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# Localization of kainate receptors to the presynaptic active zone of the rod photoreceptor in primate retina

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**Abstract**

Visual information is encoded at the photoreceptor synapse by modulation of the tonic release of glutamate from one or more electron-dense ribbons. This release is highest in the dark, when photoreceptors are depolarized, and decreases in grades when photoreceptors hyperpolarize with increasing light. Functional diversity between neurons postsynaptic at the synaptic ribbon arises in part from differential expression of both metabotropic (G-protein-gated) and ionotropic (ligand-gated) glutamate receptor. In the brain, different subunits also modulate the presynaptic active zone. In hippocampus, ionotropic kainate receptors localize to the presynaptic membrane of glutamatergic axon terminals and facilitate depolarization of the synapse (e.g. Lauri et al., 2001). Such facilitation may be helpful in the retina, where consistent depolarization of the photoreceptor axon terminal is necessary to maintain glutamate release in the dark. We investigated whether such a mechanism could be present in primate retina by using electron microscopy to examine the localization of the kainate subunits GluR6/7 at the rod axon terminal, where only a single ribbon synapse mediates glutamate release. We scored 54 rod axon terminals whose postsynaptic space contained one or more GluR6/7-labeled processes and traced these processes through serial sections to determine their identity. Of 68 labeled processes, 63% originated from narrow "fingers" of cytoplasm extending from the presynaptic axon terminal into the postsynaptic cleft. Each rod terminal typically inserts 4–6 presynaptic fingers, and we scored several instances where multiple fingers contained label. Such consistency suggests that each presynaptic finger expresses GluR6/7. The physiological properties of kainate receptors and the geometry of the rod axon terminal suggest that presynaptic GluR6/7 could provide a steady inward current to maintain consistent depolarization of the rod synapse in the long intervals between photons in the dark.

**Keywords:** Rod photoreceptor, Ribbon synapse, Glutamate receptors, Kainate receptor, Retinal ultrastructure

## Introduction

Visual information is encoded at the photoreceptor synapse by modulation of the tonic release of glutamate from specialized structures called "ribbons" that serve to funnel synaptic vesicles from a large intracellular pool to the presynaptic active zone (Rao-Mirotznik et al., 1995). The release of glutamate is highest in the dark, when photoreceptors are steadily depolarized, and decreases in grades when photoreceptors hyperpolarize with increasing light. Since photoreceptors only use glutamate as their transmitter, all visual information at any given moment is represented in the concentration of glutamate released from the synaptic ribbon. Thus, the great diversity of functional properties of individual retinal circuits depends upon how neurons postsynaptic to the photoreceptor respond to subtle changes in glutamate release.

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Converging anatomical and physiological investigations of the mammalian retina indicate that this diversity at least in part arises from the differential expression of various glutamate receptor subunits by processes postsynaptic to the synaptic ribbon. Each ribbon points between a pair of horizontal cell processes to an invagination of the terminal membrane that typically houses a central bipolar cell dendrite in a configuration called a "triad" (Calkins et al., 1996; Chun et al., 1996; Haverkamp et al., 2001). This central "invaginating" dendrite invariably arises from an ON bipolar cell, which depolarizes to an increase in photon catch and the corresponding decrease in glutamate release. These cells express the G-protein-coupled, metabotropic glutamate receptor mGluR6, characterized by its affinity for the agonist L-2-amino-4-phosphonobutyrate (Nakajima et al., 1993; de la Villa et al., 1995; Euler et al., 1996; Vardi & Morigiwa, 1997; Vardi et al., 2000; Euler & Masland, 2000). The rod axon terminal generally contains only a single synaptic ribbon and diverges to a single type of ON bipolar cell (Grünert & Martin, 1991; Rao-Mirotznik et al., 1995), while the cone axon terminal contains 20–50 ribbons and diverges to multiple types of ON bipolar cell (Calkins et al., 1996;

Chun et al., 1996; Haverkamp et al., 2001). Scores of other bipolar cell dendrites adjoin the basal surface of the cone terminal distal to the invagination (Chun et al., 1996). Most of these dendrites likely arise from OFF bipolar cells, which depolarize to a decrease in photon catch and the corresponding increase in glutamate release at the cone. These cells express one or more AMPA- ($\alpha$-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid) (GluR1-4) or kainate-sensitive (GluR5-7, KA1-2) ionotropic receptor subunits (Euler et al., 1996; Brandstätter et al., 1997; Hartveit, 1997; Morigiwa & Vardi, 1999; DeVries & Schwartz, 1999; DeVries, 2000; Qin & Pourcho, 2001; Haverkamp et al., 2001).

There is mounting evidence from regions of the brain, as well as retina, that many metabotropic and ionotropic receptor subunits also localize to and modulate the presynaptic active zone (brain: Ozawa et al., 1998; Cartmell & Schoepp, 2000; retina: Brandstätter et al., 1996; Cai & Pourcho, 1999; Koulen et al., 1999; Fletcher et al., 2000; Pourcho et al., 2001). In particular, in the brain, presynaptic ionotropic kainate receptors can bind released glutamate and sharpen depolarization of the presynaptic membrane (reviewed in Ozawa et al., 1998). In synaptosomes prepared from hippocampus, presynaptic kainate receptors enhance glutamate release (Gannon & Terrian, 1991; Malva et al., 1996). Similarly, activation of presynaptic kainate receptors depolarizes mossy fiber axon terminals in the CA3 region and Schaffer collaterals in the CA1 region of the hippocampus (Petralia et al., 1994; Chittajallu et al., 1996; Schmitz et al., 2000, 2001). These presynaptic receptors play a key role in facilitating glutamate release during long-term potentiation (Lauri et al., 2001).

In the retina, consistent depolarization of the photoreceptor axon terminal in the dark is necessary to maintain the tonic release of glutamate from the synaptic ribbon. This is especially true of the rod synapse, where steady depolarization of the axon terminal maintains a high level of glutamate release from a single ribbon during the long intervals between photons at night (Baylor et al., 1984; Rao-Mirotznik et al., 1995, 1998). A recent elegant study of kainate receptor expression in the primate retina using lightly fixed tissue detailed several sites of localization, but focused almost entirely on the postsynaptic space of the cone axon terminal (Haverkamp et al., 2001). As part of our ongoing studies of glutamate receptor expression in primate retina, we revisited the localization of kainate receptor subunits using electron microscopy to investigate whether the rod axon terminal, like axon terminals in hippocampus, includes a presynaptic mechanism that could preserve depolarization of the ribbon synapse and the integrity of glutamate release. We find that each rod inserts 4–6 narrow "fingers" of presynaptic axonal cytoplasm into its own postsynaptic cleft and the kainate subunits GluR6/7 localize consistently to these fingers. We also report several instances where similar digitations at the cone ribbon synapse also express GluR6/7. Here we discuss how the physiological properties of native kainate receptors and the geometry of the rod synapse support the idea that presynaptic GluR6/7 could provide a steady inward current to maintain consistent depolarization of the rod axon terminal in between photon absorptions in the dark.

## Methods

### *Animals and tissue preparation*

All experiments with primate tissue were conducted in full compliance with federal regulations and the policy of the University of Rochester Medical Center. We obtained retinas and brain samples from several adult *Macaca fascicularis* used in unrelated studies at Yerkes Regional Primate Research Center (Atlanta, GA) and at the University of Rochester Medical Center. Following euthanasia of the animal, isolated eyecups were cleaned of vitreous and the neural retina isolated from choroid and pigment epithelium. For Western blots, the neural retina was frozen immediately in liquid nitrogen, as were samples of brain following dissection. For immunocytochemistry, the retina was immediately immersion-fixed in 4% paraformaldehyde plus 0.05% glutaraldehyde for 2–4 h. Our fixation was therefore more stringent than the lightly fixed retina used in the study by Haverkamp et al. (2001), since we desired better preservation of synaptic ultrastructure for following processes through serial sections. We isolated and washed the retinas extensively in 0.01 M phosphate-buffered saline (PBS) and cut small pieces into 20–40 $\mu$m thick vertical sections on a vibratome. Some of these sections were embedded in paraffin for resectioning into 4–6 $\mu$m vertical sections for light-microscopic immunocytochemistry, while others were used for preembedding immuno-electron microscopy. We should note that for paraffin sections, the use of xylene as a clearing agent enhances significantly antibody penetration while preserving the gross structure of retinal layers. Small pieces of retina were also soaked in a series of 10–30% sucrose solution, embedded in tissue freezing medium, and sectioned at 10–15 $\mu$m on a cryostat for comparison with results obtained with paraffin sections. Light microscopy results were similar between vibratome sections, paraffin sections, and cryosections.

### *Antibodies*

We used rabbit polyclonal antibodies specific for the kainate-sensitive subunits GluR6/7 (Upstate Biotechnology, Lake Placid, NY) for both light-microscopic immunocytochemistry and pre-embedding immuno-electron microscopy and mouse monoclonal antibodies directed against nonspecific regions of the GluR5/6/7 subunits (Pharmingen, San Diego, CA) for light-microscopic immunocytochemistry. Both were used at 1:1000–1:2000 dilution. Previous localization experiments have demonstrated the subunit specificities of these antibodies in mammalian brain and the expression of the kainate subunits in mammalian retina (Huntley et al., 1993; Peng et al., 1995; Brandstätter et al., 1997; Morigiwa & Vardi, 1999). We also verified specificity of the polyclonal antibodies against GluR6/7 in primate brain and retina with minor modifications of a standard procedure for Western blots (Fig. 1; see also Morigiwa & Vardi, 1999). Primary antibodies were conjugated with either fluorescent (Alexa 594 or Alexa 488, Molecular Probes, Eugene, OR) secondary antibodies at 1:100 dilution (for light microscopy) or biotinylated, anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA) at 1:200 (preembedding electron microscopy). Control staining either omitting the primary antibodies or preabsorbing with the GluR6/7 peptide revealed very little or no nonspecific background binding of the secondary antibody and a complete absence of the punctate localization we describe here (not shown).

### *Immunocytochemistry and light and electron microscopy*

The sections were placed into blocking solution of 0.8% bovine serum albumin (BSA), 0.1% gelatin, and 5% normal goat serum (NGS) in 0.01 M phosphate-buffered saline (PBS) for 6–12 h at room temperature. Sections were transferred into primary-antibody solution with 0.8% BSA, 0.1% gelatin, and 1% NGS in PBS for

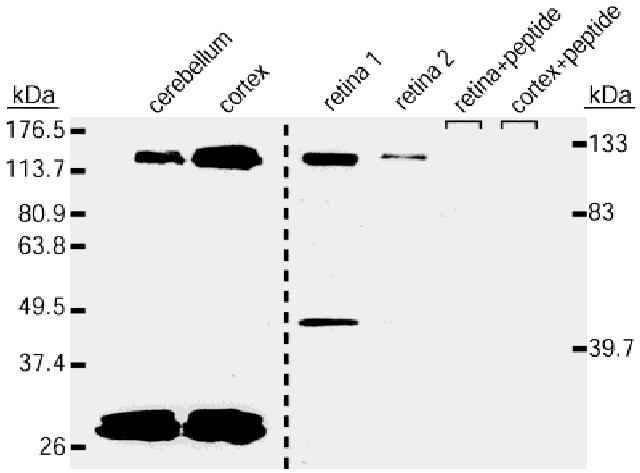


**Fig. 1.** Antibodies against kainate receptor subunits GluR6/7 are specific in primate brain and retina. Western blots of cerebellum and cortex (40 $\mu$g/lane, left marker) and of retina (15 $\mu$g/lane, right marker) demonstrate a single band at a molecular weight of 115–118 kDa. Lower bands at 29 kDa (brain) and 42 kDa (retina) represent degraded protein. These bands are highly dissimilar from preparation to preparation, as indicated in the blots of retina, and are probably due to varying levels of glycosylation and proteases in the tissue. Preabsorption with GluR6 peptide in presence of 5-fold excess of the primary antibody eliminated completely all bands recognized by the antibody (rightmost lanes).

either 6–12 h at room temperature for light microscopy (LM) or 4–6 days at 4°C for electron microscopy (EM). After rinsing in PBS, sections were placed in secondary-antibody solution for either 2 h at room temperature (LM) or overnight at 4°C (EM). All light micrographs were obtained at 20–100× magnification using an Olympus Provis AX70 digitally interfaced with a semicooled CCD camera (RT-Spot, Diagnostic Instruments, Sterling Heights, MI) and image capture software (ImagePro, MediaCybernetics, Carlsbad, CA). Using this software, pixel levels were adjusted uniformly to maximize contrast in the images.

For EM, the secondary-antibody solution contained 0.8% BSA, 0.1% gelatin, and 5% NGS in PBS. We chose to forego the use of permeabilizing agents such as Triton X-100 to optimize the ultrastructure of our tissue for serial sectioning. For monoclonal primary antibodies, normal horse serum was substituted for NGS. For visualizing the reaction product for EM, we followed with a few modifications a well-established protocol used for preembedding immuno-EM (Sassoè-Pognetto et al., 1994; Brandstätter et al., 1997; Morigiwa & Vardi, 1999). Sections for LM were rinsed in PBS and coverslipped using Mowiol or Aqua-poly mounting media. Sections for EM were incubated in Extravidin (Sigma Chemical Co., St. Louis, MO; 1:300) in PBS + 1% BSA for 1.5–2 h in the dark at room temperature, rinsed, and transferred to Tris buffer. Following conjugation with 3,3′-diaminobenzidine (DAB), sections were fixed for 45 min in 2% glutaraldehyde in cacodylate buffer and rinsed in cacodylate overnight at 4°C. To enhance the DAB product, sections were incubated in a solution of 2.6% hexamethylenetetramine, 0.2% silver nitrate, and 0.2% disodium tetraborate in double-distilled water (dd$H_2O$) for 10 min at 60°C. Sections were treated with 0.05% gold chloride in dd$H_2O$ for 3 min at room temperature, rinsed, and incubated in 2.5% sodium thiosulfate in dd$H_2O$. Sections were postfixed in 1% osmium in cacodylate buffer for 15–30 min, rinsed in cacodylate overnight, dehydrated in graded alcohols, and embedded in Epon-Araldite at 60°C for 48 h. Serial ultrathin sections were cut at 70 nm and photographed under an Hitachi 7100 electron microscope (15,000–40,000× magnification). Surface renderings of reconstructed rods were computed using *SURFdriver* software (University of Alberta).

## Results

### *Light-microscopical localization of kainate receptor subunits*

We compared the localization of kainate subunits using immunocytochemistry with two different antibodies (Figs. 2 & 3). Staining of 20–40 $\mu$m thick vertical sections with polyclonal antibodies that recognize common regions of the GluR6 and 7 subunits reveals diffuse bands of processes in the outer plexiform layer (OPL) that correspond to what are likely to be the processes of horizontal cells and dendrites of bipolar cells (Fig. 2A). In thinner cryosections, this GluR6/7 label is sometimes sufficiently intense to outline an entire bipolar or horizontal cell body (Fig. 2B). In our material, we consistently observe pockets of label that outline the axon terminals of photoreceptors (arrows in Figs. 2A & 2B); this pattern probably corresponds to processes deep within the synaptic clefts of the terminals. In these same sections, intense label in the inner plexiform layer (IPL) also appears which we did not investigate further in this study (but see Grünert et al., 2002). At higher magnifications, label in the OPL often forms clouds of discrete puncta that accent processes tightly associated with the base of the photoreceptor axon terminal, both cones (Fig. 2C), and rods (rightmost in Fig. 2D). In most of our sections, photoreceptor inner or outer segments also react to various degrees with these antibodies, which we interpret as nonspecific binding.

Because we often encountered variability from section to section in the intensity of GluR6/7 staining at the light-microscopic level, we also investigated staining using monoclonal antibodies that recognize a common epitope of the GluR5, 6, and 7 subunits. In cryosections, GluR5/6/7 label is most prominent in the OPL, outlining intermittent bands of puncta that again highlight the base of the photoreceptor axon terminal (Fig. 3A). Different layers of cell bodies in the inner nuclear layer (INL) are also stained, as are the cell bodies of ganglion cells. Micrographs obtained by combining epifluorescence and differential interference contrast (DIC) optics demonstrate a consistent row of GluR5/6/7 immunoreactive puncta along the basal surface of the cone and rod axon terminal and elsewhere in the distal portion of the OPL (Fig. 3B). With the GluR5/6/7 antibody, label also outlines discretely the photoreceptor axon terminal and the ascending axon itself, such as the cone in the middle of Fig. 3B. This staining of the axonal membrane may reflect the strong affinity of the antibody for processes deeper within the photoreceptor synaptic complex and not necessarily label within the entire axon itself (see below). At higher magnifications, the GluR5/6/7 label in the OPL appears as more discrete localization about the base of the axon terminal of individual rod and cone photoreceptors (Fig. 3C). Micrographs again applying DIC optics with epifluorescence to stained paraffin sections highlight discrete labeling within the axon terminal of both cones (upright arrow in Fig. 3D) and, even more strikingly, of rods (upside-down arrows in Fig. 3D). In cryosections, where membrane ultrastructure is more compromised than in paraffin sections, label in the OPL appears as puncta that delineate the structure of the axon terminal of both rods and cones (Fig. 3E).

Although much of the weaker labeling within the photoreceptor inner segment and axon is most likely due to nonspecific binding,

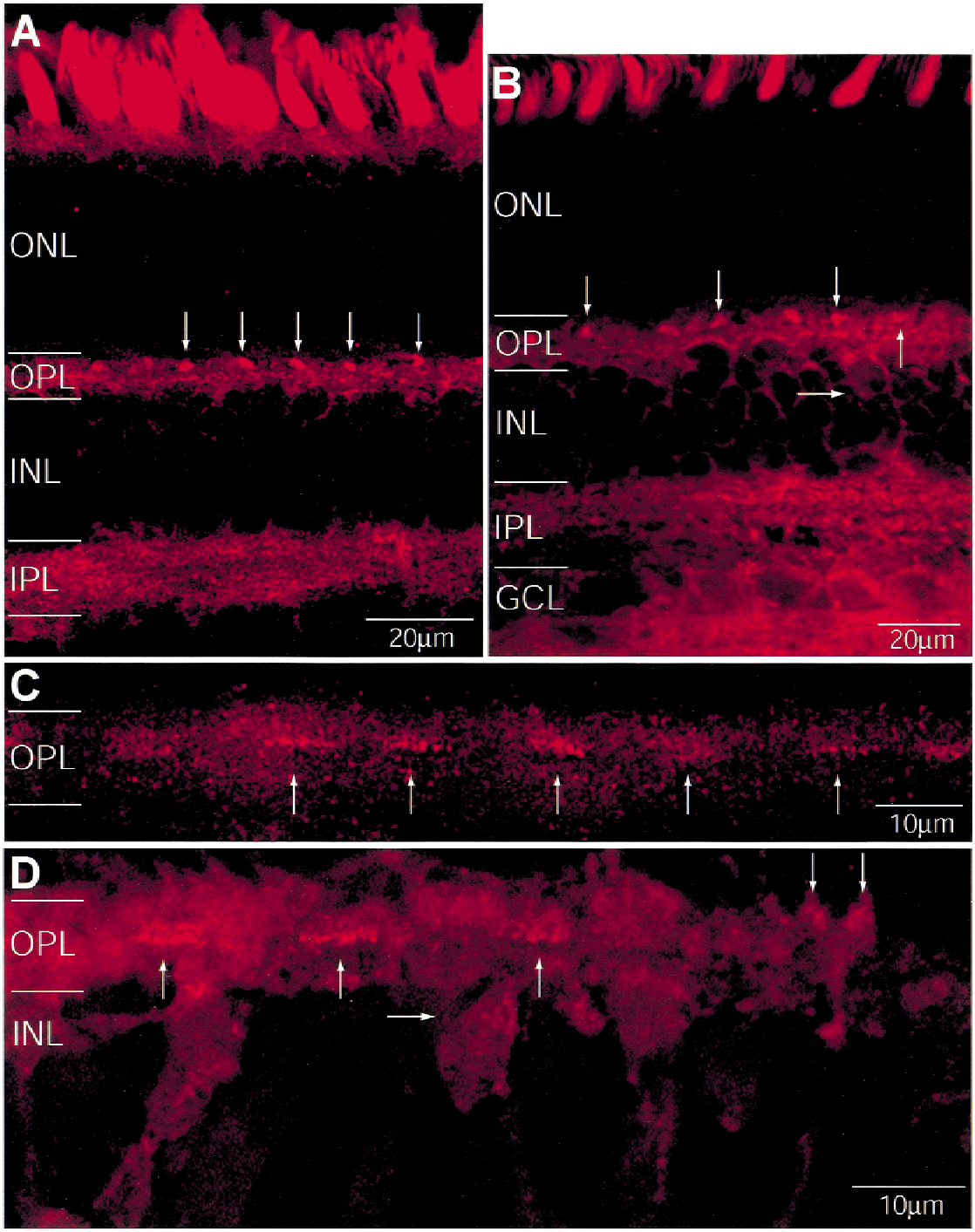


**Fig. 2.** GluR6/7 subunits localize near photoreceptor axon terminal. (A) Fluorescent micrograph of vertical thick section prepared with a vibratome labeled with polyclonal antibodies against GluR6/7. In the OPL, thin bands of labeled processes distribute below what appear to be labeled photoreceptor axon terminals, probably those of rods because of their spherical shape (arrows). Label in the IPL was less intense, delineating several thin streaks of localization. Photoreceptor inner segments (topmost structures) absorb many antibodies nonspecifically, including those against GluR6/7. (B) Fluorescent micrograph of a GluR6/7-labeled cryosection also highlights localization near or within the axon terminal of rod (upside-down arrows) and cone (upright arrow) photoreceptors. In this section, a bipolar cell with descending axon (lateral arrow) is also labeled. (C) High-magnification micrograph of OPL (cryosection) demonstrates that GluR6/7 localization beneath photoreceptor axon terminals can be extremely discrete, in this case, accentuating the base of cone photoreceptors (arrows). (D) High-magnification micrograph of paraffin section indicates clear localization of GluR6/7 at both cone (upright arrows) and rod (upside-down arrows) photoreceptor axon terminals. In this section, the immunoreaction also outlines the cell body of several horizontal and/or bipolar cells (horizontal arrow). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; and GCL: ganglion cell layer.

the label obtained with both the GluR6/7 and the GluR5/6/7 antibodies is consistently intense and punctate in nature near or within the axon terminal. Thus, at the light-microscopic level, the pattern of both GluR6/7 and GluR5/6/7 immunostaining suggests localization to processes within the synaptic cleft of both rods and cones, or perhaps localization to the axon terminal itself (see Fig. 3D). This pattern compelled us to consider the hypothesis that, in addition to documented ultrastructural sites of postsynaptic localization in primate retina (Haverkamp et al., 2001), kainate receptor subunits are expressed by the photoreceptors themselves at presynaptic sites within the axon terminal.

### *Ultrastructural localization of kainate receptor subunits*

We next investigated the precise ultrastructural localization of kainate subunits at the photoreceptor synaptic complex using the GluR6/7 antibodies we characterized by Western blot (Fig. 1) for preembedding immuno-EM. The punctate GluR6/7 label at the base of the cone terminal apparent in light micrographs (Figs. 2C, 2D, 3B, & 3D) corresponds at the EM level to several distinct foci of localization (Fig. 4). As expected, GluR6/7 localizes to bipolar cell dendrites along the basal surface of the terminal ("B" in Figs. 4A & 4B). However, label is also apparent in bipolar cell dendrites in the semiinvaginating position along the wall of the cone active zone ("S" in Figs. 4A & 4D). We also observed label in the horizontal cell processes that form the triad at cone ribbon synapses (not shown). Our general observations for the localization of kainate receptors at the cone terminal are similar to those for a recent study of primate retina (Haverkamp et al., 2001), and we will describe them quantitatively in a subsequent investigation.

What is perhaps most striking in our electron micrographs of cone terminals is frequent and intense localization of GluR6/7 to small protrusions of presynaptic membrane that extend beneath one or both horizontal cell processes at the synaptic ribbon ("P" in Figs. 4A & 4C). In our material, the GluR6/7 localization generally is confined to the very tip of the presynaptic protrusion at the cone triad (Fig. 4E). Although we did not focus on the cone terminal for this study, a preliminary count indicated that, of 75 labeled processes at 20 cone terminals, about 15% corresponded to presynaptic protrusions. This presynaptic label is generally robust and highly concentrated in a small, focal area (Figs. 4C & 4E), not at all like the random localization one would expect from nonspecific binding. These protrusions are analogous to the "collars" of axonal cytoplasm described for rods and cones in the rat retina (Fletcher et al., 2000) and to the "fingers" of cytoplasm that have been investigated in greater detail for the rod axon terminal in cat retina (Rao-Mirotznik et al., 1995). Indeed, since each rod terminal has multiple "fingers" of presynaptic cytoplasm in regular positions enclosed within its single invagination (Rao-Mirotznik et al., 1995), it is there that we now turn our attention.

The rod axon terminal generally contains only a single synaptic ribbon or at most two. Careful measurements indicate, however, that this single ribbon is quite large, comprising about 130 evenly spaced sites for vesicle docking and forming an arch that spans a sharp invagination within the axon terminal with radius of 400–500 nm (Rao-Mirotznik et al., 1995). The invagination is relatively deep, about one-third the diameter of the entire rod terminal. Even so, the number of postsynaptic processes that the invagination accommodates is limited—two horizontal cell processes and 2–4 rod bipolar cell dendrites, each probably from a different cell (Grünert & Martin, 1991; Rao-Mirotznik et al., 1995). The horizontal cells invariably flank the ribbon on either side, while the rod bipolar cell dendrites penetrate to the central positions of the invagination. The fingers of presynaptic, axonal cytoplasm insert between the dendrites of the rod bipolar cell and fill in unoccupied extracellular volume (Rao-Mirotznik et al., 1995).

We found that a cursory examination of a number of rod terminals in any single section under the EM indicated a highly dense localization of GluR6/7 within the invagination, about 500 nm from the ribbon (Figs. 5A–5C). The processes containing the GluR6/7, because of their location, could be mistaken upon first inspection as the tips of the invaginating dendrites of the rod bipolar cell. However, the labeled processes occupy locations in the invagination also similar to the positions of the rod presynaptic fingers (Figs. 5D & 5E). The cytoplasm of many of these fingers contains synaptic vesicles, like the rest of the axon terminal (for example, the fingers in Figs. 5C–5E).

Since single ultrathin sections can often be misleading, we followed numerous rod axon terminals through serial sections at high magnification under the EM. We found that many of the labeled processes in the rod invagination actually originate from cytoplasmic fingers extending from the rod axon terminal (white arrows in Figs. 6A–6F). Thus, these examples of GluR6/7-labeled processes in the rod invagination are not postsynaptic, but *presynaptic*, as suggested by our light microscopy (Figs. 2 & 3).

Our three-dimensional reconstructions of partial rod terminals demonstrate a highly regular geometry in which the rod terminal inserts multiple cytoplasmic fingers about 0.2 $\mu$m in diameter into the postsynaptic invagination, each of which expands into a bulb of roughly twice that diameter (Figs. 7A & 7B). Though not every cytoplasmic finger we examined contained GluR6/7 label (Figs. 5, 6, & 7B), the pattern of GluR6/7 expression in the rod invagination is remarkably consistent. We sampled several independent groups of serial sections from the retina of multiple animals. Each group represented 0.2–0.4 $\mu$m in retinal depth across typically 3–5 sections. We identified 54 rods with excellent fixation whose postsynaptic space included one or more processes containing GluR6/7 and traced each process at high magnification. Of 68 distinct processes that contained label, 63% definitively originated from a presynaptic finger of cytoplasm (Figs. 7C & 7D). Many of the remaining processes resembled cytoplasmic fingers in that they contained vesicles, but did not join the terminal membrane within the sample sections and therefore could not be scored as fingers. We also identified several labeled processes as horizontal cells based on their location just lateral to the synaptic ribbon (not shown). Most often, this label was confined to a single member of the pair at a particular ribbon, as in other species (Brandstatter et al., 1997; Morigiwa & Vardi, 1999; Qin & Pourcho, 2001). A complete rod terminal typically inserts 4–6 presynaptic fingers (Rao-Mirotznik et al., 1995), and we found several instances where 2–3 fingers were labeled in a particular invagination (Fig. 7C). Our samples of serial sections only rarely contained complete invaginations, which are usually 0.8–1.5 $\mu$m deep, so our counts probably underestimate the occurrence of multiple labeled fingers at the rod synapse. The examples we did find support the idea that each presynaptic finger expresses GluR6/7.

## Discussion

### *Localization of kainate receptors*

An abundance of immunocytochemical studies demonstrate widespread expression of kainate receptors across the vertebrate retina (e.g. Peng et al., 1995; Morigiwa & Vardi, 1999; Schultz et al.,

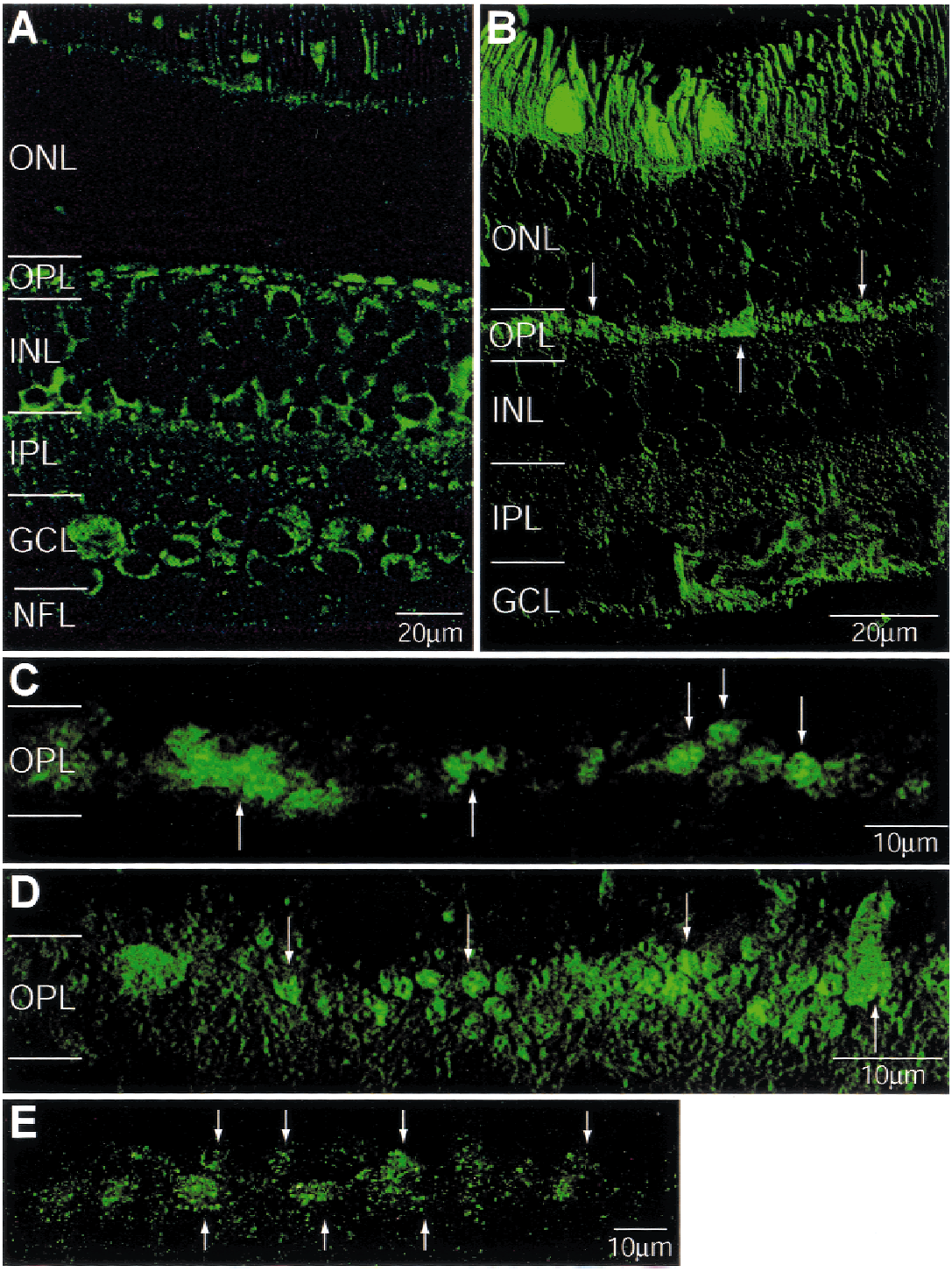


**Fig. 3.** Antibodies against GluR5/6/7 subunits localize intensely at photoreceptor axon terminal. (A) Fluorescent micrograph of vertical cryosection labeled with monoclonal antibodies against GluR5/6/7 reveals intense localization in OPL, highlighting what appear to be photoreceptor axon terminals. Ganglion cell bodies are also labeled. (B) Fluorescent micrograph viewed through DIC optics of a GluR5/6/7-labeled paraffin section. Immunoreactive puncta accent the basal surface of the axon terminal of cone (upright arrows) and rod (upside-down arrows) photoreceptors in the OPL. (C) High-magnification micrograph of GluR5/6/7-labeled paraffin section without DIC optics demonstrates discrete localization in OPL beneath the axon terminals of both cone (upright arrows) and rod (upside-down arrows) photoreceptors. (D) High-magnification of fluorescent micrograph viewed through DIC optics of GluR5/6/7-labeled paraffin section again highlights discrete labeling at a cone axon terminal (upright arrow on the far right) and also a strong signal at rod axon terminals (upside-down arrows). (E) GluR5/6/7-labeled cryosection demonstrates punctate localization at cone (upright arrows) and rod (upside-down arrows) axon terminals. This pattern is similar to that shown in Fig. 2C with GluR6/7. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; and NFL: nerve fiber layer.

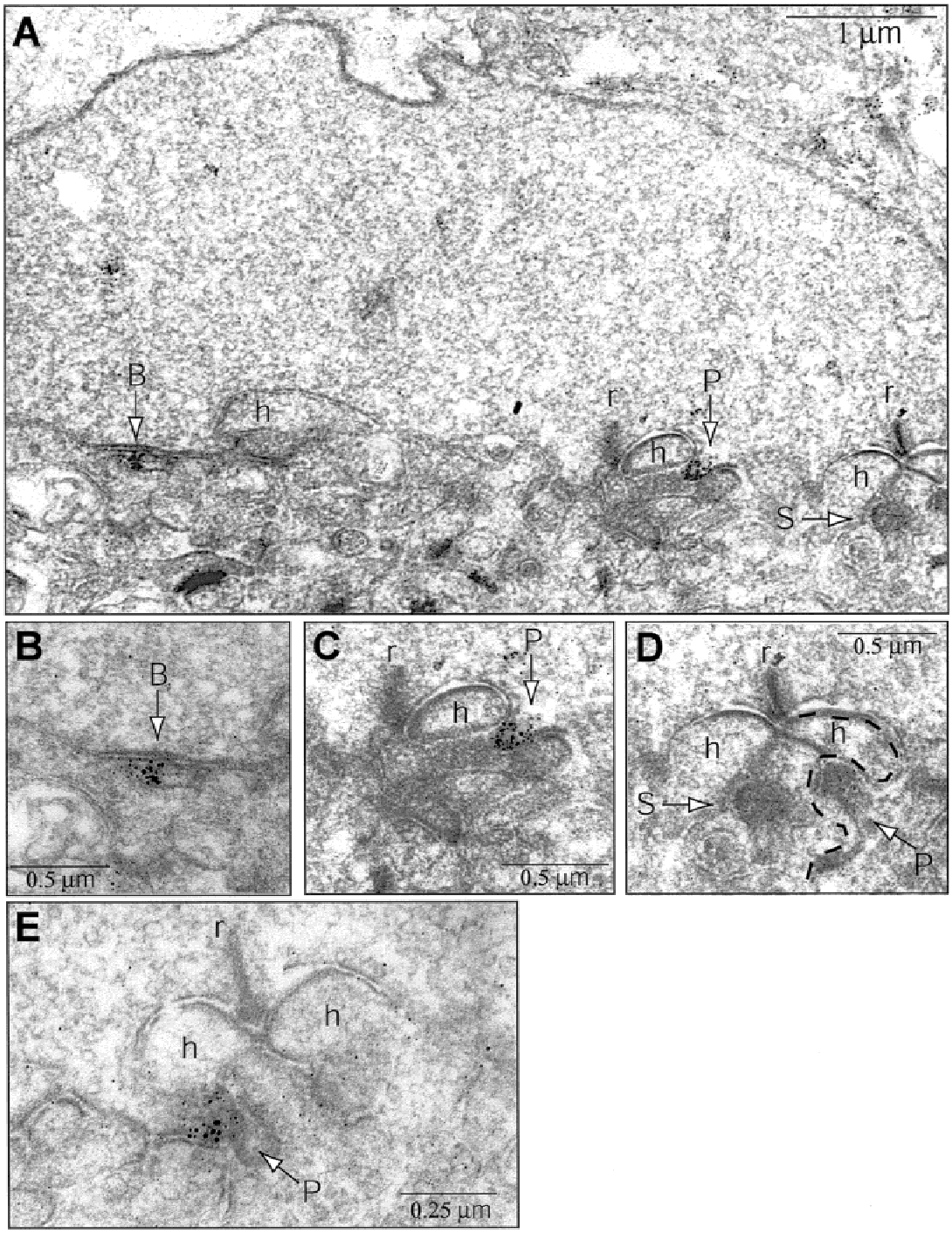


**Fig. 4.** GluR6/7 localizes at postsynaptic and presynaptic sites along the cone axon terminal. (A) Electron micrograph of cone axon terminal. GluR6/7 localizes within a bipolar cell dendrite postsynaptic to the cone along the basal surface of the terminal (B, far left) and within another bipolar cell dendrite in a semiinvaginating (S) position along the wall of a synaptic active zone. GluR6/7 also localizes within a "finger" of presynaptic cytoplasm (P) beneath a horizontal cell process (h) and proximal to a presynaptic ribbon (r). (B–D) Higher magnifications of the labeled structures in (A). In (D) is shown another example of light label contained within a presynaptic finger; the edge of the cone membrane is outlined for clarity. (E) An example of GluR6/7 within a presynaptic finger (P) near a synaptic ribbon (r) in a different cone terminal. Note the presence of synaptic vesicles in the presynaptic finger.

2001). Some of these also provide an idea of the relative abundance of particular subunits. Brandstätter et al. (1997) found in rat retina more pronounced localization of GluR6/7 than the KA2 subunit. Haverkamp et al. (2001) demonstrated in monkey retina a highly focal localization of KA2 to the OPL, with more widespread localization of GluR5 and GluR6/7; GluR6/7 was also more highly expressed than GluR5. Using subunit-specific antibodies, Qin and Pourcho (2001) found in cat retina that GluR7

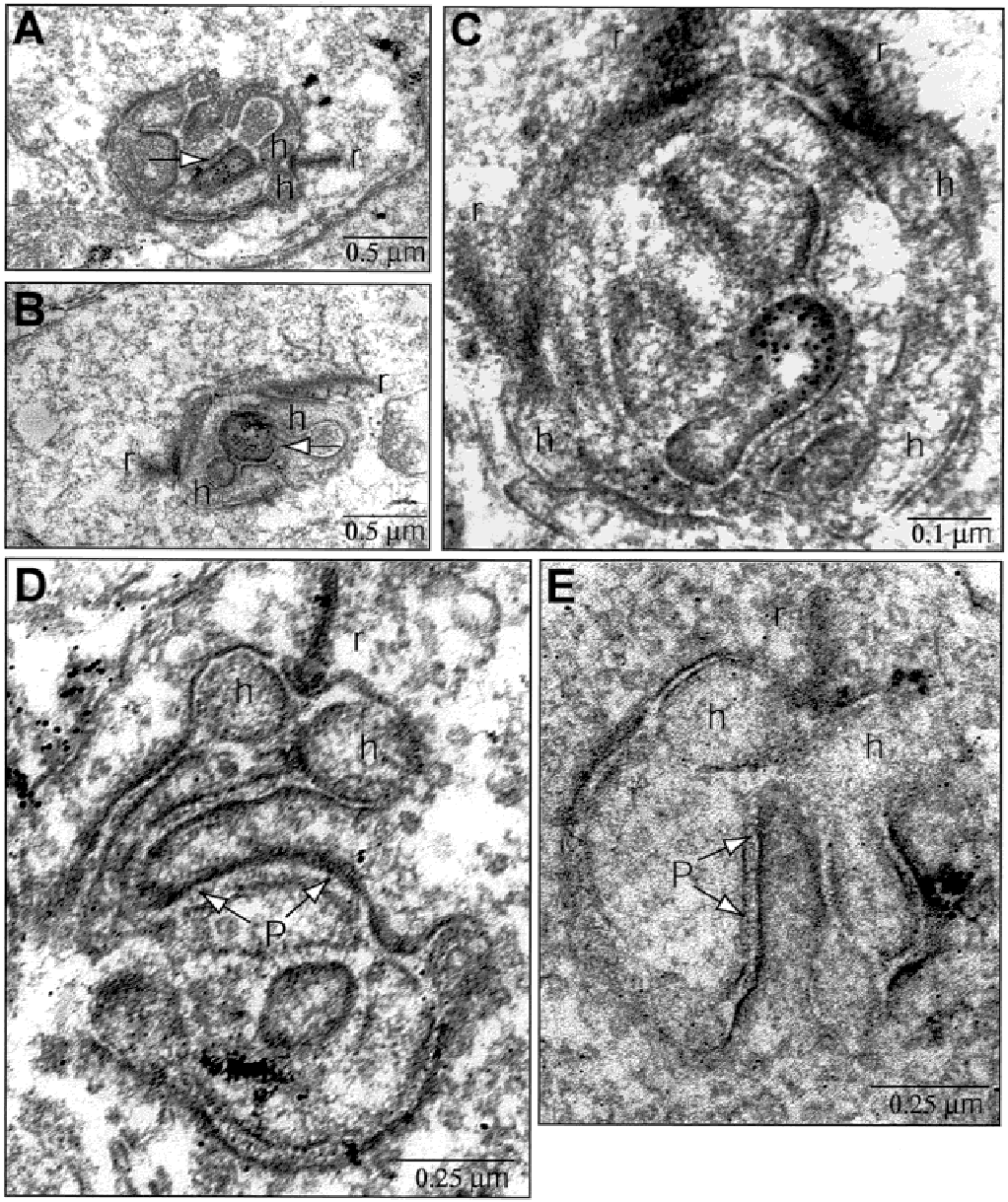


**Fig. 5.** GluR6/7 localizes within the synaptic cleft of the rod axon terminal. (A,B) Electron micrographs of two rod terminals with GluR6/7 label localized to processes within the rod invagination (arrows). Generally, a single synaptic ribbon arches over the invagination (Rao-Mirotznik et al., 1995), so that in a single section, the tips of the ribbon (r) anchor at opposite ends of the postsynaptic cleft and appear to form not one, but two active zones (as in B). Each tip of the ribbon points between a pair of inhibitory horizontal cell processes (h). (C) Higher magnification of GluR6/7 label within the invagination of another rod terminal. In this case, the rod terminal had two synaptic ribbons stretching over a single active zone. The cytoplasm of the process containing label appears to contain synaptic vesicles. (D,E) High-magnification micrographs of the synaptic active zone of two rod terminals with presynaptic fingers of axonal cytoplasm (P) extending into the postsynaptic cleft. Although the fixation of this material is not optimal for ultrastructure, the cytoplasm of the presynaptic fingers in these examples appears to contain synaptic vesicles, like the remaining presynaptic terminal.

is weakly expressed, with more prominent expression of both GluR5 and GluR6. Our labeling with antibodies against common regions of GluR5/6/7 tends to be more intense than our results using the GluR6/7 antibody (compare Figs. 2 & 3). While not quantitative, the immunocytochemical data as a whole suggest higher levels of GluR5 and GluR6 than of either GluR7, KA1, or KA2.

Our light microscopy for both GluR6/7 and GluR5/6/7 demonstrates intense staining at the photoreceptor axon terminal (Figs. 2 & 3) that we now interpret, based on our EM results, as representing both postsynaptic and presynaptic expression in the synaptic cleft. Our use of thin paraffin sections, in which membranes are compromised in the course of clearing the tissue, especially accentuates the punctate nature of the GluR5/6/7 staining (Figs. 3B

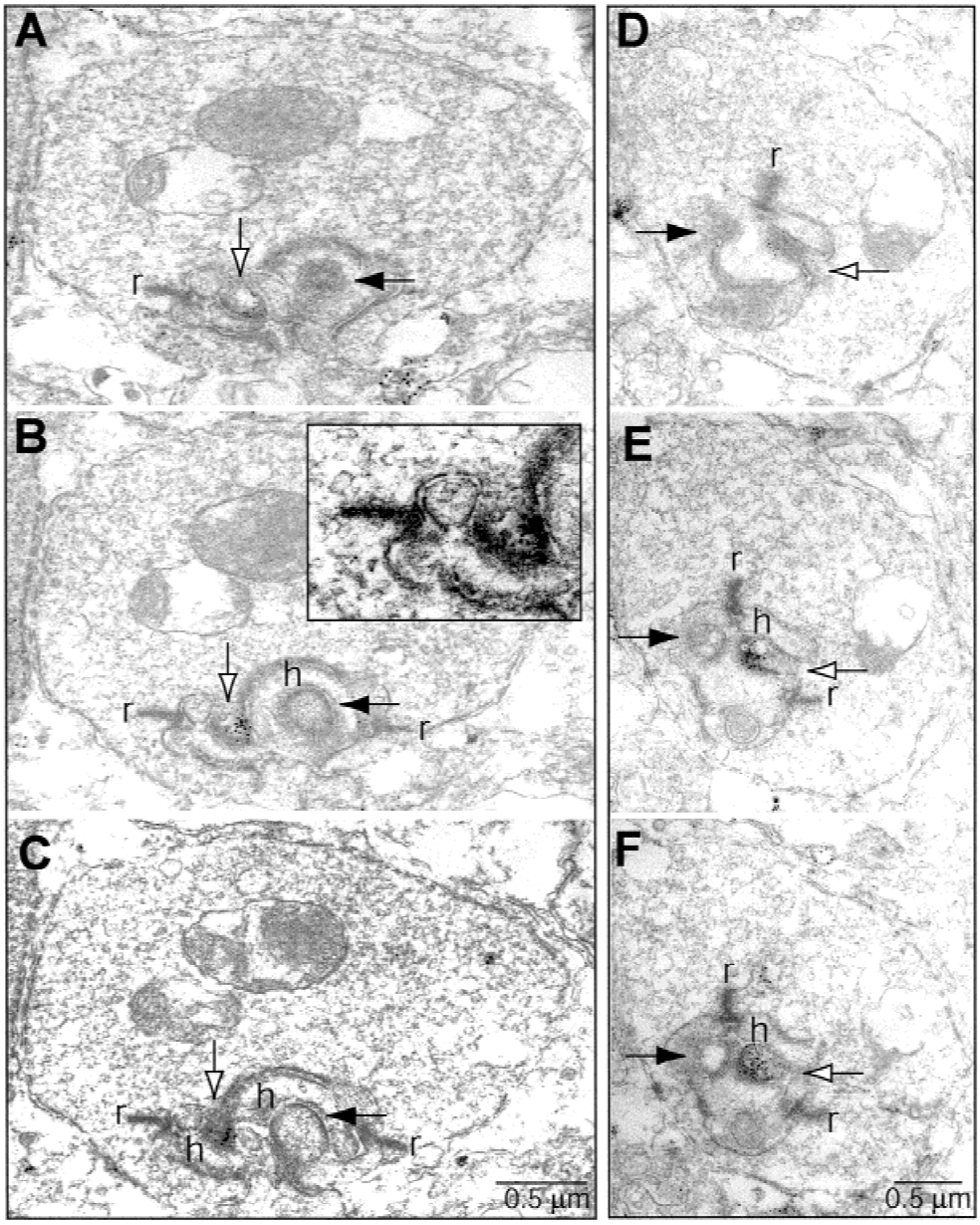


**Fig. 6.** Presynaptic fingers of rod axonal cytoplasm express Glur6/7. (A–C) Electron micrographs of a rod axon terminal traced through three serial sections. GluR6/7 distributes heavily within a finger of presynaptic cytoplasm in the rod invagination (white arrow). Fingers are 300–700 $\mu$m from the synaptic ribbon (r), roughly equidistance from each release point along the ribbon. At most rod terminals, we also observed one or more unlabeled fingers within the cleft (black arrow). Inset in (B) shows higher magnification micrograph of synaptic ribbon and labeled presynaptic finger, clearly showing vesicles within the finger. (D–F) Electron micrographs of a second rod terminal in three serial sections. Both a labeled (white arrow) and an unlabeled (black arrow) finger are apparent in these sections.

& 3D). Our results are similar in pattern, if not in resolution, to those obtained in a recent study of kainate receptors in primate retina photographed using laser confocal microscopy (Haverkamp et al., 2001). Our ultrastructural localization of GluR6/7 at the cone axon terminal (Fig. 4) is similar to the localization of kainate receptors in other studies of rat, cat, and monkey retina. In particular, for these species, one or more subunits of kainate receptor localize to the basal dendrites of putative OFF bipolar cells and, less regularly, to the processes of horizontal cells (Brandstätter et al., 1997; Morigiwa & Vardi, 1999; Qin & Pourcho, 2001; Haverkamp et al., 2001). In fish retina, kainate receptors again localize to a variety of bipolar cell dendrites (Schultz et al., 2001). Since this pattern seems to be conserved across species, it is likely to account for the kainate-receptor-mediated "OFF" response of some cone bipolar cells in ground squirrel retina (DeVries & Schwartz, 1999; DeVries, 2000) and the response of horizontal cells to kainate (Marc, 1999; Blanco & de la Villa, 1999).

We have demonstrated that in addition to postsynaptic expression, there exists a highly regular pattern of localization of Glur6/7 to "fingers" of presynaptic axonal cytoplasm that has not been

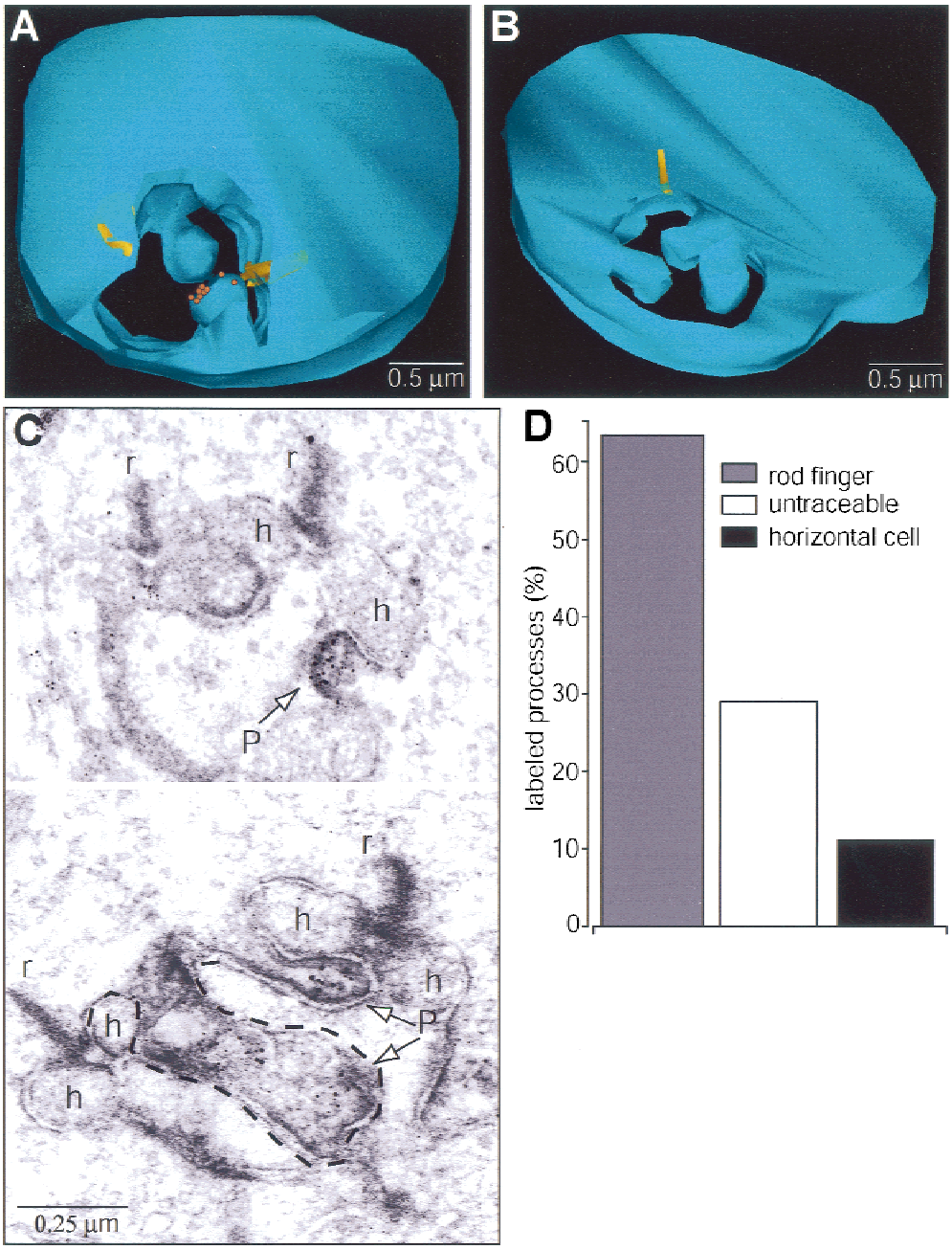


**Fig. 7.** Reconstructions of rod axon terminals and quantification of GluR6/7 label. (A) Three-dimensional rendering of a partial reconstruction through the rod terminal in Fig. 3A. Two presynaptic fingers within the sampled sections insert into the postsynaptic invagination (enclosed black space), one of which contains GluR6/7 (red dots). The reconstruction includes the tips of the synaptic ribbon (yellow) arching over the invagination. For clarity, horizontal and bipolar cell processes are not shown. (B) Partial three-dimensional reconstruction of another rod terminal. While these sections did not contain GluR6/7 label, this reconstruction demonstrates that many rods have multiple presynaptic fingers in a confined volume. Only a portion of the synaptic ribbon (yellow) appears in these sections. (C) High-magnification electron micrographs of the synaptic active zone of two rod terminals with prominent GluR6/7 label within fingers of presynaptic cytoplasm (P), which also appear to contain synaptic vesicles. In the bottom example, a large presynaptic finger whose membrane is slightly obscured is outlined for clarity. r: synaptic ribbon; and h: horizontal cell process. (D) Bar chart comparing the distribution of Glur6/7 across 68 labeled processes within the synaptic cleft of 54 rod axon terminals; 63% of these processes were presynaptic.

reported before (Figs. 4–7). Our fixation was more intense than that used in the study of primate retina by Haverkamp et al. (2001), so the penetration of our antibodies for EM was not as strong. This difference probably explains why label beneath the cone terminal was not as profound as in their study. However, because stronger fixation preserves ultrastructure, we were able to trace and reconstruct the labeled processes in the rod invagination through serial sections. Each rod inserts 4–6 presynaptic fingers, and we found

instances from samples of different retina where 2–3 fingers were labeled at a particular rod (e.g. Fig. 7C). Because of these examples, we believe it is likely that each presynaptic rod finger expresses Glur6/7.

### *A possible role for presynaptic kainate receptors at the rod terminal*

The low-affinity GluR5/6/7 subunits are able to form functional homomeric channels, while the higher-affinity KA1 and KA2 subunits are not (Chittajallu et al., 1999). Other immunocytochemical studies suggest that KA2 may combine in the retina with either GluR5 or GluR6 to create a higher-affinity functional channel (Chittajallu et al., 1999; Haverkamp et al., 2001). Indeed, KA2 localizes to processes in the rod postsynaptic cleft, but this localization has been interpreted as binding of the antibody to an unknown protein within the dendrites of the rod bipolar cell (Haverkamp et al., 2001). Our EM results raise the possibility that this localization may be real and indicative of presynaptic expression of KA2 at the rod finger.

Native kainate receptors elicit a robust but highly transient inward current that quickly decays, with desensitization of the receptor, to a steady fraction (0.2–1.5%) of its peak value (Wilding & Huettner, 1997; Paternain et al., 1998; DeVries & Schwartz, 1999). This post-desensitization "window" current arises from overlap between the kinetic processes of inactivation and activation and occurs within a narrow range of glutamate concentrations, roughly 10–700 $\mu$M. It is intriguing that the amplitude of the window current peaks near 100 $\mu$M glutamate, the estimated average concentration in the rod invagination (Shiells & Falk, 1994; Rao-Mirotznik et al., 1998). Though the residual window current is only a small fraction of the maximal transient activation current, its amplitude can be quite high. Kainate receptors on OFF cone bipolar cells produce transient glutamate currents of 200–700 pA and corresponding window currents of 7–15 pA (DeVries & Schwartz, 1999), and receptors on cultured hippocampus neurons produce maximal currents as high as 300 pA and window currents of 5–10 pA (Paternain et al., 1998). Either of these ranges would represent a substantial portion of the 18–40 pA standing dark current in the rod (Baylor et al., 1984).

There is growing evidence from other brain regions that presynaptic kainate receptors can bind glutamate and modulate its release (reviewed in Ozawa et al., 1998). In synaptosomes prepared from hippocampus, presynaptic kainate receptors enhance glutamate release by depolarization of the presynaptic membrane (Gannon & Terrian, 1991; Malva et al., 1996). Similarly, activation of pre-synaptic kainate receptors depolarizes mossy fiber axon terminals in the CA3 region and Schaffer collaterals in the CA1 region (Petralia et al., 1994; Chittajallu et al., 1996; Schmitz et al., 2000, 2001; Lauri et al., 2001). At the rod axon terminal, by inserting near the center of the invagination (Fig. 7), each presynaptic finger places the cluster of Glur6/7 roughly equidistance from each release site along the ribbon. At this location, the spatial variation in the rate of release from different release points should be minimal, and the inward current subject to less variability. Were the Glur6/7 placed nearer the ribbon, the concentration of glutamate would be 2–3 orders of magnitude greater (Rao-Mirotznik et al., 1995), well beyond the 500 $\mu$M or so upper limit for a meaningful window current (Paternain et al., 1998). While recovery from desensitization is slow, on the order of seconds (Paternain et al., 1998; DeVries & Schwartz, 1999), the sustained inward current from kainate receptors is quickly shut down in response to a dramatic drop in glutamate (DeVries & Schwartz, 1999). Thus, the Glur6/7 at the rod presynaptic membrane should aid in quickly reducing vesicle release after a photon event. With recovery of the rod after a photon event, the large transient current mediated by the Glur6/7 would more quickly depolarize the terminal and "reload" the synapse. The fact that we also observed occasional presynaptic Glur6/7 near the ribbon synapse in cone terminals indicates that the glutamate-induced window current could be a more regular feature of tonic synapses.

Though their precise function is not known, presynaptic kainate receptors may work in concert with other glutamate receptors known to localize to the photoreceptor presynaptic membrane. The second-messenger-gated mGluR1$\alpha$ and mGluR8 subunits localize heavily throughout the axon terminal of some photoreceptors (cat retina: Cai & Pourcho, 1999; rat retina: Koulen et al., 1999), as do the ligand-gated N-methyl-d-aspartate (NMDA) subunits NR2B and NR1C (rat: Fletcher et al., 2000; cat: Pourcho et al., 2001). The subcellular localization of the NR1C subunit at the rod terminal in rat retina includes the entire axon terminal, but, like GluR6/7, NR1C is also heavily concentrated in presynaptic fingers (Fletcher et al., 2000). Even so, the localization of GluR6/7 to presynaptic fingers within the rod invagination we describe here is virtually unique in its precision and near-perfect confinement to the very tips of the fingers (e.g. Fig. 7C). This implies that the position of the GluR6/7—equidistant from each release point along the curvature of the synaptic ribbon—is critical to its function as an "autoreceptor".

Our broad hypothesis is that GluR6/7 subunits at the rod presynaptic finger are generally maintained in a desensitized state, due to high levels glutamate in the postsynaptic cleft. In this state, the GluR6/7 would maintain a steady inward current to promote depolarization of the rod membrane and the influx of calcium, as in neurons of the brain. With the absorption of a photon, the window current would quickly decrease due to the sharp drop of glutamate in the rod invagination (DeVries & Schwartz, 1999). Interestingly, the effect of presynaptic second-messenger-gated mGluR8 subunits on photoreceptors is precisely the opposite configuration: these receptors in rat retina decrease calcium influx to the photoreceptor terminal to presumably inhibit the release of glutamate (Koulen et al., 1999). How mGluR8 and GluR6/7 would interplay with presynaptic mGluR1 and NMDA subunits is unkown (Cai & Pourcho, 1999; Fletcher et al., 2000). Cone photoreceptors in salamander retina respond strongly to their own glutamate, with an outward current that has been attributed to a glutamate-transporter-gated chloride channel (Picaud et al., 1995). Our results and the results of other ultrastructural studies suggest that any such response in mammalian photoreceptors is likely to arise at least in part from interactions between a variety of glutamate receptor subunits expressed directly at the presynaptic active zone.

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