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Interplexiform Cells of the Goldfish Retina

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ABSTRACT

Dopaminergic and glycinergic interplexiform cells (IPCs) in the goldfish retina were impregnated by using two new Golgi protocols. The two cell types have markedly different morphological characteristics: Dopaminergic IPCs have primary dendrites that descend into and stratify in the inner plexiform layer, where they give rise to processes that project to the outer plexiform layer. Conversely, glycinergic IPCs have primary dendrites that ascend to the outer plexiform layer and from this dendritic arbor, many processes then project into the inner plexiform layer. The apparent coverage of dopaminergic IPCs is almost four times that of glycinergic IPCs. Even so, the coverage of each glycinergic IPC in the outer plexiform layer allows it to provide an accurate copy of the S-space to the inner plexiform layer. Considering the known GABAergic and glycinergic synaptologies in the inner plexiform layer, the glycinergic IPC must form a major element in the retinal circuitry of the goldfish.

Key words: Golgi impregnation, autoradiography, dopamine, glycine, retinal circuitry

The vertebrate retina is composed of six major classes of neurons. Photoreceptors, bipolar cells, and ganglion cells form the vertical "through" pathways; horizontal cells and amacrine cells form the lateral pathways. Interactions among these cells at the outer plexiform layer (OPL) and the inner plexiform layer (IPL) initiate the task of encoding visual information. The bipolar cells appear to be the major cell class connecting the two plexiform layers, forming a vectorial "through" pathway from photoreceptors to ganglion cells. However, it is now well established that a sixth class of retinal neuron, the interplexiform cell (IPC), also provides connections between the outer and inner plexiform layers of the vertebrate retina (Ehinger and Falck, '69; Ehinger et al., '69; Gallego, '71; Boycott et al., '75; Dowling and Ehinger, '75, '78; Kolb and West, '77; Oyster and Takahashi, '77; Smiley and Basinger, '88).

The name "interplexiform cell" is a purely morphological appellation that refers to retinal neurons arborizing in both plexiform layers, as do bipolar cells, but that express several unique features compared to bipolar cells: (1) extensive lateral arborizations in both plexiform layers, (2) somas that are decidedly nonbipolar in the vertical axis and strongly multipolar in the horizontal plane, (3) unique synaptic contacts with horizontal cells, bipolar cells, amacrine cells, other IPCs, possibly ganglion cells, and processes in the nerve fiber layer, but not photoreceptors, (4) apparent content of inhibitory neurotransmitters such as glycine or GABA, or the modulatory neurotransmitter dopamine, whereas bipolar cells appear to use glutamate (Gallego, '71; Boycott et al., '75; Dowling and Ehinger, '75, '78; Kolb and West, '77; Marc and Lam, '81; Pourcho and Goebel, '83; Marc and Liu, '84; Ehinger et al., '88; Smiley and Basinger, '88; Yazulla and Zucker, '88; Marc et al., '89). The classification of a neuron as an IPC does not reveal whether the cell forms part of a centripetal or centrifugal pathway; several obviously different cells that meet the above four criteria have been called IPCs (Gallego, '71; Boycott et al., '75; Dowling and Ehinger, '75, '78; Kolb and West, '77; Oyster and Takahashi, '77; Marc and Lam, '81; Marc and Liu, '84; Smiley and Basinger, '88; Kalloniatis and Marc, '89).

The goldfish retina appears to contain two classes of IPCs based on fluorescence microscopy, immunoreactivity, or autoradiography: the dopaminergic and glycinergic IPCs (Dowling and Ehinger, '75, '78; Marc and Lam, '81; Marc, '82; Marc and Liu, '84; Yazulla and Zucker, '88). Of all the retinal neurons described in fish retinas, IPCs have been very difficult to impregnate by the Golgi method or inject with dyes (e.g., Teranishi and Negishi, '86, '88), although Ramón y Cajal (1893) described a cell in the perch that would fit the definition of an IPC. Density estimates range from 84 to 92 cells/mm^2 for dopaminergic IPCs and 20 to 50 cells/mm^2 for glycinergic IPCs in medium-size goldfishes (10 to 12 cm in body length; Marc, '82; Marc and Liu, '84). The density values for the dopaminergic IPCs compare favorably with those of catecholamine-accumulating cells reported by Negishi ('81) for similar size goldfish, provided a 17% shrinkage factor is incorporated in the calculations (Negishi et al., '81). Although the densities of both these cell types and some aspects of their synaptologies are known, knowledge of their detailed morphologies and dendritic field sizes

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is incomplete. In this work we describe some detailed characteristics of both classes of IPCs as observed in Golgi-impregnated material. Considering the known patterns of GABAergic and glycinergic synaptic contacts in the IPL, we conclude that, like dopaminergic IPCs, glycinergic IPCs must form a major element in the circuitry of the teleost retina.

MATERIALS AND METHODS

Medium-size (10 to 12 cm body length) goldfishes (*Carassius auratus*) were maintained in a communal aquarium and were sacrificed by cervical transection and pithing. The eyes were enucleated and the retinas removed under normal room illumination. For neurotransmitter uptake experiments, retinas were incubated in an oxygenated physiological saline containing ≈5 μM [^{3}H] glycine at an activity of 100 μCi/ml. After the incubation, the retinas were rinsed in saline, fixed in 1% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, 0.01% $CaCl_2$ in a 0.1 M phosphate buffer (pH 7.4) for 10 to 30 minutes at room temperature and overnight at 4°C. Some retinas were incubated in oxygenated physiological saline without radioisotopes and fixed; others were fixed immediately after removal. The retinas were kept in fixative for several days before Golgi procedures were performed.

Although many Golgi techniques are available (Ramón y Cajal, 1893; Colonnier, '64; Stell, '65; Stell and Lightfoot, '75; Leeper, '78), the preparations in this study were obtained using two new procedures modified from Izzo et al. ('87): protocols K1 and K2. The procedures proved useful in providing well-isolated and extensively impregnated cells in the goldfish retina. Good Golgi-impregnated cells were observed in virtually all goldfish preparations with protocol K1 and either protocol permits easy flat mounting.

Golgi protocol K1

After a phosphate buffer rinse, pieces of fixed retina were sandwiched between two glass slides and placed upright in a shallow bath of 9.5% aqueous potassium dichromate and 0.1% osmium tetroxide (a 19:1 mixture of 10% dichromate and 2% osmium) for 1 to 4 days. Increasing the time of this osmication step improved the quality of impregnation (cf. Leeper, '78). The retinas were briefly rinsed in 10% potassium dichromate, sandwiched again, and placed in 8% potassium dichromate and 5% glutaraldehyde for a further 4 to 7 days. The retinas were then placed between two clean glass slides and transferred to 1% aqueous silver nitrate for 1 to 2 days in the dark. Throughout the protocol, the glass slides were placed upright, 2 to 3 mm into the solution in a plastic beaker covered with Parafilm (TM) to minimize evaporation. Capillary action was sufficient to keep the glass slides together. Air bubbles were carefully removed by placing a razor blade between the slides and allowing the solution to form a uniform layer.

The retinas were removed from the silver nitrate solution, lightly brushed in deionized water to remove the silver chromate precipitate (Fregerslev et al., '71) from the retinal surface, and then examined by light microscopy to determine the degree of impregnation. It was usually necessary to repeat the dichromate:glutaraldehyde and silver nitrate step once to obtain a reasonably large sample of cells. The dichromate:osmium step was not repeated. We were unsuccessful in obtaining good material using a short dichromate: glutaraldehyde step, and it was only after the mixture became murky and precipitates formed (after 4 to 5 days) that good Golgi impregnated material was assured.

Golgi protocol K2

The second protocol also used glass slides to sandwich the retinas but had different potassium dichromate/silver nitrate cycles. An osmium:dichromate step of approximately 4 hours was followed by a silver nitrate step of 4 to 24 hours (concentrations as indicated above). The retinas were recycled once omitting osmium and using 10% potassium dichromate. This Golgi procedure provided extensive impregnation of the IPL, but we frequently failed to obtain impregnated material. Another characteristic of this protocol was the rough appearance of Golgi-impregnated cells, in contrast to the smooth appearance of cells impregnated with protocol K1. Although we favored the use of protocol K1, two of the dopaminergic IPCs in this report were obtained with protocol K2.

Well-impregnated retinas were dehydrated in cold graded methanol washes and embedded in Medcast (Pelco, Redding, CA) between two large epoxy blocks for vertical sectioning or flatmounted in Medcast on glass slides. The vertically embedded tissue was sectioned at 90 μm on a sliding microtome and mounted in plastic on a glass slide (West, '72; Stell and Lightfoot, '75). If the large blocks with sandwiched retinas were sectioned immediately after removal from the curing oven, they remained pliable and did not require surface heating to avoid chipping after each 90-μm section. Suitable cells in vertical sections or flatmounts were photographed and drawn by camera lucida. Two presumed glycinergic IPCs from tissue that had been incubated in [^{3}H] glycine were carefully removed from the slide by first inscribing a small rectangle containing the cell. After gently heating the slide, the rectangle containing the cell was easily removed and placed in a drop of resin between two Medcast discs. The Medcast discs were faced and mounted on an ultramicrotome for semithin sectioning (modified from Stell and Lightfoot, '75).

Autoradiography of Golgi-impregnated cells

Several methods are available to conduct light microscopic autoradiography on Golgi-impregnated material (Feigin and Naoumenko, '76; Geisert and Updyke, '77; Somogyi et al., '81; Goebel and Pourcho, '82; Hitchcock and Hickey, '83). Each has advantages and disadvantages, and none completely met our needs. We therefore developed a protocol (modified from Haase et al., '85) to convert the silver chromate to a chromatic dye product, which allowed for direct identification of Golgi-impregnated somas and autoradiographic silver grains. The dye conversion technique involves the substitution of Golgi material (silver chromate) to a silver halide, which in turn is converted to a chromatic dye.

One or two 0.5-μm sections through the somas of the Golgi-impregnated cells were floated onto beads of deionized water on clean glass slides, dried on a hotplate, and deplasticized using sodium methoxide (Marc and Lam, '81; Marc and Liu, '84). Because products of the dye conversion are all alcohol-soluble (Bertsch et al., '88), the reactions must be carried out as postembedding steps. Our protocol for optimal dye coupling in 0.5-μm sections or lightly Golgi-impregnated somas involved the following steps. 1. Deplasticize in sodium methoxide, followed by four 2-minute methanol washes and a 5- to 10-minute deionized water wash. 2. Immerse in 0.5 M sodium bromide for 5 to 10

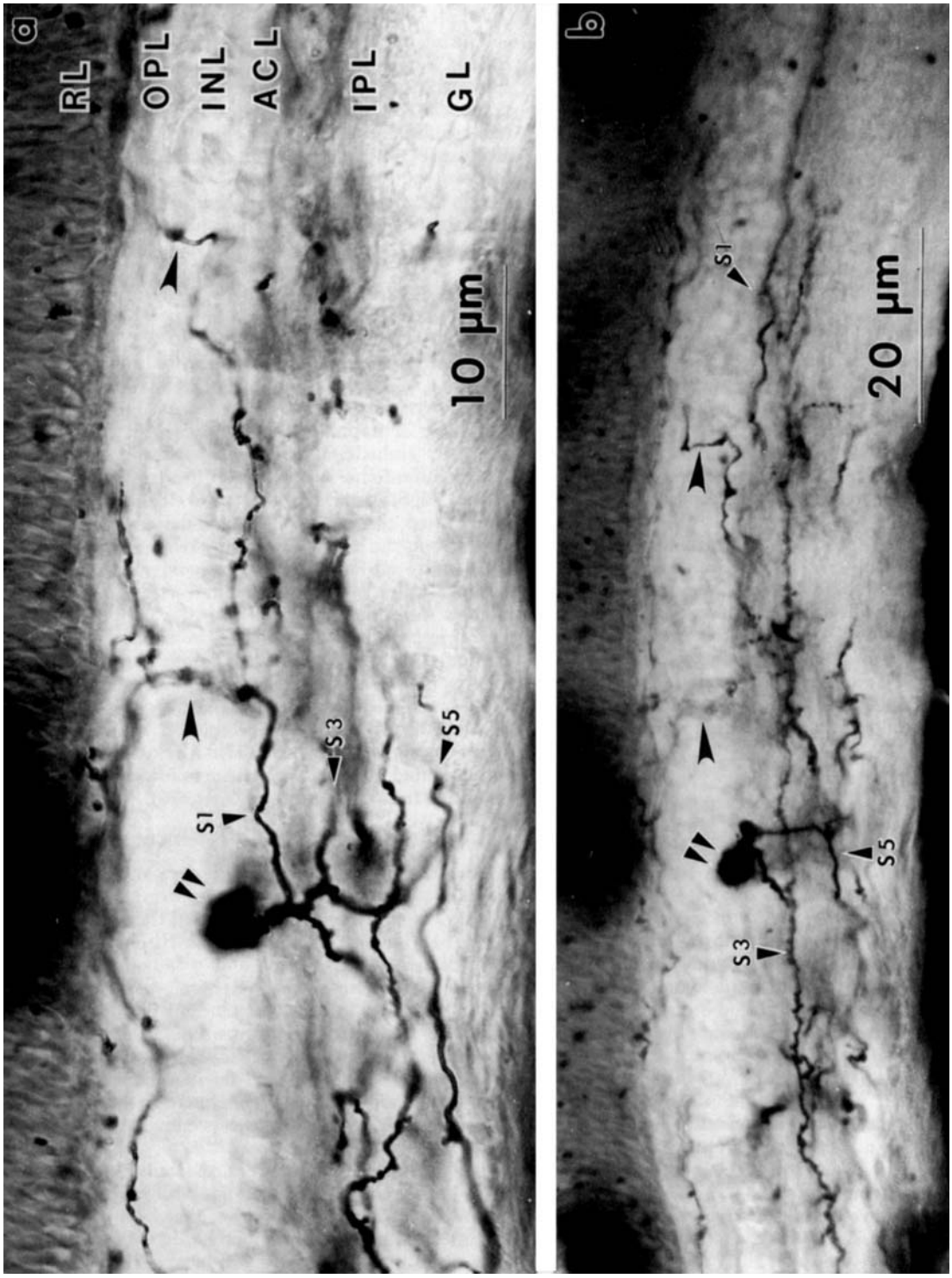

Fig. 1. A Golgi-impregnated dopaminergic IPC (DA-1 at two focal planes and two magnifications) showing the cell body (double arrowhead) in the amacrine cell layer and major processes in the OPL and IPL. Processes extending from sublayer 1 of the IPL to the OPL are indicated by the single arrowhead, and examples of the stratification in sublayers 1 (S1), 3 (S3), and 5 (S5) are also indicated. In (**a**), parts of both visible ascending processes are in focus and in (**b**) the more distal one is shown to arise from a process in sublayer 1. RL—*receptor layer*; OPL—*outer plexiform layer*; INL—*inner nuclear layer*; ACL—amacrine cell layer; IPL—inner plexiform layer; GL—ganglion cell layer.

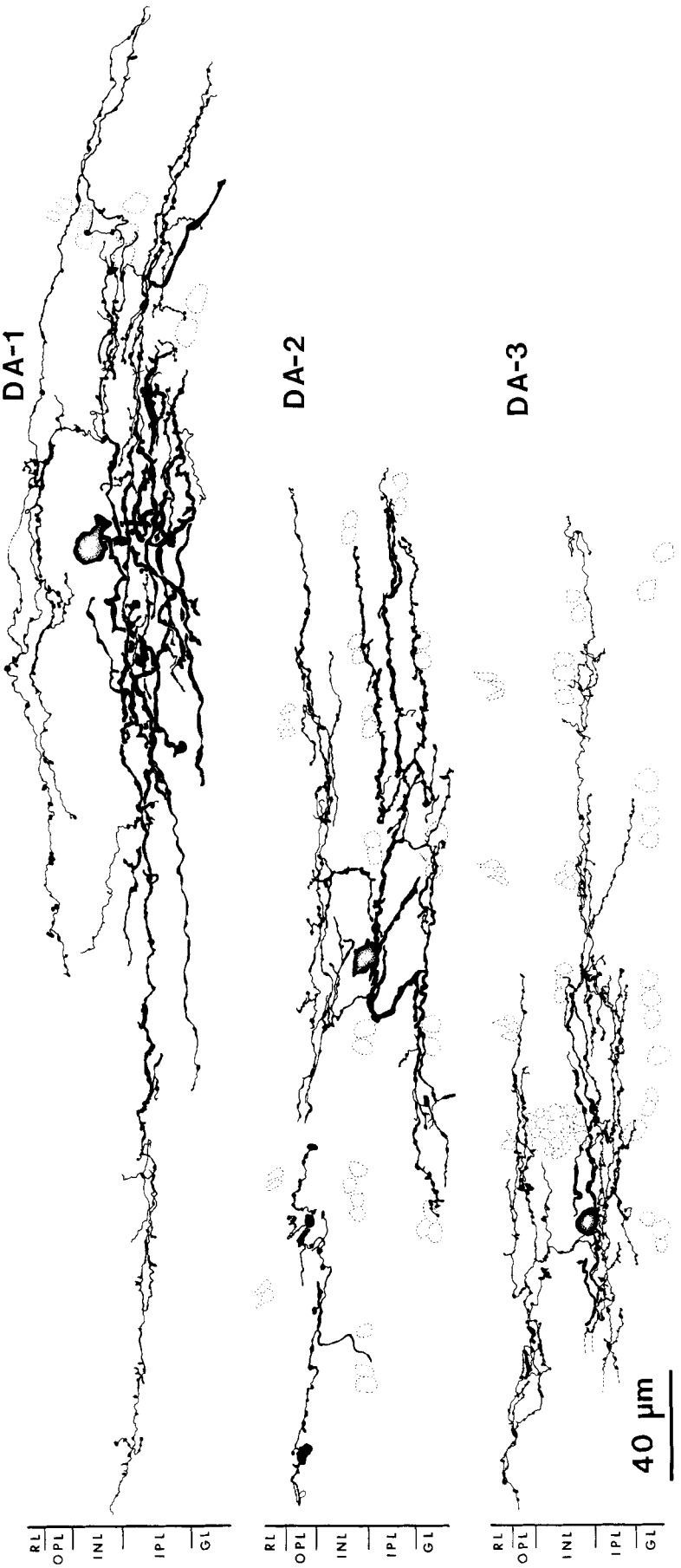

Fig. 2. Camera lucida drawing of three Golgi-impregnated dopaminergic IPCs. The cell bodies are generally oval in shape but may be irregular in appearance (DA-1 and DA-2). Characteristically there are few ascending processes to the OPL; they branch profusely in the distal third of the INL. DA-1 has four ascending processes (two of which have not impregnated extensively), DA-2 three and DA-3 one (the DA-3 OPL arbor is clearly underimpregnated). In the IPL, the stout ropey processes near the cell body give way to smoother smaller processes studded with varicosities. DA-1 and DA-2 were impregnated using protocol K2, and DA-3 with protocol K1. Abbreviations as in Figure 1.

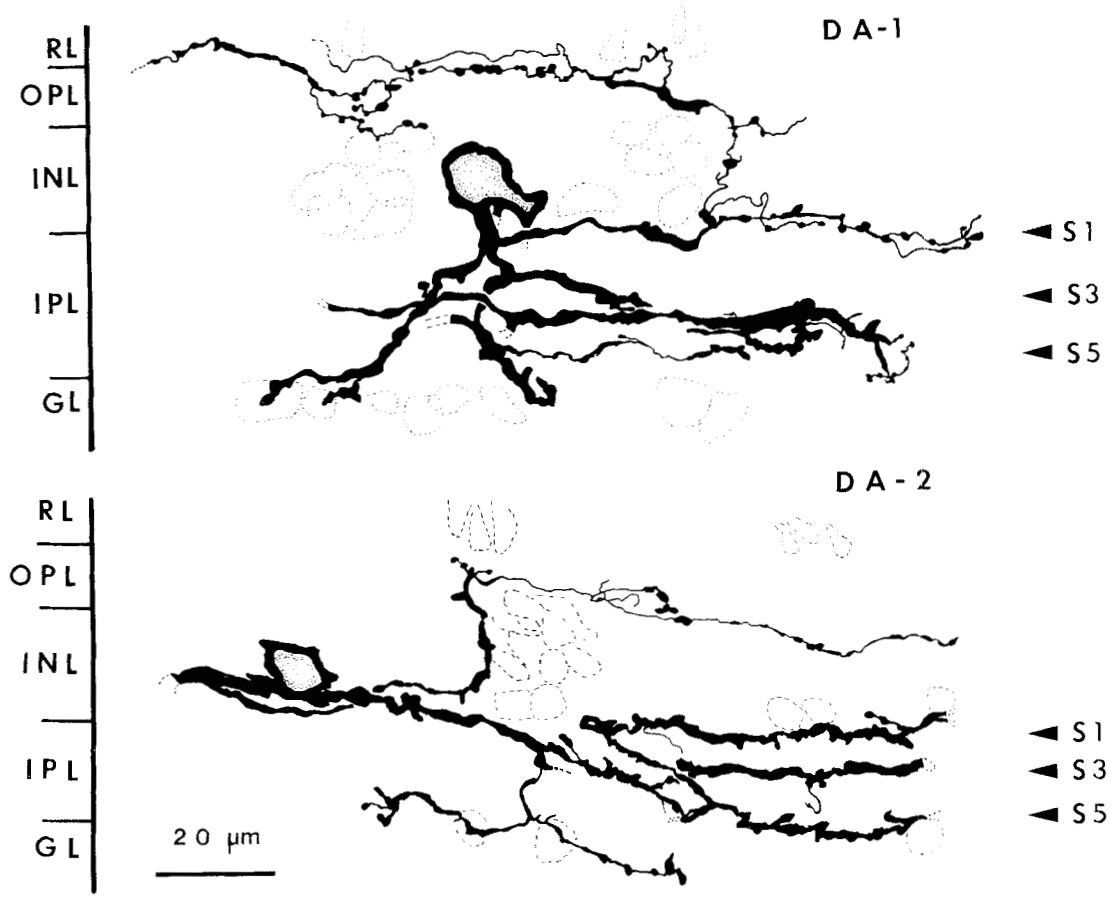

Fig. 3. A camera lucida drawing of the discriminant features of DA-1 and DA-2. Note the stratification in sublayers 1 (S1), 3 (S3), and 5 (S5) of the IPL. DA-1 was in a tilted section which made the stratification difficult to perceive in Figures 1 and 2. Abbreviations as in Figure 1.

minutes with slight agitation. 3. Wash in deionized water for 5 minutes. 4. Immerse in a combined (50 ml) solution of dye coupler plus color developer for 5 to 10 minutes with slight agitation. (Color developer solution: 45 ml deionized water, 100 mg sodium sulfite, 100 mg color developer CD2 [Eastman Kodak # 171 4567], 50 mg potassium bromide, and 1 g sodium carbonate; dye coupler solution: 0.2 N sodium hydroxide in 5 ml ethanol, 100 mg dye coupler M-38 [Eastman Kodak # 179 6341], C-16 [# 155 8220], or Y-55 [# 188 5177], for magenta, cyan, or yellow reaction products, respectively.) The color developer and dye coupler solutions should be prepared and combined just before use. 5. Wash for 5 minutes in deionized water. 6. Fix for 30 to 60 minutes with slight agitation (24 g sodium thiosulfate and 15 g sodium sulfite in 100 ml deionized water), rinse in deionized water, and air dry. The fixing step with sodium thiosulfate also ensures that any remaining silver chromate or silver bromide precipitate is removed (Feigin and Naoumenko, '76).

Slides may then be processed for autoradiography (e.g., Marc and Lam, '81; Marc and Liu, '84). For thicker sections (1 μm) or densely impregnated somas, we first converted silver chromate directly to elemental silver with 100% Kodak D-19, then oxidized the silver to silver bromide according to Haase et al. ('85), followed by the dye conversion protocol beginning at step 3.

Control experiments on Golgi-impregnated tissue previously labelled with [^{3}H] glycine ensured that minimal chemography was associated with this dye conversion procedure. Magenta dye converted somas in the INL were examined after 1 day of autoradiographic exposure (in 1-μm sections). The silver grain density was very low (mean = 3.2 grains/soma, SD = 1.2, N = 13) and showed little increase after 7 or 8 days exposure in nonradioactive dye converted somas in the INL (mean = 7.1 grains/soma, SD = 2.1, N = 7). Amacrine cells exhibiting high affinity [^{3}H] glycine uptake in the same preparation had much higher grain densities after 7 or 8 days exposure (mean = 73 grains/soma, SD = 16, N = 18) making the distinction between the lightly chemographic somas and radioactive somas definitive. This protocol does not wholly eliminate chemography, but it renders it at least one order of magnitude less potent than our standard light microscope autoradiographic preparations for [^{3}H] neurotransmitter uptake.

RESULTS

Dopaminergic interplexiform cells

The classification of cells as dopaminergic IPCs was based on earlier morphological reports (Dowling and Ehinger, '75, '78; Yazulla and Zucker, '88), and results in our laboratory

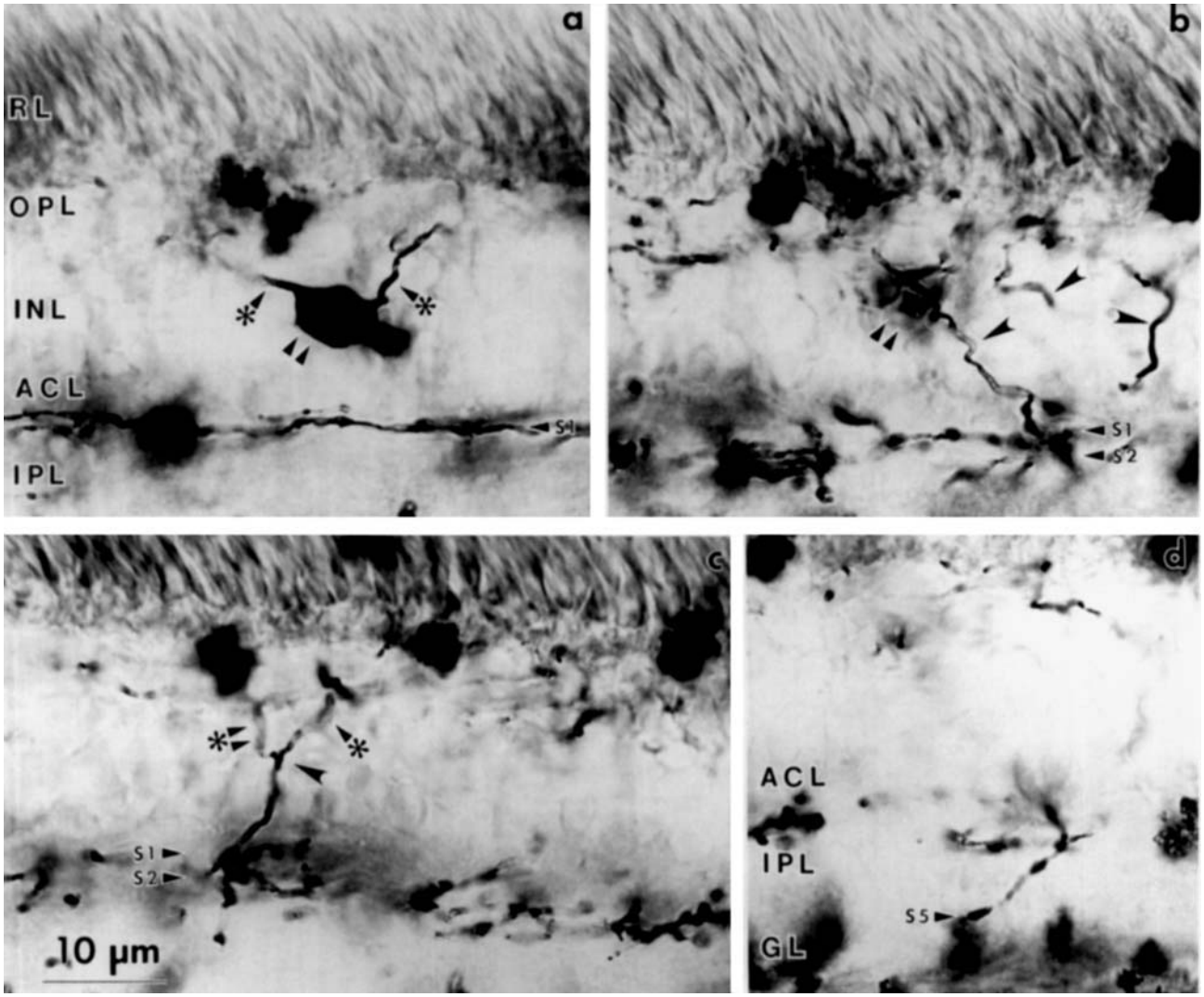

Fig. 4. A Golgi-impregnated glycinergic IPC (Gly-2) shown in vertical section. (**a**) The cell body (double arrowhead) is in the middle of the INL, and two major dendrites approach the OPL (arrowhead with asterisk). A process stratifying in sublayer 1 of the IPL is also visible. (**b**) Several descending processes are indicated with a single arrowhead, and one process shows extensive arborization in sublayers 1 (S1) and 2 (S2) of the IPL. The position of the out of focus cell body is indicated by the double arrowheads. (**c**) A characteristic Y-shaped dendrite (arrowhead) in the middle of the INL. The primary dendrite (arrowhead with asterisk) can be traced to the cell body, and the other process (double arrowhead with asterisk) proceeds to the OPL. The IPL processes can be dense in S1 and S2 with many branches and varicosities. (**d**) Some glycinergic IPC processes project to sublayer 5 (S5). All glycinergic IPCs shown in this study were impregnated using protocol K1. Abbreviations as in Figure 1.

using tyrosine hydroxylase and dopamine immunocytochemistry (Van Haesendonck and Marc, in preparation). These reports characterized dopaminergic IPCs as having (1) cell bodies located in the amacrine cell layer, (2) major dendrites arborizing in the IPL, and (3) processes ascending from the major dendrites to the OPL where they formed dense terminal arbors.

Five presumed dopaminergic IPCs were impregnated, three being well isolated and extensively impregnated (Figs. 1–3). To better appreciate their general morphologies, Figure 2 shows camera lucida drawings of these three cells. The IPL dendritic field is at least 520 μm wide in one cell, and there is extensive arborization in both plexiform layers. The oval cell body lies directly in the amacrine cell layer, and three or more primary dendrites extend toward the IPL. Processes extending to the OPL arise from peripheral branches of dendrites in sublayer 1 and branch repeatedly when they reach the distal third of the INL and the OPL. The number of ascending processes found for the five dopaminergic IPCs were 1, 3, 4, 5, and 6. The discriminant characteristics of these cells are best depicted by drawing those processes close to a single focal plane (Fig. 3), where stratification of large ropelike dendrites, studded with varicosities, is especially evident in sublayers 1, 3, and 5 of the IPL. In contrast, the OPL processes are finer, though richly adorned with varicosities.

The three dopaminergic IPCs have dendritic extents of 268, 380, and 520 μm in the IPL, in vertical sections. Assuming that these values provide an accurate minimum representation of a radially symmetric dendritic field size, a

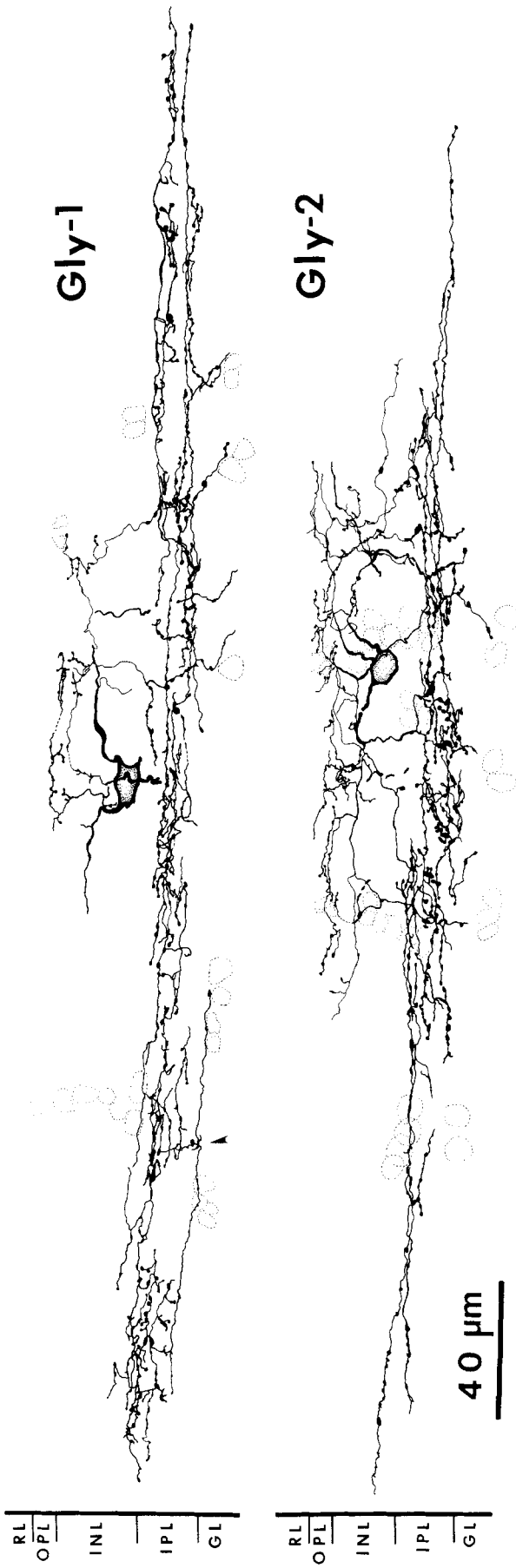

Fig. 5. Camera lucida drawing of two extensively impregnated glycinergic IPCs (Gly-1 and Gly-2). The arrowhead on Gly-1 indicates a process that branches and extends approximately 110 µm along sublayer 5 of the IPL. Note the characteristic Y-shaped appearance of the slender descending processes in the middle of the INL, the bifurcation of dendrites in the amacrine cell layer just before they reach the IPL, and the dense arborization in sublayers 1 and 2 of the IPL.

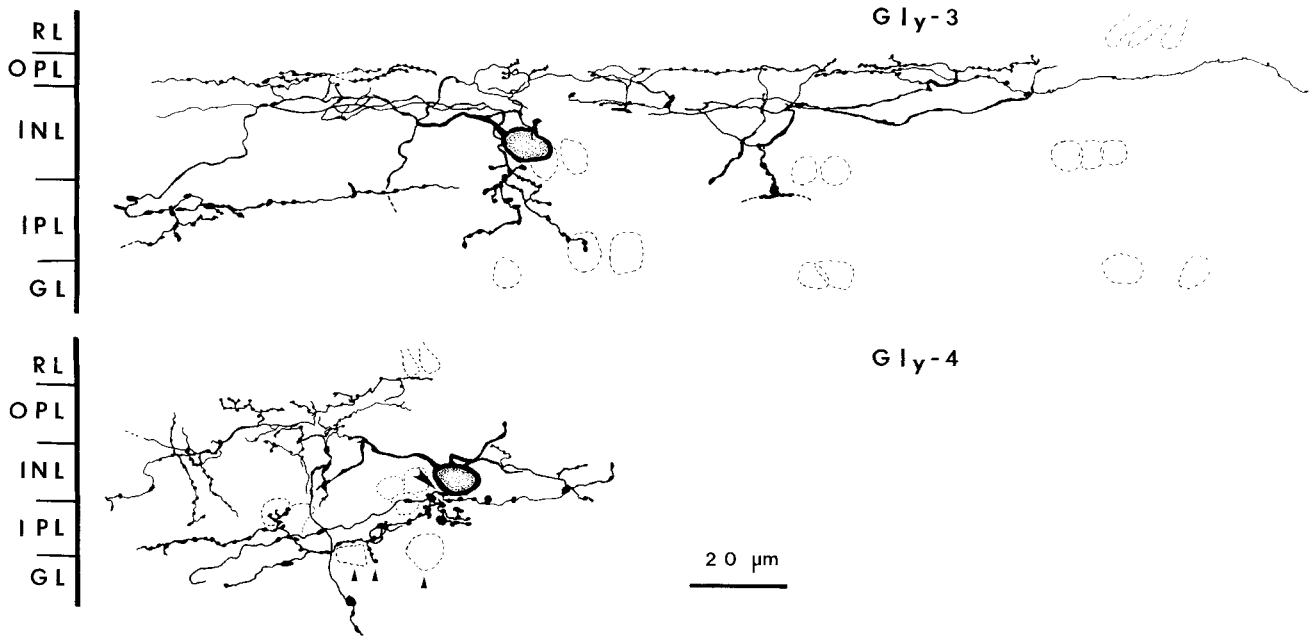

Fig. 6. Camera lucida drawing of two glycinergic IPCs showing some unique characteristics. Gly-3 has an extensively impregnated OPL dendritic tree extending almost 240 μm, and Gly-4 has a process extending from the cell body to the IPL (large arrowhead). Gly-4 was in an oblique section and the relative scaling of the retinal layers reflects this tilt. The three triangular arrowheads indicate that the glycinergic IPC process and the two somas in the ganglion cell layer were in the same focal plane.

mean cell density of 88 cells/mm^2 yields coverage factors (dendritic field size × cell density) of 5, 10, and 18.7 (mean = 11.2, SD = 7) in the IPL. In the OPL, the dendritic extents of the three cells were 223, 226, and 354 μm, yielding minimum coverage factors of 3.4, 3.5, and 8.7 (mean = 5.2, SD = 3).

Glycinergic interplexiform cells

We identified four cells in flatmounts and 16 cells in vertical sections having the structural characteristics of glycinergic IPCs. Of these, two from the flatmounts and four from vertical sections are shown. The somas are located in the middle of the inner nuclear layer (INL), and two or three primary dendrites extend toward the OPL (Figs. 4–6), just as described for glycinergic IPCs (Marc and Lam, '81; Marc and Liu, '84). Rarely is there a process extending to the IPL from the soma; only one example has been found (Gly-4 in Fig. 6). Note that Gly-4 has three primary dendrites extending toward the OPL. In the proximal part of the OPL, these large processes rapidly taper and branch, becoming beaded

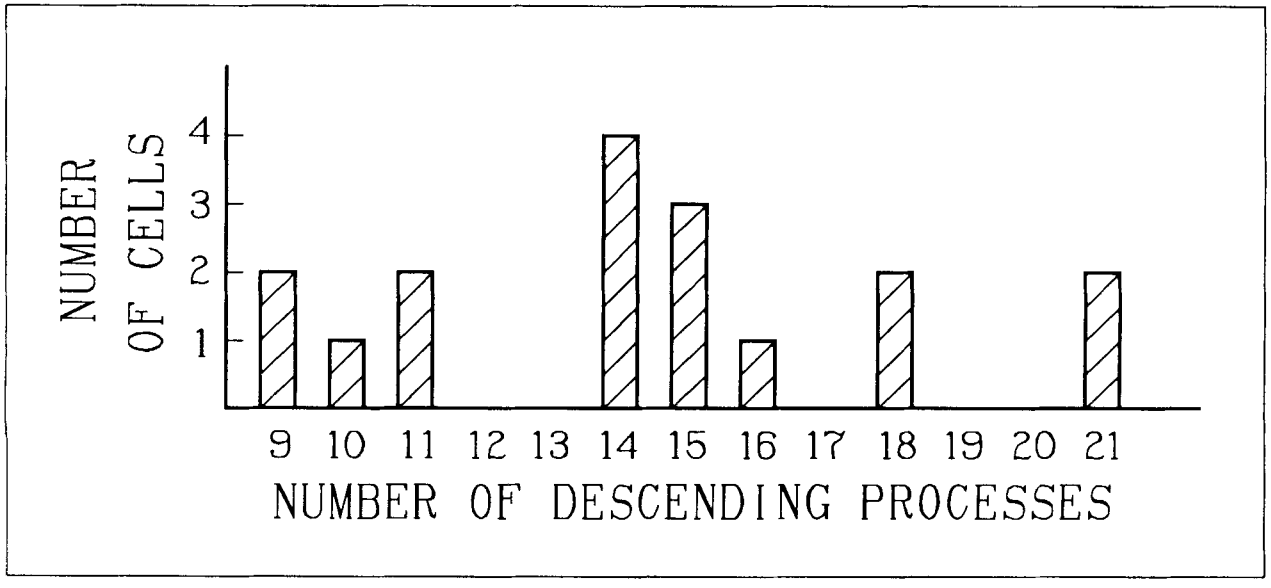

Fig. 7. Frequency histogram illustrating the number of glycinergic IPCs encountered (using protocols K1 or K2) that displayed multiple descending processes.

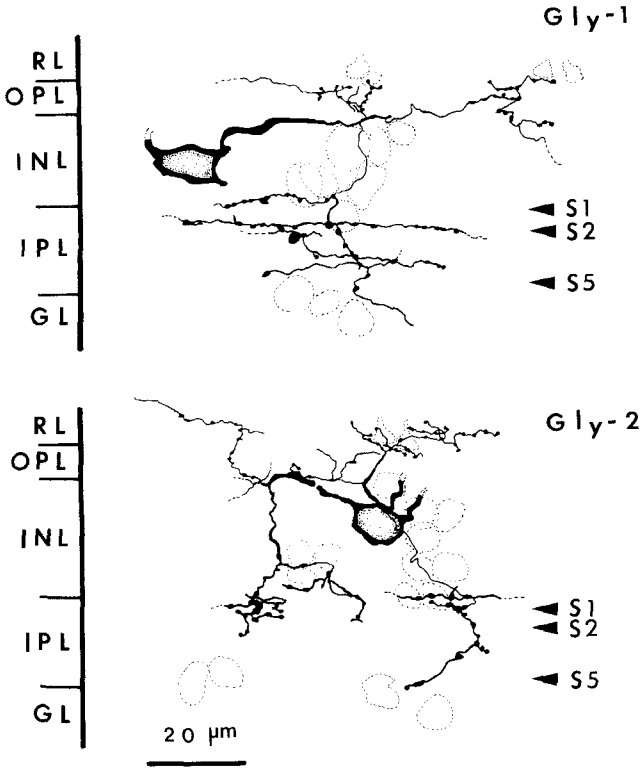

Fig. 8. Camera lucida drawing of the discriminant features of Gly-1 and Gly-2. The cell body is located in the middle of the INL and two or three major dendrites project to the OPL where they arborize in distal OPL. Note the slender processes connecting the OPL to the IPL, the beaded terminals, and the extensive arborization in sublayer 1 (S1), sublayer 2 (S2), and deep in sublayer 5 (S5) of the IPL.

and finally arborizing in distal OPL. These OPL dendrites often send small processes proximally, descending directly through the INL to the IPL. The branching in the OPL can be very dense (Figs. 5, 6), with many processes being sent to the IPL. As a rule, glycinergic IPC processes and the beaded varicosities are smaller and form denser clusters than dopaminergic IPC processes.

The processes descending from the OPL to the IPL may arise from either one of the large primary dendrites, forming a characteristic Y-shaped junction in mid-INL (Fig. 4c) or from very fine processes distant from the cell body. We have found up to 21 descending processes associated with a single glycinergic IPC. Figure 7 illustrates the frequency distribution of descending processes for 16 presumed glycinergic IPCs. A glycinergic IPC will send, on average, approximately 14 processes from the OPL to the IPL. Once these processes reach the IPL, they branch, become beaded, and arborize extensively in sublayers 1 and 2. Some processes extend deep into sublayer 5, where they further branch and stratify. A good example of the branching in sublayer 5 is shown by Gly-1 (Fig. 5). The striking characteristic of the IPL stratification is the very dense distribution of processes and the large beaded appearance of the terminals. Glycinergic terminals identified by autoradiography have a beaded appearance throughout the IPL (Marc and Lam, '81; Muller and Marc, '90). Figure 8 illustrates the discriminant features of the glycinergic IPCs in vertical section by depicting the major processes near the cell body close to one focal plane.

Two flatmounted cells are shown in Figures 9 through 11. Figure 10 is a camera lucida drawing of the somas, major processes in the INL, and processes in the OPL. Arrows indicate the loci from which descending processes arise. The IPL dendrites of these cells are shown separately in Figure 11. Though not as extensively impregnated as cells in vertical sections, the flatmounted glycinergic IPCs have impregnated in all the retinal layers identified in the vertical sections. The small arrows indicate the origins and stars the apparent ends of processes, which stratify in sublayer 5 (Fig. 11). It is clear that there is additional branching in sublayer 5, and processes meander up to 60 to 100 μm (cf. Gly-1 in Fig. 5).

To obtain an idea of the minimum coverages of these cells, two cells were digitized, randomly rotated, and copied onto a representative distribution of somas at a density of 35 cells/mm^2 (Fig. 12). The cell does not appear to provide a uniform coverage of the OPL, but the coverage appears a bit more even in the IPL. We stress that Figure 12 is clearly a *minimum* representation of the coverage. Even so, the minimum coverage factor for glycinergic IPCs in the IPL, based on the assumptions of radially symmetrically dendritic field diameters ranging from 190 to 520 μm (mean = 319, SD = 120, N = 9) and a cell density of 35 cell/mm^2, is 0.8 to 7.4 (mean = 3.1, SD = 2.2). In the OPL, the estimated dendritic extent of 140 to 354 μm (mean = 209, SD = 75, N = 17) implies a coverage of 0.5 to 3.5 (mean = 1.3, SD = 1). Dopaminergic IPCs appear to provide a mean coverage approximately four times greater than that provided by glycinergic IPCs in the IPL and OPL. We emphasize that it is not possible to estimate the degree of impregnation of IPCs, although we suspect that the larger fields represent cells whose extents should be close to true values. Even so, it is evident that the dendritic field sizes of glycinergic IPCs and dopaminergic IPCs are really very similar and that the well-established differences in cell densities account for most of the differences in coverage.

Light microscope autoradiography

To confirm that the presumed glycinergic IPCs were in fact the same INL cells as those showing high affinity uptake of [^{3}H] glycine, two cells from those shown in Figures 5 and 6 (Gly-1 and Gly-3) were sectioned for light microscope autoradiography. Both cells exhibited high affinity uptake for [^{3}H] glycine (Fig. 13), and we thus conclude that the Golgi-impregnated IPCs with somas in the INL are the neurons showing high affinity uptake for glycine in the INL (Marc and Lam, '81; Marc and Liu, '84). Because of the low rate of impregnation of dopaminergic IPCs, attempts to obtain double labeling with [^{3}H] dopamine have not been successful.

DISCUSSION

Morphological characteristics of IPCs

In the goldfish retina, two classes of IPCs arborize in both plexiform layers and exhibit fundamentally different morphologies. The dopaminergic IPC has its soma located directly in the amacrine cell layer and sends its primary dendrites into the IPL. Relatively few processes extend to the OPL and they generally arise from dendrites in sublayer 1. Five Golgi-impregnated IPCs were identified as dopaminergic IPCs based on close morphological similarities to IPCs revealed by monoamine histofluorescence (Ehinger et al., '69; Dowling and Ehinger, '75, '78), immunocytochemistry or autoradiography (Sarthy and Lam, '79; Marc, '82; Yazulla and Zucker, '88; Van Haesendonck and Marc, in

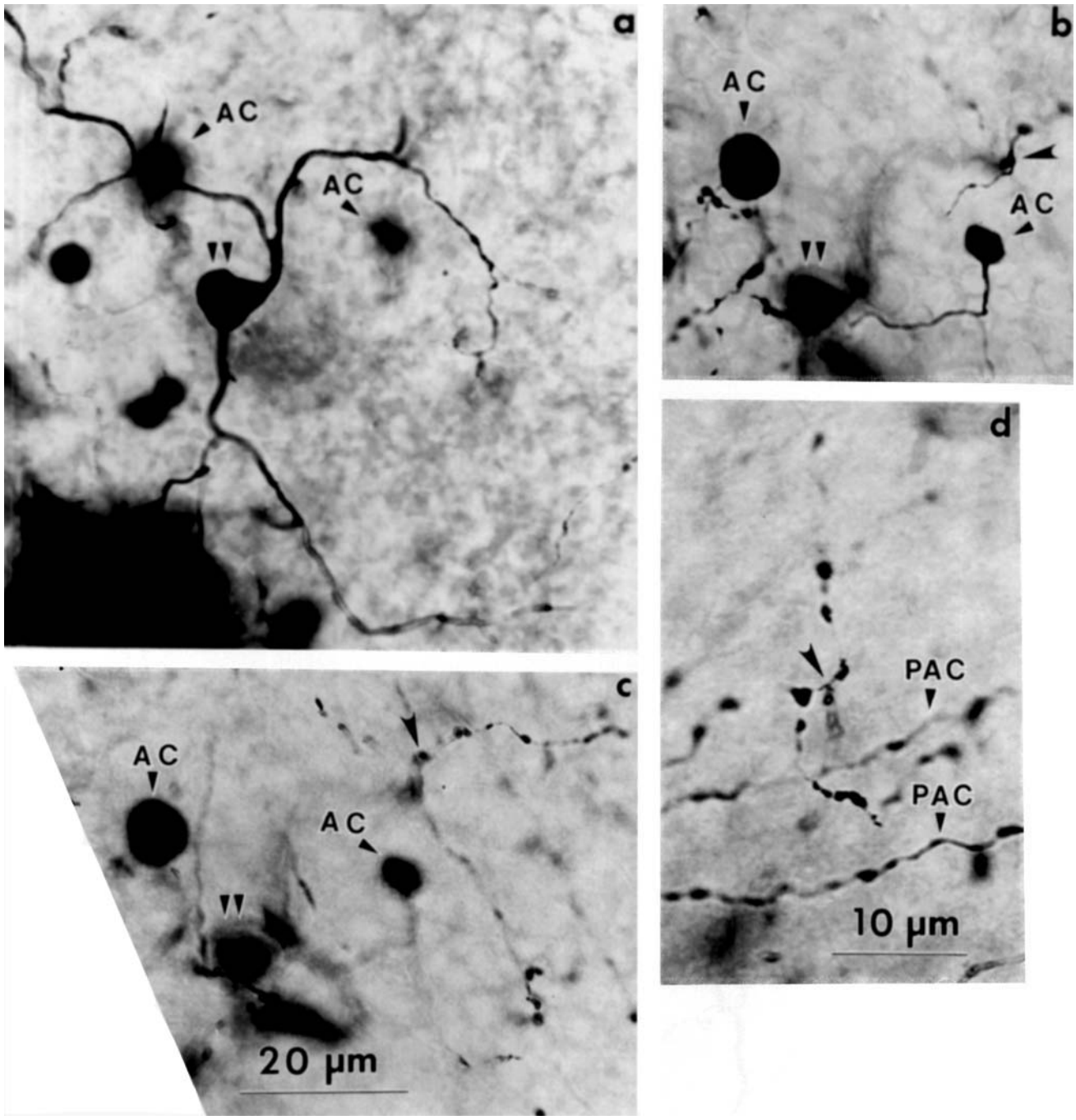

Fig. 9. A Golgi-impregnated glycinergic IPC (Gly-5) shown in a flatmounted preparation. (**a**) Two major processes extend from the soma (double arrowheads) and exhibit dichotomous branching as they proceed to the OPL. The focal plane is approximately in the middle of the INL, although the section is slightly tilted. Two out of focus amacrine cell (AC) somas are indicated. (**b**) The single arrowhead indicates a bifurcation of a glycinergic IPC process in the amacrine cell layer. The outlines of cell bodies in this layer are evident as are the two impregnated amacrine cells. The glycinergic IPC soma (double arrowhead) is now out of focus. (**c**) The focal plane is approximately in sublayer 2 of the IPL and thus the amacrine cell bodies and glycinergic IPC soma (double arrowhead) are out of focus. The single arrowhead indicates a glycinergic IPC process branching and stratifying in this sublayer. (**d**) The single arrowhead indicates the bifurcation of one of the glycinergic IPC processes branching in sublayer 5 of the IPL. Note the processes from a pyriform amacrine cell (PAC) that also stratify in sublayer 5, are in the same focal plane. (a), (b), and (c) are at the same magnification.

preparation). The glycinergic IPC has its soma in the middle of the INL (we have never found one in the amacrine cell layer) and sends primary dendrites to the OPL where they profusely arborize in the distal OPL. The dendrites spawn frequent descending processes, which cross the INL, and these may arise from either primary dendrites extending from the cell body or the fine processes in the OPL. Only one of 20 glycinergic IPCs displayed a process entering the IPL directly from the soma. These findings are similar to earlier autoradiographic results showing major glycinergic IPC

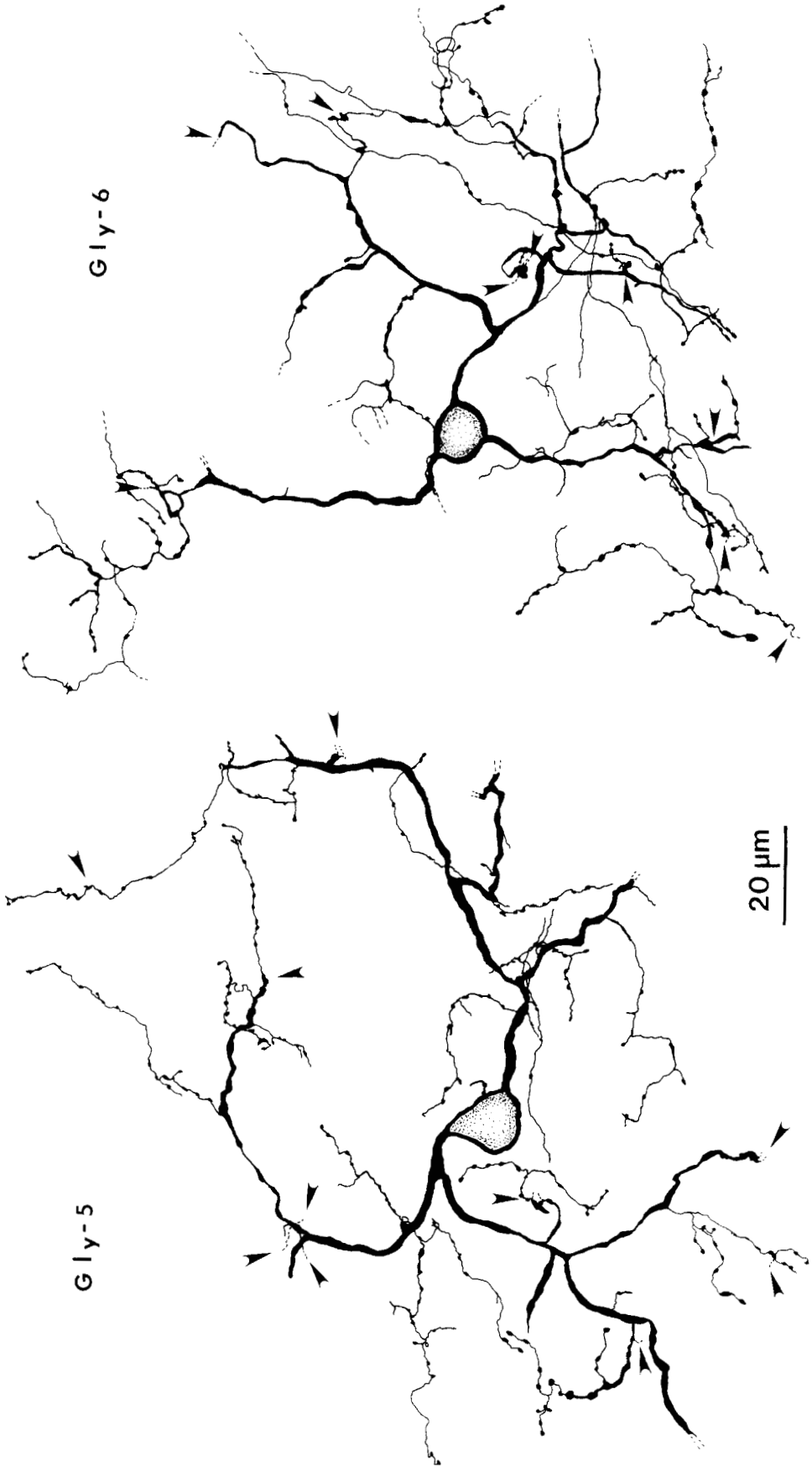

Fig. 10. Camera lucida drawings of two flatmounted glycinergic IPCs (Gly-5 and Gly-6) showing the major processes in the middle to distal third of the INL and fine-beaded varicosities in the OPL. The arrowheads indicate the position from which processes began their descent into the IPL.

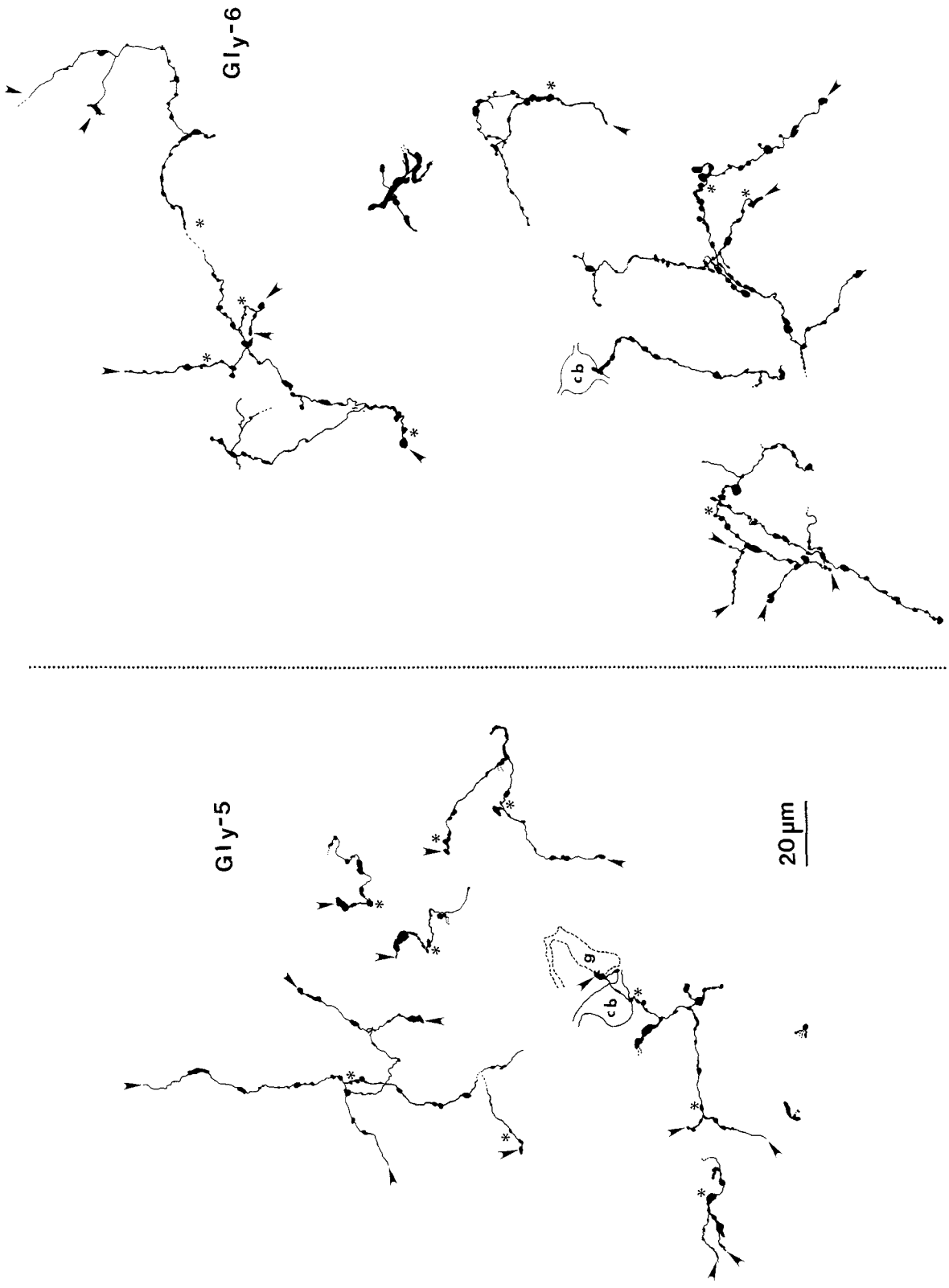

Fig. 11. Camera lucida drawings of the IPL processes of the same two glycinergic IPCs shown in Figure 10. The asterisk indicates the beginning and the arrowhead the apparent end of processes in sublayer 5 of the IPL. The outlines of the cell bodies (cb) are shown as is the outline of a partially impregnated soma in the ganglion cell layer (g). One of the glycinergic IPC processes terminates very close to this soma.

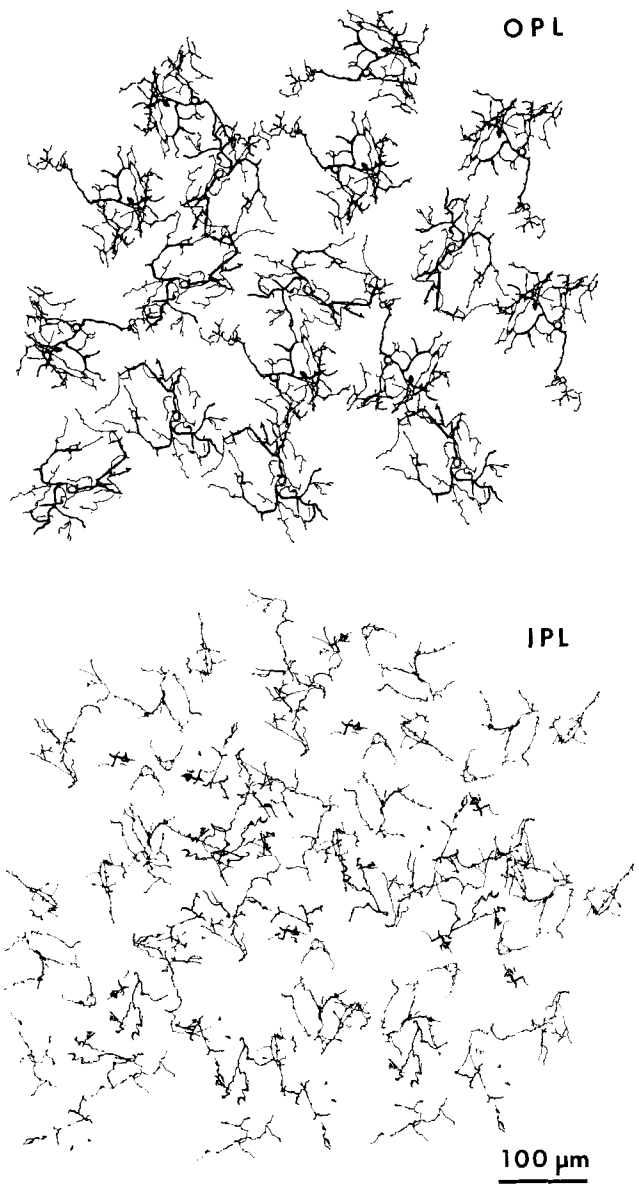

Fig. 12. Digitized representative coverage of the glycinergic IPC in the OPL and IPL. The OPL distribution shows the soma and major processes in the proximal third of the INL and the smaller processes in the distal part of the OPL. There are large areas in both the OPL and IPL that are apparently not covered. The cell density used was 35 cells/mm^2 and the two flatmounted cells (Gly-5 and Gly-6) were randomly oriented in one of eight positions. Abbreviations as in Figure 1.

processes proceeding toward the OPL and direct dendritic interconnections of the OPL and IPL without an interposed soma (Marc and Lam, '81; Marc, '82; Marc and Liu, '84). Evidence for a glycinergic IPC is further supported by the results of Studholme and Yazulla ('88) using immunocytochemistry with antiglycine immunoglobulins.

The IPCs drawn by Ramón y Cajal (1893) for perch retina have smaller somas than goldfish glycinergic IPCs and have many processes arising from the cell body and descending to the IPL. The morphological appearance of the cell in the perch retina resembles a "starlike" arrangement, and thus the term "stellate cell" is befitting, although the cell also fits the definition of an IPC (see introductory section). However, the glycinergic IPC in the goldfish retina does not have a stellate appearance, but has the characteristics of an IPC. Wagner (1976) described neurons similar to Cajal's perch IPCs in *Nannacara anomala* (a cichlid), which possessed large somas located in the INL and a few oblique dendrites projecting to the OPL, but, as in perch, these neurons also had some processes entering the IPL directly from the soma. If these IPC-like cells from various teleostean orders are all glycinergic, they clearly have significant variations in detailed form. This further strengthens the case for recognizing their common IPC-like attributes rather than presuming they are different cells because they have subtle differences in dendritic disposition.

Coverage factor of IPCs

Attempts to visualize the full dendritic morphologies of dopaminergic IPCs using intracellular injection of Lucifer Yellow in the carp retina have resulted only in partial fills (Teranishi and Negishi, '86, '88). Golgi methods also underestimate the true dendritic field sizes of cells, but the coverage factors for the dopaminergic IPCs in our samples ranged from 3.4 to 8.7 in the OPL and 5 to 18.7 in the IPL. The mean IPL value of 11.2 is almost four times the mean coverage of 2.9 found in large carp (33 cm body length; Teranishi and Negishi, '88). The dendritic extent reported by Teranishi and Negishi ('88) is similar to that reported here, but the cell density they employed is less than half that of the 10 to 12 cm goldfish (Marc, '82). This implies that the dendritic field size of this cell may not change significantly with increase in body length, but the cell densities change dramatically (Negishi, '81; Negishi et al., '81). Therefore, the coverage factor may change considerably with increasing body size in cyprinids, which raises the possibility that the coverage of the dopaminergic IPC may not be a sensitive metric of function. For example, the turtle retina lacks dopaminergic IPCs, but dopamine released from dopaminergic amacrine cells diffuses to the OPL from the IPL and regulates electrical coupling between horizontal cells (Piccolino et al., '84, '87).

The coverage factor of the glycinergic IPCs appears to be much smaller than that of the dopaminergic IPCs. Marc and Liu ('84) demonstrated glycinergic IPCs to be heavily postsynaptic to type H1 horizontal cells via somatodendritic and less heavily through axo-dendritic contacts. Although our flatmounted preparation appeared to show uneven coverage of the OPL, the population of glycinergic IPCs may still provide a faithful copy of the S-space to the IPL if it samples from a coupled sheet. To illustrate the sampling of H1 horizontal cells by the glycinergic IPC population, the OPL processes shown in Figure 12 were digitally superimposed on an idealized H1 horizontal cell soma tiling (Fig. 14). Assuming that each process in the OPL proper can contact a horizontal cell (e.g., Marc and Liu, '84) if it traverses a tile, we estimate that at least 54% of the horizontal cell somas within the rectangular grid are contacted by the glycinergic IPCs. The soma contacts are illustrated by the broader lines outlining the horizontal cell tiles sampled by the glycinergic IPCs (Fig. 14).

Figure 15 illustrates the result of digitally expanding the sampling field of each horizontal cell soma from 20 μm to 400 μm (400 μm is the space constant of uncoupled H1 horizontal cells determined by Teranishi et al., '84). It is evident that the IPC processes in the distal part of the OPL can provide an accurate copy of the S-space (Fig. 15), irrespective of whether the horizontal cells are coupled or

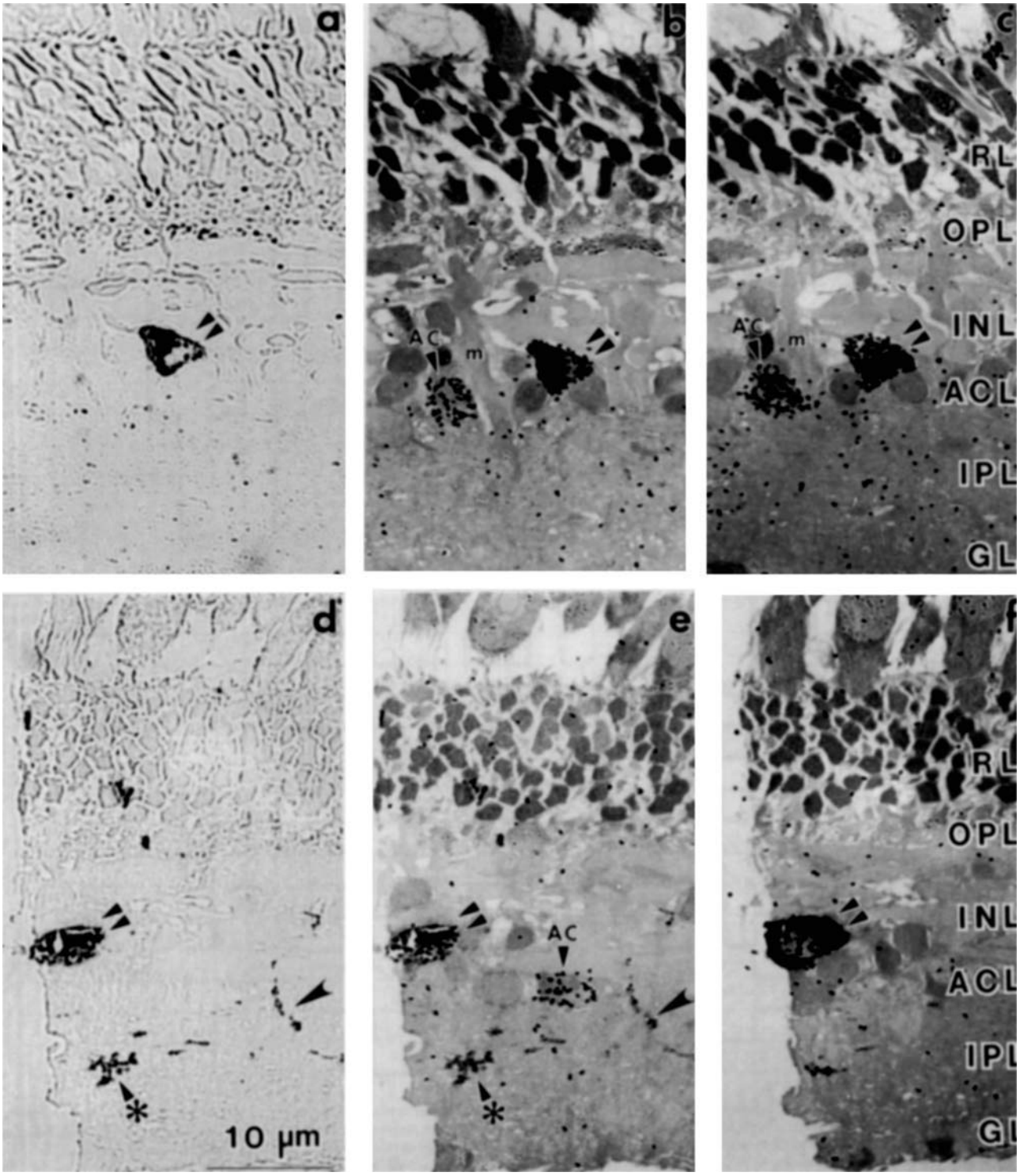

Fig. 13. Light microscope autoradiographs of Gly-1 (**a, b,** and **c**) and Gly-3 (**d, e,** and **f**). Both cell bodies demonstrate high affinity uptake of [^{3}H] glycine. The cell bodies were photographed before (a and d) dye conversion and then lightly stained with toluidine blue after autoradiography (b and e); (c) and (f) were in adjacent sections. Note the autoradiographic grains over the nucleus in (b) and (f). The arrowhead with the asterisk (in d and e) indicates Golgi material that had been dye converted and shows no chemography, and the single arrowhead (in d and e) indicates a glycinergic IPC process in the IPL. Glycinergic amacrine cells (AC) and a process from a Muller cell (m) are also marked. Exposure time ranged from 7 to 14 days. Abbreviations as in Figure 1.

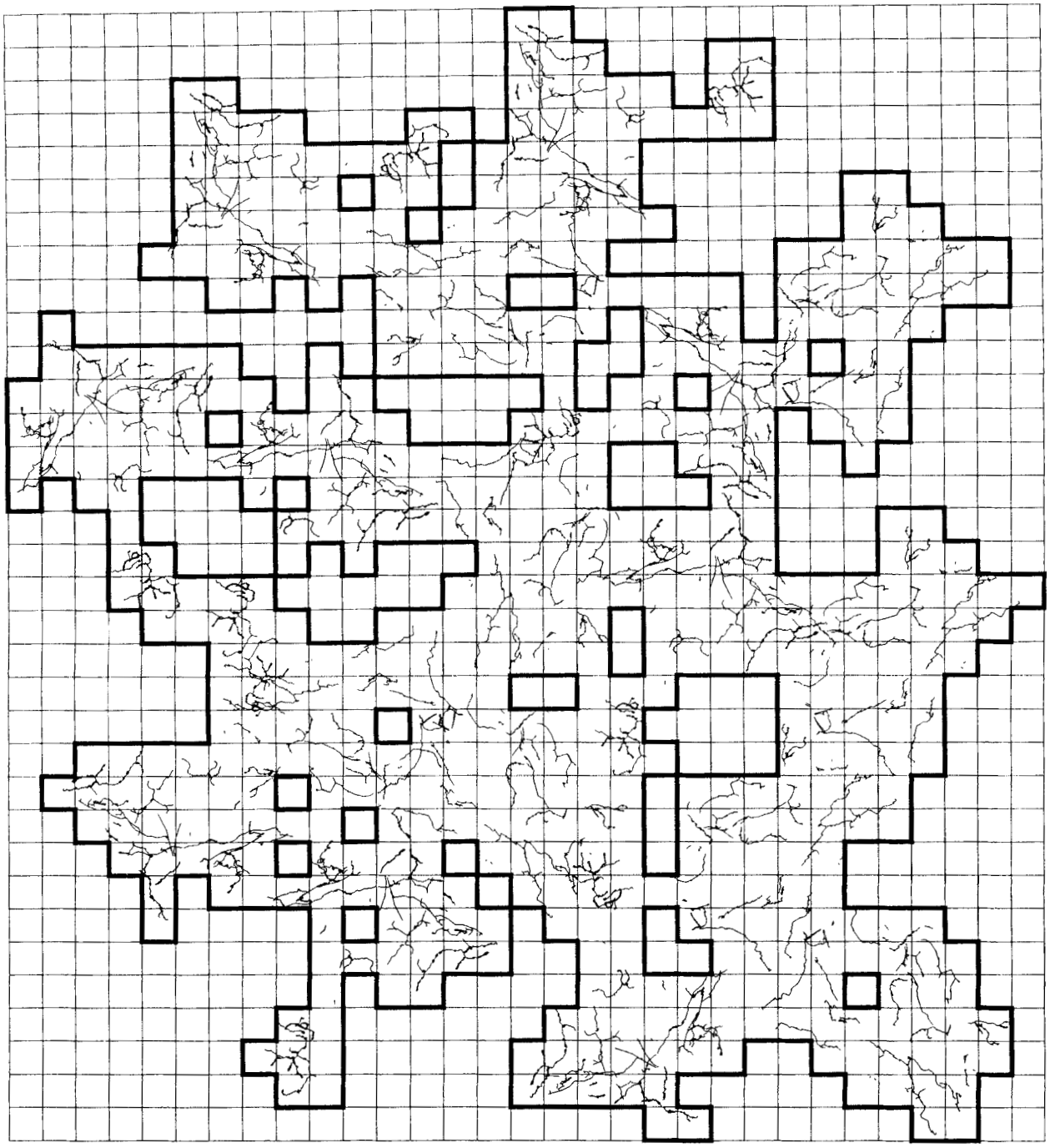

Fig. 14. Possible horizontal cell contacts of glycinergic IPC distal dendrites. An idealized H1 horizontal cell tiling was constructed with a square cell diameter of 20 μm and a cell density of 2,000 cells/mm^2 (Marc, '82). The distal OPL processes of glycinergic IPCs Gly-5 and Gly-6 (see Fig. 12) were superimposed on this tiling. The broader lines outlining the horizontal cell grid indicate the horizontal cells that could be contacted by the IPC field. Up to 54% of the horizontal cell somas in the grid are potentially contacted by the glycinergic IPCs. Using 56 μm as the mean dendritic extent of H1 horizontal cells for goldfishes of the same size (Stell and Lightfoot, '75), the sampling efficiency of the glycinergic IPCs is 82% of the horizontal cells within the rectangular grid.

uncoupled and irrespective of the fact that our impregnations are probably incomplete. Although the impregnation of the cells may be incomplete, it also seems evident that glycinergic IPCs do not form radially symmetrical fields of dendrites in the OPL. The fact that glycinergic IPCs display adequate S-space sampling removes the need for uniform dendritic dispersal in development and implies once more that the differences in coverage factors between the two IPCs may not be terribly significant.

Synaptic characteristics of IPCs

Dopaminergic IPCs form presynaptic junctions in the OPL (Dowling and Ehinger, '78). The actions of exogenously

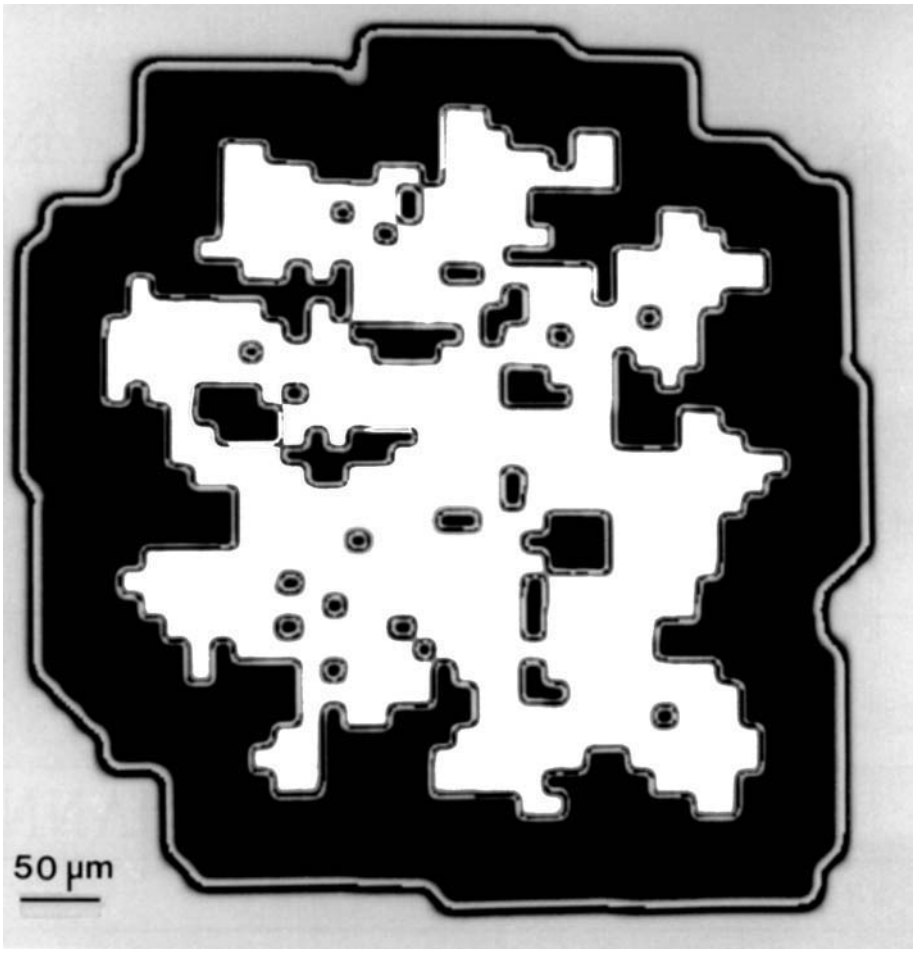

Fig. 15. Visualization of the estimated receptive fields sampled by glycinergic IPCs from a coupled sheet. The aggregate contact field of the array of glycinergic IPC dendrites shown in Figure 14 was idealized as an irregular shape or jigsaw puzzle: the *white* area represents the collection of all the horizontal cell somas presumably contacted by the glycinergic IPCs in this field. Note the presence of holes in the white field, which indicates horizontal cells bypassed by glycinergic IPC dendrites. Since each horizontal cell is expected to have a receptive field of at least 400 μm in diameter, the functional receptive field of the glycinergic IPC contacts was estimated by symmetrically enlarging each 20-μm horizontal cell tile with a digital dilation algorithm until it had grown to 400 μm in diameter. This enlarged field is shown in *black* with the original white contact field superimposed. Notice that all of the holes in the white array are filled in and the size of the functional field extends far beyond the borders of the glycinergic IPC dendrites. Therefore, when sampling from a coupled sheet, uniform receptive fields may be constructed from sparse, nonuniform patterns of dendritic contact.

applied dopamine and dopamine analogues have indicated that dopaminergic IPCs are associated with several possible functions in the vertebrate retina: (1) potentiation of the bipolar cell center responses via reduction of lateral inhibitory actions (Heddon and Dowling, '78), (2) uncoupling of electrical junctions between horizontal cells (Teranishi et al., '83, '84); Piccolino et al., '84), and (3) control of photoreceptor retinomotor functions (Pierce and Besharse, '85; Dearry and Burnside, '86). Although many dopaminergic pre- and postsynaptic contacts are found in the IPL (Dowling and Ehinger, '78; Yazulla and Zucker, '88), their functions are unclear. There are, however, abundant gap junctional contacts in the IPL among various neurons (Marc et al., '88), and it is plausible that the dopaminergic IPC may play some role in regulating coupling in the IPL.

The morphological data provided in this study allow a new perspective from which existing electron microscopic data of goldfish glycinergic contacts in the IPL can be evaluated. Glycine is a transmitter in sign-inverting synapses in the central nervous system and the retina (for reviews see Marc '85, '88). In the OPL, red hyperpolarizing GABAergic H1 horizontal cell bodies and axon terminals make extensive conventional synapses with glycinergic IPCs (Marc and Liu, '84; Marshak and Dowling, '87). Since GABA typically mediates sign-inverting synaptic transmission (see Fig. 16 for details), it would be expected that glycinergic IPCs depolarize in response to light. Though the specific connectivity of the glycinergic IPC in the IPL is still unknown, several aspects of glycinergic contact patterns have been determined (Marc and Lam, '81; Marc and Liu, '84; Muller and Marc, '90): (1) there are no known synapses between glycinergic neurons, (2) for all practical purposes, there are no synapses between bipolar cells and glycinergic neurons, (3) there are a few rare glycinergic synapses onto bipolar cells in sublamina *a*, although the bipolar cell type is unknown, (4) there are extensive synaptic contacts between glycinergic profiles and nonglycinergic amacrine cells, and (5) there are extensive glycinergic inputs to ganglion cells

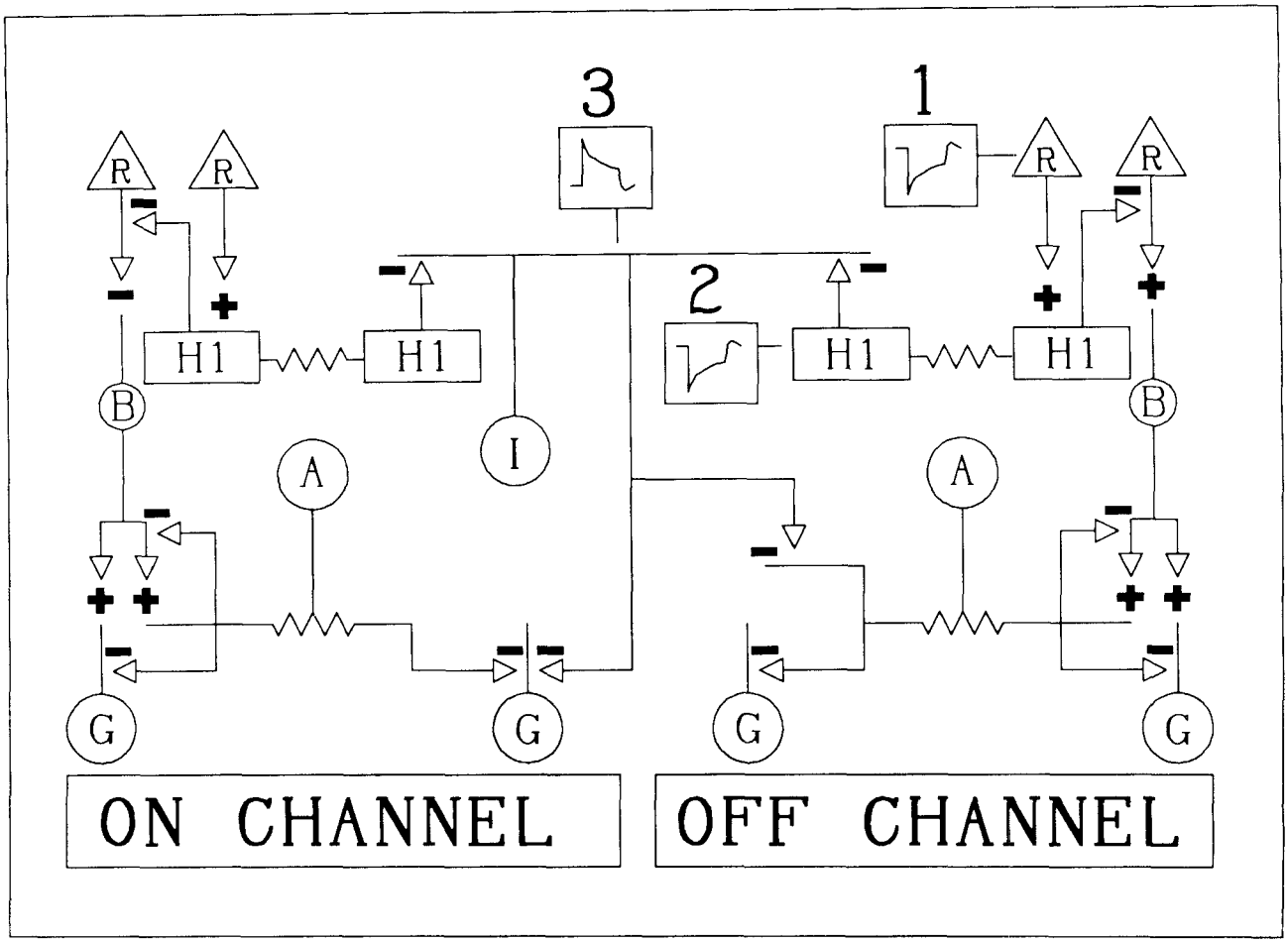

Fig. 16. Circuit diagram of the ON and OFF retinal pathways incorporating an hypothesis for glycinergic IPC connectivity. H1 horizontal cells (H1) receive input from long-wavelength (R) cones via sign-conserving synapses (+). Idealized hyperpolarizing responses to a light increment by R cones and H1 horizontal cells are shown in boxes 1 and 2. If the GABAergic H1 horizontal cell behaves in a similar way to most GABAergic synapses in the central nervous system, i.e., acting via a sign-inverting synapse (−), light-evoked hyperpolarization of the horizontal cell would result in a depolarization of the glycinergic IPC (box 3). The spatial response characteristics of the glycinergic IPC should resemble those of the H1 horizontal cell. If the outputs of glycinergic IPCs (I) behave like glycinergic synapses in the central nervous system, then depolarization of this cell would result in an increase of an inhibitory postsynaptic current in OFF-amacrine cells or ON-center ganglion cells. Therefore, the glycinergic IPC can directly provide an additional hyperpolarizing surround to the ON-center ganglion cells. To effect an additional depolarizing surround in OFF-center ganglion cells, the hyperpolarizing amacrine cells depicted in the figure (most likely GABAergic) would need to contact the OFF-center ganglion cell or hyperpolarizing-bipolar cell via sign-inverting synapses. There is abundant anatomical evidence of GABAergic amacrine cell synaptic contacts onto bipolar cells and ganglion cells (see Marc, '89; Muller and Marc, '90). The hyperpolarizing response of the presumed GABAergic amacrine cell would result in a decrease of GABA release and a decrease in the dark-maintained inhibitory postsynaptic current in the OFF-center ganglion cell leading to a depolarizing surround. Similarly, a decrease of GABA release from the amacrine cell would depolarize the hyperpolarizing-bipolar cell resulting in an increase of glutamate release, and thus an increase in the excitatory postsynaptic current in the OFF-center ganglion cells leading to a depolarizing surround. A—amacrine cell; G—ganglion cell; B—bipolar cell.

throughout the IPL. It is likely that the glycinergic processes contact GABAergic amacrine cells since GABAergic and glycinergic amacrine cells together comprise at least 85% to 90% of the amacrine cell population in the goldfish retina (Marc, '82, '88, '89; Muller and Marc, '90). Considering the abundant contributions of glycinergic IPC dendrites to sublayers 1, 2 and 5 of the IPL, it is likely that many of the glycinergic contacts previously described in these sublayers arise from IPCs (Marc and Lam, '81; Marc, '85; Muller and Marc, '90).

Glycinergic IPCs in *Xenopus* retina make many synapses with amacrine cells in sublayers 1 and 2 of the IPL, and deep in sublayer 5 they are postsynaptic to amacrine cells and presynaptic to unidentified processes (Smiley and Basinger, '88), similar to contact patterns inferred for glycinergic IPCs in the goldfish retina. However, the contacts of amphibian glycinergic IPCs in the OPL are not similar to those of glycinergic IPCs of the goldfish (Marc and Lam, '81; Marc and Liu, '84; Smiley and Basinger, '88), thus the possible homology of the two glycinergic IPCs remains unsubstantiated.

Functional role of glycinergic IPCs

Glycinergic IPCs could have at least two modes of transferring S-space signals to the IPL, consistent with the

known surround properties of sustained ganglion cells (Fig. 16). In sublamina *a*, the presumed depolarizing glycinergic IPCs may directly contact hyperpolarizing GABAergic amacrine cells via a sign-inverting synapse. These amacrine cells are presynaptic to OFF-center ganglion cells (Muller and Marc, '90) and, via a sign-inverting action, can provide an additional depolarizing surround for this ganglion cell class (see Fig. 16 caption). In sublamina *b*, the presumed depolarizing glycinergic IPCs could contribute to the hyperpolarizing surround properties of ON-center ganglion cells via direct sign-inverting contacts.

As local signals pass through the photoreceptor-bipolar cell-ganglion cell chain, the intensity-response curves of successive elements become steeper, displaying greater differentiative properties (e.g., Thibos and Werblin, '78; Naka et al., '79). However, when the average illumination is very bright, the *local* surround may not be potent enough to reset the ganglion cell at an appropriate membrane potential to faithfully encode local intensity variations. The glycinergic IPC could constitute a means whereby a *large*, bright background (as opposed to local surround stimuli) could activate fast, large field adaptation to reset the operating curve of ganglion cells. This model predicts the existence of both GABAergic and glycinergic components to the surround mechanisms of some ON-type and OFF-type ganglion cells, consistent with the observations of Negishi et al. ('78).

Neurophysiological data from the catfish retina indicate that direct current injection into horizontal cells can drive amacrine cells and ganglion cells (Naka, '77; Naka et al., '78; Sakuranaga and Naka, '85; Sakai and Naka, '87a), but the polarities of those interactions are not those predicted by our circuit. The neurophysiological data are fully consistent with the model that the current driven responses are mediated by bipolar cells rather than the horizontal cell axon terminals directly driving amacrine cells (Sakai and Naka, '87b) or the horizontal cell somas driving IPCs directly. It seems improbable that just a few horizontal cell synapses could inject enough current into an amacrine cell to evoke a detectable voltage change. A single glycinergic IPC can potentially sample a minimum of 30 horizontal cells and because each glycinergic IPC is an extremely large neuron, the current injected into a glycinergic IPC from a single horizontal cell would probably evoke a small voltage change (cf. Miller and Bloomfield, '83). Conversely, the likely higher input resistances of cones and bipolar cells (if only because they are smaller) coupled with the high synaptic gain of photoreceptors (Ashmore and Falk, '76; Ashmore and Copenhagen, '83; Capovilla et al., '87) render the horizontal cell-photoreceptor-bipolar cell chain particularly sensitive to current injection. The interruption of horizontal cell to ON-center ganglion cell transmission in rabbit retina by 2,4 APB (Mangel and Miller, '87) strongly supports the dominance of the bipolar cell pathway for *local* horizontal cell inputs. We propose that the glycinergic IPC transfers *global* horizontal cell signals to the IPL.

Summary

We have identified two morphologically distinct IPCs in the goldfish retina through Golgi impregnation protocols. The known circuitry of the dopaminergic IPC appears to provide, in part, a *centrifugal* pathway for information flow in the vertebrate retina. Considering the sampling possibilities of the glycinergic IPC, it is likely that this cell provides a copy of the S-space to the IPL; it is thus a *centripetal* pathway that bypasses the bipolar cell spatial filter. Based on the morphology and known synaptology of glycinergic pathways in the goldfish retina, we are proposing that this cell provides large field surround antagonism for certain ON-center and OFF-center ganglion cells. These surround properties provided by glycinergic IPCs should be very linear and their frequency responses similar to horizontal cells rather than the high pass characteristics of the responses derived from bipolar cells.

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NOTE ADDED IN PROOF

While this manuscript was in press, Yazulla and Studholme (1990) presented direct evidence of large glycine immunoreactive somas in the inner nuclear layer and "Y" shaped dendritic branches connecting the inner and outer plexiform layers of the goldfish retina. These features are identical to those we describe for Golgi impregnated glycinergic IPCs. Reference: Yazulla, S. and Studholme, K. (1990) Multiple subtypes of glycine-immunoreactive neurons in the goldfish retina: Single- and double-label studies. Visual Neuroscience *4*:299–310.

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