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Neuronal Coupling in the Developing Mammalian Retina

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During the first 3 weeks of postnatal development in the ferret retina, cells in the ganglion cell layer spontaneously generate waves of electrical activity that travel across the retina in the absence of mature photoreceptors (Meister et al., 1991; Wong et al., 1993). Since few chemical synapses are present at the earliest stages when waves are present, we have explored whether gap junctions could act to correlate the activity of cells in the immature ganglion cell layer. Retinal ganglion cells in a living *in vitro* preparation from postnatal day 1 (P1) to P45 were intracellularly injected with the tracer Neurobiotin and the fluorescent dye Lucifer yellow, molecules that are known to pass through gap junctions. Lucifer yellow consistently filled only the injected cell, whereas Neurobiotin filled not only the injected cell but also passed to a constellation of neighboring cells. Coupling revealed by Neurobiotin is seen as early as P1, but, at this stage, it was not possible to identify the various morphological types of cells that were coupled. Thereafter, α ganglion cells showed homologous coupling to other α cells and to both conventionally placed and displaced amacrine cells. Likewise, γ ganglion cells appeared coupled to other γ cells and to amacrine cells. However, β ganglion cells never showed tracer coupling in the neonatal or in adult retinas. The percentage of α and γ cells that were coupled to other cells increased progressively with age. By the end of the third postnatal week, the pattern of Neurobiotin coupling in the ferret retina was adult-like, with virtually every injected α cell showing tracer coupling. Our observations suggest that intercellular junctions able to pass Neurobiotin are present in the inner plexiform layer during the period when the firing of retinal ganglion cells is highly correlated. Such junctions could contribute to synchronization of the activity of subsets of neighboring ganglion cells during development, but it cannot be the sole mediator of this activity because β cells, which also participate in the correlated activity, showed no coupling at any stage. In addition, the continued presence of coupling in the adult retina implies that other changes in retinal circuitry are likely to contribute to the disappearance of the waves.

[Key words: gap junctions, Neurobiotin coupling, dye coupling, retinal ganglion cell development, activity-dependent development, spontaneous retinal activity, retinal waves]

Patterned neuronal activity is known to be required for the formation of precise sets of connections in the developing nervous system (Goodman and Shatz, 1993). In the vertebrate visual system, the correlated firing of neighboring retinal ganglion cells is thought to provide activity-dependent cues used by retinal axons to form orderly topographic maps and eye-specific layers within their target structures, the lateral geniculate nucleus (LGN) and optic tectum (reviewed in Udin and Fawcett, 1988; Constantine-Paton et al., 1990; Shatz, 1990a,b). In the early development of the mammalian visual system, these correlations must be provided by the spontaneous firing of retinal ganglion cells, rather than by vision itself, because the photoreceptors are not yet mature (Greiner and Weidman, 1981; Galli and Maffei, 1988; Maffei and Galli-Resta, 1990). When the activity of many ganglion cells of the ferret retina is observed simultaneously using multielectrode or optical recording techniques, groups of neighboring cells are indeed seen to fire action potentials synchronously (Meister et al., 1991; Wong et al., 1993) or to increase levels of intracellular calcium (Wong et al., 1992a). Moreover, the pattern of activity resembles a wave that travels across the retina at about 100 μm/sec and involves cells situated within approximately 300 μm of each other (Meister et al., 1991; Wong et al., 1993).

A major question is how the retina generates this particular spatiotemporal pattern of correlated activity. One possibility is that the diffusion of an extracellular excitatory agent acts to synchronize the firing of neighboring cells. However, it could also be that these waves of correlated activity are generated by a specific neural circuit. In view of the immaturity of the retina during the period in which the waves exist, such a circuit is unlikely to involve extensive synaptic connections (Greiner and Weidman, 1981; Maslim and Stone, 1986). Another possibility is that gap junctions between retinal cells could provide a morphological substrate for the correlations (for review, see Dermietzel and Spray, 1993; Vaney, 1994). Such junctions are known to be present in the developing cerebral cortex, where they are required for generating localized domains of spontaneous neuronal activity *in vitro* (Yuste et al., 1992; Peinado et al., 1993). It seemed likely that the retina might also contain similar junctional networks during development (Mastronarde, 1989), especially because gap junctions have been found in the inner plexiform layer of the adult mammalian retina (Kolb, 1979; Raviola and Raviola, 1982; Dacheux and Raviola, 1986; Cohen and Sterling, 1990; Strettoi et al., 1990).

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Here we have used intracellular injections of Neurobiotin to investigate whether ganglion cells in the developing retina are connected to each other and to other cell types by intracellular junctions. Although many previous studies have demonstrated that Lucifer yellow (457 Da) passes through gap junctions in some retinal cell types (Stewart, 1978; Jensen and DeVoe, 1982; Piccolino et al., 1982; Teranishi et al., 1984; Kouyana and Watanabe, 1986) and in some developing systems (LoTurco and Kriegstein, 1991; for review, see Guthrie and Gilula, 1989), the junctions present in the mammalian inner retina both in adults and in development do not appear to pass this dye (Dann et al., 1988; Ramoa et al., 1988; Wong, 1990; Vaney, 1991, 1994; Wingate et al., 1992). However, in the adult retina these junctions do pass Neurobiotin (286 Da), a smaller tracer molecule (Vaney, 1991, 1994; Hampson et al., 1992). Therefore, we maintained retinas *in vitro* and injected single retinal ganglion cells with Neurobiotin.

Ferret retinas were studied from postnatal day 1 (P1) through P21, the period before eye opening when the waves of correlated activity are present and activity-dependent remodeling of ganglion cell axon terminals is taking place within the LGN (Linden et al., 1981; Cucchiaro and Guillery, 1984; Shatz and Stryker, 1988; Sretavan et al., 1988; Hahm et al., 1991; Meister et al., 1991). Retinas were also examined at later ages, when the waves have disappeared (P30), the eyes have opened (P33), and retinal ganglion cells are functioning in an adult manner.

Materials and Methods

In vitro retinal preparation. To examine tracer coupling between cells in the developing ferret, retinas from P1 to P45 ferrets were isolated and maintained alive *in vitro*. A total of 56 separate retinas had well-filled ganglion cells that were analyzed. The ferrets were anesthetized with Nembutal (20 mg/kg). The retina was dissected from the eyecup in oxygenated Ames medium (Sigma, A 1420) with 20 mM HEPES (Sigma, H-3375). Fetal calf serum (0.1%; GIBCO) was added to the oxygenated media in approximately half the experiments. While it had no detectable effect on tracer coupling, it did appear to prolong the period of time that the tissue could be maintained in a healthy state *in vitro* (approximately 5–7 hr) as judged by the appearance of the Lucifer yellow fills and the maintenance of adequate cellular resting potentials (e.g., −50 to −70 mV). After dissection, the whole retina or a hemisected retina was mounted on a piece of filter paper (Millipore, HABP 045), ganglion cell side up. Holes 1 mm^2 in the filter paper allowed the retinal cells to be examined with transilluminated light when the filter paper was secured in a perfusion chamber mounted on the stage of an upright fluorescent microscope. These windows for illumination were placed midway between the optic nerve head and the retinal edge. Changes in ganglion cell morphology with eccentricity and proximity to the visual streak are far less pronounced in the ferret than in the cat; indeed, few positional changes are observed (Vitek et al., 1985; Wingate et al., 1992). Thus, for our study, it was sufficient to note that at all ages, the injected cells were located in midperipheral retina. The tissue was maintained by superfusion at approximately 1 ml/min with the same media used for dissection warmed to 33–37°C by two Peltier devices mounted under the stage of the microscope. This method has also been described elsewhere (Wong et al., 1991, 1992b).

Intracellular injections. Intracellular electrodes were filled with a solution containing 3% Neurobiotin [*N*-(2-aminoethyl)-biotinomide hydrochloride, Vector Labs #SP1120] and 1% Lucifer yellow CH (Sigma) in 0.1 M Tris buffer, pH 7.6 (Vaney, 1991). Because Neurobiotin is colorless, the addition of some Lucifer yellow to the pipette enabled us to assess if the targeted cells were successfully penetrated. This also allowed us to identify the morphology of the injected cell due to the faint staining of the dendritic tree with an initial brief pulse of Lucifer yellow. In several initial experiments, 2.5% carboxyfluorescein (Kodak, #9953) in 0.1 M potassium acetate was used instead of Lucifer yellow. The Neurobiotin/fluorescent dye solution was made fresh for each experiment and sonicated before use. Electrode resistance needed to be between 100 MΩ and 200 MΩ to achieve good intracellular fills. A new electrode was normally used for each injection. Cells in the ganglion cell layer were injected under visual control using a 40× objective and Hoffman optics. Cellular resting potentials of approximately −50 mV could be measured prior to injection. A single cell was iontophoretically filled with fluorescent dye (−1 to −3 nA, continuous) for several seconds to check that only it had been impaled, before Neurobiotin was injected (+1 to +3 nA, continuous) for 30 sec to 2 min, depending on the size of the cell. Increasing the current or length of filling beyond these parameters led to a very poor yield of well-filled cells, probably due to cell death. Great care was taken to penetrate and fill only one cell at a time. During the injections, the morphology of the injected cell and its approximate location were noted. At all ages, the injected cells were always located in the midperipheral retina. After a number of ganglion cells were injected (6–15 per window), the tissue was left in the chamber for at least 15 min following the last injection to allow the tracer to spread to any coupled cells. The tissue was then removed from the chamber and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 2 hr.

Enhancement of Neurobiotin-filled cells. To visualize cells containing Neurobiotin, the tissue was removed from the filter paper and first incubated in 0.5% Triton-X for 2–4 hr (increasing the length of time according to the age of the tissue). It was then incubated for 4 hr in Streptavidin (Amersham RPN.1231) diluted 1:500 in 0.1 M sodium phosphate buffer and visualized with 0.05% DAB (3,3′-diaminobenzidine tetrahydrochloride) reacted with 0.01–0.02% hydrogen peroxide in 0.1 M Tris buffer, pH 7.6.

Two techniques were used to enhance the DAB staining. Osmication was achieved by placing the processed tissue in a shallow well containing 0.1 M phosphate buffer and placing the well in an enclosed box containing an open container of osmium tetroxide to expose the tissue to the vapor for approximately 1 hr. This method produced good enhancement of the DAB, but the surrounding tissue also darkened and the retina became fragile. Alternatively, DAB staining was enhanced by photochromatic intensification in the presence of nitro blue tetrazolium (NBT) (Vaney, 1992). The retina was incubated in 0.1% NBT in 0.1 M Tris buffer, pH 8.2, for 5 min and then coverslipped in the same solution. Areas with injected neurons were then illuminated with light from a Lucifer yellow filter set (Zeiss filter set 5) through a 10× or 20× objective. The extent of intensification could be monitored during this period and maximal intensification was achieved after 30–45 sec. Tissue was then thoroughly rinsed in 0.1 M Tris buffer prior to whole-mounting. Care was taken to photograph and draw the cells enhanced with NBT immediately because a precipitate occasionally formed in the tissue over several months that greatly decreased the contrast between Neurobiotin-filled cells and the surrounding tissue. All retinal tissue was mounted on gelatinized slides, dehydrated, cleared with xylene, and coverslipped in Permount prior to photography and camera lucida drawings.

Analysis. Injected cells were included for analysis only if there was good axonal filling and the dendritic tree appeared complete. A cell was considered to be tracer coupled to the injected cell if Neurobiotin also filled its soma and it was located within the dendritic tree or just beyond the distal dendrites of the injected cell. This criterion allowed for the inclusion of cells that might be coupled by fine dendrodendritic contacts too small to visualize under these conditions. Axons of ganglion cells filled indirectly with Neurobiotin by tracer coupling were only occasionally visible and dendritic staining was quite variable.

Results

The results are presented in two main sections. First, to establish the basis for the technique used to explore early anatomical connections in the ferret retinal ganglion cell layer, we demonstrate that the ganglion cells of mature ferret retina show Neurobiotin coupling resembling that seen in the adult cat (Vaney, 1991). Second, we explore the developmental pattern of Neurobiotin coupling of different morphological classes of retinal ganglion cells and show that coupling is present during the first 3 postnatal weeks at the same time that correlated activity occurs in the ganglion cell layer. We also report that there are substantial alterations in the pattern of coupling during these weeks of development.

Retinal ganglion cells in the mature ferret retina can be identified on the basis of their dendritic morphology and soma size

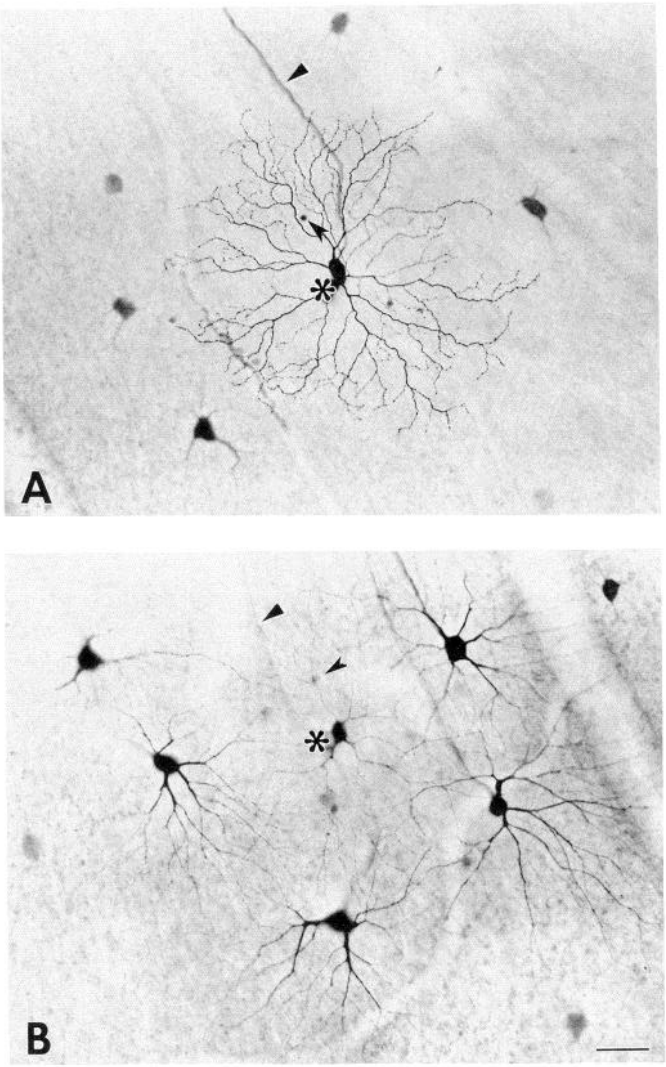

Figure 1. Coupling pattern for α ganglion cells in P45 ferret retina. *A* and *B*, The central α ganglion cell (marked by *asterisk*) has been injected with both Lucifer yellow (not shown) and Neurobiotin. Neurobiotin processing and subsequent photochromatic intensification revealed that in each case the α cell was coupled to a halo of other α ganglion cells, as well as to amacrine cells. *A*, This α ganglion cell has an extensively branched, well-filled dendritic tree surrounded by six coupled α ganglion cells (the sixth faintly stained cell is in the *lower right corner*). The cell is also coupled to amacrine cells, some of which are displaced into the retinal ganglion cell layer (small cells marked by *notched arrowhead*). *B*, Another α ganglion cell has thicker, less-branched dendrites and is coupled to other α cells of the same morphological subtype. Note here that Neurobiotin has spread to a second ring of faintly labeled α ganglion cell somata lying beyond the halo of α cells immediately surrounding the injected cell. (*Notched arrowhead* indicates a conventionally placed amacrine cell.) *Larger triangular arrowheads* in *A* and *B* indicate location of the axon of the injected cell (out of focal plane in *B*). Scale bar, 50 μm.

(Henderson, 1985; Vitek et al., 1985; Wingate et al., 1992). As in the cat, three major classes—α, β, and γ—are apparent (for review, see Wässle and Boycott, 1991). α cells have the largest somata and large dendritic fields made by long, sparsely branching dendrites; β cells have intermediate-size somata and small dendritic fields made by short, extensively branched dendrites; and γ cells, while more heterogeneous than the former two classes, have the smallest somata and moderate-size dendritic

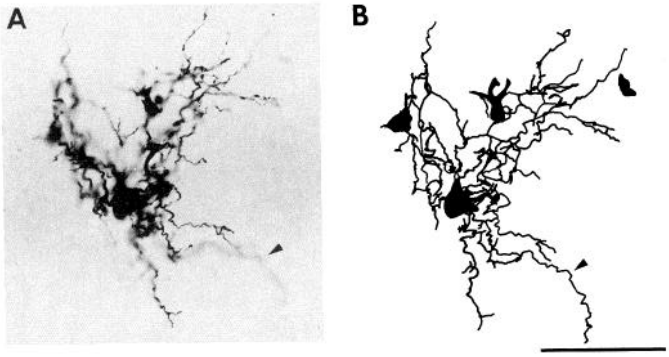

Figure 2. A ganglion cell at P1 injected with Neurobiotin. *A*, Bright-field photomicrograph of the injected cell shows the immature branching pattern at P1. Three coupled cells are visible here within the dendritic tree of the injected cell, but are in slightly different focal planes within the ganglion cell layer. *B*, Camera lucida drawing revealing the dendritic branching pattern of the injected cell and showing the coupled cells more clearly. While the injected cell is too morphologically immature to be identified by subtype, it can be identified as a ganglion cell by the presence of an axon (indicated by *arrowhead*). Scale bar, 50 μm.

fields made by long, less-branched dendrites (e.g., see Fig. 4). Since previous intracellular recordings have suggested that all three classes are likely to participate in the wave activity (Wong et al., 1993), we have examined the coupling patterns of each of these cell types.

Neurobiotin coupling in the mature ferret retina

The first set of experiments demonstrate that Neurobiotin coupling is present in the mature ferret retina (Fig. 1; see also Fig. 7). After P30, the ferret retina is essentially anatomically mature (Greiner and Weidman, 1981), the retinal ganglion cell axons are appropriately connected in the LGN (Linden et al., 1981; Cucchiaro and Guillery, 1984), and eye opening is about to occur (P33). Figure 1 shows two examples of injected cells in a P45 retina. Only the central cell in each field was injected with Neurobiotin and Lucifer yellow, but subsequent processing revealed that it was coupled to a number of neighboring cells. Based on their morphology, each injected cell in Figure 1 (marked with an asterisk) can be identified as an α ganglion cell. The injected α cells were seen to be coupled to several neighboring α ganglion cells. In addition, these ganglion cells were also coupled to many cells with very small somata, both within the ganglion cell layer (notched arrowhead, Fig. 1*A*) and within the inner nuclear layer (notched arrowhead, Fig. 1*B*). These cells are highly likely to be amacrine cells based on their distinctively small soma size, their laminar distribution (reviewed in Wässle and Boycott, 1991), and the fact that this pattern of amacrine cell labeling is similar to that seen in the adult cat retina (Vaney, 1991). All α cells injected in retinas greater than postnatal week 3 showed this pattern of tracer coupling (see Fig. 7 for quantitation).

It is also notable that the two injected α cells shown here reflect two slightly different dendritic branching patterns within the α cell class. Figure 1*A* shows an α cell with many fine higher-order branches, and the α cells coupled to it appear to resemble the central cell (when examined under higher magnification to reveal the faint dendritic processes). On the other hand, the injected α cell in Figure 1*B* (which is slightly out of the plane of focus) has thicker, less-branched processes (and again the coupled cells surrounding it have dendrites that resemble those of the central cell).

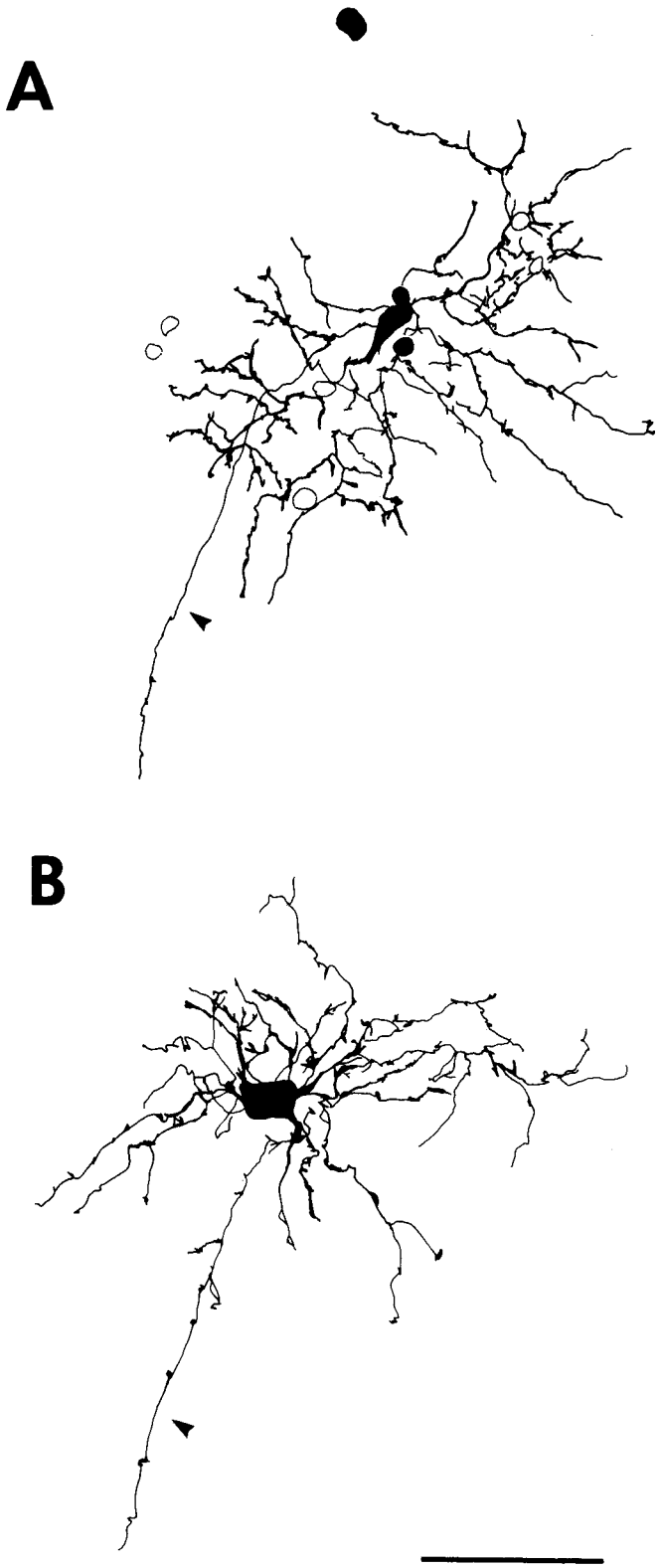

Figure 3. Camera lucida drawings of representative Neurobiotin-filled ganglion cells from postnatal week 1 retinas. Cells shown in *A* and *B* were injected under identical conditions in the same retina at P5 and are morphologically similar. *A*, This cell has several likely cells coupled to it. All the *black somata* lie within the ganglion cell layer, as does the injected cell, while the *white somata* lie in the inner nuclear layer. *B*, This cell showed no evident coupling despite an equally well-filled axon and dendritic tree. *Arrowheads* indicate the axon. Scale bar, 50 μm.

The comparison of Figure 1, *A* and *B*, also serves to point out the variability of dendritic labeling of the cells secondarily filled with Neurobiotin. The etiology of this variability is unclear; it does not appear to relate to the amount of Neurobiotin injected (in Fig. 1 the cells were injected under the same conditions and appear equally well filled), but may instead relate to the density of junctional connections, the size of junctional connections, or the electrotonic properties of these connections (see Discussion).

Only two γ cells were injected in mature retinas (see Fig. 7), but given the pattern of resulting Neurobiotin coupling and the developmental pattern described below, it appears that, like α cells, γ cells also are coupled to cells of the same class and probably to amacrine cells as well. β cells showed no Neurobiotin coupling in retinas older than P30 (see Fig. 7), a result similar to that found in the cat (Vaney, 1991), demonstrating that Neurobiotin coupling is not ubiquitous among ferret retinal ganglion cells.

Developmental changes in Neurobiotin coupling

The developmental sequence of Neurobiotin coupling was next examined in P1–P21 retinas, the period during which spontaneous correlated activity is present and activity-dependent segregation of retinal ganglion cell axons occurs within the LGN.

Postnatal week 1 (P1–P7)

During the first postnatal week the retina is very immature, consisting primarily of a thick ganglion cell layer of uniform density, a developing inner nuclear layer, and a large germinal layer called the ventricular zone. Cells injected in the ganglion cell layer cannot be reliably identified on the basis of their dendritic morphology during the first postnatal week and show many immature morphological properties, such as excessive branching and exuberant dendritic spines (Ramoa et al., 1988). However, they can always be identified as ganglion cells by the presence of an axon.

Neurobiotin coupling between retinal cells was present at the earliest ages examined. As shown in the camera lucida drawing of Figure 2*A*, a ganglion cell injected in a P1 retina is coupled to three other cells within its dendritic tree. These cells are approximately the same size as the injected cell, but lie in a slightly different plane of focus, which is not surprising because the ganglion cell layer is several cells thick at this stage of development (Greiner and Weidman, 1981). It should be noted that the coupled cells do not lie next to the injected cell's soma, nor are many cells in the vicinity filled with Neurobiotin; thus, even at this early age, the passage of Neurobiotin appears quite specific in its pattern of transfer.

Yet not all cells injected during postnatal week 1 were Neurobiotin coupled. Figure 3 shows a camera lucida drawing of two cells with very similar dendritic morphology that were injected under identical conditions (in the same series of injections in a single retina). The top cell was clearly coupled to a few cells within or near its dendritic tree (Fig. 3*A*, black and white somata), while the bottom cell was not coupled. The majority of the cells injected during the first postnatal week were not coupled (see Fig. 7 for quantitation). There was also never any clear coupling seen with Lucifer yellow dye fills, as expected from previous studies (Ramoa et al., 1988; Vaney, 1991), again indicating that when there are junctions between the immature ganglion cells they pass Neurobiotin but not Lucifer yellow.

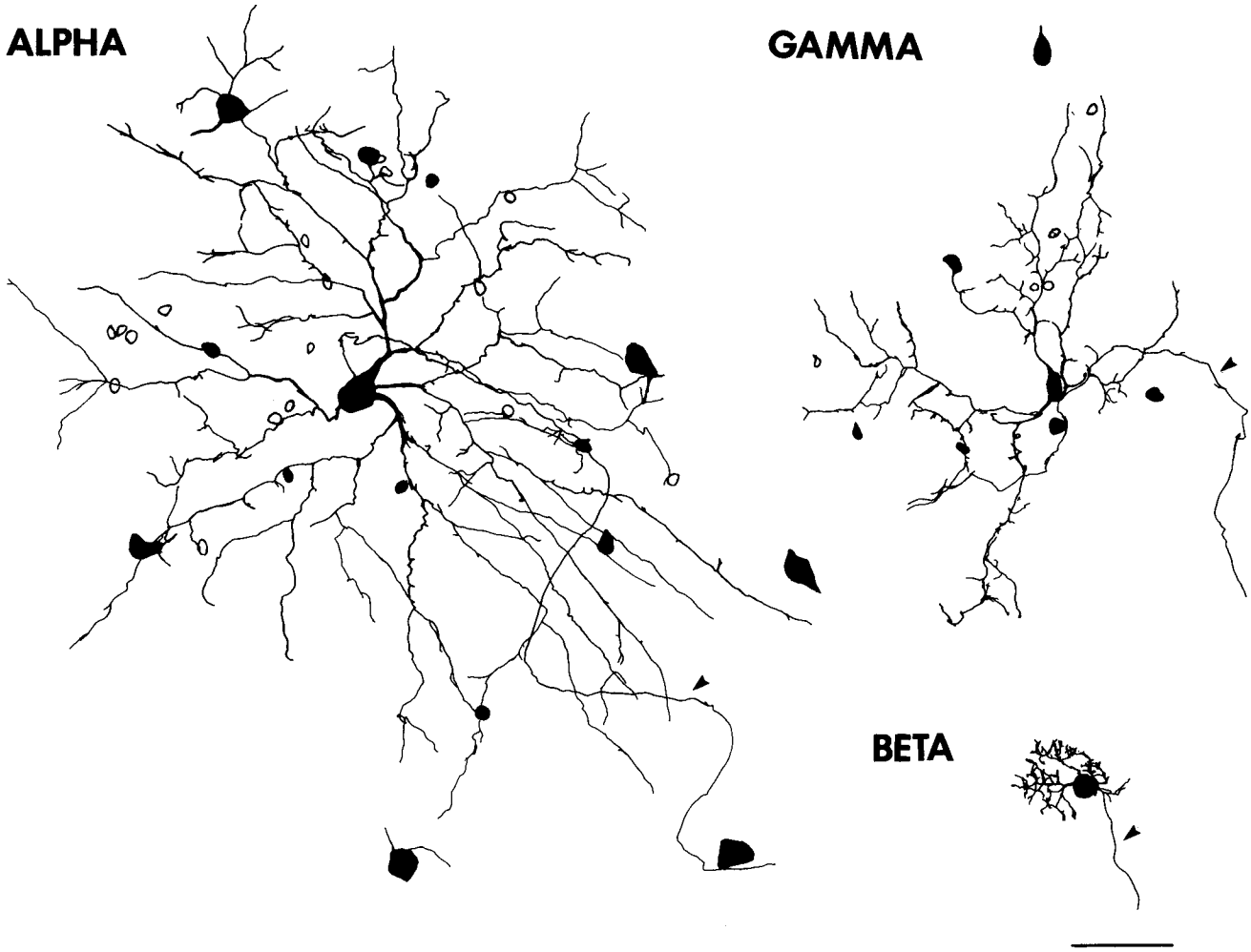

Figure 4. Camera lucida drawings of ganglion cells from postnatal week 2 (P8–P14). Typical examples of the three major classes of retinal ganglion cells (α, β, and γ) are shown. *Black somata* are coupled cells located within the ganglion cell layer, while the *white somata* are located within the inner nuclear layer. The α ganglion cell is coupled to a ring of six other α cells (*large black somata*) as well as to many smaller cells, presumably amacrine cells based on soma size and position (some of which are located in the inner nuclear layer; *white somata*). The γ ganglion cell is coupled to several cells of similar soma size lying in the same plane as the injected cell (e.g., cell at 12 o'clock). It is also coupled to cells with smaller somata lying both within the ganglion cell layer and the inner nuclear layer. The β cell shown here was not apparently coupled to any other cells. *Arrowheads* indicate the axons. Scale bar, 50 μm.

Postnatal week 2 (P8–P14)

During the second and third postnatal weeks, Neurobiotin coupling was progressively more prominent. Beginning at P8, some cells could be classified on the basis of dendritic morphology as described for mature ferret ganglion cells. By this age, α ganglion cells had somata that vary in size from 10 to 15 μm and can be identified as well by the striking size and branching pattern of their dendritic trees. β cells had somata that ranged from 7 to 10 μm and had small, highly branched dendritic arbors. γ cells had 6–7-μm-diameter somata and long, infrequently branched dendrites. Cell soma size varied slightly on the basis of eccentricity, but this gradient was found to be shallow, consistent with earlier reports (Henderson, 1985), so cells could be identified reliably using a combination of cell body size and dendritic morphology. This ability to identify particular retinal ganglion cell classes even at these early times in development correlates well with previous studies of the developing cat retina (Dann et al., 1988; Ramoa et al., 1988), and many of the transient morphological features of the immature retinal ganglion cells noted in those studies, such as spines and an excessive number of branches, are still present during this week in the ferret retina (compare Figs. 3, 4 with Figs. 1, 5).

Figure 4 shows representative α, β, and γ cells following Neurobiotin injections. Even at this early stage, some cells identified morphologically as α cells had acquired the pattern of Neurobiotin coupling described above for mature α cells—a ring of approximately five neighboring α cells and conventionally and displaced amacrine cells are coupled within the dendritic tree of the injected cell shown in Figure 4 (α)—but only about a third of the α cells filled during postnatal week 2 showed coupling (see Fig. 7). The rest had full dendritic trees, well-filled axons, and no coupling of any type. β ganglion cells were also identifiable but were not apparently coupled. A heterogeneous population of γ cells could also be identified on the basis of their very small soma size and long, irregular dendritic branching; a substantial portion of these cells were coupled to cells of similar

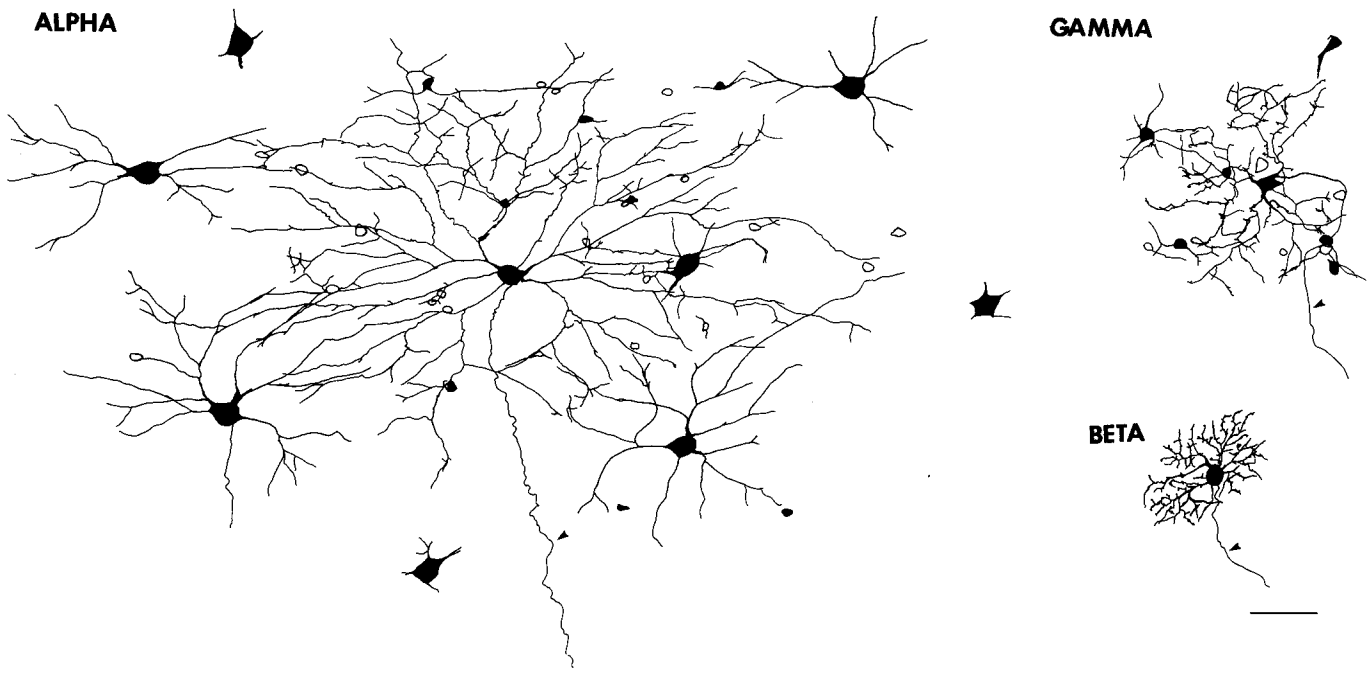

Figure 5. Camera lucida drawings of ganglion cells from postnatal week 3 (P15–P21). Conventions are as for Figure 4. The α ganglion cell is coupled to a halo of other α cells. Of the eight coupled α cells, six appear to surround the injected cell, while an additional two (*on the right*) lie beyond this ring. The α cell is also coupled to many smaller cells, presumably amacrine cells, with somata in both the ganglion cell layer and the inner nuclear layer. The γ ganglion cell is coupled to several cells of similar soma size that lie in the same plane as the injected cell, as well as to cells with smaller somata that lie both in the ganglion cell layer and the inner nuclear layer. The β cell was not apparently coupled. Scale bar, 50 μm.

soma size and to amacrine cells (based on their location and size). During the first few days of postnatal week 2, there were still cells that could not be reliably identified according to ganglion cell class, and some of these cells were also found to be Neurobiotin coupled (see Fig. 7, week 2, "unident").

Postnatal week 3 (P15–P21)

During postnatal week 3, the morphology of retinal ganglion cells further matured and all well-filled cells could be identified reliably according to cell class. The cells have increased in soma size (α soma size is 12–16 μm; β, 8–12 μm; γ, 8–9 μm) and dendritic extent (compare Figs. 4, 5), and many of the immature features seen in retinal ganglion cells from the previous week have disappeared. Neurobiotin coupling resembled that seen in the adult, both in pattern and in percentage of cell types showing coupling (see Figs. 5, 7). All well-filled α ganglion cells were coupled to a halo of other α cells of similar morphology and to a number of displaced and conventionally placed amacrine cells (Fig. 5, α). Again β cells were not coupled, while there was a further increase in the percentage of γ cells that showed tracer coupling (see Fig. 7).

The pattern of Neurobiotin coupling during postnatal week 3 is further illustrated in Figure 6. Here a central α cell was injected with Neurobiotin and Lucifer yellow. Later processing revealed the presence of many Neurobiotin-labeled amacrine cells within the dendritic arbor, as well as coupled α cells. Two of these amacrine cells are marked (Fig. 6*A*, notched arrowheads). One of the them (notched arrowhead, lower left) may even have acquired its labeling via coupling to a ganglion cell that itself is part of the halo of secondarily (noninjected) filled cells. A number of other cells apparently coupled in this way can also be noted in Figure 6*A*. This observation suggests that an extensive network of coupled α cells and amacrine cells may tile the entire retina. However, only a portion of this network is revealed in any single injection, presumably due to the limited quantity of Neurobiotin that can be iontophoresed into a ganglion cell that subsequently diffuses slowly into junctionally coupled cells.

This extensive coverage of the retina could occur either through the presence of dendrodendritic contacts or by dendrosomatic contacts. A high-magnification view reveals apparent dendritic overlap between two α cells (Fig. 6*B*, triangular arrowheads)—the injected cell and another cell whose soma lies slightly beyond the edges of the dendritic field of the injected cell. Indeed, processes from two coupled α cells were often so closely opposed, at least at the tips, that the origin of the process could not be determined with certainty. On the other hand, we rarely saw the dendrites of the injected cell extending all the way to the somata of the surrounding halo of Neurobiotin-labeled cells (see Figs. 1*A*, 4, 5). In contrast, dendrites of the injected α cell often passed directly over the somata of coupled amacrine cells (Fig. 6*B*, notched arrowhead). Electron microscopy is necessary to define further the contact points between cells that show coupling on the basis of Neurobiotin transfer.

Quantitative analysis of the coupling pattern

A total of 170 tracer-injected cells were analyzed for this study. The percentage of injected cells that showed Neurobiotin coupling is presented graphically by cell type and retinal age in Figure 7. The percentage of coupled cells present during each postnatal period is broken down by morphological class, except for postnatal week 1, because, as noted above, at the earliest

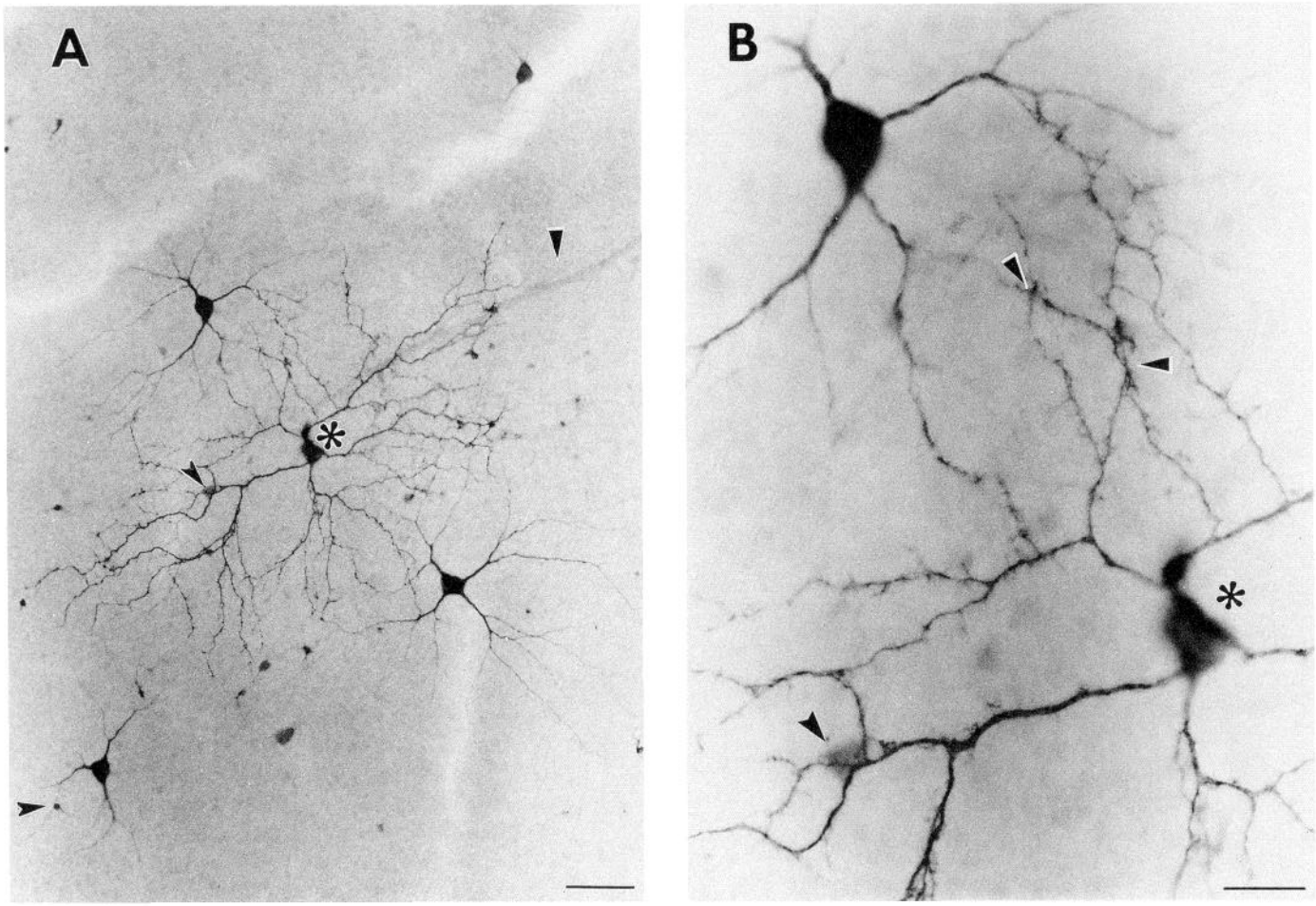

Figure 6. Photomicrographs showing Neurobiotin coupling of a postnatal day 16 α ganglion cell. *A,* The injected α ganglion cell (*asterisk*) is coupled to five other α cells, as well as to many amacrine cells. The array of filled ganglion cells is also coupled to amacrine cells, both displaced and conventionally placed (e.g., *notched arrowhead, lower left corner*). The axon of the injected cell is also indicated (*large triangular arrowhead*). *B,* Higher-magnification view of the injected cell and its upper left neighbor; dendrodendritic crossing points are visible (*triangular arrowheads*). Note also that one dendrite of the injected cell runs directly above a coupled amacrine cell (*notched arrowhead*; same amacrine cell as shown at *notched arrowhead* near center of *A*). Scale bars: *A*, 50 μm; *B*, 20 μm.

ages the cells are too immature to be identified reliably according to ganglion cell class. Table 1 summarizes the numbers of injected cells analyzed at each age, and indicates the types and numbers of other cell types coupled to the injected cell. All 170 cells included here had well-filled dendritic arbors and axons that could be traced toward the optic nerve head.

In retinas from postnatal week 1 (P1–P7), 49 cells that met the criteria for analysis were examined, and only 20% were found to pass Neurobiotin to other cells. The number of cells coupled to the injected cell during postnatal week 1 ranged from 2 to 15. Since cells at this early age could not be identified morphologically, it was impossible to determine if the variability of numbers of cells in a coupled group is a function of cell type or determined by some other parameter.

By postnatal week 2, most cells could be identified morphologically. As at earlier ages, only a moderate number of injected cells showed Neurobiotin coupling at this age: 31% of the α cells, 40% of the γ cells, and 25% of the cells still too immature morphologically to be identified reliably were coupled. β cells were never seen to be coupled. With regard to γ cells, since most of the coupled cells were situated in the same layer as the injected γ cell and were of approximately the same soma diameter (6–7 μm), we believe at least some are likely to be other γ cells. It is also possible that the γ cells are coupled to amacrine cells, since the amacrines have only slightly smaller cell soma diameters at this age and there was some coupling to cells located in the same focal plane as the conventionally placed amacrines (as noted above; see Fig. 3). Likewise, the morphologically unidentified cells that showed coupling (25%) were predominantly coupled to cells of a similar size in approximately the same focal plane as the injected cell.

There was a dramatic rise in the percentage of cells showing Neurobiotin coupling during postnatal week 3. For example, 95% of the α cells were coupled, similar to the percentage of coupled α cells found in the mature retina. The average number of α cells found coupled to an injected α cell ranged from 2 to 7 (see also Figs. 4, 5). The number of coupled amacrine cells was quite variable, with conventionally placed amacrine cells ranging in number from 1 to 21 and displaced amacrine cells ranging from 1 to 16. There was one α cell coupled only to other α cells, but not to any conventional or displaced amacrine cells. Like the α cells, a high percentage (78%) of the γ cells showed

Table 1. Summary of Neurobiotin coupling by age and cell type

Age	Proportion of coupled cells	Type and # cells (range) coupled to injected cell[a]
P1–P7	Unidentified,[b] 5/16	2–15 (unidentified)
(n = 49)		
P8–P14	α, 5/16	α, 1–6; amacrine, 1–17; displaced amacrine, 2–8
(n = 45)	γ, 6/15	γ and/or amacrine, 2–4[c]
	β, 0/6	β, 0; amacrine, 0
	Unidentified, 2/8	Unidentified, 1–8
P15–P21	α, 22/23	α, 2–7; amacrine, 1–21; displaced amacrine, 1–16
(n = 46)	γ, 7/9	γ and/or amacrine, 2–5[c]
	β, 0/14	β, 0; amacrine, 0
>P21	α, 21/21	α, 4–7; amacrine, 1–5; displaced amacrine, 1–9
(n = 30)	γ, 2/2	γ and/or amacrine, 2–3[c]
	β, 0/7	β, 0; amacrine, 0

[a] For example, if an injected cell was an α cell, then the numbers refer to the numbers of α cells coupled to the injected α cell, and to the numbers of conventionally placed and displaced amacrine cells coupled to the injected α cell.

[b] Unidentified: at these early ages, it was not possible to identify the cells according to ganglion cell class.

[c] Many of these cells had somata of the same size and laminar location as the injected cell; we have tentatively classified them as γ cells, although the possibility remains that some are also amacrine cells. See text for further details.

Neurobiotin coupling during postnatal week 3. Again, β cells were never found to be coupled.

At ages greater than postnatal week 3, when the spontaneous waves of correlated retinal activity have disappeared, our analysis focused principally on α cells, as can be seen in Figure 7 and Table 1. Since a previous report by Vaney (1991) showed that α cells in the adult cat retina had a particular pattern of coupling, we used this information to assess whether the ferret retina had a similar pattern of coupling at older postnatal ages as presented above. Thirty cells from retinas P22 or older were analyzed. All of the α cells showed coupling to both α cells and amacrine cells in a pattern similar to that shown for α cells in the adult cat retina.

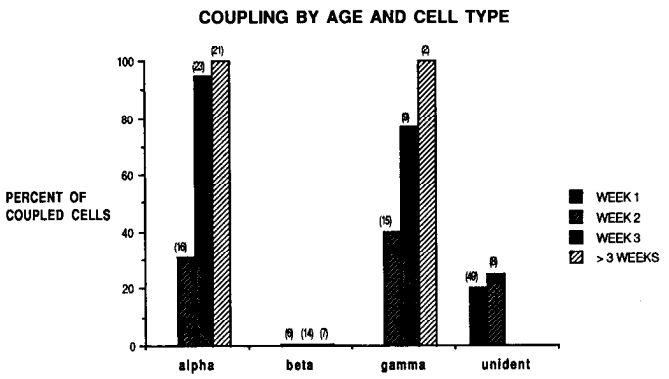

Figure 7. Histogram to illustrate developmental changes in the percentage of injected cells in each ganglion cell class demonstrating Neurobiotin coupling. For postnatal weeks 1 and 2, some cells were too immature to identify morphologically and were therefore categorized as unidentified (*unident*). Note that the absolute percentages of unidentified cells at weeks 1 and 2 are not directly comparable because the "unidentified" category includes all cells analyzed for postnatal week 1, but only a small percentage of the cells for week 2. The total number of cells of each type filled for a given postnatal period is given in *parentheses* above each column.

Discussion

In this study we have used intracellular injections of Neurobiotin (Vaney, 1991, 1992) to examine whether cells in the ganglion cell layer of the developing ferret retina are junctionally coupled to each other during the same period in which neighboring ganglion cells are known to fire in synchrony and generate waves of activity. Our results demonstrate that Neurobiotin coupling indeed exists as early as P1. In fact, coupling becomes more extensive, so that by the end of the second postnatal week, many cells were Neurobiotin labeled as a result of the injection of a single retinal ganglion cell. Not only are ganglion cells apparently intercellularly connected to each other, they are also tracer coupled to many neighboring amacrine cells. This is particularly evident when α ganglion cells are injected. In contrast, β ganglion cells were never found to be coupled to any other cell, suggesting that while there is a morphological substrate that could contribute to the correlated firing of retinal ganglion cells, intercellular junctions alone cannot account entirely for the waves and synchronous firing present during development.

Significance of coupling

Several lines of evidence suggest that the tracer coupling observed here represents true connectivity between cells rather than an artifact of the injection technique. First, the coupling was revealed by the passage of Neurobiotin but not by intracellular filling with fluorescent dyes. Lucifer yellow or carboxyfluorescein, injected in order to monitor visually the quality of the fill, were seen to label only the injected cell, indicating that dye leakage due to damage or nonspecific uptake did not occur. In addition, if tracer coupling resulted from nonspecific uptake of tracer from the injection site, then one would not expect the staining to be cell-type specific. Second, the array of labeled somata resulting from a Neurobiotin injection was almost always distant from the injected soma, but generally within the area of its dendritic tree, suggesting that the coupling is mediated by cellular contacts rather than by accidental penetration of nearby cells or their processes. We did occasionally observe Lucifer yellow transfer into cells whose somata were located

deep in the ventricular zone and radially beneath the cell being filled in retinas P21 and younger (data not shown). We believe that this labeling results from the accidental penetration of the end feet of neuroepithelial cells or glial cells because similar patterns can be produced without impaling any cell bodies, but merely by penetrating the vitreal surface of the retina with a dye-filled electrode. The artifactual nature of this type pattern is further supported by several observations: cell processes that pass very close to a ganglion cell soma can occasionally be identified; injecting Neurobiotin and Lucifer yellow directly into the surface of the ganglion cell layer without filling a ganglion cell body can cause immediate Lucifer yellow filling of several cell somata located in the ventricular zone (and subsequent processing reveals Neurobiotin-labeled processes that run from the ventricular zone to the retinal surface); and injecting rhodamine dextrans, which are far too large to pass through gap junctions (MW 10,000), produces the same pattern of intracellular labeling. We cannot completely rule out the possibility that some transfer of Lucifer yellow between cells in the ventricular layer may in fact indicate genuine coupling, as has been reported previously by LoTurco and Kriegstein (1991) for cells in the ventricular zone of the developing cerebral cortex. However, this radial pattern of labeling should be distinguished from the tangential pattern of Neurobiotin coupling described above, which cannot be produced artifactually by any of these methods.

We observed that the frequency of cells with Neurobiotin coupling was low at the earliest ages studied, and increased progressively with age. While this could result from technical problems such as a greater lability of coupling in more immature cells or greater susceptibility to cell damage during the injections (these cells are often less than 10 μm in diameter), it is also possible that this progressive change represents a real developmental sequence in which the extent of coupling increases to achieve the adult levels. This seems reasonable in view of the fact that the first 3 postnatal weeks encompass a period of extensive growth and development within the retina (Greiner and Weidman, 1981; Henderson et al., 1988). For instance, the more extensive coupling in older animals may be due to changes in the types of connexin proteins that form the gap junctions (Demietzel and Spray, 1993). In the immature retina, the diameter of the intercellular channel could be considerably smaller in size, or the number of channels significantly lower, restricting the amount of Neurobiotin that can spread from cell to cell.

Comparison with neocortical gap junctional coupling

The observation here that the pattern of tracer coupling in the retina becomes more established with age contrasts with the finding in neocortex, where tracer coupling between cortical cells exists only transiently during development (LoTurco and Kriegstein, 1991; Peinado et al., 1993). However, some similarities between the retinal and cortical patterns of coupling are evident. First, coupling usually involves neurons of the same class; for example, in the retina, α cells are coupled to α cells, and not to β or γ cells, and in the neocortex, pyramidal cells are specifically interconnected with each other (and not to stellate cells). Another similarity is that in the developing cortex, the extent of the spread of the tracer and therefore the number of cells that are coupled did not appear to be a function of the duration of tissue incubation after cell injection (provided at least 15 min was allowed for tracer passage) (Peinado et al., 1993). Likewise, no relationship was found between the time when, during the course of an experiment, the retinal cells were injected and the number of Neurobiotin cells labeled, but a distinct dropoff in intensity of labeling beyond the injected cell was always seen, as in the neocortex. This implies that the localized transfer of Neurobiotin probably reflects a consistent parameter that restricts coupling (such as the number or size of gap junctions).

In view of the above considerations, we believe that the presence of Neurobiotin coupling at these early ages implies that there are gap junctions between cells in the developing inner retina and that progressively more cells become coupled with maturation. It is noteworthy that the retinal ganglion and amacrine cells, like the cortical neurons (Yuste et al., 1992), also exhibit spontaneous correlated calcium bursting (Wong et al., 1992a), although no waves are seen in the cortex. In the neocortex, both the correlations and the Neurobiotin coupling can be abolished by agents that are thought to close gap junctions, such as halothane and octanol (Peinado et al., 1993). If the tracer coupling seen in the immature ferret retina is mediated by gap junctions, then the pattern of coupling seen here should also be susceptible to similar manipulations. Indeed, in preliminary experiments in which we have lowered intracellular pH indirectly, a technique also known to produce uncoupling of gap junctions (Bennett et al., 1978; DeVries and Schwartz, 1989; Peracchia, 1990; LoTurco and Kriegstein, 1991), the transfer of Neurobiotin from injected retinal ganglion cells (five of five α cells) was abolished; α-to-α cell coupling could be restored following return to pH 7.4 (eight of eight α cells). Thus, while these observations strongly suggest that gap junctions are present in the developing mammalian retinal ganglion cell layer, electrophysiological experiments are needed to confirm the presence of these junctions between the immature ganglion cells and amacrine cells. In addition, it will be important to determine which connexins immature and adult ganglion cells use to construct these junctions (Beyer et al., 1990).

Are junctions needed for correlations/waves?

The results of this study indicate that gap junctions are very likely to be present between ganglion and amacrine cells during the same period in development in which the discharges of the retinal ganglion cells are highly correlated and spatially organized into traveling waves. However, two major observations of our study suggest that the junctional contacts between ganglion cells and amacrine cells cannot account entirely for the propagation of the waves.

If coupling between ganglion cells contributes to the stereotyped correlated waves of firing in the retina, then we must account for the fact that the waves disappear after the third week of postnatal life in the ferret, whereas the Neurobiotin coupling becomes progressively more robust. It is certainly possible that the coupling has nothing to do with the mechanism that generates the waves. However, a possibility we consider more likely is that the morphological substrate responsible for the waves remains in the adult retina, but other retinal circuits become dominant and are responsible for the physiological properties of the adult ganglion cells, much in the same way that the physiological properties of neurons in invertebrate circuits such as the lobster stomatogastric ganglion can be radically altered by simple changes in inputs from modulatory neurons (Marder and Weimann, 1992). Indeed, careful physiological recordings of the adult cat retina in the dark have shown that ganglion cells with overlapping receptive fields of the same subtype are correlated in their spontaneous firing (Mastronarde, 1989). The sharp correlations of these adult ganglion cells, which can spontaneously

fire within milliseconds of each other, differ from ganglion cells in the neonatal ferret retina, which fire within much broader time intervals of 0.5–1.0 sec of each other (Wong et al., 1993). This difference could be achieved by a progressive increase in the extent of coupling. However, in the adult these sharp correlations are rarely seen because they are obscured by the strength of the vertical inputs to ganglion cells derived from the photoreceptors via the bipolar cells. During the period when the waves are present, few if any of these vertical inputs have even formed, and it is not until the time that the waves disappear that bipolar-to-ganglion cell synapses are clearly present (Greiner and Weidman, 1981; Maslim and Stone, 1986). Consequently, we suggest that whatever mechanisms bring about the disappearance of the waves, the coupling seen in the adult retina could represent the persistence of an early retinal circuit that, rather than being dismantled, is incorporated during ensuing development into the functional circuitry that subserves visual information processing.

The second discrepancy is that while there is extensive coupling involving α ganglion cells, amacrine cells, and γ cells, we found no evidence for coupling of β cells at any time. Yet β cells comprise almost half of the retinal ganglion cell population (Hughes, 1985; Wässle and Boycott, 1991), and during development they are known to fire spontaneous action potentials in bursts similar to those produced by other ganglion cell classes (Wong et al., 1993). In addition, if the correlated firing is required for the activity-dependent refinement of patterned connections within retinorecipient targets (Meister et al., 1991; Mooney et al., 1993; Wong et al., 1993), then it is difficult to see how the β retinal ganglion cells could undergo axonal remodeling without somehow participating in the correlated activity. While gap junctions may play a role in correlating retinal activity, other mechanisms must also contribute. A close analysis of the correlated activity has indicated that fast excitatory synapses are unlikely to be involved in the wave-like spread of activity, but it has certainly not ruled out the possibility that a combination of both intracellular and extracellular signals contributes (Wong et al., 1993). One way in which this could occur is if some ganglion cells, for instance the β cells, were *recruited* into waves by receiving an extracellular diffusible signal such as neurotransmitter or other signaling molecules released from amacrine cells.

If gap junctional coupling in the immature retina does contribute to the propagation of waves, then our observation that amacrine cells are labeled following Neurobiotin injection of a ganglion cell implies that these interneurons could be involved in producing or spreading the correlated activity. Amacrine cells are generated at the same time or slightly later than ganglion cells (Zimmerman et al., 1988; M. Kliot and C. J. Shatz, unpublished observations). Preliminary intracellular dye injection studies suggest that they mature morphologically at approximately the same rate as many ganglion cells (Campbell et al., 1987). The suggestion that amacrine cells participate in the wave generation is supported by our preliminary observations of the activity of these cells *in vitro*. Using calcium-sensitive dyes to monitor simultaneously the neuronal activity of amacrine and ganglion cells, we have found that some amacrine cells show periodic elevations in intracellular calcium that are correlated with those of neighboring ganglion cells (Wong et al., 1992a). Whether these spontaneously active amacrine cells are equivalent to those that show tracer coupling to the ganglion cells needs further investigation. Our current impression is that the spatial distribution of simultaneously active interneurons and ganglion cells seen with calcium imaging is very similar to that produced by Neurobiotin coupling, implying that gap junctions may indeed be part of a functional network that contributes to the correlation of spontaneous activity. While it appears that some amacrine cells could act to correlate the activity of ganglion cells by means of gap junctions, it is also possible that they could do so through nonsynaptic extracellular signaling. Indeed, amacrine cell cultures from the neonatal rat retina can release neurotransmitters such as ACh (Lipton, 1988). Thus, in the model we are proposing, both gap junctions and an extracellular mechanism would be required for waves; neither alone would be sufficient.

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