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# Transient Morphological Features of Identified Ganglion Cells in Living Fetal and Neonatal Retina

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**The function and morphology of retinal ganglion cells in the adult mammalian visual system has been well studied, but little is known about how the adult state is achieved. To address this question, the morphological changes that retinal ganglion cells undergo during development were studied. Ganglion cells were first identified by retrograde labeling with rhodamine latex microspheres deposited in retinorecipient targets in fetal and early postnatal cats. The structure of ganglion cells was then revealed by intracellular injection of Lucifer yellow in living retinas removed and maintained in vitro. As early as 2 weeks before birth, a morphologically diverse assortment of ganglion cells is present, some of which resemble the α, β, and γ classes found in the adult. However, in contrast to the adult, developing ganglion cells exhibit several transient features, including excessive axonal and dendritic branching and exuberant somatic and dendritic spines. These morphological features indicate that there is a transient network of connectivity that could play an important role in the final determination of retinal ganglion cell form and function.**

IN THE ADULT MAMMALIAN CENTRAL nervous system there is an enormous diversity of cell types and connections. For example, the retina of the adult cat contains a variety of retinal ganglion cell types that are well characterized on the basis of their form, function, and unique pattern of connections (*1, 2*). At least three distinct classes have been extensively studied and can be distinguished from each other on morphological grounds: the α, β, and γ cells. Alpha cells have the largest cell bodies and long, sparsely branched dendrites; β cells have medium-sized cell bodies and short, extensively branched dendrites; and γ cells, although possibly heterogeneous both morphologically and physiologically, have small cell bodies and long, relatively unbranched dendrites (*1, 2*). Although the exact size and shape of ganglion cells vary with their distance from the central retina, at each retinal locus cells of all three classes can be identified (*1*). What developmental events lead to the establishment of these ganglion cell classes? We have begun to answer this question by studying the morphological development of ganglion cells during fetal and neonatal life to determine if and when morphological remodeling of ganglion cells plays a role in the acquisition of adult morphology.

The morphology of ganglion cells was revealed by intracellular injection of Lucifer yellow in living fetal and neonatal cat retinas maintained in vitro. We studied 187 ganglion cells in 16 retinas from 14 animals between embryonic day 36 (E36) and birth (E65), and between postnatal day 3 (P3) and the adult. Fetal animals were anesthetized via the maternal circulation: mother cats received a mixture of halothane and nitrous oxide [see (*3*) for complete details]. Postnatal animals were anesthetized with Nembutal (intraperitoneal, 30 mg per kilogram of body weight) and were subsequently killed with an overdose of Nembutal. Eyes were removed and immediately placed in cold Ringer solution (*4*). Retinas were dissected free from the pigment epithelium and lens and placed in a tissue-slice chamber mounted on the stage of a compound microscope so that ganglion cells could be injected intracellularly with Lucifer yellow.

Retinal ganglion cells were identified by the retrograde transport of rhodamine-labeled latex microspheres (*5, 6*) that had been injected into the central targets of the retinal projection. To do so in the fetal animals, the head was exteriorized by cesarean section,

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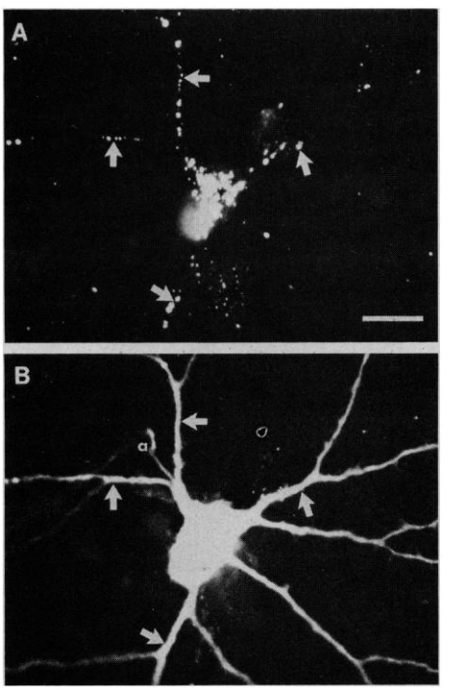


**Fig. 1.** Example of a retinal ganglion cell (**A**) retrogradely labeled in vivo with rhodamine microspheres and (**B**) subsequently injected intracellularly with Lucifer yellow in vitro at P7. Multiple injections (0.2 μl each) of microspheres [Tracer Technology, used as supplied (*5*)] were made into both the lateral geniculate nucleus and superior colliculus at P5. Two days later, the retina was removed, placed in a tissue-slice chamber, and rapidly perfused with an oxygenated Ringer solution at 23°C (see text) so that the same cell could be injected intracellularly with Lucifer yellow. When placed in vitro, retinas were viewed either in (A) rhodamine (Zeiss filter set 48 77 14) or (B) Lucifer yellow (Zeiss filter set 48 77 09) fluorescence. The microspheres usually filled not only the soma but also many of the proximal dendrites [arrowheads in (A)]. (Microspheres contained within the somata of other retrogradely labeled cells can also be seen.) The Lucifer yellow injection reveals many distal dendrites, fine structural details, and the axon [a in (B)], only part of which is visible in the focal plane of this photomicrograph. Corresponding regions of the same labeled dendrite are indicated by arrows. Cells were injected with Lucifer yellow–CH (Sigma, 20% in 0.1*M* LiCl) immediately after the recording of a membrane resting potential according to the method of Katz, Burkhalter, and Dreyer (*5*). (Conditions in the chamber were sufficient to maintain resting potentials of up to 60 mV for 6 to 8 hours.) After several ganglion cells had been injected successfully, retinas were fixed by immersion in 4% paraformaldehyde in 0.1*M* sodium phosphate buffer, whole-mounted onto gelatinized slides (*19*), dehydrated, and cleared, and a cover slip was applied. Cells were viewed in a fluorescence microscope, photographed, and then drawn with a camera lucida attachment. A cell was considered to be reliably injected if distal dendrites were well filled and if the processes were not excessively beaded or broken. Calibration bar, 12 μm, applies to (A) and (B).

and a 1-μl syringe containing the microspheres was used to make five or six injections (0.1 μl each) in the lateral geniculate nucleus and superior colliculus. The fetus was returned to the uterus, and 48 hours later it was removed for the in vitro experiment. A typical example of a retinal ganglion cell retrogradely labeled with rhodamine microspheres and subsequently injected with Lucifer yellow at P7 is shown in Fig. 1.

During fetal development, it is difficult to distinguish retinal ganglion cells from other cell types in the ganglion cell layer by standard histological criteria (*7*). The use of rhodamine microspheres ensures the study of cells whose axons have reached central targets. Moreover, the technique permits nearby ganglion cells of all soma sizes to be injected with Lucifer yellow, thereby allowing direct comparison of cell types at similar eccentricity. We believe that the Lucifer yellow injection technique used in conjunction with living retinas has many advantages over the methods of Golgi impregnation or extracellular horseradish peroxidase injection. For example, in healthy preparations, as judged by membrane resting potentials, it is possible to reveal the fine morphological details of ganglion cells, including delicate dendrites, spines, growth cones, and filopodia without the beading usually associated with damage or the effects of fixation. Moreover, axons can be filled routinely and can be distinguished readily from dendrites by their smooth contour and by their location in the optic fiber layer.

At the earliest ages studied (E36 and E45), Lucifer yellow injections of ganglion cells revealed that the majority are morphologically immature, and it is not possible to classify them in a reliable fashion (*8*). From E52 onward, injections of adjacent retinal ganglion cells revealed a great diversity of form. When injected cells at similar eccentricity were compared, we could place some of them into one of three morphological classes on the basis of relative soma size, dendritic branching pattern, and dendritic spread: class I, cells with large somata and extensive, highly branched dendrites; class

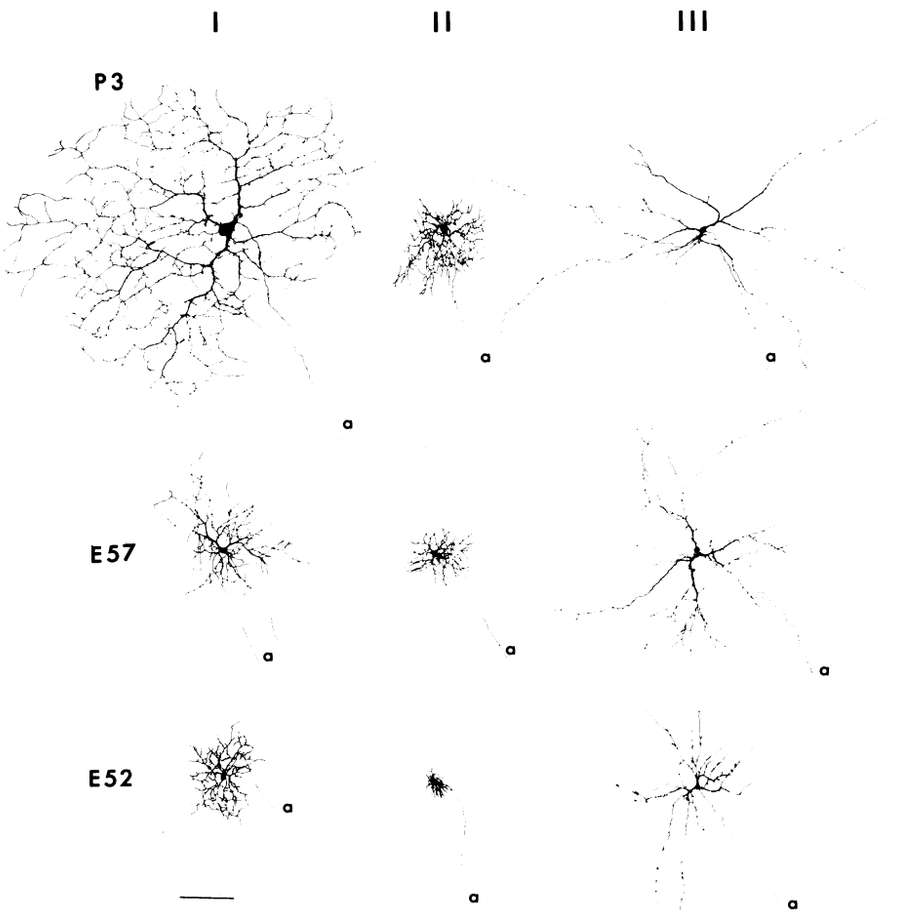


**Fig. 2.** Camera lucida drawings of sets of three adjacent retinal ganglion cells injected with Lucifer yellow at E52, E57, or P3. Every cell here has an axon (a) that travels into the optic fiber layer toward the optic disk. The cells at E57 and P3 were also retrogradely labeled with rhodamine microspheres. At each age the cells illustrated could be assigned to one of three general morphological classes (class I, class II, and class III, see text) based on soma size, dendritic branching pattern, and dendritic spread. Calibration bar, 100 μm. For the class III cell at P3, calibration bar, 165 μm.

II, cells with medium-sized somata and short, highly branched dendrites; and class III, cells with small somata and very long, sparsely branched dendrites. Camera lucida drawings of three sets of nearby ganglion cells injected at E52, E57, and P3 that display characteristics of each class are shown in Fig. 2. In addition, Fig. 2 illustrates that between E52 and P3 there is a steady growth in soma size and dendritic spread. These observations suggest that retinal ganglion cells can achieve a certain degree of morphological maturity even before birth and can resemble the α (class I), β (class II), and γ (class III) classes of the adult with respect to the relation between soma size and dendritic extent.

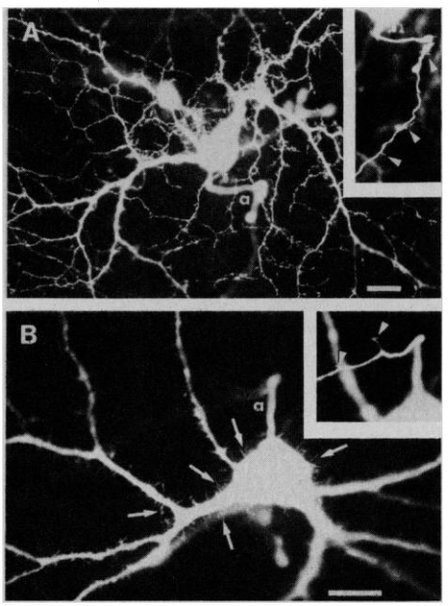


**Fig. 3.** Two Lucifer yellow–injected cells at P3 illustrating several transient features of morphology. (Both cells were also labeled with microspheres.) (**A**) The complexity of dendritic branching and dendritic protrusions (spines) characteristic of many cells at this age. (**B**) The delicate spines and filopodia that stud the soma and proximal dendrite (arrows). Insets to both figures show in a focal plane at the level of the optic fiber layer that the proximal portions of both axons carry a number of short side branches (arrowheads). In both figures the initial part of the axon is indicated by the letter a. Calibration bars (A and B), 14 μm.

In other respects ganglion cells exhibit several transient features of immaturity that are especially evident between E57 and P7, and are gone by P31. For instance, the dendrites and occasionally the somata of many cells are studded with many delicate spines, which gives them a hairy appearance, as shown at P3 in Fig. 3 (*9*). Quantitative work in progress (*8*) has confirmed this impression. For example, at E57, 58% (14 of 24) of ganglion cells have more than 100 spines per neuron; at P3, 77% (20 of 26) of ganglion cells have more than 100 spines per neuron; in the adult, this figure is 0% (0 of 20). In addition, as shown in Fig. 2 (P3) through Fig. 4, the dendrites of many cells appear to be more highly branched than those of ganglion cells in the adult: the proportion of cells with over 100 branch points is 33% (8 of 24) at E57, 54% (14 of 26) at P3, and only 5% (1 of 20) in the adult.

The technique of intracellular labeling with Lucifer yellow also permitted us to examine morphological features of intraretinal axonal development. In so doing, we found that the axons of ganglion cells in fetal and neonatal retinas are immature in several respects. As shown in the insets in Fig. 3, A and B, the proximal portions of axons frequently (75 ganglion cells out of 130 between E36 and P7; none in the adult) carry short, simple side branches reminiscent of those seen along the preterminal portions of retinal ganglion cell axons within the lateral geniculate nucleus during fetal life (*10*). Some ganglion cells also have bifurcating axons (Fig. 4). In this example from a P7 retina, one axon collateral is directed correctly toward the optic disk (Fig. 4, arrow), whereas the other collateral runs diametrically opposite toward the ciliary margin. Ganglion cell axons can also give off two collaterals at 90° with respect to each other, or even two collaterals both of which head toward the optic disk. Between E36 and P7, 11 of 130 ganglion cells had bifurcating axons whereas at P31 and adult, none of the 34 injected cells had such axons (*11*).

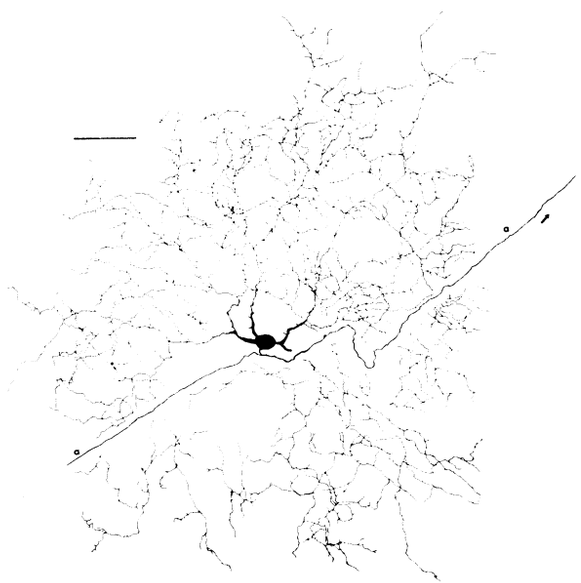

**Fig. 4.** Camera lucida drawing of a double-labeled cell at P7 that has a bifurcating axon (a). Both branches travel well beyond the dendritic spread of the cell within the optic fiber layer. However, only one branch is directed toward the optic disk (arrow). For purposes of clarity, lines delineating the axons were thickened and nearby dendritic branches were deleted. Calibration bar, 50 μm.

The results of our study show that, although there is great diversity in the morphology of ganglion cells during prenatal development, by 2 weeks before birth some cells can be assigned to one of three developmental classes. Since some of the basic structural features of ganglion cells belonging to each class resemble those of the α, β, and γ types of the adult cat retina (*1, 2*), we think it is likely that ganglion cells of classes I, II, and III mature into α, β, and γ cells, respectively. If so, then it is remarkable that ganglion cells acquire their basic structure within the retina not only well before vision is possible (*12*), but also many weeks before their terminal arborizations within target nuclei take on their characteristic morphologies (*10, 13*).

We have also demonstrated that the form of adult ganglion cells is achieved by a conjoint process of progressive growth and remodeling. Cells are likely to acquire their adult form by the addition of membrane to their somata and dendrites and by the retraction of the spines and axonal side branches that characterize the immature state. Previous studies in the cat have suggested that ganglion cells resembling all three adult classes can be identified neonatally (*14*) and as early as E50 prenatally (*15*). However, these studies did not report the presence of transient morphological features—somewhat surprising in view of the many studies of development in other systems demonstrating that neurons can undergo remodeling (*16*). In our own study, we have shown that remodeling does indeed occur during ganglion cell development. Moreover, such remodeling involves not only the retraction of spines and filopodia and the simplification of dendritic trees—expected phenomena, on the basis of analogy with studies in other systems—but also involves the elimination of intrinsic axon collaterals and side branches.

The transient features of axonal and dendritic morphology can be correlated with a number of developmental events that have

been well studied in the cat retina. Ganglion cells exhibit morphological exuberance beginning roughly at E50, a time when synaptogenesis within the inner plexiform layer has just commenced (*17*), and reach their peak complexity at about the time of eye opening (1 week postnatal). Then there is a decline to nearly adult levels by about 1 month after birth, which suggests that visual experience may play a role in the loss of morphological exuberance. The period of decline also coincides with the time during which retinal ganglion cells are capable of rearranging their dendrites in response to the destruction of their neighbors (*18*). Thus, these transient processes may represent morphological correlates of the competition for presynaptic inputs that Perry and Linden (*18*) have suggested underlies the phenomenon of dendritic remodeling. Retinal ganglion cells could communicate through these processes with each other directly or via other cell types, thereby providing a morphological basis for local synaptic interactions that may play a role in the establishment of retinal ganglion cell form and function.


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6. The rhodamine-labeled microspheres have no cytotoxicity in a cortical brain slice preparation (*5*). Nevertheless, we examined whether the retrograde transport of the microspheres had any adverse effect on the morphology of ganglion cells. This seems unlikely, since the morphology of ganglion cells is similar regardless of the apparent density of microspheres inside the cell body. Moreover, in additional animals that were not injected with microspheres, Lucifer yellow revealed morphological features of ganglion cells similar to the ones we describe here.
7. It is possible to confuse ganglion cells with glial cells or displaced amacrine cells early in development. However, neither of these cell types can be labeled by the injection of microspheres into retinorecipient targets, either in adults or in fetal animals. In addition, amacrine cells were never labeled in postnatal animals that had been injected with microspheres when of fetal age—thus suggesting that amacrine cells never project an axon centrally (*8*).
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20. We thank L. Katz for assistance in developing the intracellular injection techniques; M. Konishi for helping to arrange for the necessary microscope modifications and chamber construction; D. Baylor, B. B. Boycott, E. I. Knudsen, L. Peichl, R. W. Rodieck, and H. Wässle for their useful criticisms of the manuscript; J. J. M. Chun and M. W. Siegel for help with the fetal surgeries; and C. Thomas for word processing. Supported by grants from the National Institutes of Health (EY 02858), the National Science Foundation (BNS 8317228), the March of Dimes, and the McKnight Foundation.

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# A Defined Medium for a Fastidious Spiroplasma


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**A defined medium (H-1) was developed for cultivation of the suckling mouse cataract agent, *Spiroplasma mirum*, a fastidious member of the class Mollicutes that causes cataracts and chronic brain infection in inoculated neonate mice. The H-1 medium was used to show the importance of sphingomyelin as a growth factor for the culture of the spiroplasma in vitro. The growth of *Spiroplasma mirum* and the pathology it induces in sphingomyelin-rich tissues in vivo may be related to this dependency.**


THE SUCKLING MOUSE CATARACT agent (SMCA) was isolated in 1961 from a pooled extract of rabbit ticks, *Haemaphysalis leporispalustris*, collected from a cottontail rabbit (*1*). Thought to be a slow virus prior to 1973 (*1*) and a mycoplasma-like organism thereafter (*2, 3*), SMCA was shown in 1976 to be a spiroplasma (*4*) (Mollicutes, Spiroplasmataceae) (*5*), and was eventually named *Spiroplasma mirum* (*6*).

Primary isolates of *S. mirum* were originally obtained by inoculating embryonated chicken egg yolk sacs with tick extracts; the cultured organisms killed the eggs in 4 to 10 days (*1*). High titers of the agent were demonstrated in the eyes and brains of intracerebrally inoculated newborn mice; cataracts, retinitis, and chronic brain infections resulted. The severity of symptoms varied and appeared to depend on the *S. mirum* strain used (*1, 7*). In 1971, *S. mirum* was cultivated in rabbit lens organ culture (*8*) and in 1984, in rabbit lens cell culture (*9*). Recently, Kotani *et al.* showed that *S. mirum* could infect and transform mammalian cells in vitro; malignant tumors were observed in mice inoculated with the transformed cells (*10*).

Although initial attempts to cultivate SMCA in conventional liquid or solid media were unsuccessful, Tully *et al.* (*11*) succeeded in 1977 in cultivating *S. mirum* from the allantoic fluid of embryonated chicken eggs in a newly developed medium (SP-4). This medium, based on a defined cell culture medium [CMRL 1066 (*12*)], also contained complex undefined additives such as peptones, yeast hydrolysate, fresh yeast extract, and serum. Strain SMCA growing in SP-4 medium reached titers of $10^8$ to $10^9$ infectious units per milliliter, as determined by end point titrations after 5 to 7 days of incubation. The growth rate of *S. mirum*, very slow in comparison to most spiroplasma species, suggested that *S. mirum* and similar spiroplasmas possess specialized nutritive requirements.

The SP-4 formulation was subsequently found to be an excellent medium for other spiroplasmas, particularly those associated with ticks (*13*), but it also proved to be useful for isolation and cultivation of certain slow-growing nonhelical mollicutes such as *Mycoplasma pneumoniae* (*14*) and *M. genitalium* (*15*). Once this unanticipated growth enhancement of human mollicutes had been discovered, a search began for factors in the SP-4 medium that were responsible for growth enhancement.

Initially, we analyzed the effect of deletion


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