Local Order among the Dendrites of an Amacrine Cell Population¹

MASAKI TAUCHI* AND RICHARD H. MASLAND*‡²

*Department of Neurosurgery, Massachusetts General Hospital, Boston, Massachusetts 02114 and ‡Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115

Abstract

The cholinergic amacrine cells of the rabbit retina branch within a narrow stratum of the retina's inner synaptic layer, and their dendritic fields overlap as much as 70-fold. Because each cell's dendrites have many branches, the overlap must create a dense meshwork of cholinergic dendrites. To learn how the overlapping dendrites are positioned with respect to each other, we filled the dendrites of groups of neighboring cells with Lucifer Yellow CH. The cholinergic amacrine cells were selectively stained by intraocular injection of the fluorescent molecule 4,6-diamidino-2-phenylindole. The retinas were then fixed with 2% paraformaldehyde and 0.01% glutaraldehyde. The stained cells were penetrated under visual control by Lucifer Yellow-filled micropipettes. A systematic arrangement of the dendrites was observed. When a pair of cells was injected, their dendrites were often seen to lie alongside each other. In the terminal dendritic region, there are virtually no dendrites that do not end in apposition to a dendrite of a neighboring cholinergic amacrine cell. When small clusters of nearby cells were injected, an ordered microstructure appeared. The dendrites of the cells join together to form curving bundles, which enclose spaces that rarely contain any cholinergic dendrites: the appearance of the dendritic mosaic is that of a lattice with a repeating unit roughly 10 μm in diameter. The significance of this ordering is not certain, but it is possible that the repeating structural unit participates in a modular functional arrangement.

The cholinergic neurons of the rabbit retina (and most other retinas) are a group of amacrine cells located in nearly equal numbers on both sides of the inner plexiform layer. The cell population has been studied by autoradiography (Masland and Mills, 1979), by staining with a silver method (Vaney et al., 1981), and by labeling with the fluorescent molecule 4,6-diamidino-2-phenylindole (DAPI), which was shown by several independent methods to stain these neurons selectively (Masland et al., 1984b). There are about 1600 cholinergic neurons/mm² in the central retina and 350 neurons/mm² in the far periphery. Their shape was learned by injecting them with Lucifer Yellow CH (Tauchi and Masland, 1983, 1984; Vaney, 1984), and they have also been stained by the Golgi method (where they

were called a "starburst" cell; Famiglietti, 1983). They send two to seven primary dendrites to the neuropil of the inner plexiform layer. The dendrites then branch in two narrow strata, one containing the dendrites of the conventionally placed cholinergic amacrine cells, and a second containing those from the cell bodies in the ganglion cell layer. Their dendritic field diameter ranges from 230 μm in the central retina to 800 μm in the periphery. The spacing of the cells and the extent of their dendritic spread indicate that their dendritic fields overlap extensively. A point on the retinal surface is covered by the dendritic fields of 30 to 70 of each type (conventional and displaced) of cholinergic amacrine cell, depending on the retinal region. This contrasts strikingly with other retinal neurons thus far studied, which cover the retina efficiently: the overlap among cells of the same functional class is slight (Wässle and Riemann, 1978; Wässle et al., 1981a, b; Peichl and Bolz, 1984). It seems clear that the physiological function of the cholinergic neurons is somehow reflected in the apparent redundancy of their coverage of the retinal surface.

The primary dendrites of the cholinergic amacrine cells branch many times to end in as many as 80 dendritic terminals. Because the branching is quite profuse and the overlap of the cells very great, the narrow strata where the dendrites lie must contain a dense plexus of cholinergic dendrites. The plexus can be modeled graphically, by printing the image of one of the cells over and over on the same paper, at the density of cells known to exist at the retinal location where that cell was encountered. In the resulting composite (Tauchi and Masland, 1984), the largest dendrite-free spaces are (in the central retina) less than 1.5 μm in diameter. Such modeling, however, assumes that the dendrites of the cells are positioned randomly with respect to each other. Because it may provide clues to the function of the cells, we were curious to see what the real arrangement of the dendrites is. We therefore sought a way to visualize the plexus of dendrites directly.

In our previous study (Tauchi and Masland, 1984), the cholinergic cells were stained with DAPI and were injected under visual control with Lucifer Yellow CH. Penetration of the cell and injection of the dye were done in lightly fixed tissue. Because one selects the cell to be injected visually, and because the fixed tissue is mechanically stable, a large population of cholinergic cells could be completely filled with the dye. Subsequent improvements in technique (notably in the conditions of fixation) have increased the yield of successfully filled neurons still further. This suggested that we might be able to fill enough overlapping cells to reveal the actual dendritic pattern.

Materials and Methods

The techniques used for this work were fundamentally those used previously (Tauchi and Masland, 1984). They will be described briefly here, with emphasis on recent improvements that made it possible to inject clusters of the cholinergic neurons.

Retinal preparations. Adult New Zealand White rabbits were used. To label the cholinergic neurons, 0.5 μg of DAPI (Sigma) was injected intraocularly. One to 2 days later the animals were deeply anesthetized with sodium pentobarbital (20 mg/kg) and diazepam (2 mg/kg), and the eye was enucleated. It was hemisected at the equator and the lens and vitreous humor were

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² To whom correspondence should be addressed, at Department of Neurosurgery, Warren Building 455, Massachusetts General Hospital, Boston, MA 02114.

lifted away. The posterior eyecup was everted over the round head of a Teflon rod under Ames medium (Ames and Nesbett, 1981) equilibrated with 95% O₂ and 5% CO₂. The retina was gently teased away from the pigment epithelium and the optic nerve stump was trimmed closely.

Four cuts were made to allow the retina to lie flat, in the shape of a Maltese cross. In a medium-filled Petri dish it was spread across a piece of Whatman no. 40 ashless filter paper and was then sandwiched under a second piece of the same paper. The retina was gently flattened by a glass slide. The medium was drained from the dish and replaced by the fixative mixture to be described below. After fixation the tissues were rinsed five times in large volumes of 0.1 M phosphate buffer and were stored in it at 4°C. Neurons in these preparations can be successfully injected with Lucifer Yellow for many weeks. Penicillin (100 units/ml) and streptomycin (0.1 mg/ml) were added to the buffer to inhibit bacterial growth.

In the previous experiments (Tauchi and Masland, 1984) we fixed the retinas with 4% paraformaldehyde in 0.1 M phosphate buffer. In those preparations, however, dye injection sometimes revealed what appeared to be broken dendrites. These appeared after injection as blunt stumps; for the thicker dendrites, Lucifer Yellow could sometimes be seen diffusing from the open end. Since our purposes required complete filling of the dendritic tree, even a small number of these events would have been a serious limitation. Broken dendrites were rarely seen when cells in living tissue were injected (unpublished experiments), and we reasoned that the breakage might have resulted from mechanical damage after fixation; fixation would be expected to make the tissue more brittle. One way to reduce the breakage is by taking great care to avoid mechanical disturbance of the tissue. It is also lessened when a small amount of glutaraldehyde is added to the fixative mixture. The minimum effective concentration of glutaraldehyde was between 0.01 and 0.005%, in combination with 2% paraformaldehyde (in 0.1 M phosphate buffer.) Fixation was generally for 1 hr, although periods as short as 30 min were sometimes used.

During Lucifer Yellow injection the tissue was mounted in a small Petri dish placed on the stage of a fluorescence microscope. The arrangement of the tissue was designed to hold it flat and keep it moist. A piece of black (non-autofluorescent) filter paper (Schleicher and Schuell) lay at the bottom of the dish, with the retina lying upon it. Above the retina was a piece of coarse (5-mm openings) nylon mesh, and above the mesh was a doughnut-shaped piece of moist filter paper. A similarly shaped piece of parafilm lay above the filter paper, to retard evaporation, and the whole multilayered sandwich was held down by small weights placed on its edges.

Micropipettes were pulled from Pyrex glass capillary tubing using a Kopf vertical puller. They were filled with a solution of 2 to 4% Lucifer Yellow CH and 0.25 M KCl in 50 mM Tris-HCl (pH 7.5). The resistance of the micropipettes ranged from 20 to 50 megohms when measured in 0.1 M phosphate buffer. Dye was injected by passing 100-msec pulses of negative current at 5 to 20 nA.

The tissue and electrode were viewed by conventional epifluorescence optics (Leitz), using filter sets that permit visualization of DAPI, Lucifer Yellow, or both simultaneously. Microscope objectives with very long working distances (Nikon $\times$ 40, 10 mm; Leitz $\times$ 20, 6 mm) were used. The electrode was positioned coarsely using a micromanipulator. Final alignment of the electrode over the desired cell was achieved by moving the stage, which was controlled by fine micrometer screws. The electrode was advanced by a hydraulic drive. The encountering of the cell soma by the micropipette was visible in the microscope as displacement or dimpling of the cell's surface. Penetration was usually achieved by oscillating a capacitance compensation circuit, as previously described (Tauchi and Masland, 1984).

In order that they may be easily compared, all of the figures in this paper show neurons from the central retina, less than 4 mm ventral to the visual streak. The spacing of the cells, and the spacing of their dendrites, increase from central retina to periphery. The findings were qualitatively similar, however, at all eccentricities. All of the cells studied were from the population of cholinergic amacrine cells with cell bodies in the ganglion cell layer. Indirect evidence (from the distribution of silver grains seen in autoradiography—see "Discussion") indicates that the arrangement of the dendrites of the conventionally placed cholinergic amacrine cells is the same as for the displaced ones, but this will require direct confirmation.

Müller cells (Fig. 5) were filled by injecting Lucifer dye into their cytoplasm at the level of the ganglion cell layer. It was not necessary to visualize individual Müller cells in order to inject them, because the Müller cell feet fill a large fraction of the space within the ganglion cell layer; when the micropipette tip was at the depth of the ganglion cell bodies it had very often penetrated a Müller cell. The injected Müller cells were viewed in whole mounts or in 50- to 100- μ m vertical sections of agar-embedded tissue (Tauchi and Masland, 1984).

Other neurons and other tissues. An appendix to this paper describes experiments aimed at learning the extent to which the methods that we

developed for study of the cholinergic amacrine cells could be generalized to other situations. Three examples are shown. For these, differing methods of fluorescent staining and tissue preparation were used.

Rabbit horizontal cells were injected following staining of their cell bodies by intraocularly injected Lucifer Yellow CH. The rabbit was anesthetized and 1.5 mg of Lucifer Yellow were injected. Two days later, the rabbit was again anesthetized and the retina was removed, fixed, and prepared for injection as described above. In the rabbit, intraocularly injected Lucifer Yellow accumulates in the cell bodies of the horizontal cells. They were visualized for injection using Leitz filter set H2 (exciting wavelengths, 390 to 490 nm; viewing wavelengths, 515 nm and longer).

Cat retinas were isolated and fixed in the same way as rabbit retinas. The cell bodies were stained by immersion of the fixed tissue for 5 min in 1 μ M acridine orange, in 0.1 M phosphate buffer. After staining had occurred, the tissue was rinsed in buffer and was mounted in the microscope. The cell bodies and microelectrode were visualized by use of Leitz filter set H2.

Rat hippocampal slices were prepared from young Long-Evans rats by methods conventional for physiological experiments on these preparations (Teyler, 1980). The hippocampal slices were kindly donated by Dr. Barry Sandrew. The animal was stunned by a blow to the neck and quickly decapitated. The brain was exposed and the hippocampus was dissected free. It was cut into slices approximately 400 μ m thick using a Stoelting tissue slicer. The slices were transferred to wells of a commercial incubation chamber (Wright) filled with Ames medium and kept at 37°C. They were maintained in the chamber for 1 hr, after which they were removed and fixed. The conditions of fixation were the same as those used for retinas. (Incubation under physiological conditions improved the preservation of neurons near the surface of the slice. It appeared as though the cut dendrites at the surface "healed" during the incubation but could not do so after fixation.) The slices were stained with acridine orange as for cat retinas, and neurons were injected by the usual method.

The selection of micropipettes depended somewhat on the class of cell that we desired to inject. High resistance pipettes were used for the smaller cells. Although larger micropipettes could be used for cells with larger cell bodies, these tend to damage the cell at the point of entry. The hole made by the micropipette then fails to reseal when the electrode is withdrawn, so that the injected dye leaks out of the cell. The tendency for the cells to leak varied somewhat from preparation to preparation. For large neurons it was rarely an issue, but in occasional tissues leakage prevented successful injection of the smallest ones. For cells located relatively deep within the preparation, mechanically strong (short shank) pipettes were preferable, because they can be advanced toward the target cell without bending. For cells deep within the tissue it is also useful for the electrode to enter the tissue at an angle as near as possible to the vertical—hence the use of long working distance objectives.

Results

The shape of the cholinergic neurons, as revealed by injecting Lucifer Yellow into their DAPI-labeled cell bodies, is shown in Figure 1. The proximal dendrites run quickly into the inner plexiform layer, where they curve to branch almost on a plane. The dendrites bear small twigs and varicosities. These are especially numerous in the distal third of the dendritic tree, but are present to some extent throughout.

Because the cell body to be injected was selected visually, it was possible systematically to inject cells with overlapping dendritic trees. We began by injecting pairs of cells, three of which are shown in Figure 2. To a superficial glance, the dendrites of these pairs seem to overlap irregularly. A moment's closer examination, however, shows that the overlapping dendrites are arranged in a fashion that is far from random.

The evidence is perhaps most striking when the overlap of the dendritic fields is partial. For the cells shown in Figure 2A, for example, the region of overlap is only about 5%. At the borders of the region of overlap, there are pairs of dendrites that appear to have sought out each other: they cross a large expanse of neuropil to end in exact apposition. In one case a varicosity terminates each of the two branches, so that the varicosities abut each other precisely. As one moves from the edges of the region of overlap toward its center, the dendrites are seen to run alongside each other. Often they are so closely intertwined that only the doubled brightness of the fluorescence confirms that two dendrites are still present. Remarkably, almost all of the dendritic terminals end in

Figure 1. The shape of the cholinergic amacrine cells. Two nearby cells have been injected with Lucifer Yellow CH. (Many other cholinergic amacrine cells, which were not injected, lie between them.) Scale bar, 100 μm .

apposition to dendrites of the neighboring cell. Within the region where the dendritic fields overlap, there are only a handful of instances where the terminal end of a dendrite can be clearly visualized. The ends of most of the dendrites are obscured by their intertwining with dendrites of the neighboring cell.

In Figure 2B, two cells with greater overlap between their dendritic fields are shown. Because of the spacing of the cells, there are necessarily cases where the dendrites cross at right angles. However, there are still striking instances (Fig. 2B, arrows) where they turn to run together. One also sees that the joined dendrites begin to enclose dendrite-free spaces, the significance of which will become plain later. Figure 2C shows the dendritic fields of two cells whose cell bodies are located only 40 μm apart, so that their dendritic fields overlap very substantially. Because the cell bodies lie close together, the dendrites are once again more or less parallel, as they were for the relatively distant cells shown in Figure 2A. As for the cells shown in Figure 2A, there is a marked tendency for the dendrites to run together in pairs. Because of the pairing, spaces in the dendritic mosaic are created. They are larger than would be expected were the dendrites of the two cells positioned randomly with respect to each other.

If the individual elements of the dendritic mosaic obey systematic rules, one might expect that the final meshwork would also show signs of order. To investigate this possibility, we attempted to inject a larger fraction of the cells whose dendrites overlay a point on the retinal surface. Figure 3 shows one such experiment. In this case five cholinergic amacrine cells, with overlapping dendritic fields, were injected, as shown in the *inset*. The mosaic of cholinergic dendrites appears as a regular lattice. Groups of up to five dendrites join together to form bundles. The bundles curve or form irregular polygons to enclose spaces that do not include any fluorescent dendrites. Although the lattice is not perfectly regular, it appears to

be constructed of a repeating unit that has a diameter of roughly 10 μm . In retrospect, the gaps in the dendritic trees of cell pairs like those shown in Figure 2 would seem to foreshadow the unit of size around which the final mosaic is built.

In calling the image shown in Figure 3 a regular lattice we are assuming, of course, that the pattern shown there is different from the pattern that would occur if the dendrites were positioned at random. As a test of that proposition, we performed the following experiment. A drawing was made of a cell that had the shape and dendritic field diameter of the cells injected for Figure 3 (the size and morphology of the dendritic fields, for a constant retinal eccentricity, varies very little from cell to cell—Tauchi and Masland, 1984). Five copies of that image were made on transparent plastic. The copies were positioned in exact proportion to the locations of the cell bodies of the neurons whose dendritic mosaic is shown in Figure 3. At the region of overlap corresponding to the field shown in Figure 3, a tracing of the dendrites was made. The resulting "random" dendritic mosaic is shown in Figure 4. Figure 4 also shows the actual dendritic mosaic traced directly from Figure 3. The difference is obvious.

We wondered whether the gaps in the lattice might be due to the stalks of the Müller cells, which pass through the synaptic plane of the cholinergic amacrine cells. From published data, however, it appeared that the Müller cell stalks are too small to account for the gaps, and this was confirmed by injecting Müller cells with Lucifer Yellow. A drawing of one of the injected Müller cells is shown in Figure 5. There is a second way to visualize the Müller cells as they pass through the inner plexiform layer. If one ejects Lucifer Yellow from a micropipette placed within the neuropil, it diffuses throughout the extracellular space but is excluded from the Müller cells. Since the Müller cells in whole mounts are viewed end-on, they are revealed by their negative image; they appear as dark holes surrounded by

Figure 2. Pairs of cholinergic amacrine cells have been injected, with varying degrees (A, B, C) of dendritic overlap. Arrows in B and C indicate points at which dendrites of the two cells join in apposition. Scale bars, 50 μm .

Lucifer fluorescence. Figure 5 also shows the results of such an experiment. In this case the electrode was withdrawn after injection of dye into the plexiform layer, and a cholinergic amacrine cell above the injection site was then filled, to illustrate the scale of the two cells. The diameter of the Müller cell stalks, viewed either by injection or by negative staining, was 3 to 5 μm —too small to account for the gaps in the lattice of cholinergic dendrites.

Because there was an ordered arrangement of the dendrites in the lateral plane, we sought evidence of an organizational principle in the vertical (scleral-vitreous) dimension. Although the dendrites of the cholinergic neurons lie within narrow strata, the width of these strata (from autoradiography they are estimated to be 2 to 4 μm

thick) would still allow for systematic vertical arrangement of the dendrites. For example, there might be sublamination, so that the cells are layered upon each other. For these studies we used pairs of injected cells. We observed a consistent relationship between the location of the proximal dendrites and that of the more distal ones. For the cholinergic neurons with cell bodies in the ganglion cell layer, the inner third of the dendritic tree always lies more vitread than the remainder. Once the dendrites have reached their final plane, however, there is no regular pattern that we can discern. Although it is difficult to resolve small differences in the depth of the most closely apposed of the dendrites, clear instances were seen in which the dendrites of the two cells were interwoven, such that first one cell

Figure 3. The mosaic formed at the intersection of the dendritic fields of five nearby cholinergic amacrine cells. The *drawing* below shows the overall extent of the dendritic arbors of the filled cells. In the drawing, the field shown in the micrograph is outlined by a dotted circle. Scale bar, 20 μ m.

and then the other was uppermost. An example of such an analysis (for two cells with an intermediate separation, as in Fig. 2B) is shown in Figure 6.

Discussion

The repeating lattice created by the dendrites of the cholinergic amacrine cells reflects a systematic ordering of their position with respect to each other. It is not due simply to the need to bypass the

stalks of the Müller cells; these fill only a fraction of the space enclosed by bundles of cholinergic dendrites. There is evidence quite independent of the present studies that the lattice structure is the actual arrangement of the cholinergic dendrites. Eckenstein and colleagues (Eckenstein and Thoenen, 1982; Eckenstein et al., 1983; F. Eckenstein and R. W. Baughman, manuscript in preparation) have carried out careful immunohistochemical studies of the cholinergic system in the rat retina (which has the same cholinergic amacrine cell as the rabbit) using a well characterized antiserum to choline acetyltransferase and sensitive histochemical methods. They found reaction product distributed in a repeating pattern that is exactly as would be expected from the dendrites shown by our injections. One could not reach a firm conclusion about its meaning because of uncertainty that the antibody had revealed the whole dendritic tree of the neurons. (Current immunohistochemical methods for detecting neurotransmitter enzymes almost invariably stain proximal dendrites more strongly than distal ones.) There is no reason to think, however, that Eckenstein's methods did not stain all of the cells at a given location to the same degree. The immunohistochemical results thus provide complementary evidence that the dendritic lattice revealed by complete filling of a smaller number of neurons is real.

There is another way to cross-check the present findings. In a

Figure 4. The lattice of dendrites shown in Figure 3 has been traced (*left*). It is compared with a mosaic created by positioning the same number of cells irregularly with respect to each other (*right*). Note that it is rare to see a free dendritic ending in the actual case, but quite common in the synthetic one. The difference between the actual and the synthetic case is actually underestimated in this representation; some dendrites in the actual case run so closely parallel that they could not be resolved and were drawn as a single line.

previous study Masland and Mills (1979) used a rapid-freezing method to autoradiographically identify [^3H]acetylcholine synthesized within the rabbit retina from extracellular [^3H]choline. Vertical sections $2\ \mu\text{m}$ thick were autoradiographed. Within the planes where the dendrites of the cholinergic neurons are now known to lie, bands of [^3H]acetylcholine were seen in the autoradiograms. The bands are nearly continuous; that is, they are present in all autoradiographed sections and run from one end of the section to the other. That finding (which had also been obtained in the chicken retina—Baughman and Bader, 1977) was actually the first indication that the cholinergic neurons overlap so extensively. A $2\text{-}\mu\text{m}$ vertical section is thin relative to the dimensions of retinal dendritic trees; in order for the band of radioactivity to be present across every section, it is necessary that the cholinergic neurons branch widely enough and densely enough to blanket the retina quite evenly (Masland and Mills, 1979; Masland, 1980). However, there are gaps in the bands of acetylcholine, and it is of interest to see how the gaps correspond to the spaces in the mosaic revealed by the present work. One can do this by imagining a $2\text{-}\mu\text{m}$ section taken vertically through the mosaic of cholinergic dendrites shown in Figure 3. This section is then considered to be autoradiographed: wherever dendrites are present in the section, silver grains will appear in the autoradiogram. If one allows for the spread of beta particles that occurs during autoradiography, it is seen that the size and frequency of gaps in the hypothetical section are quite similar to the gaps observed when actual sections are autoradiographed (see Masland and Mills, 1979, Figs. 4, 8, 12, 13, and 15; Masland et al., 1984a, Fig. 8; Masland et al., 1984b, Figs. 1, 6, and 11.)

Taken together, the evidence thus indicates that a repeating lattice is the actual arrangement of the cholinergic dendrites, and one immediately asks how such a structure could be created. The question is perhaps best addressed from the point of view of pairs of cells, as shown in Figure 2, for which the dendrites have systematically become aligned. We can imagine three ways in which this might happen. Each has obvious precedent in analyses of neural development, and each implies a mechanism that has ramifications which would require extensive discussion (for review, see Magistretti et al., 1984). Without denying the importance of such issues, we will state the possibilities in shorthand form.

First, the cholinergic dendrites might seek each other out, through adhesive specificity or tropic interactions. This would be especially likely if the cells synapse on each other. (One should remember that for amacrine cells the “dendrite” both receives synaptic input and makes synaptic output.) There are instances (see Fig. 2A) in which a varicosity located at the extreme end of a process of one cell is located end-to-end with a varicosity on a identical terminal of a neighboring cell. These cases cry out for electron microscopic examination.

Figure 5. The shape and distribution of the Müller cells. *Left*, Drawing of a Müller cell that had been injected with Lucifer Yellow. *i.p.l.*, inner plexiform layer. *Right*, The size and distribution of Müller cell stalks within the inner plexiform layer. They are made visible by Lucifer dye that was injected into the extracellular space of the neuropil, and appear as dark spaces. To show the relative sizes and proportions of the Müller cells and the cholinergic amacrine, one of the latter has been injected intracellularly. Only the proximal dendrites of the cholinergic amacrine are shown, as they pass from the cell body into the neuropil; they lie above the focal plane of the micrograph. The scale bar at lower right represents $30\ \mu\text{m}$ for the whole mount and $14\ \mu\text{m}$ for the injected Müller cell.

Figure 6. Two cholinergic amacrine cells were injected and the relative depth of their dendrites within the inner plexiform layer was studied. *Cell A* has been drawn by a solid line, and *cell B* is drawn by a dotted line. In some cases it was not possible to decide which of the dendrites lay deeper. In others, however, the distinction could be made clearly: at the points of dendritic crossing, instances where cell A was deeper are indicated by a solid arrow and instances where cell B was deeper are indicated by an open arrow. Neither cell is always uppermost; the dendrites are interwoven.

Alternatively, the dendrites could run together because they follow a common track. This could be an attractive element in the extracellular matrix, or simply a path of least resistance (channel) among the other processes of the inner plexiform layer.

Finally, the processes could run together because they are attracted to a common neuronal target. The obvious candidate, of course, would be the dendrite of the retinal ganglion cell, upon which the cholinergic amacrine cell is known to synapse (Masland and Ames, 1976; Ariel and Daw, 1982; Famiglietti, 1983). The repeating unit in the dendritic mosaic of the cholinergic amacrine cells, however, is far smaller than the dendritic fields of the ganglion cells. For example, the dendritic field of the directionally selective ganglion cells injected by Amthor et al. (1983, 1984) would be covered by roughly 150 of the lattice units. Important functional interactions occur on a spatial scale smaller than the ganglion cell dendritic field. The "local subunit" of the directionally selective cell is a famous case in point; but the directionally selective cell is actually a special case within the wider class of ganglion cells that are sensitive to the movement of stimuli within subareas of their receptive fields (see Masland et al., 1984a). One wonders if the cholinergic dendrites might combine with others that impinge upon the lattice to create a repeating functional unit—a "module" that carries out a multineuronal integrative action on a local scale.

The cholinergic neurons are an unusually favorable case for observing order among the dendrites. A pattern exists among dendrites of a single cell class, and its dendrites lie in a single plane. To see order in a three-dimensional structure would have been harder. There is reason, however, to think that it might also be present elsewhere—among dendrites of similar neuronal classes and among those of dissimilar ones. The degree of order in the

position of retinal cell bodies is striking (Wässle and Reimann, 1978; Wässle et al., 1981a, b), and the retina is a tissue in which a relatively small number of functions are replicated over and over in the lateral plane. From electron microscopy it is known that certain synaptic interrelationships recur among inner retinal neurons (Dowling and Boycott, 1966; Kolb, 1979; McGuire et al., 1984). It would be surprising if recurring interrelations in the larger arrangement of their dendrites did not exist as well.

Appendix

We were naturally curious to see whether our approach to studying the cholinergic amacrine cells could be used for other neurons of the mammalian central nervous system. We therefore carried out experiments in which we injected a variety of neurons of the rabbit retina, the cat retina, and the rat hippocampus.

Figure 7. A, Horizontal cells of the rabbit retina, injected after staining of the cell bodies by intraocularly injected Lucifer Yellow. The cell bodies of some of the cells that were not injected are visible in the field. B, Beta-type ganglion cells of the cat retina, injected after staining of the cell bodies with acridine orange. The uninjected cell bodies are not visible because their staining by acridine orange is less bright than the Lucifer dye that was injected into the beta cell (and fades more quickly during photography). C, Granule cells injected in a rat hippocampal slice stained with acridine orange. Scale bars, 40 μ m.

Several different methods for fluorescent staining of the neuronal cell bodies were used. In some cases the dye, granular blue, was applied to the cut axons of the optic tract (see Hayden et al., 1980; Masland et al., 1984b), and after retrograde transport had occurred the retinas were fixed and the labeled ganglion cells were injected with Lucifer Yellow. This gives a specific labeling, the cells being defined by the location of termination of their axons. We found, however, that it is not always necessary to have a method that gives perfectly specific staining. A variety of fluorescent molecules, when applied to living tissue, stain cells with some degree of specificity (de Monasterio et al., 1981; Sarthy et al., 1982; Wilcox and Francheschini, 1984). One of these is Lucifer Yellow itself. When Lucifer Yellow is injected into the rabbit eye (see "Materials and Methods"), it is accumulated by the cell bodies of horizontal cells and some ganglion cells. After fixation, the cells can be injected with Lucifer Yellow to reveal their whole shape. A pair of injected horizontal cells, from a rabbit retina stained *in vivo* by Lucifer Yellow, is shown in Figure 7.

Other molecules label most neurons but still allow visually guided penetration of the cell bodies. Fluorescent nucleic acid stains are particularly useful in this regard, because the nuclear staining that they produce yields lower background fluorescence than do molecules which stain a more widely distributed component of the cell. These dyes need not be applied *in vivo*; the tissue can be stained after fixation (Schmued et al., 1982). Molecules particularly useful for this purpose were Nuclear Yellow and bisbenzimidazole (Hoechst 33258). Acridine orange applied to fixed tissue stains both the nucleus and the cytoplasm, and was particularly useful. Because we began this work from the point of view of "specific" staining, we only gradually realized that cells can be discriminated to a considerable degree on the basis of staining features other than brightness—provided that the stain, as with the nucleic acid-binding molecules, reveals distinctive features of the cell clearly. The cells can be discriminated on the basis of location, cell body size, cell body shape, and nuclear morphology. There are also subtle differences in the brightness and pattern of nuclear staining that are reliably correlated with cell type. The final correlation with cell type is made, of course, by trial and error: because Lucifer Yellow injection under the microscope gives instant information about the cell's dendritic shape, one quickly learns the soma features associated with specific dendritic morphologies. One can then preselect for injection, from the appearance of the soma, a particular morphological class of neuron. For cells with very distinctive cell body staining (the alpha type ganglion cell of the cat retina), the accuracy of preselection is virtually perfect. For even the least distinctive cells one can increase the odds of injecting a member of the desired neuronal class to well above chance. Neighboring beta type ganglion cells, from a cat retina stained with acridine orange, are shown in Figure 7. The figure also shows a pair of granule cells injected in a rat hippocampal slice that had been fixed and then stained with acridine orange.

In principle these methods allow one to learn the shape of any neuron whose cell body can be fluorescently labeled in the living or lightly fixed tissue. Because cell filling is directly observed, one learns immediately when a successful fill has occurred. Over- and under-filling are avoided. After a successful injection, one can select the location and type of the next cell to be injected. This makes possible systematic study of pairs (or more) of nearby neurons. Such has been done here for cells of similar type, but dissimilar pairs can also be injected.

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