have included an S cone because the sample was small and the S cone is sparsely distributed. Therefore, we identified S-cone terminals by immunostaining for the S-cone opsin. We applied the mouse antibody to mGluR6 to the same sections. Bipolar dendritic tips postsynaptic to S-cone terminals were clearly stained (Fig. 7A). However, the S-cone bipolar cell is not the only ON bipolar contacting the S cone (Calkins, personal communication), so an additional approach was needed.

We stained the S-cone bipolar cell with an antibody to CCK precursor (Kouyama and Marshak, 1992; Grünert et al., 1994) and observed staining for mGluR6 using the

TABLE 1. All On Bipolar Dendritic Tips are Stained.

	Periphery[1]			Fovea[1]		
Pedicle number	1	6	7	1	2	4
Serial sections (no.)	13	15	31	40	40	40
Pedicle diameter (μm)	7.5	7.5	7.5	6.2	6.5	6.2
Reconstructed terminal (%)	15	17	35	55	55	55
Central elements, stained (no.)	8	7	10	17	18	16
Central elements, unstained (no.)	0	0	0	2	2	1
Basal elements, stained (no.)	1	0	1	12	18	5

mouse antibody. Because the mouse antibody did not stain somas, the test for co-localization could only be made in the dendrites. This proved difficult because in the dendritic tips, where mGluR6 staining is strongest, CCK staining is weakest. Nevertheless, we were able to follow some CCK-stained dendrites almost to their tips, where at their very ends, there was stain for mGluR6 (Fig. 7B).

Fine localization of mGluR6 in the distal dendrite

We had expected mGluR6 staining to localize at the extreme tip of the ON bipolar dendrite, where it most closely approaches the active zone. However, in more than 30 examples of ON cone bipolar dendrites, this region was devoid of stain or else stained only weakly. Instead, stain was concentrated 200–600 nm more proximally. Where an ON dendrite ascended vertically to penetrate the invagination, stain was concentrated on the membrane just inside the mouth of the invagination (Fig. 8A,B). Where an ON dendrite traveled laterally to reach the invagination, stain was concentrated partially within the invagination and partially outside (Fig. 8C,D). In the fovea, where ON dendrites formed basal contacts, stain was concentrated at the region of that contact (Fig. 8E). In a few cases stain did extend from its usual proximal location into the very tip of the dendrite. This might be caused by overstaining or diffusion of the reaction product.

At each site where the ON dendrite stained for mGluR6, the membrane of the photoreceptor terminal (both rod and cone) exhibited a dense specialization (Fig. 8). This usually occurred at the mouth of the invagination, but it also occurred at the base of the foveal cone terminal where an ON dendrite formed a basal contact. We also tried to assess the precise localization in the rod bipolar dendritic tips. This was more difficult because staining in these tips was intense, and the membrane of rod bipolar dendrite that apposes the horizontal cell process is short. For a few examples (especially in cat), however, it appears that the rod bipolar membrane apposing the horizontal cell is unstained.

DISCUSSION

Evidence that the mGluR6 receptor is a single isoform

Antibodies prepared against the C-terminus of rat mGluR6 recognize two proteins near the expected molecular weight (95 kDa) and two bands at twice these weights (Nomura et al., 1994). Similarly, antibodies prepared against human mGluR6 recognize a band at about 85 kDa apparent molecular weight and another one at twice that weight. The light bands have been explained as the plain monomer and a posttranslational modified form. The heavy bands are probably dimers (Nomura et al., 1994). It seems unlikely that there are other isoforms because probing of both rat and human libraries has identified only one isoform in each species (Nakajima et al., 1993; Ueda et al., 1997; Hashimoto et al., 1999; present study). Moreover, as shown here, antibodies specific for the C-terminus bind to all known types of ON bipolar cell in primate retina: the single type of rod bipolar and four types of cone bipolar cell: midget, diffuse types (DB5 and DB6); and the S-cone bipolar cell. Similarly, the rat's four to five types of ON bipolar cell all express the same mGluR6 (Vardi and Morigiwa, 1997; Vardi et al., 1998).

It seems somewhat surprising that a single isoform of the mGluR6 receptor can serve such different spatiotemporal components of the photoreceptor output. For example, the rod synapse onto rod bipolar cell carries slow signals caused by a single or just few photoisomerization (R*); whereas cone synapses onto cone bipolar cells carry faster signals generated by multiple, simultaneous R*. Also, different cone bipolar types divide the temporal bandwidth, some carrying high and others low temporal frequencies (Freed, 2000a,b). Moreover, temporal responses for short wavelengths are slower than for middle and long wavelengths (Brindley et al., 1966; Mollon and Polden, 1977; Williams et al., 1981), but the origin of this difference is unknown (Nelson, 1985; Schnapf et al., 1990; Stockman et al., 1993; Chichilnisky and Baylor, 1999). One anticipates from other central nervous system circuits that such different functions would require different isoforms of the postsynaptic receptor with different binding constants, kinetics, desensitization rates, channel conductances, etc. (e.g., Gardner et al., 1999). That one mGluR6 isoform serves all ON bipolar types suggests that their functional differences are generated further downstream and that it excludes glutamate receptors from being the origin of the slow responses in the short wavelength perception.

Fig. 5. In monkey fovea, mGluR6 localizes to central elements of the cone triad and also to the cone base. **A:** Radial view of cone pedicle number 2 (shaded area) with stained dendritic tips traced over seven serial sections. **B:** Tangential view (90° rotation) of the same pedicle reconstructed from 40 sections. Dendritic tips stained for mGluR6 are outlined from the horizontal projection. Dendrites outlined in red were central elements, and those outlined in blue terminated at the cone base and did not participate in a triad. Ribbons are black, and regions occluded by the dendritic tips are dotted. Brackets designate sections shown in radial view in A. Dendrites marked by * and ** are shown in Figure 8D and E, respectively.

Fig. 6. The diffuse bipolar cell type 3 (DB3) does not express mGluR6. Monkey retina stained for calbindin (red) and mGluR6 (green). Somatic staining for mGluR6 is restricted to Golgi apparatus. Somatic staining for calbindin labels the whole soma. DB3 somas never stained for mGluR6. Ped, cone pedicle stained for calbindin. Confocal optical section: ×40 oil immersion.

Fig. 7. The S-cone bipolar cell expresses mGluR6. **A:** S-cones contact dendrites that express mGluR6. Monkey peripheral retina. Red shows immunostain for S opsin; green shows immunostain for mGluR6. Three cone pedicles (dotted) are present, two of which are S-pedicles (S ped); all are contacted by dendrites expressing mGluR6. **B:** Red shows immunostain for CCK, an established marker for the S-cone bipolar cell. Green shows immunostain for mGluR6. Dendrite from an S-cone bipolar cell (S bip) forms multiple twigs at base of a cone pedicle. Each such twig (arrows in inset) is stained at its tip for mGluR6 (arrowheads). Double arrowheads, stained dendrites run laterally to reach other S-cones. Confocal optical section: ~0.4-μm resolution in Z axis, ×100 oil immersion, 1.4 N.A.

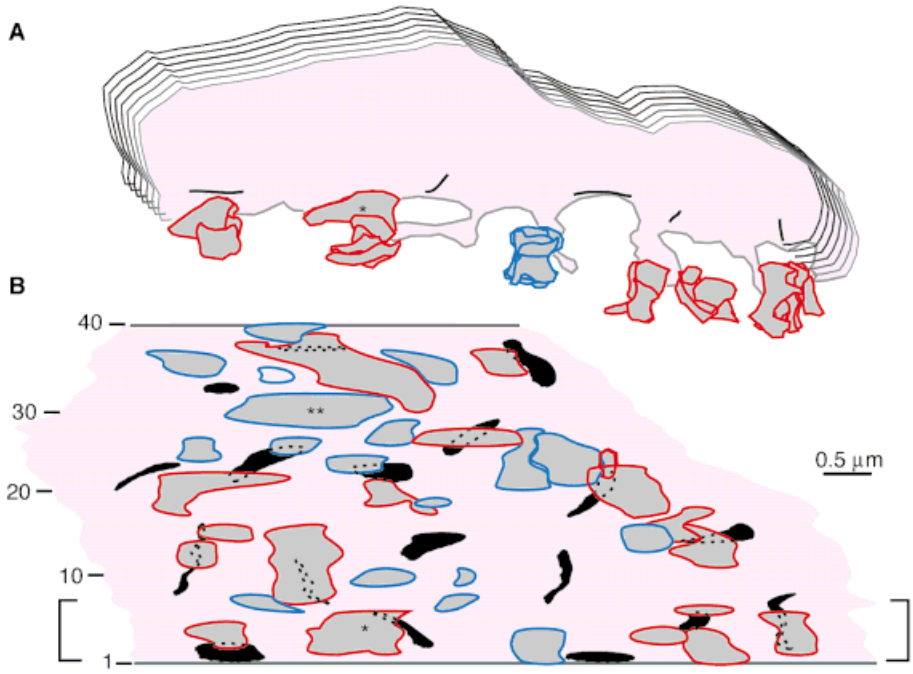


Figure 5

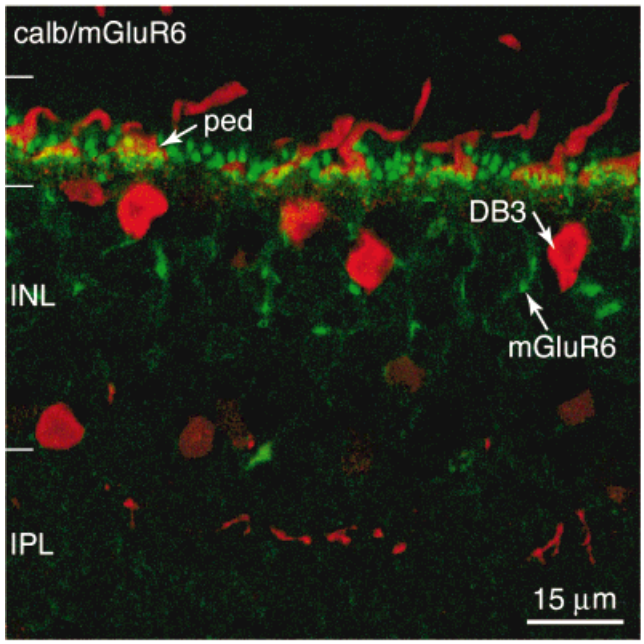


Figure 6

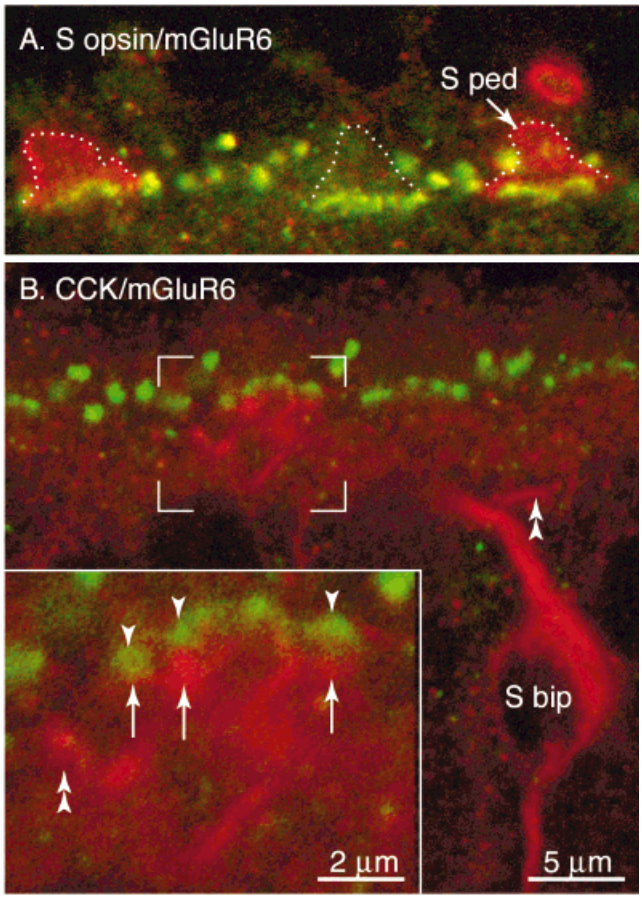


Figure 7

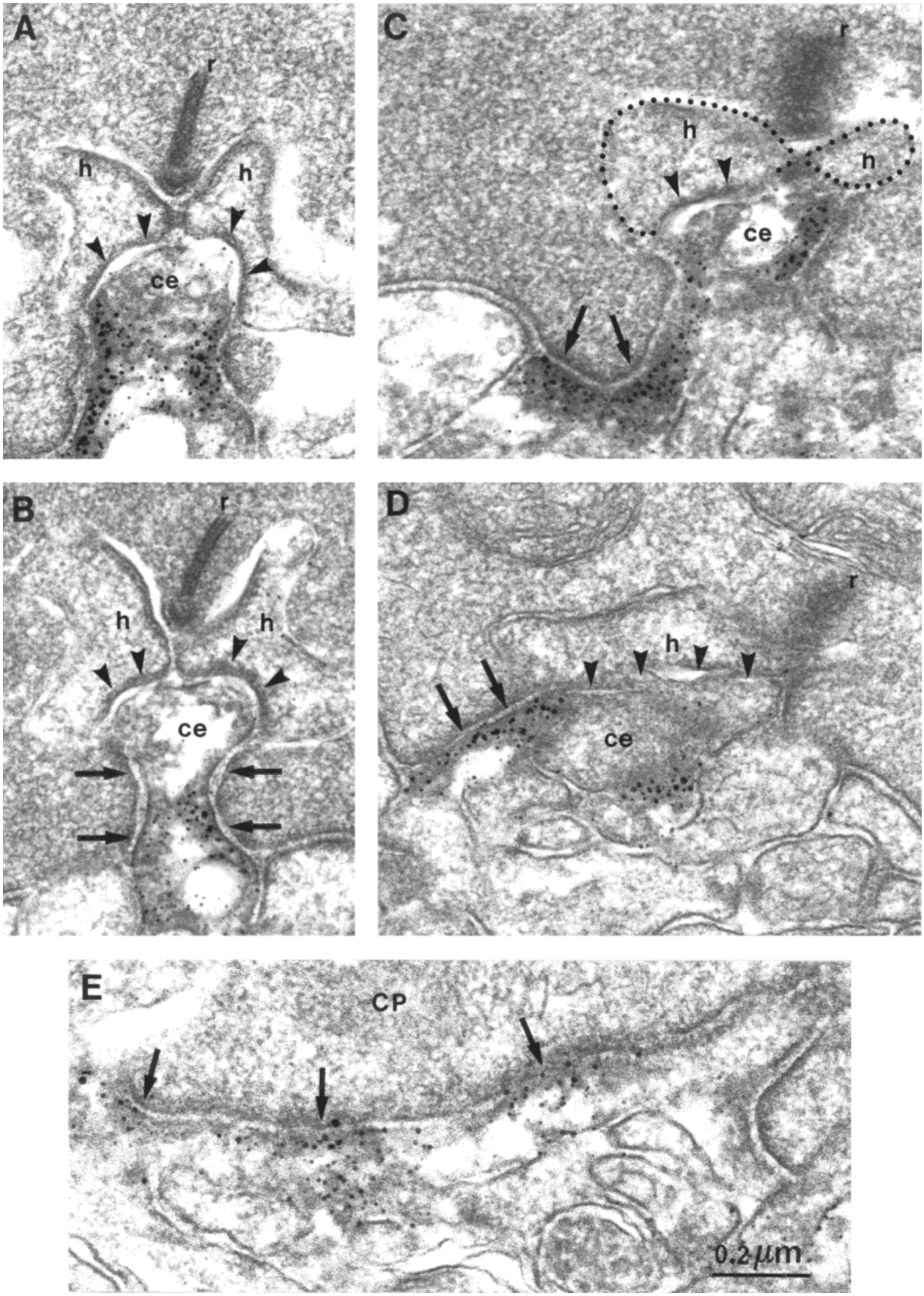


Fig. 8. mGluR6 concentrates not at the tip of the central element, but more proximally where it faces electron-dense region of cone pedicle. **A,B:** Distal tip of central element faces horizontal cell membrane (arrowheads), about 100 nm from vesicle release sites. This region was largely unstained. Proximal region of central element faces electron-dense cone membrane (arrows). This region stained intensely. **C,D:** Central element approaches parallel to base of pedicle. Where tip faces horizontal cell (arrowheads), stain was absent; where proximal region faces dense cone membrane (arrows), stain was concentrated. The cone in D is foveal (pedicle 2, Table 1), and the central element is designated by * in Figure 5. **E:** Foveal cone (pedicle 2, Table 1). Dendrite located at the pedicle base did not participate in a triad but formed a "basal" contact. At this contact, where it faces the electron-dense pedicle membrane (arrows), it is stained. CP, cone pedicle; h, horizontal cell lateral element; r, ribbon; ce, central element.

mGluR6 receptors cluster far from the active zone

At a conventional CNS synapse, postsynaptic receptors face the presynaptic release sites across a ~20-nm cleft. Over this distance a transmitter quantum diffuses rapidly and creates a steep temporal and spatial gradient (Clements et al., 1992; Rao-Mirotznik et al., 1998; Rusakov and Kullmann, 1998). In contrast, the distal dendritic tips of ON bipolar end at least 100 nm from the release sites. Surprisingly, mGluR6 is not present there, but on the segment of dendrite near the mouth of the invagination or at the base of the cone terminal, at a distance of 400–800 nm from the neurotransmitter release sites. This resembles the distance from release site to the AMPA/kainate receptor of OFF bipolar dendrites (500–950) (Calkins et al., 1996; Brandstätter et al., 1997; Morigiwa and Vardi, 1999; Qin and Pourcho, 1999). Over this distance the temporal and spatial gradient of glutamate concentration is shallow (Rao-Mirotznik et al., 1998). Release from multiple active zones (adjacent invaginations) may further reduce the difference in glutamate concentration at ON and OFF dendrites. Glutamate transporters probably shape the peak and decay of the quantal glutamate "puff" (Eliasof and Werblin, 1993; Picaud et al., 1995; Eliasof and Jahr, 1996; Vandenbranden et al., 1996), but the effects for ON and OFF bipolar cells are probably similar.

We noticed that the rod and cone membranes that face the mGluR6 receptor always display a "fluffy" density. This density is so distinctive that it could be used as a marker for the location of the mGluR6 receptor in ON dendrites that are not immunostained. Given that many of the densities and particles originally described for these synapses (Raviola and Gilula, 1975) correspond to specific ion channels, receptors, etc. (Vardi et al., 1998), one would expect this density to also represent some important macromolecules.

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