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Exclusionary dendritic interactions in the retina of the goldfish

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Summary

The retina of the goldfish grows throughout its life, in part, by the addition of new neurons at the margin. New ganglion cells added at the margin tend not to grow their dendritic arbors into the older, central retina. Hitchcock and Easter (*J. Neurosci.* 6, 1037–1050 (1986)) proposed that the dendrites of the new cells were prevented from extending centrally within the inner plexiform layer by the dendrites of the previous generations of cells. This proposal was tested by first killing existing ganglion cells with a retrogradely transported neurotoxin (propidium iodide; PI), and then observing the orientation and branching pattern of the dendrites of ganglion cells added subsequently at the margin. Dendrites were stained in retinal wholemounts by intracellular injections of Lucifer yellow. The data showed that cells added subsequent to the PI treatment grew their dendritic arbors preferentially toward central retina consistent with the hypothesis. It is concluded that interactions among adjacent ganglion cells regulates dendritic growth.

Key words: plasticity, neurotoxin, Lucifer yellow, intracellular injection, propidium iodide, neuron, goldfish, retina, dendritic growth.

Introduction

During development interneuronal interactions shape the precise synaptic connections that are characteristic of the mature central nervous system. These interactions generally occur relatively late in neural development, and serve to refine initially diffuse connections (e.g. Sretevan and Shatz, 1986), modulate neuronal survival (e.g. O'Leary *et al.* 1986), or control the outgrowth of neuronal processes (e.g. Deitch & Rubel, 1984). Most studies have focused upon interactions that occur between adjacent axon terminals, or between terminals and their targets. Recent evidence suggests that interactions occur between the growing dendrites of neighboring cells as well (e.g. Wässle *et al.* 1981; Perry & Linden, 1982). In this study, the role played by neighboring ganglion cells in modulating each others' dendritic growth is investigated in the retina of the goldfish.

New ganglion cells are continually added at the margin of the goldfish retina (Johns, 1977; Meyer, 1978). Each cohort of new cells grows their dendrites in a characteristic manner: initially parallel to the retinal margin, then toward the margin as the margin recedes by the addition of yet newer cells (Hitchcock & Easter, 1986). Relatively few dendrites grow toward the center of the retina (see also Kock & Reuter, 1978). This relative lack of dendritic growth into central retina by each generation of new cells led to the hypothesis that the dendrites of the older, centrally located cells could exclude the dendrites of the younger, peripherally located ones (Hitchcock & Easter, 1986). This hypothesis was similar to the original proposal of dendritic competition (Perry & Linden, 1982) with the added constraint that in the fish older cells have an advantage over younger ones.

The study described here experimentally tested the hypothesis of exclusionary dendritic interactions (Hitchcock & Easter, 1986). The hypothesis predicts that cells added to the margin of a retina depleted of ganglion cells should grow their dendrites centrally to a greater extent than those cells added to a retina with a normal complement of cells. The prediction was fulfilled. Further, many of these cells had dendrites with abnormal branching patterns.

These data have been presented previously in abstracts (Hitchcock, 1988; Hitchcock & Bernhardt, 1988).

Materials and methods

Because ganglion cells in the goldfish can regenerate their axons, it was impossible to kill these cells by simply severing the optic nerve, as can be done with mammals (e.g. Perry & Linden, 1982). Therefore, ganglion cells were neurotoxically killed by retrograde, 'suicide' transport of propidium iodide (PI) applied to the orbital portion of the optic nerve. Propidium iodide, which fluoresces red when using a filter set for rhodamine (490–540 nm excitatory wave length), has various uses such as a vital stain for cell sorting *via* flow cytometry (e.g. Sasaki *et al.* 1987), or as a retrogradely transported tract tracer (e.g. Kohler & Steinbusch, 1982).

Propidium iodide is toxic to neurons over time, presumably because it intercalates between the base pairs of double stranded nucleic acids (Tas & Westerneng, 1981). Initially, several compounds were screened for neurotoxicity (e.g. doxorubicin; Koda & Van der Kooy, 1983), but PI proved to be the most reliable.

Counts of neurons within the inner nuclear layer (INL) showed that PI did not cause any transneuronal retrograde cell death, and qualitative inspection of the inner plexiform layer (IPL) at the ultrastructural level showed that amacrine cell processes, bipolar terminals, and Müller cells were all normal in appearance (Hitchcock, unpublished data; see also Osborne & Perry, 1985; Wässle *et al.* 1987).

Propidium iodide was applied in the following manner. All surgery was done after the fish (3–4 inch standard length) were deeply anesthetized in 0·1 % tricaine methanesulfonate. The left orbit was opened dorsally, the optic nerve exposed and sectioned bloodlessly and completely. A pledget of Gelfoam soaked in 2 μl of 15 % PI in 2 % dimethylsulfoxide (DMSO) and 1·45 units ml^{-1} of phospholipase A2 (from *Naja naja* venom; Sigma), to solubilize axonal membranes, was immediately applied to the stump. At concentrations below 15 % the extent of ganglion cell death seemed more variable, whereas at concentrations above 15 % the PI treatment produced lethal kidney damage. The orbit was then closed and sealed with cyanoacrylate glue, and the animal was returned to its home tank. Right, untreated retinae served as controls.

The dendritic arbors were stained by intracellular injections of Lucifer yellow (LY) (see Tauchi & Masland, 1984). After survival times of 6–13 months, ganglion cells were retrogradely labelled with 10 % PI in 2 % DMSO as described above. 36–48 h later, the fish were dark adapted for 1 or more hours, and both the experimental and control retinae were isolated from the eyecup (Hitchcock & Easter, 1986), fixed without agitation for 30 min in fresh, room temperature 2 % paraformaldehyde in 0·1 M-phosphate buffer, pH 7·2–7·4. Retinae were wholemounted ganglion cell-side up onto non-fluorescing filter paper (Schleicher & Schuell, cat. #05710), which provided mechanical support, and the vitreous was gently brushed away. The filter paper/retina was transferred to a plexiglass chamber that was clamped to the stage of an Olympus fluorescent microscope, modified with a fixed stage. Cells were viewed with a 50× objective (Olympus, ULWD MS Plan) with an 8 mm working distance. PI-labelled cells were selected for injection using the green filter set, which was exchanged for the blue filter set for viewing LY (400–490 nm wavelength), and the electrode was visually guided to the cell. There was enough residual fluorescence in the PI-labelled cells to visualize them with the blue excitatory light (see Figs 2 and 5). The tips of the electrodes (1·0 mm o.d., 0·05 mm i.d.; resistance = 100–140 MΩ with 0·1 M-phosphate buffer) were filled with 3–10 % aqueous Lucifer yellow CH (Sigma) and backfilled with 0·25 M-KCl dissolved in 0·05 M-Tris–HCl buffer, pH 7·4. The electrode was positioned using a three-dimensional hydraulic micromanipulator attached to a mechanical one (Narashige), and cells were penetrated by modulating the capacity compensation circuit of the amplifier (buzzing) when the tip of the electrode came into contact with the cell membrane. Lucifer yellow was iontophoretically ejected for 1–5 min with 2–10 na of negative current of 100 ms duration at 1–4 Hz. During the experiments, the retinae were kept under a thin layer of the phosphate buffer. After injecting cells, the retinae were removed from the filter paper, mounted onto gelatinized slides, and coverslipped with phosphate-buffered glycerine (9 parts buffer: 1 part glycerine). Retinae and LY-filled cells were either photographed or traced using a drawing tube attached to a fluorescent microscope.

The injections of cells in the experimental retinae was less consistently successful than in the controls, even when both were isolated and fixed in parallel. In particular, large somata were sometimes leaky, which made it difficult to fill their arbors with LY. In a few experiments no cells could be successfully filled in the experimental retina, although virtually every cell attempted could be well filled in the control retina that had been processed in tandem. This finding perhaps suggests that cells in the experimental retinae were relatively unhealthy, or that they were more susceptible to the toxic affects of the PI used to prelabel them. Subsequent experiments have shown that more cells can be successfully injected at shorter survival times (24 h or less; Hitchcock, unpublished observations). The reason for these differences is unknown.

The distribution of the dendritic arbors about the soma was quantified using a modified Scholl analysis as shown in Fig. 1. A template of concentric circles, divided into 8 sectors was placed on the drawing of each cell, centered on the soma, and the number of intersections between dentrites and rings was tabulated for each sector. Because the dentritic lengths of the ganglion cells vary dramatically (see Fig. 3B & Hitchcock & Easter, 1986), values were expressed as a per cent of arbor in each sector. For statistical purposes, a Chi-square test was employed to compare the per cent of arbor lying in the four sectors nearest the margin and the four nearest the optic disc for cells in the control and experimental retinae. The quantitative data described below are from upon 54 and 66 well-

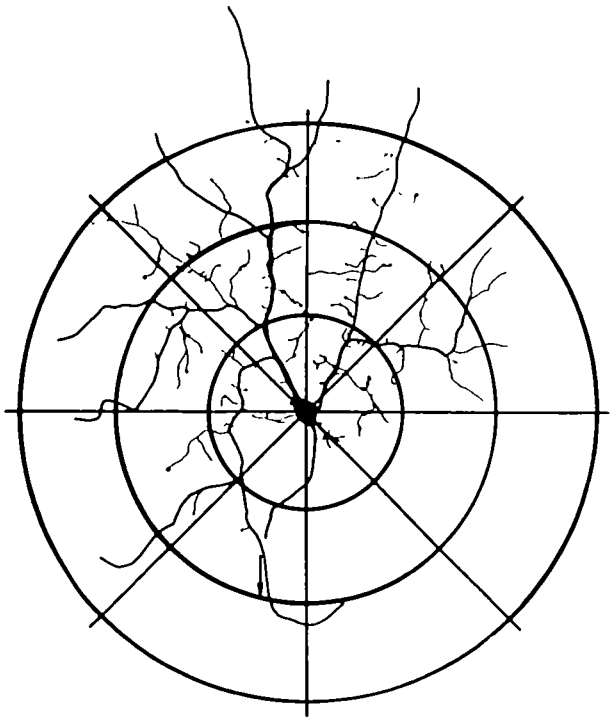


Fig. 1. Tracing of a LY-filled ganglion cell from a normal retina overlaid with a template of concentric rings, spaced 50 μm apart and divided into eight sectors. Dendritic orientation was quantified by counting the number of intersections between dendrites and rings in each sector (see Figs 4 and 8). The margin is toward the top; the optic disc is toward the bottom.

filled cells from eight fish in control and experimental retinae, respectively.

As part of another study, the dorsotemporal quadrants of the retinae studied here were fixed separately for light and electron microscopy. These were mounted ganglion cell-side up on gelatinized slides, counterstained with 0·25 % toluidine

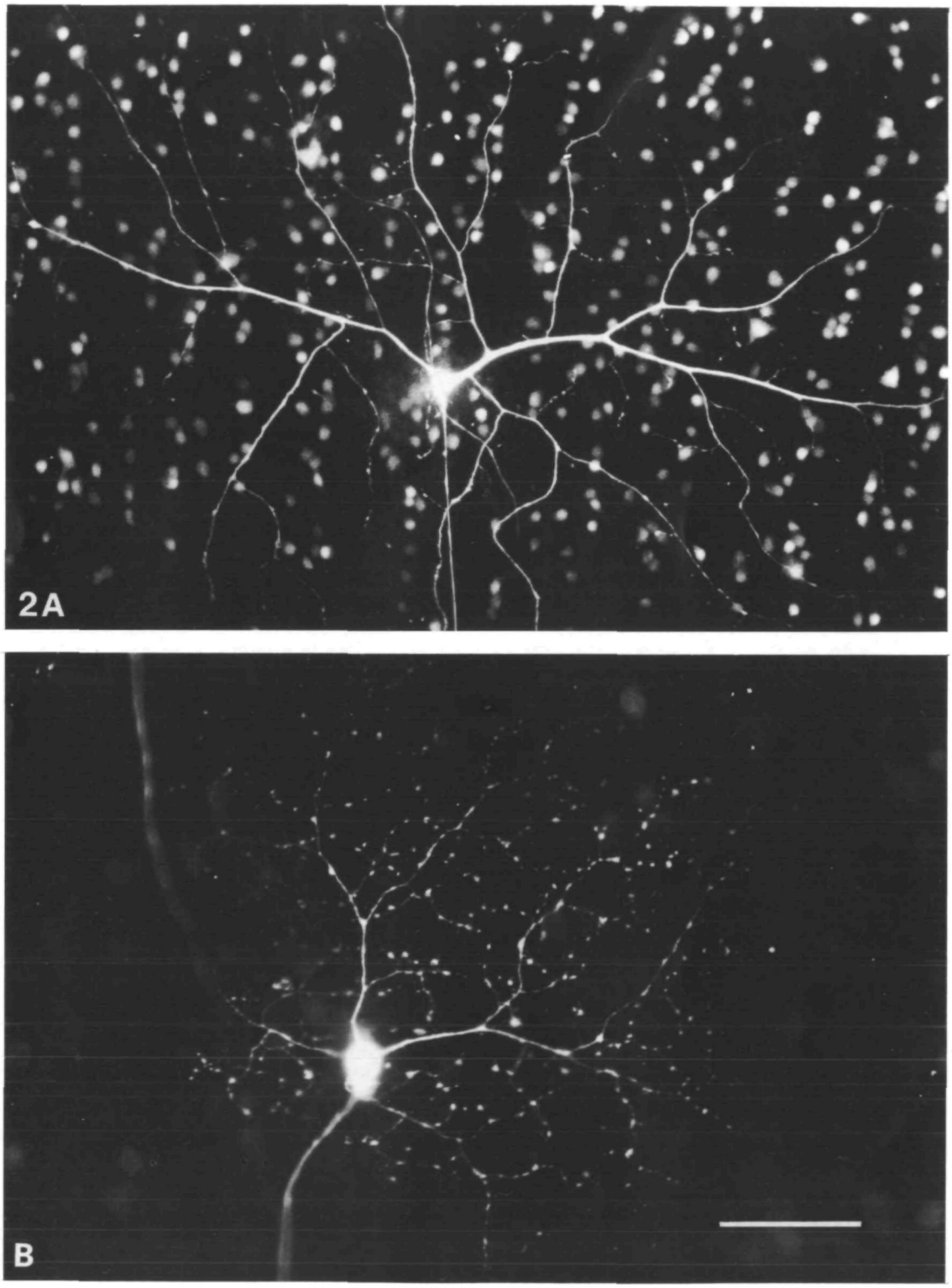


Fig. 2. Photomicrographs of ganglion cells intracellularly injected with LY in a normal, wholemounted retina. (A) Large field ganglion cell (type 1, see Hitchcock & Easter, 1986); (B) small field ganglion cell (type 2). Both of these cells were retrogradely labelled with PI then intracellularly labelled with LY. Note the faint PI-labelled cells in the background. The optic disc is toward the bottom of each photograph. Scale bar equals 100 μm for A and 50 μm for B.

blue, dehydrated in alcohols, cleared in xylenes, and coverslipped. To quantitatively assess the degree of ganglion cell depletion in the PI-treated retinae, the ganglion cells in these retinal quadrants were counted in 5 non-adjacent fields, at 500× magnifications midway between the retinal margin and the optic disc (see Raymond *et al.* 1988 for details of cell counting). The counts were averaged for each retina, and the per cent difference in the number of cells counted in experimental and control retinae was determined for four individuals (8 retinae).

Results

Normal retinae

Fig. 2 illustrates two ganglion cells in a wholemounted control retina that were prelabelled with PI and intracellulary injected with LY. Both cells are oriented such that the optic disc is toward the bottom of the figure, and the margin is toward the top. Panel (A) is a Type 1 (large-field) cell (Hitchcock & Easter, 1986); panel (B)

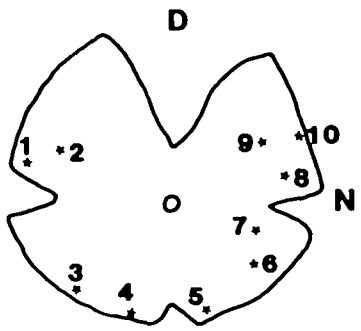
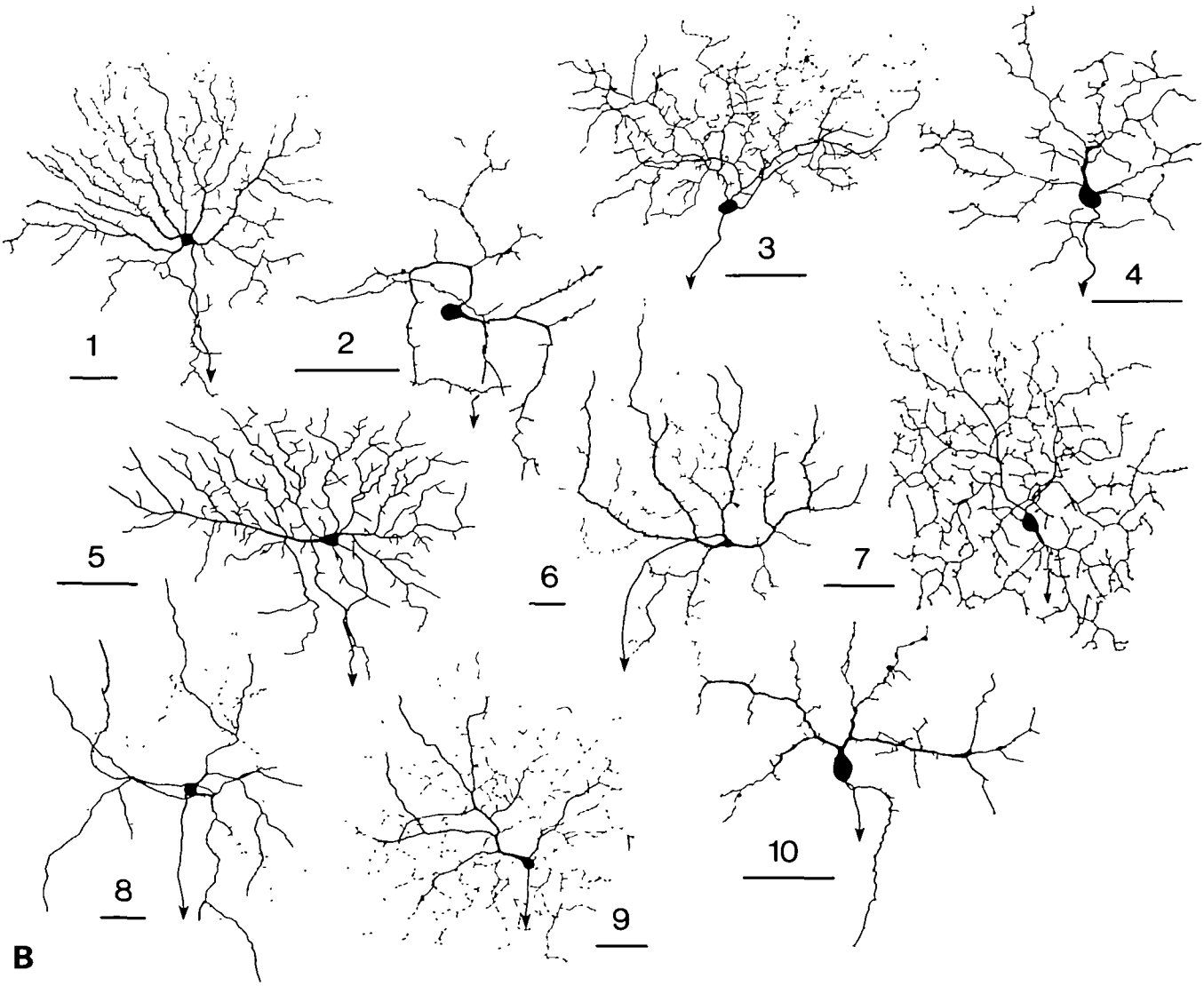


Fig. 3. (A) Representative retinal wholemount. The numbers refer to the cells shown in B. (B) Composite of LY-filled ganglion cells in control retinae. All scale bars equal 50 μm. To conserve space, the cells were reproduced at the same absolute size. Note the differences in lengths of scale bars, and therefore, the differences in the sizes of the cells. Orientation of the cells is such that the optic disc is toward the bottom of the figure. Axons are tipped with arrowheads.



is a Type 2 (small-field) cell (Hitchcock & Easter, 1986). Ganglion cells labelled with PI can be seen in the background of both photomicrographs. The intracellular injections filled both the dendrites and small appendages with the LY. This technique revealed more fine processes than retrograde filling with horseradish peroxidase (Hitchcock & Easter, 1986). The only shortcoming was that for the largest cells (e.g. Fig. 2A), some of which have dendritic fields greater than 0·5 mm in radius, the distalmost processes were usually only lightly filled. Attempts to densely fill the distal processes of large cells, either by increasing the injection time or passing more current, resulted in LY leaking out of the injection site and obscuring the soma. This was not a problem with small cells (Fig. 2B), however.

Fig. 3 includes a composite of 10 drawings of LY-filled ganglion cells from control retinae. This figure shows examples of the various types of ganglion cells in the goldfish (Hitchcock & Easter, 1986), and illustrates the shape and orientation of their dendritic arbors. Note that many of the dendritic arbors are displaced toward the retinal margin, relative to the cell body.

Experimental retinae

Fig. 4 shows low-magnification photomicrographs taken of Nissl-stained, wholemounted retinae at the level of the ganglion cell layer from the central region of a normal retina (A), and from the center (B) and margin (C) of a retina treated with PI six months previously. Comparisons of panels (A) and (B) show the degree to which the PI depleted this retina of ganglion cells. The degree of cell killing by the PI was variable. In some retinae, ganglion cell depletion was comparable to that shown in Fig. 4B, whereas in others the ganglion cell layer appeared to have a normal number of cells. Data were collected only from retinae in which it was obvious prior to beginning the cell injections that the vast majority of ganglion cells had been killed. Cell counts showed that the PI killed an average of 85 % of the ganglion cells (range, 70–98 %) within the central regions of the experimental retinae. Fig. 4C shows the thin rim of new tissue (between the arrows) added subsequent to the PI treatment to the retina shown in Fig. 4B. There was generally a gradual transition in the number of ganglion cells from the old, PI-treated retina to the rim of new retina. This observation suggested that those ganglion cells residing near the margin at the time of the PI treatment were less effectively killed than the more centrally located ones. This may be due to various factors, including the absence of an axon in the orbital optic nerve, or an ineffective dose of the PI taken up by the extremely fine axons of the marginal cells (see Easter *et al.* 1984). Those cells near the margin are referred to below as new cells, born after the PI treatment, though some may have been born shortly before.

Fig. 5 illustrates two photomicrographs of LY-filled ganglion cells from an experimental retina. Both are oriented as in Fig. 2. In contrast to the cells in Figs 2 and 3B, the cells in Fig. 5 extend the majority of their dendrites toward central retina. Fig. 6B includes a

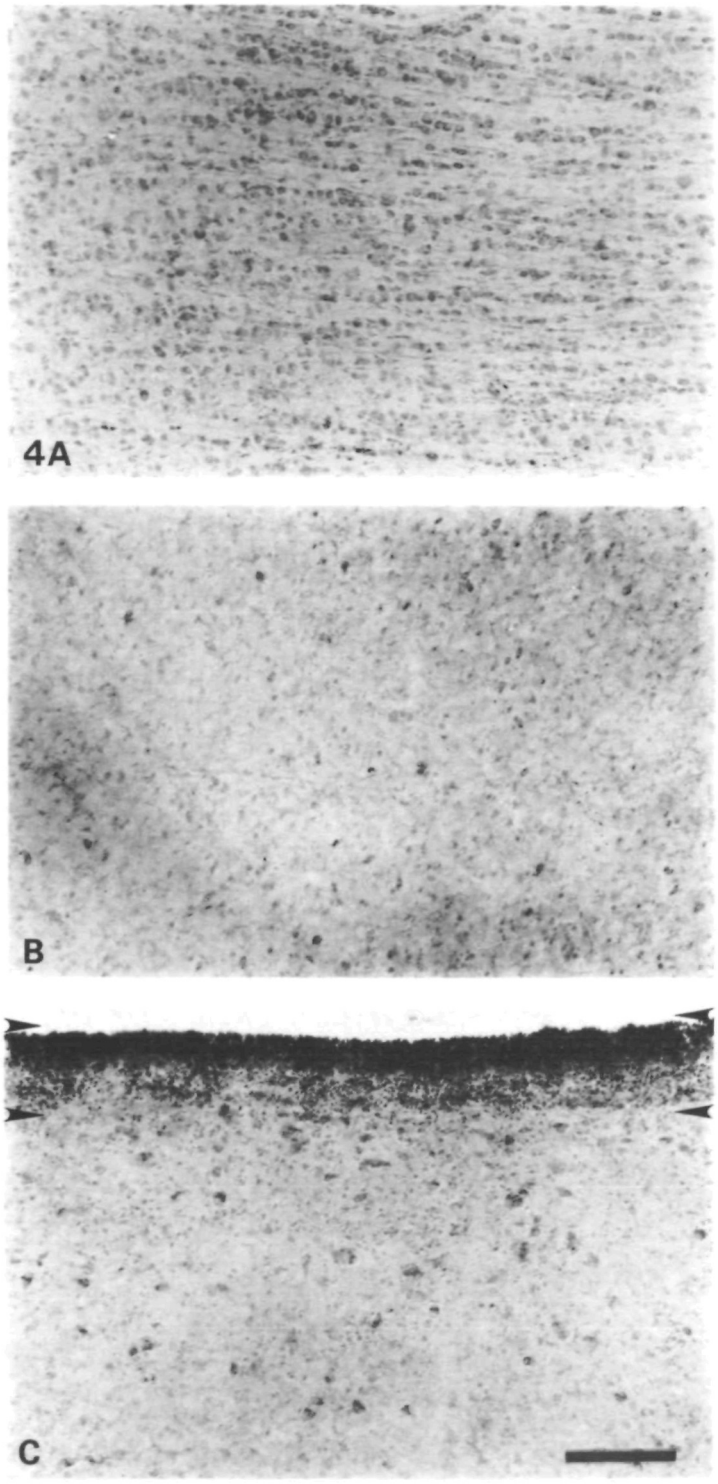


Fig. 4. Photomicrographs through the ganglion cell layer in wholemounted retinae from (A) a normal retina and (B) a retina six months after the application of PI to the orbital optic nerve. (C) A photograph of the margin of the retina shown in B. In C, the optic disc is toward the bottom of the figure. The arrowheads indicate retina added at the margin subsequent to the PI treatment. Scale bar equals 100 μm.

composite of 11 drawings of cells from experimental retinae. Comparing Figs 3B and 6B demonstrates two striking differences between cells in the control and experimental retinae. First, the dendritic arbors of cells in the experimental retinae were preferentially oriented toward central retina. Second, the dendritic branching patterns of many cells in the experimental retinae were abnormal (see also Leventhal *et al.* 1988). Some had

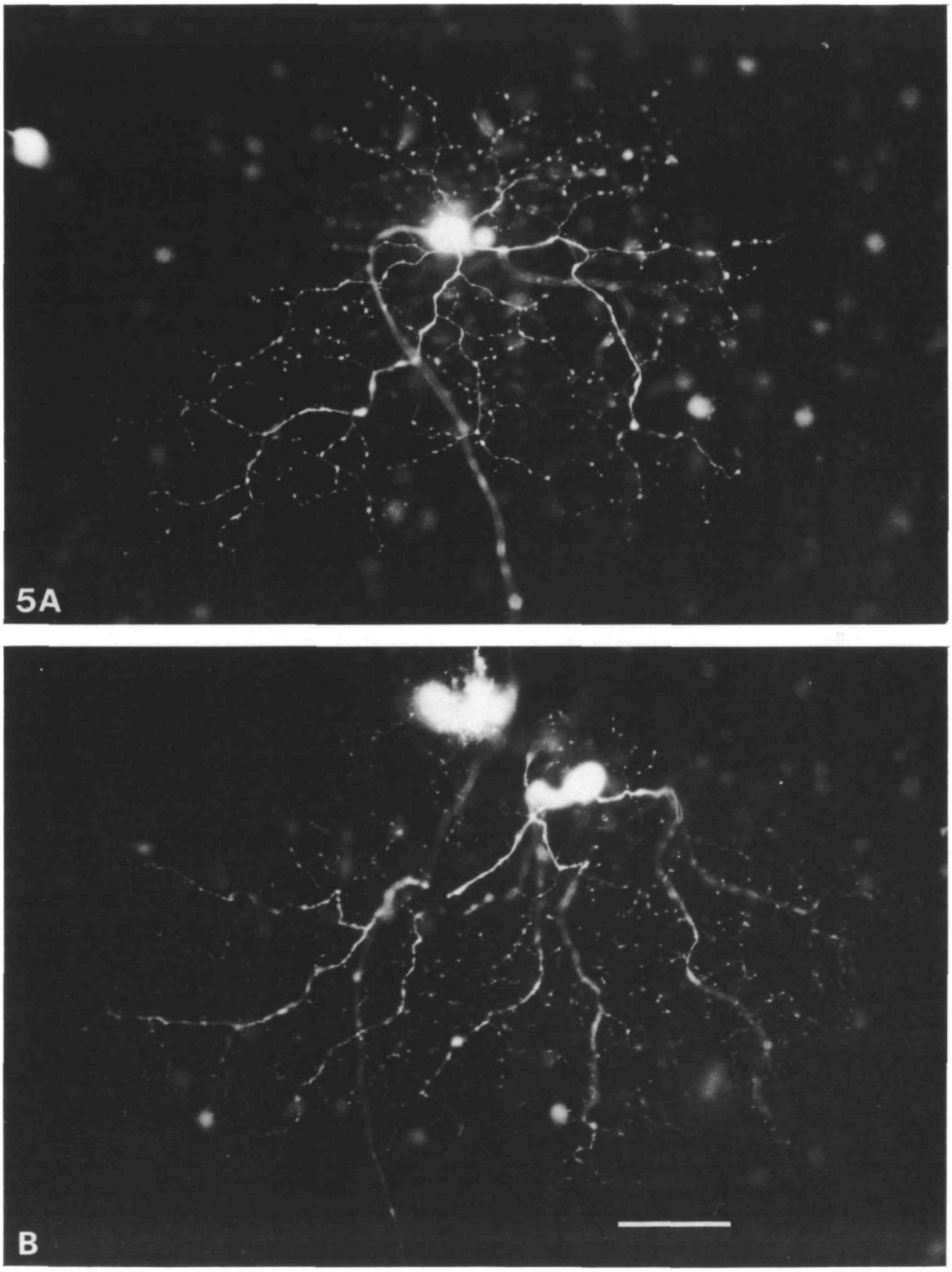


Fig. 5. Photographs of ganglion cells intracellularly filled with LY in a retina treated with PI. The optic disc is toward the bottom of each panel. Note the out-of-focus axon coursing downward from the cell in A. In B two somata are filled with LY, but only one dendritic arbor. Compare the orientation of the dendritic arbors of these cells and those illustrated in Figs 2 and 3B. Scale bar equals 50 μm for both panels.

one or two major, centrally directed dendrites that gave rise to fine, radially extending processes, e.g. Fig. 6B, cells 1 and 7. Others had only thin dendrites that formed very sparse arbors, e.g. Fig. 6B cells 3 and 8. The dendritic arbors of some of cells, however, appeared relatively normal, e.g. Fig. 6B, cell 5 and 11.

Fig. 7 summarizes the quantitative data from cells in the control (left) and experimental (right) retinae. In both panels, the length of each ray extending from the idealized soma represents the average percentage of dendritic arbor found within the corresponding sector illustrated in Fig. 1 (see also Methods). In control retinae, 65% of the dendritic arbor extended toward the margin and 35% toward central retina. In the experimental retinae these values were nearly reversed, 36% and 64% respectively. Comparing these figures illustrates that cells in the experimental retinae grew less arbor toward the margin and more toward central retina than those in the control retinae. This difference was statistically significant ($\chi^2 = 36{\cdot}9$, df 1, $P < 0{\cdot}001$).

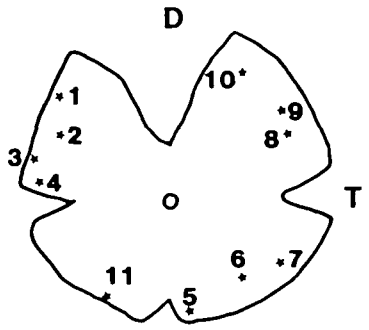
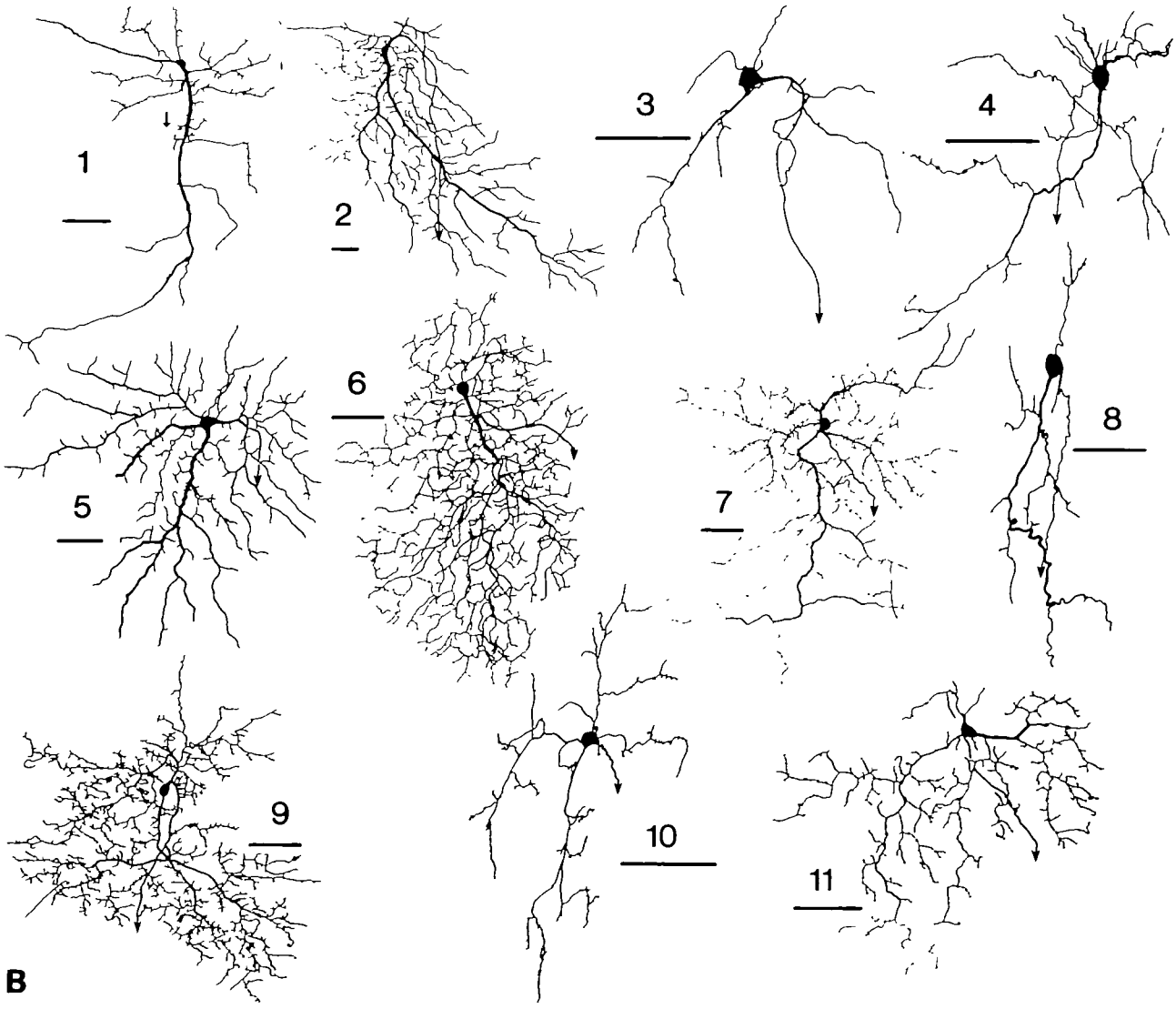


Fig. 6. (A) Representative retinal wholemount. The numbers refer to the cells shown in B. (B) Composite of LY-filled ganglion cells from retinae treated with PI. Cells are oriented as in Fig. 3B. Note the differences in orientation and branching patterns of the dendrites of the cells illustrated here and in Fig. 3B. Axons are tipped with arrowheads. All scale bars equal 50 μm.



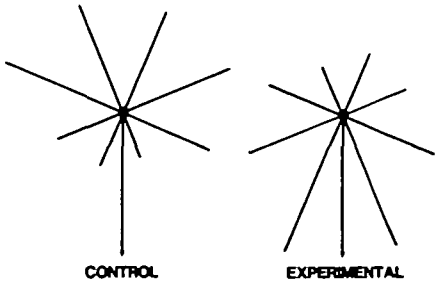


Fig. 7. Polar plot of the relative amount of dendritic arbor in each sector (See Fig. 1) for cells in control (left) and experimental (right) retinae. The radial lines originate at an imaginary soma, whose axon, tipped with an arrow, indicates the direction of the optic disc. The linear distance from the soma along a radial line indicates the percent of dendrite located in each sector. (See Fig. 1).

Discussion

In the normal goldfish retina, new ganglion cells near the proliferative margin tend not to grow their dendrites toward central retina (Hitchcock & Easter, 1986). However, when the more central neighbors of these cells are depleted, they alter their dendritic outgrowth and preferentially extend their arbors centrally. These data suggest that in the normal retina each generation of cells respects the dendritic territories established by the previous generation, and only in the absence of the older cells can the dendrites of the younger ones grow appreciably toward central retina. This implies that once an older dendrite is in place it cannot be displaced by a less mature one. This finding is qualitatively similar to the experimental results in mammals in which ganglion cells preferentially grow their dendrites toward a ganglion cell-free patch (see below), and suggests that the marginal region of the goldfish's retina is continuously engaged in developmental events common to mammalian retinae.

Wässle *et al.* (1981) first proposed that the dendrites of adjacent ganglion cells interact during development. They (see also Wässle & Reiman, 1978) observed that in the cat's retina the somata of individual classes of ganglion cells were arrayed in a mosaic across the retinal surface, and the dendritic fields of these cells overlapped slightly to completely cover, or 'tile' the retina. Further, they demonstrated that the dendritic coverage of a given functional class of cells was independent of all others. Based upon these observations of normal retina, they proposed that the growing dendrites of adjacent ganglion cells interacted locally to regulate the size and shape of their dendritic fields. This would account for both the relatively low redundancy of the dendritic coverage, and the inverse relationship between cell density and dendritic field area, characteristic of ganglion cells in the cat's retina. This proposal (Wässle *et al.* 1981) has been tested in two complementary ways. First, when small patches of ganglion cell-free retina were created in newborn rats, nearby ganglion cells preferentially grew their dendrites into these regions (Perry & Linden, 1982). Subsequent studies in the cat (Eysel *et al.* 1985; Leventhal *et al.* 1988) have confirmed and extended these results. Similar results have also been shown for amacrine cells in goldfish (Negishi *et al.* 1982). Second, as would be predicted, experimentally increasing the planimetric density of ganglion cells in one eye by prenatal enucleation of the other resulted in dendritic arbors that were significantly smaller than normal (Kirby & Chalupa, 1986).

The data cited above lead to the idea that in the normal retina the dendrites of ganglion cells compete for territory, and as a consequence create a uniform coverage of the retina with their dendritic fields. Dendritic competition, by definition (see Guillery, 1987), requires that there is a factor (or factors) in limited supply that the dendrites of ganglion cells seek, and once this factor is harvested locally by a given dendrite, others nearby are forced to grow elsewhere. It was originally proposed (Perry & Linden, 1982) that ganglion cells compete for synapses. Although a subsequent study (Perry & Maffei, 1988) showed that alterations in dendritic outgrowth can occur largely in the absence of synapses within the inner plexiform layer. No one knows the underlying cell biology of the dendritic plasticity seen in the developing retina, and, therefore, 'dendritic competition' remains merely a phenomenon (Perry & Maffei, 1988).

The results of the present study and those described above are taken as evidence that, during normal development of the retina, interactions between adjacent ganglion cells at the level of their dendritic arbors regulate their dendritic growth. There are two classes of alternative interpretations, however. First, once could conclude that experimentally induced alterations in dendritic growth do not reveal events that occur during normal development, but are lesion induced. For example, the death of nearby ganglion cells could cause the release of a factor, not normally expressed, that has a chemotropic effect on the dendrites of the cells adjacent to the lesion. One argument against this is that different experimental paradigms (cf. Perry & Linden, 1982 and Kirby & Chalupa, 1986) give different but predictable results that can be interpreted similarly. Second, if adjacent cells do interact during normal development, these interactions may not occur between dendrites. To induce the reorientation of dendritic growth, one must deplete both dendrites and somata. Ganglion cells are initially crowded together; perhaps cell–cell interactions at the somatic level, analogous to those that regulate cell proliferation (see Reh & Tully, 1986), control the direction of dendritic outgrowth. Although these alternative interpretations cannot be explicitly excluded, one can conclude that dendritic outgrowth by cells in the developing retina can be influenced by the presence or absence of neighboring cells.

Recent studies of axonal outgrowth have focused on the critical role played by the growth cone, the specialized structure at the tip of the growing axon that provides motility and directs growth in response to specific environmental cues (see Dodd & Jessell, 1988).

If one speculates that growing dendrites rely on growth cones to guide their growth as well, the study described here suggests that in the goldfish's retina extant ganglion cells prevent the dendritic growth cones of immature cells near the margin from extending centrally, presumably by inhibiting their movement. This exclusion could be mediated by contact inhibition (Kapfhammer *et al.* 1986; Kapfhammer & Raper, 1987; although see Perry & Maffei, 1988), local changes in the neurotransmitter milieu (Cohan *et al.* 1987; Haydon *et al.* 1984; Kater *et al.* 1988; Lankford *et al.* 1988; Lipton *et al.* 1988; Mattson, 1988; Mattson *et al.* 1988), or local alterations in the extracellular matrix (Krystosek & Seeds, 1981; Monard, 1988; Pittman, 1985). Whether or not common cellular mechanisms are utilized by both growing dendrites and axons is unknown; however, the known mechanisms of axon growth should provide useful clues to guide future studies of dendritic growth.

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