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Foveal Cones form Basal as well as Invaginating Junctions with Diffuse ON Bipolar Cells

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The response of a mammalian bipolar cell is generally thought to be determined by the location and morphology of synapses from the cone terminal: ON bipolar cells are believed to be depolarized strictly at invaginating contacts and OFF bipolar cells hyperpolarized at basal contacts. This hypothesis was re-investigated in the macaque fovea (1 deg nasal) using electron micrographs of serial sections. We determined the number of invaginating sites available and then identified the contacts to bipolar cells with axons in the ON level of the inner plexiform layer. A cone terminal forms about 20 active zones marked by ribbons. A few active zones house two invaginating dendrites, so there are 22 invaginating sites per cone. A midget ON bipolar cell collects 18 invaginating contacts from one cone, thus only about four invaginating sites remain for diffuse ON bipolar cells. Two diffuse ON cells were reconstructed; each collects about 25 contacts from an estimated 10 cones. Only three or four of these contacts are invaginating; the rest are basal, adjacent to the triad. This suggests that basal contacts can be depolarizing. The distance from the vesicle release site at active zones to an invaginating contact is 140 ± 40 nm; to a basal contact adjacent to the triad is 500 ± 160 nm, and to the next nearest basal contact is 950 ± 370 nm.

Cone Bipolar cell Fovea Basal synapse Invaginating synapse

INTRODUCTION

The cone synaptic terminal forms two types of junction with bipolar cell dendrites. At the invaginating junction, a bipolar cell dendrite plus two horizontal cell processes form a "triad" and invaginate the base of the pedicle to approach closely the sites of synaptic vesicle release just beneath the synaptic ribbon; at the basal junction, the bipolar cell dendrite simply abuts the base of the cone terminal (Missotten, 1965; Dowling & Boycott, 1966). It is widely believed that in mammals the invaginating contact conveys the ON response, and the basal contact conveys the OFF response (reviewed in Kolb, 1994; Hopkins & Boycott, 1995). This hypothesis arose from the observation that certain bipolar cells with axons at the ON level of the inner plexiform layer receive exclusively invaginating contacts (e.g., the midget and "blue cone" bipolar cells), while bipolar cells with axons in the OFF sublamina receive basal contacts (Kolb, 1970; Mariani, 1981, 1984; Kouyama & Marshak, 1992; Hopkins & Boycott, 1995). The idea has persisted in the face of contrary observations. For example, in cat the most common ON bipolar cell (shown by recordings to be depolarizing) does not invaginate but forms basal contacts just lateral to the triad (Nelson & Kolb, 1983; McGuire *et al.*, 1984; Cohen & Sterling, 1990). Also, in other vertebrate retina some depolarizing bipolar cells form either predominantly basal or a mixture of basal and invaginating contacts (Stell, 1976; Lasansky, 1978; Dacheux, 1982).

The "ON = invaginating" hypothesis might encounter serious difficulty in the macaque fovea where the number of active zones (and thus sites for invagination) per cone terminal is modest (calculable from Kolb, 1970; reviewed by Chun *et al.*, 1996), while several types of ON bipolar cell vie for the invaginating position. Thus, there is a midget ON bipolar cell, known to collect multiple invaginating contacts from a single cone (Missotten, 1965; Dowling & Boycott, 1966; Kolb, 1970; Herr *et al.*, 1995), plus several types of diffuse ON bipolar cell that collect unknown numbers of contacts from multiple cones (Boycott & Dowling, 1969; Mariani, 1981; Boycott & Wässle, 1991; see also Polyak, 1941). So, either the number of midget ON bipolar cell dendrites is restricted (to free sites for diffuse bipolar cell dendrites); or the invagination can house several

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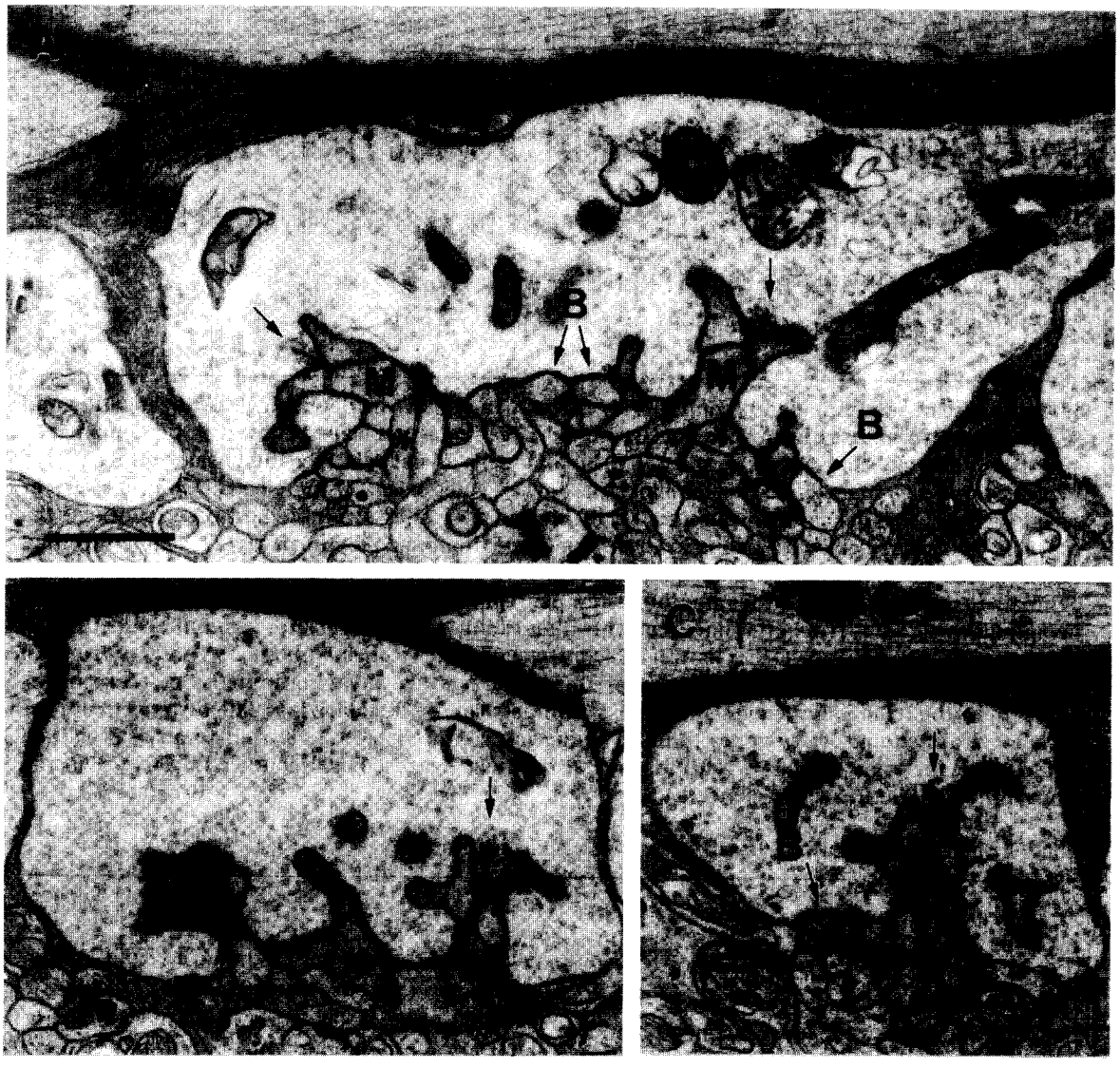

FIGURE 1. (A) Electron micrograph of a cone terminal in vertical view. Two triads are shown (arrows), each with a pair of horizontal cell processes and an invaginating midget bipolar cell dendrite (M). Dendrites from diffuse ON bipolar cell number 1 (asterisks; Table 2, Fig. 4) receive basal contact adjacent to the triads. (B) Electron micrograph of cone terminal 4 (Table 1) in vertical view. A triad is shown (arrow) with an invaginating dendrite (D) from the diffuse ON bipolar cell number 5 (Table 2, Fig. 5). (C) Same as (B), but with an invaginating midget bipolar cell dendrite (M) and a dendrite from diffuse ON bipolar cell number 5 (asterisk) receiving basal contact adjacent to the triad. Basal contacts (B) more distal from the invaginations are also shown. Scale = 1 μm.

dendrites; or some diffuse ON bipolar cells (like those in other species) receive contacts outside the invagination — at basal junctions. We investigated this problem quantitatively in a small patch of macaque fovea by tracing the dendritic branchlets of midget and diffuse ON bipolar cells that collect synapses from the same patch of cones.

METHODS

A retina was obtained from an adult male *Macaca fascicularis* and prepared for electron microscopy (Tsukamoto *et al.*, 1992). Consecutive sections (319) were cut vertically at 90 nm along the horizontal meridian of nasal fovea. A portion of each section containing the outer plexiform layer was photographed at 5000–12,000× and printed with an additional magnification of at least 2.8×. At these magnifications, the electron dense ribbon defining a cone active zone was readily identified (Figs 1–2). The cone pedicles whose bipolar cell connections we studied were located at 500–540 μm eccentricity. Each pedicle was displaced from its inner segment by 305–325 μm, and the retinal magnification factor for this retina was 216 μm/deg. Thus, the inner segment eccentricity was 0.9–1.0 deg.

Our investigation proceeded along two converging directions. First, starting from four neighboring pedicles, we traced the central element in every triad to its source, either a midget or diffuse ON bipolar cell. The dendrite of a diffuse bipolar cell was slender and was contacted by multiple cones, while the dendrite of the midget ON bipolar cell was much thicker and was contacted by only

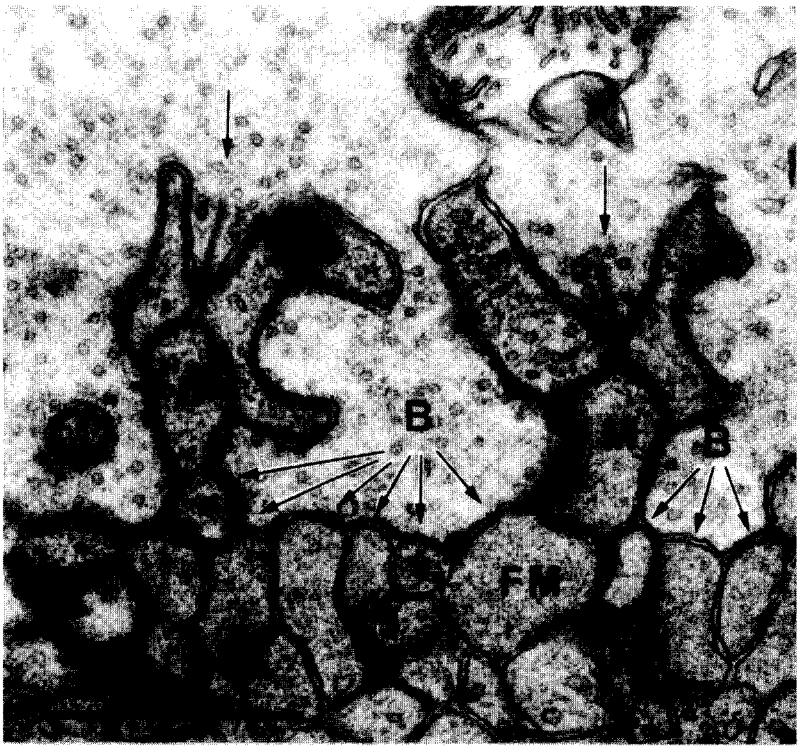

FIGURE 2. Electron micrograph at a higher magnification of a cone terminal in vertical view. Two triads are shown (arrows), housing the dendrite of either a diffuse (left, D) or midget (right, M) ON bipolar cell. Sites of basal contact (B) to the midget OFF bipolar cell (the so-called "flat" midget, FM), to diffuse OFF bipolar cells (asterisks, Calkins *et al.*, 1995) and to other unidentified dendrites are ultrastructurally identical regardless of their location on the terminal (see also Raviola & Gilula, 1975; Hopkins & Boycott, 1995). Scale = 1 μm.

a single cone (Figs 1–3). Second, starting from dendritic stalks, we reconstructed midget and diffuse bipolar cells that were contacted by the four neighboring pedicles, tracing their dendrites through the tissue and identifying the types of synapse they received (Figs 3–5). The tracings were transferred to acetate sheets, then digitized and stacked by computer (Montage software package, Smith, 1987; Cohen & Sterling, 1990, 1992; Calkins *et al.*, 1994).

RESULTS

Organization of the cone terminal

We quantified the pre- and postsynaptic organization of four cone terminals (Table 1). Each had about 20 ribbons (19.8 $\pm$ 1.3), in agreement with other work (Kolb, 1970; Herr *et al.*, 1995; Chun *et al.*, 1996), and each of these was associated with an active zone pointing between a pair of horizontal cell processes to an invagination of the presynaptic membrane (Figs 1–2). The active zones were clustered toward the center of the cone terminal's basal surface within a roughly circular region, about 6 μm across [Fig. 3(B)]. The active zones were rather evenly spaced [Fig. 3(B)]: taking the midpoint of each ribbon as the center of an active zone, the nearest neighbor distance was about 1 μm (1.06 $\pm$ 0.13). Thus, the active zones at the secretory face of the cone terminal have about the same spacing as that of the giant boutons in the brain (Trussell *et al.*, 1993).

Most of the 20 invaginations at a cone terminal (18.0 $\pm$ 2.2) housed a single dendritic twig, and a few invaginations (1.8 $\pm$ 1.0) housed two dendritic twigs, but none housed more than two. Thus, each terminal provided 21.5 $\pm$ 0.6 sites for invaginating contacts to bipolar cells. Most of these sites were occupied by midget ON bipolar cell dendrites (18.0 $\pm$ 1.8). For example, the midget cell shown in Fig. 3 provided a twig to 19 of the 20 invaginations of the cone terminal. This tendency left only a few remaining invaginating sites (3.5 $\pm$ 1.3) free for diffuse bipolar cell dendrites.

The number of distinct midget bipolar cell dendrites penetrating each cone terminal was smaller than the number of midget central elements (14.5 $\pm$ 1.0), indicat-

TABLE 1. Organization of four cone terminals

Presynaptic		Postsynaptic invaginating processes		
Terminal No.	Active zones	Midget ON	Diffuse ON	Total
1	21	20	2	22
2	18	17	4	21
3	20	16	5	21
4	20	19	3	22
Mean $\pm$ SD	19.8 $\pm$ 1.3	18.0 $\pm$ 1.8	3.5 $\pm$ 1.3	21.5 $\pm$ 0.6

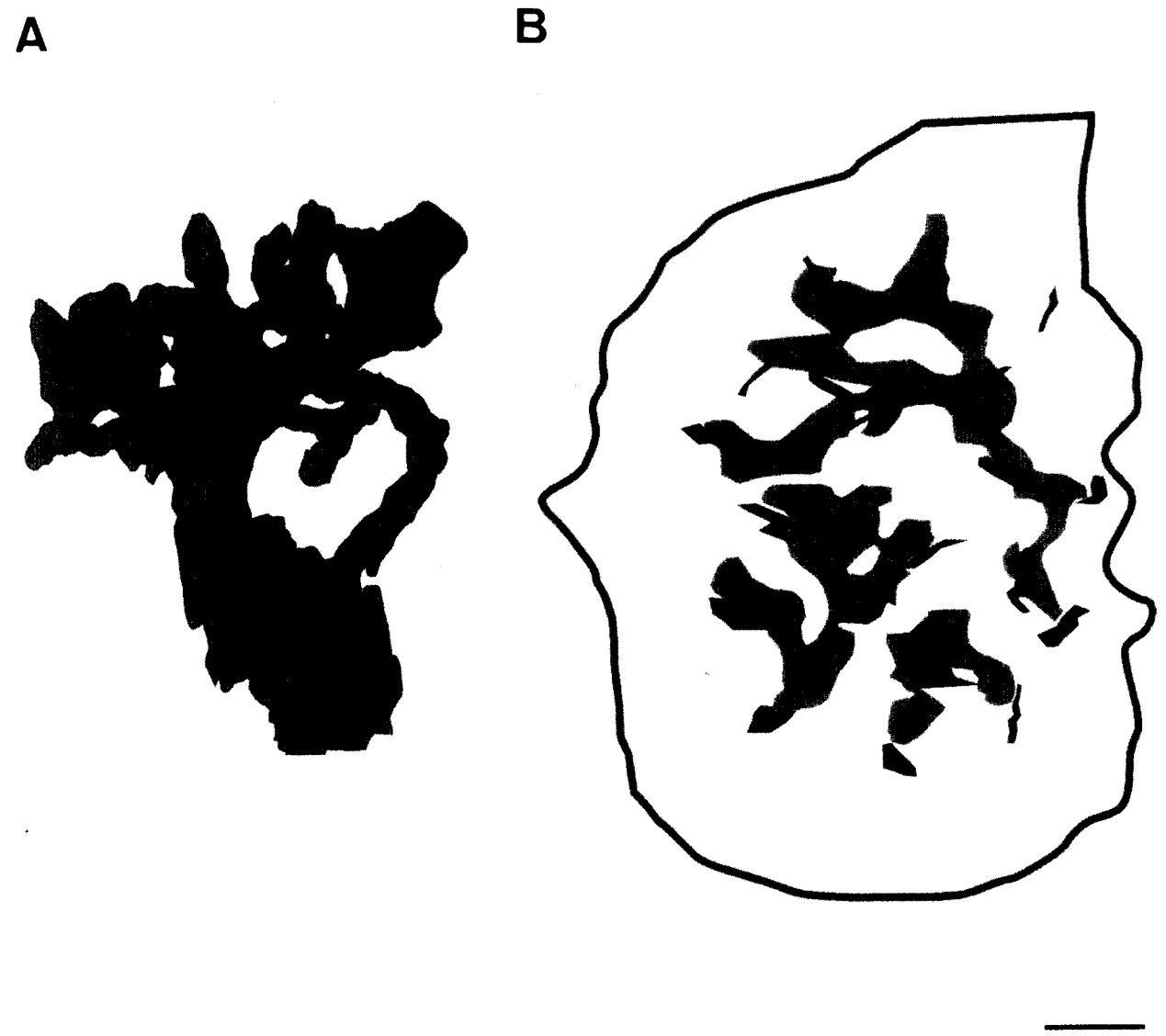

FIGURE 3. (A) Reconstructed dendritic tree of a midget ON bipolar cell in vertical view. The number of distinct midget bipolar dendrites for four cells was 14.5 ± 1.0. Therefore, some dendrites contributed twigs to two active zones; they would appear in the horizontal plane as the butterfly synapses shown by Chun *et al.* (1995). (B) Same dendritic tree in horizontal view (gray) superimposed on the basal surface of pedicle 4 (outline only). Solid marks indicate reconstructed synaptic ribbons (20), each associated with a separate active zone and invagination. The midget ON bipolar cell dendrites protrude into 19 of the invaginations, leaving only one (upper right) entirely free for a different bipolar cell (number 5 in Table 2). Scale = 1 μm.

ing that 3–4 dendrites formed the invaginating process at two active zones. These cases would appear in the horizontal plane as the "butterfly" synapses shown in Chun *et al.* (1996). In no case did a single diffuse bipolar cell dendrite contribute the central element to two invaginations.

Identification of diffuse ON bipolar cells

In a patch of retina containing 24 cone terminals we identified every diffuse bipolar cell by tracing its dendrites to multiple cones. We then traced the axon of each bipolar cell to its arborization in either the OFF or ON sublamina of the inner plexiform layer. There were 38 diffuse bipolar cells for a ratio of 1.6 per cone terminal, in good agreement with previous estimates by Golgi staining and immunocytochemistry (Boycott & Wässle, 1991; Grünert *et al.*, 1994). Twenty-three of these cells sent axons to the OFF layer and 15 sent axons to the ON layer. As reported by Klug *et al.* (1991), the OFF somas were pale and located just above the amacrine cell tier of the inner nuclear layer, whereas the ON somas were dark and located just above the OFF somas.

TABLE 2. Diffuse bipolar cell dendritic processes

Cone terminal 3			Cone terminal 4		
Cell No.	Invaginating	Basal	Cell No.	Invaginating	Basal
1	2	4	5	1	3
2	1	1	6	1	2
3	1		7	1	4
4	1	1			

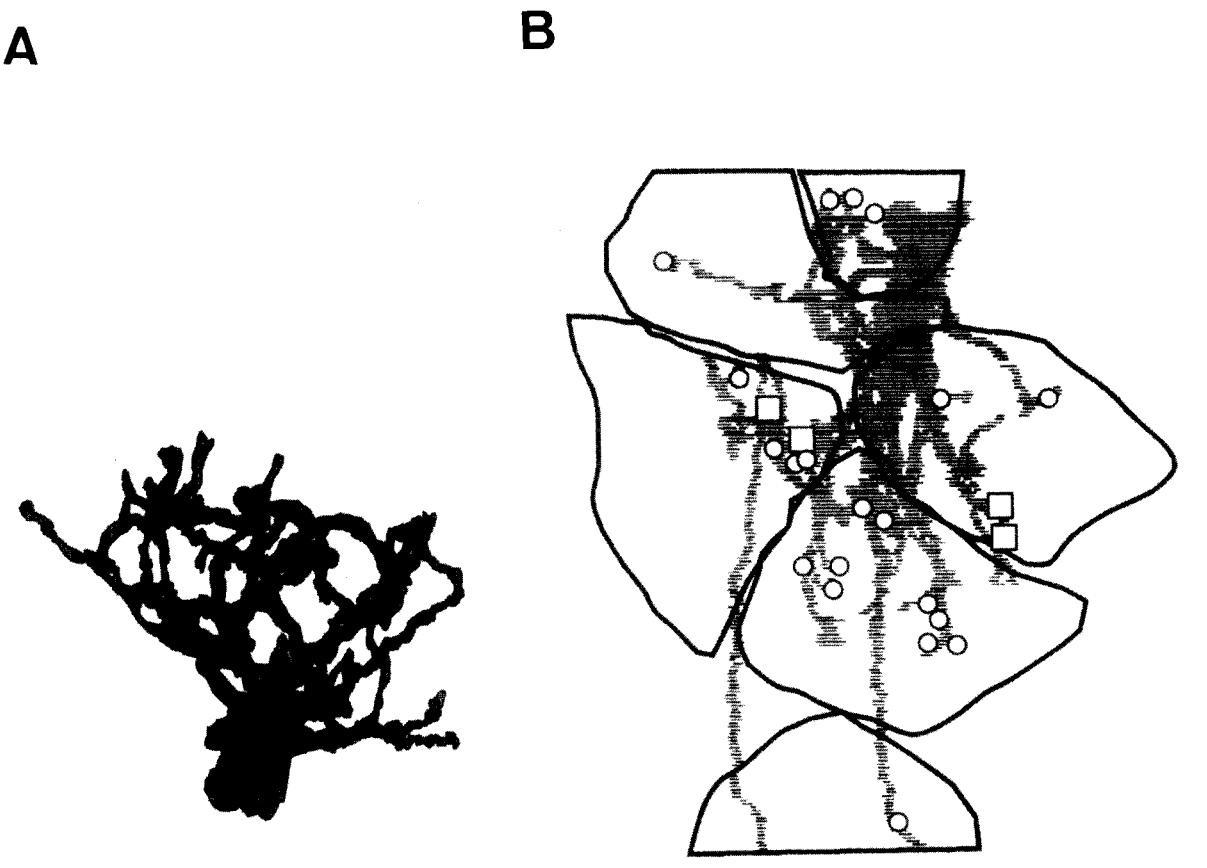

FIGURE 4. (A) Vertical view of the reconstructed dendritic tree of diffuse ON cell number 1 (Table 2). (B) Tangential view of the same tree (gray) and profiles of the overlying cone terminals. Squares mark four sites of invaginating contact; circles mark 20 sites of basal contact just lateral to triads. The bottom and top terminals mark the beginning and end of our series with two dendrites running out. The dendrites under the top three terminals continue but could not be traced further. Scale = 10 μm.

Divergence to diffuse ON bipolar cells

We considered next whether the 3–4 invaginating sites per cone terminal available for diffuse ON bipolar cells are directed at a single cell or whether in this respect there is divergence. For two cone terminals (numbers 3 and 4 in Table 1) we traced each invaginating diffuse bipolar cell dendrite back to an identifiable soma (Table 2). Five of these cells were identified as ON by their axons in the ON layer of the IPL; the other two sent axons beyond the territory of our series but were identified as ON by their soma positions and dark cytoplasm (see above). Cone terminal 3 contributed one invaginating contact to each of three diffuse bipolar cells and two invaginating contacts to a fourth cell. Cone terminal 4 contributed one invaginating contact to each of three diffuse bipolar cells. Thus, six of seven diffuse bipolar cells (of unknown types) collected only one invaginating contact from a given cone. Terminals 3 and 4 also contacted these same diffuse bipolar cells via 1–4 basal contacts (Figs 1–2; Table 2).

Convergence to diffuse ON bipolar cells

We reconstructed in detail two diffuse bipolar cells (numbers 1 and 5 in Table 2) whose axons had been traced to the ON region of the inner plexiform layer. Although a few dendrites extended beyond the series or could not be traced, each cell appeared to collect synapses from every cone terminal within its dendritic field. Thus, the cell in Fig. 4 collected definitely from six cones, and the cell in Fig. 5 collected definitely from eight cones. We estimate the full convergence of cones onto these diffuse ON bipolar cells to be about ten. One exception to this "connect-all" rule is shown in Fig. 5(B). There the starred terminal fails to contact any of the reconstructed diffuse bipolar cell dendrites that run beneath it, even though the dendrites are contacted by neighboring cone terminals. This terminal is an S cone based on its contact with dendrites of "blue cone" bipolar cells and its lack of a midget ON bipolar cell (Klug *et al.*, 1992). Thus, at least one type of diffuse ON bipolar cell seems to avoid S cone input, but whether this is true for all types is unknown.

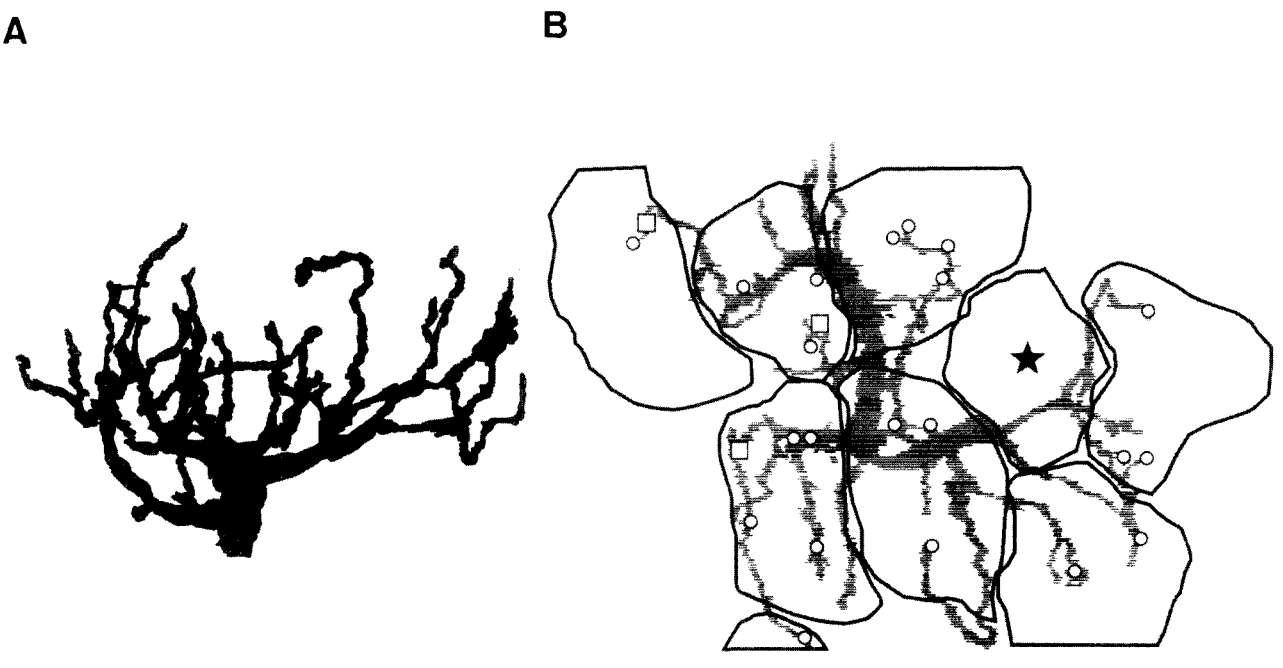

FIGURE 5. (A) Vertical view of the reconstructed dendritic tree of diffuse ON cell number 5 (Table 2). (B) Tangential view of the same tree (gray) and profiles of overlying cone terminals. Squares mark three invaginating contacts; open circles mark 21 basal contacts just lateral to the triads. The star marks an S cone terminal (Klug *et al.*, 1992) that fails to contact the dendrites running beneath it. Five of eight terminals contact this diffuse ON bipolar cell solely at basal junctions. The dendrites at upper left continued but could not be traced. Scale = 10 μm.

The reconstructed diffuse ON bipolar cells in Figs 4 and 5 received 24 contacts each, with a range of 1–9 contacts from each cone. Most of these (83 and 88%) were basal contacts, and they always occupied the position adjacent to the invaginating bipolar process that contributes to the triad. Thus, in foveal retina diffuse ON bipolar cells may collect from the same cone an invaginating contact plus several basal contacts, specifically those termed "triad-associated" by Boycott & Hopkins (1991) and discussed in detail in Hopkins & Boycott (1995). Also in Figs 4 and 5 certain cones make no contact at invaginations, but exclusively at these triad-associated basal junctions. The cone terminals in Fig. 4(B) and Fig. 5(B) include both M and L types (Calkins *et al.*, 1994), but no spectral specificity was apparent in the distribution of invaginating and basal contacts.

Basal junctions adjacent to the triad and those further removed were essentially identical in structure (Fig. 2). We could discern no consistent difference between the specializations of the pre- or postsynaptic membrane, nor between the widths of the postsynaptic cleft for the two locations of basal junction. This agrees with earlier observations (e.g., Lasansky, 1972; Raviola & Gilula, 1975).

Distance from active zone to postsynaptic sites

In the brain the standard distance from the presynaptic site of vesicle release to postsynaptic receptors is about 20 nm, and in the mammalian rod it is about the same from release site to the invaginating horizontal cell processes (Rao-Mirotznik *et al.*, 1995). However, in a rod the distance from the release site to the invaginating bipolar cell dendrite is much greater: 130–640 nm (Rao-Mirotznik *et al.*, 1995). The specific values may be important because the spatial concentration gradient from a point source of transmitter is exponential. Therefore, for cone terminal number 3 we measured the shortest extracellular distance from each active zone to postsynaptic contacts of each type. The invaginating dendrites, whether from midget or diffuse ON cells, penetrate closer to the active zone than in the rod: 80–240 nm (mean $\pm$ SD: 140 $\pm$ 40 nm, $n = 21$). The basal contacts adjacent to the triads are somewhat farther from the active zone: 110–920 nm (500 $\pm$ 160 nm, $n = 21$). The next nearest set of basal contacts beyond those adjacent to the triads, are still more distant and greatly variable: 270–1840 nm (950 $\pm$ 370 nm, $n = 21$). While there is considerable overlap between the ranges of these distances, the mean distance from the active zone to each type of contact differed statistically from the mean distance to the other two types (t-statistic, each $P < 0.001$).

DISCUSSION

Basal synapses can be depolarizing

Bipolar cells innervating the inner half of the inner plexiform layer depolarize to light increments and excite

ON ganglion cells (Nelson *et al.*, 1978; reviewed by Shiells & Falk, 1995). In mammals, the only exception to this rule so far is the cat diffuse bipolar cell CB6 reported by Nelson & Kolb (1983) to stratify in the ON sublamina and hyperpolarize to light increments. However, only one recording exists, so whether this cell constitutes a genuine exception awaits further work (Sterling *et al.*, 1995). Thus, to classify bipolar cells as ON by tracing their axons to this region seems reasonable. Diffuse bipolar cells identified in this way are shown here to receive both invaginating and basal contacts. Therefore we suggest that the basal synapses to these cells are likely to be depolarizing. One can imagine patterns of connection that would have pointed to a different conclusion. For example, if some cones had contributed only invaginating contacts to a particular diffuse bipolar cell and other cones had contributed only basal contacts, one might suspect antagonistic actions from the two types of contact. Spectrally antagonistic inputs to bipolar cells have been demonstrated in fish (Kaneko & Tachibana, 1981) or, if one particular diffuse ON cell collected only basal contacts, one might suspect it to be hyperpolarizing. Yet, there was no hint of such specific patterns; rather, some cones provided both invaginating and basal contacts to the same diffuse bipolar cell, and six of seven diffuse bipolar cells in Table 2 received a mixture of basal and invaginating contacts.

The tips of diffuse bipolar dendrites do not appear to distinguish between the invaginating and the adjacent basal positions. Therefore, it is unclear why there is so little variation in the ratio of basal/invaginating contacts per cone to these diffuse cells, although another putative ON cell does show such variation (see DB4 in Hopkins & Boycott, 1995). The simplest idea may be that the locations for ON cells are determined by a developmental sequence in which the midget ON bipolar cell dendrites grow out first to fill (or induce) the invaginations. The diffuse ON cell dendrites would grow in later, occupying what remains of the invaginating sites, and then accept basal contacts at adjacent locations (see also Boycott & Hopkins, 1991; Hopkins & Boycott, 1995). Indeed, this sequence would also fit in peripheral retina where a cone terminal provides 40–50 active zones for invagination, and the midget bipolar cell only occupies about half (Chun *et al.*, 1996). There one would expect diffuse ON bipolar cells to occupy the remaining invaginations and so receive fewer basal contacts; this does prove to be so (Boycott & Hopkins, 1993).

Mammalian ON bipolar cells are thought to express a metabotropic glutamate receptor because application of 2-amino-, 4-phosphonobutyric acid (APB) blocks the light response of all ON ganglion cells so far studied (reviewed by Wässle & Boycott, 1991), and this pharmacology has been associated with a G-protein-PDE-cGMP mechanism (Nawy & Jahr, 1990; Shiells & Falk, 1990; Yamashita & Wässle, 1991; reviewed by Shiells & Falk, 1995). The corresponding receptor molecule (mGLUR6) has recently been identified at the tips of rodent rod bipolar dendrites (Nomura *et al.*, 1994), but whether the identical molecule is also employed by all types of ON cone bipolar cell remains to be determined. If so, one expects to see immunocytochemical staining at both invaginating and basal synapses on these cells. However, it may be well to keep in mind that in fish and salamander, additional mechanisms have been shown for ON bipolar cells, including a glutamate-gated ion channel with a negative reversal potential (Saito *et al.*, 1979; Nawy & Copenhagen, 1990; Grant & Dowling, 1995). Given that the mGLUR mechanism is conserved from elasmobranchs onward (Shiells & Falk, 1990), one might expect other channels arising early in evolution to be similarly conserved.

Width of synaptic cleft

The distances from the vesicle release sites at the ribbons to the tips of bipolar dendrites raise an entirely different issue. Whereas, at conventional synapses, the synaptic cleft is narrow and invariant, here it is wide and variable. The mean measured here for the invaginating contacts is 7-fold greater than in the brain, and it varies by a factor of 3 (80–240 nm), so the maximum distance can be 12-fold greater! The mean distance measured here for the basal contacts to diffuse ON bipolar cell dendrites is still greater (500 nm). However, it is within the range found for the invaginating rod bipolar cell dendrites (Rao-Mirotznik *et al.*, 1995). The apparent interposition of the horizontal cell processes between the release sites and the ON bipolar cell dendrites (Figs 1 and 2) presents no barrier to diffusion of transmitter to the bipolar cell dendrite because the cleft is huge relative to the size of the glutamate molecule (Kuffler & Nicholls, 1966).

For small cleft widths the glutamate concentration due to one synaptic vesicle reaches high levels ($\sim$1 mM) and decays rapidly ($<$ 1 msec) (Rao *et al.*, 1992; Clements *et al.*, 1992). Thus, the ionotropic glutamate receptors on the invaginating horizontal cell processes (Figs 1 and 2) would see fast, intense pulses of transmitter while metabotropic receptors at invaginating contacts would see slower, weaker pulses ($\sim$1 msec; $\sim$10–100 μM; Rao *et al.*, 1992). This arrangement appears to match what is known so far of the binding affinities of the two receptor types (reviewed by Rao-Mirotznik *et al.*, 1995). Thus, it seems plausible that the invaginating and basal synapses to ON bipolar dendrites could employ the same receptor type and respond to single transmitter quanta. On the other hand, the distances from vesicle release sites to the farther basal contacts (to diffuse OFF bipolar cells, Fig. 2; Boycott & Hopkins, 1993; Calkins *et al.*, 1995) are so great (up to 1800 nm) that they would tend to erase any temporal gradient in glutamate concentration due to a single vesicle. Ostensibly, an occasional vesicle could fuse to the presynaptic membrane at basal junctions — but without even a rudimentary active zone to dock a population of vesicles (Figs 1–2), such events probably would be rare and contribute little to signal transmission. Thus, how light-modulated temporal gradients of glutamate are established at basal junctions of OFF bipolar dendrites is quite mysterious.

One idea is that glutamate is released at these sites by reversal of a glutamate transporter (Schwartz, 1987). Another idea — virtually the opposite — is that high rates of vesicular release at 20 active zones (calculated to be 100 quanta/active zone/sec; Rao *et al.*, 1994) would deliver glutamate to the basal surface faster than it could diffuse away or be removed by glutamate transporters present, for example, on OFF bipolar cell dendrites (Grünert *et al.*, 1994). Suppression of glutamate release by light would decrease its maintained concentration in the cleft. This idea has bearing on the loss of a light response in horizontal cells upon blocking the glutamate transporter (Eliasof & Werblin, 1993).

The idea that transmitter from 20 active zones accumulates at the base of the terminal might seem to receive support from observations in brain that quanta from closely spaced release sites can sum (Faber & Korn, 1988) and desensitize (Trussell *et al.*, 1993). However, those geometries differ from the present case: in brain quanta from adjacent sites are released onto a continuous sheet of postsynaptic membrane — so the only avenue for diffusion is at the edges of the large synapse (Fig. 7, Trussell *et al.*, 1993). But at the cone terminal numerous dendritic twigs (probably more than 100) ascend to the secretory face, thereby creating extensive drainage channels (Fig. 1). So, it is hard to see how transmitter could accumulate unless there were a barrier within these channels to diffusion. Since half of the input to the visual system must cross this set of junctions, a better understanding of how they work would be valuable.

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