Immunocytochemical Localization of Opsin in the Cell Membrane of Developing Rat Retinal Photoreceptors

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ABSTRACT   Mature retinal rod photoreceptors sequester opsin in the disk and plasma membranes of the rod outer segment (ROS). Opsin is synthesized in the inner segment and is transferred to the outer segment along the connecting cilium that joins the two compartments. We have investigated early stages of retinal development during which the polarized distribution of opsin is established in the rod photoreceptor cell. Retinas were isolated from newborn rats, 3–21 d old, and incubated with affinity purified biotinyl-sheep anti-bovine opsin followed by avidin-ferritin. At early postnatal ages prior to the development of the ROS, opsin is labeled by antiopsin on the inner segment plasma membrane. At the fifth postnatal day, as ROS formation begins opsin was detected on the connecting cilium plasma membrane. However, the labeling density of the ciliary plasma membrane was not uniform: the proximal cilium was relatively unlabeled in comparison with the distal cilium and the ROS plasma membrane. In nearly mature rat retinas, opsin was no longer detected on the inner segment plasma membrane. A similar polarized distribution of opsin was also observed in adult human rod photoreceptor cells labeled with the same antibodies. These results suggest that some component(s) of the connecting cilium and its plasma membrane may participate in establishing and maintaining the polarized distribution of opsin.

Development and maintenance of cell polarity is a crucial function of neuronal and epithelial cells. The specific distribution of membrane components to one or more surfaces of the cell may arise as a result of localized synthesis, vectorial transport of newly synthesized molecules from sites of synthesis to sites of function, localized reorganization of membrane components by translation along the membrane, or by redistribution of discrete portions of the cell membrane after insertion into the plasma membrane (15, 17, 20, 22). Visual cells of the vertebrate retina are highly compartmentalized and serve as a useful model for the study of the biogenesis of polarized membrane protein distribution. A unique photosensitive membranous organelle at one end of the cell, the rod outer segment (ROS)[1] has membranes composed largely of

the integral membrane protein rhodopsin. Early biochemical studies and more recent immunocytochemical studies of mammalian and amphibian rod photoreceptors using antiopsin antibodies have demonstrated that the distribution of opsin is highly polarized: the disks and plasma membrane of the ROS contain abundant opsin whereas the inner segment is nearly unlabeled by antiopsin and contains little spectroscopically detectable rhodopsin (17, 26).

Polarity of opsin's distribution is maintained throughout adult life while the photoreceptor cell continuously renews the ROS membranes. Synthesis of opsin is confined to the rod inner segment (RIS) compartment which is connected by a nonmotile cilium to the ROS. New disk membranes are formed at the base of the outer segment by the expansion of the plasma membrane at the outer portion of the connecting cilium (19, 25, 27). The ROS plasma membrane remains continuous with the disk membrane until the disk becomes separated above the base of the ROS. The ROS plasma

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[1] *Abbreviations used in this paper:* AvF, avidin-ferritin; IMP, intramembranous particle; PE, pigment epithelium; RIS, rod inner segment; ROS, rod outer segment.

membrane is also continuous with the plasma membranes of the connecting cilium and inner segment. Despite this continuity, opsin is not randomly distributed over the surface of the photoreceptor. Clearly some barrier to redistribution of opsin on the photoreceptor's plasma membrane exists. The plasma membrane of the connecting cilium and the recently described periciliary ridge complex, a highly ordered domain of the apical plasma membrane of the inner segment (19), may serve as this barrier. Recent studies by Andrews and Cohen (1) of cholesterol distribution also implicate lipid phase boundaries in this process.

In prior studies of antiopsin binding to frog rods we observed high labeling density on the surface of ROS but not on the inner segment plasma membrane (14, 17). Although the plasma membrane of the connecting cilium adjacent to the ROS was heavily labeled, there was almost no antiopsin binding on the basal ciliary plasma membrane adjacent to the periciliary ridge complex of the inner segment (14, 19, 24). Similar distribution of opsin in the rat connecting cilium plasma membrane was noted although the border of unlabeled and labeled regions was not as well demarcated (17, 24). Jan and Revel (11) also observed labeling of mouse and cattle rod connecting cilia and rod inner segments using peroxidase-conjugated Fab anti-bovine ROS.

The connecting cilium and outer segment are among the last domains of the photoreceptor cell to differentiate in the developing retina. During embryonic and neonatal development, the photoreceptor cells become progressively elongated and project a cilium beyond the apical surface of the RIS plasma membrane. The distal plasma membrane of the cilium becomes elaborately folded and then reorganizes into the lamellar array of disks characteristic of the adult retina's ROS (Fig. 1). Some component within the connecting cilium plasma membrane may actively maintain the orderly distribution of opsin. This function of the photoreceptor connecting cilium has been proposed in studies of its structure (13, 21) and may also apply in more general studies of cell membrane polarity in other ciliated cells (23). To evaluate the role of the connecting cilium in generating and maintaining the polarity of opsin distribution in photoreceptors, we studied the localization of opsin in the plasma membrane of rods during early stages of photoreceptor maturation in the developing rat retina.

MATERIALS AND METHODS

Tissue Preparation: Retinas were isolated from newborn normal pigmented RCS p⁺ rdy⁺ (12) rats 1–21 d old from the same litter. Comparable experiments were conducted with albino Sprague-Dawley rats 4-, 7-, and 8-d old from three separate litters. The animals were kept in a 12-h light/12-h dark cycle and sacrificed 30 min before the onset of light. Retinal tissue was separated from the adjacent pigmented epithelium, rinsed in 0.01 M phosphate buffered saline pH 7.4 (PBS) and fixed with 1% glutaraldehyde in 0.15 M phosphate buffer pH 7.0 for 60 min at room temperature as previously described (14). A human retina obtained from a surgically removed eye was comparably fixed.

Cytochemical Reactions: Fixed retinas were rinsed with 0.05 M glycine followed by 2% bovine serum albumin in PBS then incubated with 95 µg/ml biotinyl-sheep anti-bovine opsin for 90 min at room temperature (14). After rinsing in PBS for 60 min, the bound antibodies were visualized by incubating the tissue with 0.2 mg/ml avidin-ferritin (AvF) diluted in bovine serum albumin-PBS for 90 min at room temperature. Control reactions consisted of incubation of the retinas in biotinyl-nonimmune IgG followed by AvF. Affinity purified biotinyl-antiopsin and AvF were prepared as described previously (10, 16, 18).

Electron Microscopy: Following the cytochemical reactions, the retinas were postfixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.0 for 15
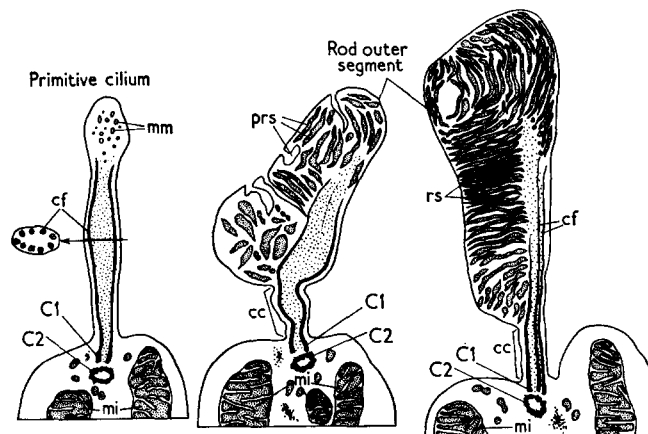

FIGURE 1 Diagram of the ultrastructure of developing rat photoreceptors (reproduced with permission from *J. Gen. Physiol.*, 43:1–13 [Suppl.]).

min at 4°C, dehydrated, and embedded in araldite. Thin sections, without additional staining, were viewed in a Jeol 100-B or Phillips EM-300 electron microscope.

RESULTS

In 3–4-d-old rats, rod outer segments (ROS) are not yet developed. The immature photoreceptor consists of inner segments and budding cilia (6). Antiopsin labeling revealed the presence of opsin molecules in the rod inner segment (RIS) plasma membrane (Fig. 2).[2] Complete absence of antiopsin binding to the adjacent pigment epithelium (PE) layer is indicative of the specificity of the immunocytochemical reaction (Fig. 2).

Opsin was not uniformly detectable on the plasma membranes of inner segments of all photoreceptors. Often, antiopsin labeled one cell while an adjacent rod remained unlabeled (Fig. 3). Whenever an unlabeled RIS was observed, its connecting cilium was also not labeled (Fig. 4). Thus, there was no preferential early accumulation of opsin in the newly developed connecting cilium. Since labeled cells were located immediately next to unlabeled cells (Figs. 3 and 4) it is unlikely that absence of labeling of some cells was a result of inaccessibility of the antibodies to the unlabeled cells. By the fifth postnatal day rudimentary outer segments began to appear on the tips of many of the connecting cilia. At this state antiopsin bound to the surface of the newly formed ROS and the connecting cilium as well as to the surface of the inner segments (Fig. 5). Once an outer segment began to organize, its connecting cilium was always labeled.

From the onset of ROS formation, a differential labeling pattern of the connecting cilium was observed: the labeling density on the proximal portion adjacent to the inner segment was substantially lower than the density on the distal region of the conecting cilium. The reduced labeling density on the proximal surface of the connecting cilium plasma membrane was evident even in comparison with the adjacent, more heavily labeled inner segment plasma membrane (Figs. 5 and 6). It is interesting to note that on the fifth postnatal day, unlabeled inner segments were still seen adjacent to labeled photoreceptors (Fig. 5).

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[2] Figs. 2–8 are micrographs of developing rat retinas (3–21 postnatal days old) labeled by sequential immersion in biotinyl-antiopsin and AvF.
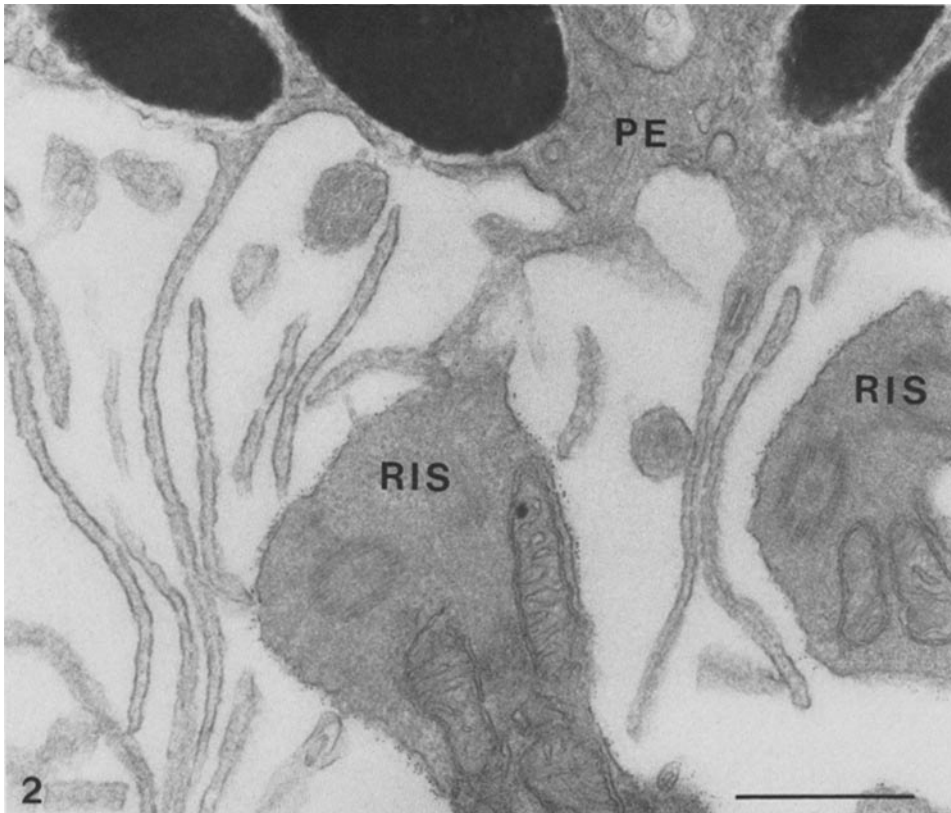
FIGURE 2 4-d-old retina. Although the retinal layer was separated from the pigment epithelium (PE), some PE cells remained attached to the rudimentary photoreceptor cells, which at this stage consist only of incompletely developed rod inner segments (RIS). Antiopsin binds only to the RIS plasma membrane. The long PE cell processes are unlabeled. The presence of a few attached PE cells closely associated with the immature photoreceptors did not affect the accessibility of the antibody and AvF to the RIS plasma membrane which is heavily labeled. Bar, 0.5 µm. × 42,600.
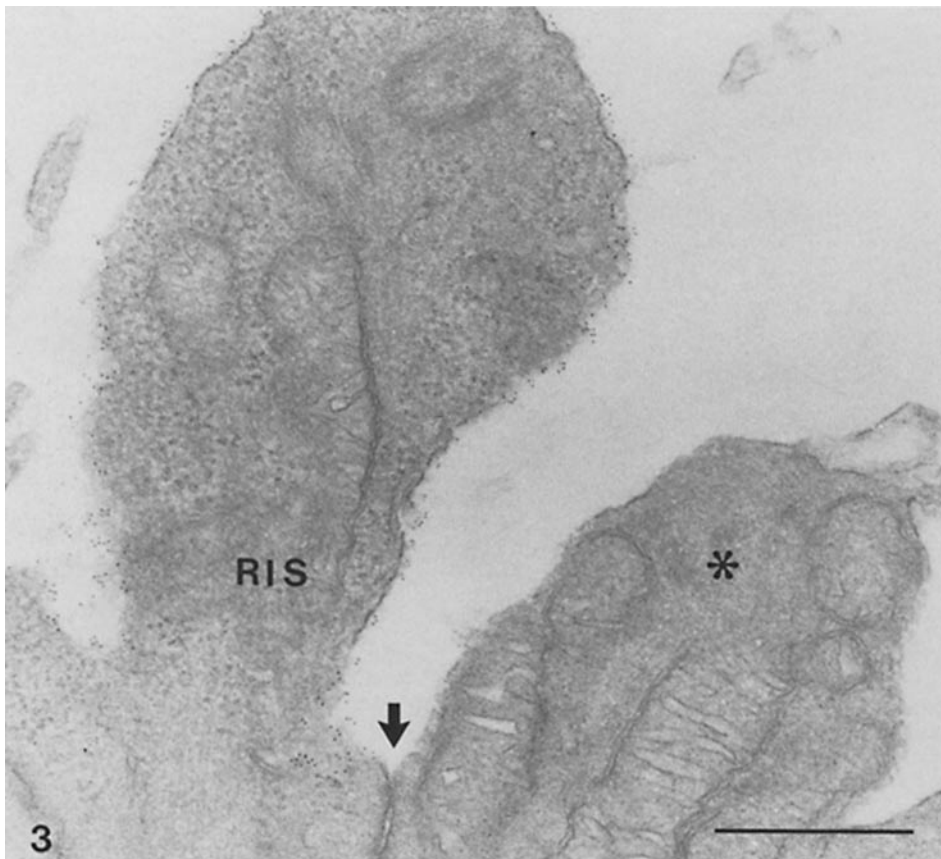

FIGURE 3 3-d-old retina. Photoreceptor cells vary in their degree of differentiation. While one rod cell contains opsin in its inner segment (RIS) plasma membrane, an adjacent cell's plasma membrane (*) is not labeled. Labeling of RIS is abruptly terminated at the outer limiting membrane (arrow) formed by a tight junction. Bar, 0.5 µm. × 49,300.

FIGURE 4 3-d-old retina. Budding of a connecting cilium does not coincide with the onset of opsin insertion into the inner segment plasma membrane. An elongated cilium (C) and rod inner segment (IS) plasma membrane are not labeled whereas the RIS plasma membranes of adjacent cells are heavily labeled (*). Bar 0.5 μm. × 60,700.
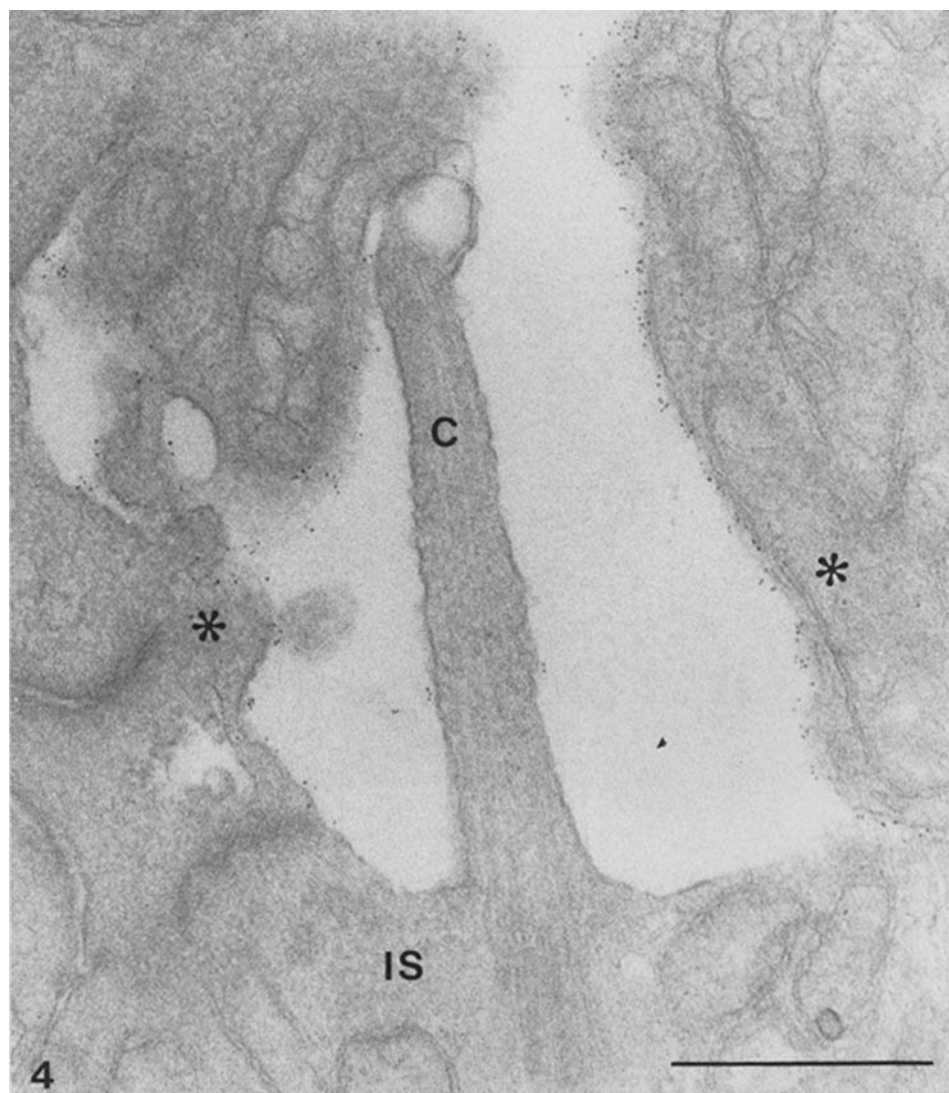
The region of low antiopsin labeling along the proximal connecting cilium had a relatively abrupt border. No characteristic morphological feature was apparent in the areas of lower labeling density, however. We occasionally observed the presence of longitudinally aligned membranes in the interior of the connecting cilium. These may represent axially aligned new disks forming beyond the plane of section (Fig. 7).

Upon maturation the antiopsin labeling of rat rod inner segment plasma membranes was drastically reduced. In a 21-d-old rat the inner segment plasma membrane was almost completely devoid of bound antiopsin. There was also a substantial reduction in the connecting cilium labeling especially its proximal portions (Fig. 8). Absence of antiopsin labeling of rod inner segment and proximal connecting cilium plasma membrane labeling was also noted in a human retina. Anti-bovine opsin cross-reacted with human opsin and intensely labeled the ROS plasma membrane (Fig. 9). The connecting cilium and inner segment, however, were free of bound antiopsin. Control reactions of retinas incubated with nonimmune serum were unlabeled. Comparison with another strain of albino Sprague-Dawley rats revealed no differences from the results with normal pigmented RCS p$^+$ rdy$^+$ congenic rats. All illustrations are from the RCS p$^+$ rdy$^+$ rats.

DISCUSSION

Shortly after birth the rat retina rapidly matures. Maturation is centrifugal since the central retina matures earlier than the lateral retina. The number of rat retinal rod cells rapidly increases during the first postnatal days. Initial budding of outer segments is seen by the fifth postnatal day (8). We detected opsin on the inner segment plasma membrane of the majority of photoreceptors at early developmental stages by the third day, prior to the formation of outer segments. Spectroscopic analysis of detergent extracts of immature mouse and rat retinas detected rhodopsin 7–10 d after birth at the time coinciding with significant major ROS development (2, 4, 5, 7). Immunocytochemistry was highly sensitive and clearly demonstrated opsin at even earlier stages of development. Indeed, labeling of a single photoreceptor's inner segment plasma membrane was observed in a 17-h postnatal rat. Numerous unlabeled cells with unlabeled connecting cilia were observed in these neonatal rats. Since labeled and unlabeled cells were observed adjacent to each other, photoreceptors are locally heterogeneous and not synchronized in their onset of opsin synthesis or its insertion into the plasma membrane. The growth of a cilium by itself does not signal the onset of distribution of opsin onto the ciliary plasma
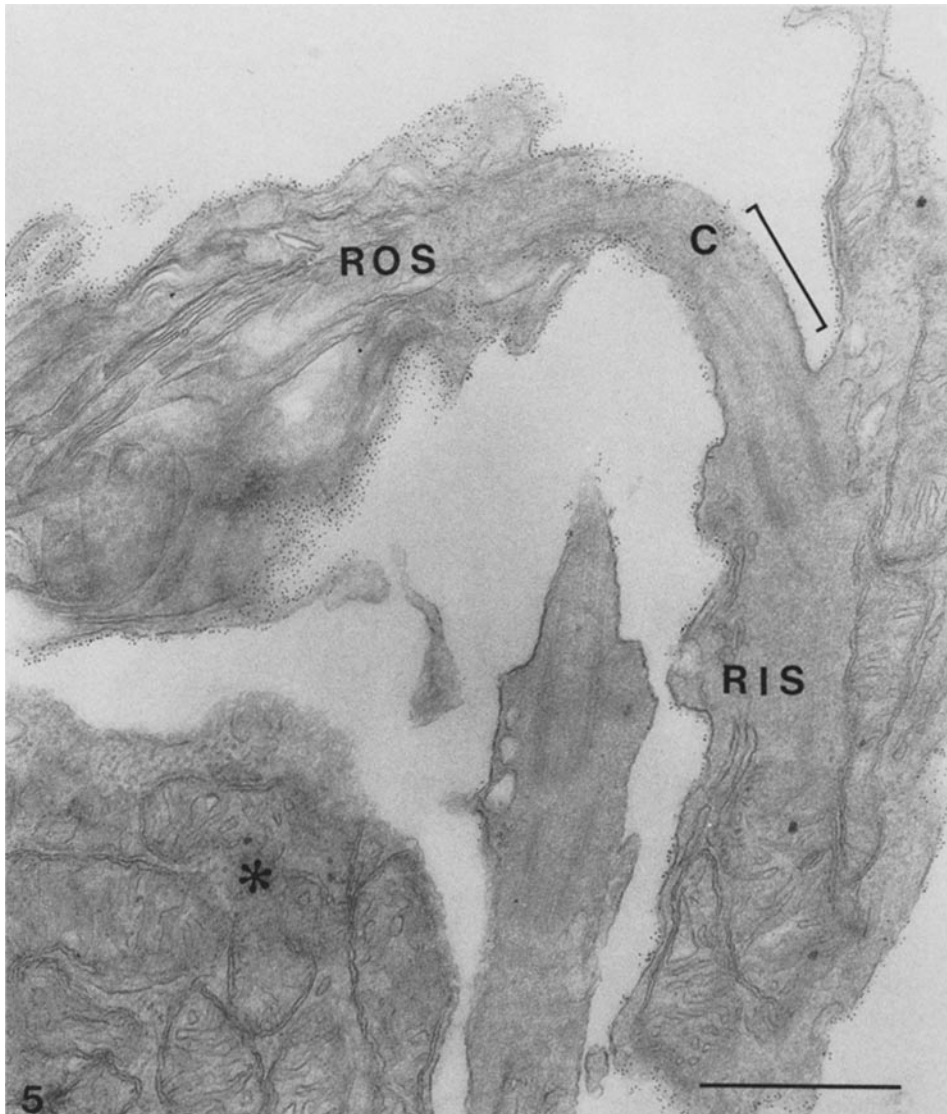
FIGURE 5   5-d-old retina. As rod outer segment (ROS) morphogenesis begins, disks are axially aligned. ROS, distal connecting cilium (C), and RIS plasma membranes are densely labeled. The proximal portion of the cilium appears to be less densely labeled (bracket). At this developmental stage, an adjacent inner segment (*) is still unlabeled. Bar, 0.5 μm. × 46,700.

membrane since some cells with fairly elongated cilia were unlabeled if their inner segments were unlabeled. Labeling of cilia was always seen in cells in which budding of ROS at the distal end of the cilium had begun by the fifth day. Transitions between these stages may exist but were not observed.

Absence of antiopsin labeling on the surface of connecting cilium at early developmental stages points to a biosynthetic sequence in which the ciliary membrane is first formed prior to the onset of opsin transfer to the developing ROS. This observation resembles the events associated with ciliary membrane renewal in *Tetrahymena* in which new immature ciliary membrane is relatively undifferentiated and contains bulk lipid bilayer components with few large assemblies of intramembranous particles (23).

Once opsin becomes a component of the connecting cilium plasma membrane, a typical differential pattern of labeling was noted: the proximal portion of the ciliary plasma membrane was labeled at low density whereas the labeling density of more distal cilium approached that of the ROS. This pattern resembled the labeling of rod cilia on thin sections of rat retinas (17, 24). Thus both immersion and thin section techniques yielded comparable results which supports the interpretation that the lower labeling density of the basal cilium was not caused by limitation of diffusion of the reagents onto the cilium surface.

Freeze fracture studies of the connecting cilium in rat retina revealed the presence of intramembranous particles (IMPs) that were arranged in numerous circumferential rows (13, 21). This particular arrangement of IMPs is seen at the base

Figure 7   8-d-old retina. In some connecting cilia (C) which were observed at early stages of ROS morphogenesis, the region of high labeling density on the plasma membrane of the distal connecting cilium overlays a region that contains axially oriented membranes. These membranes probably are new disks (arrow). Bar, 0.2 μm. × 60,700.
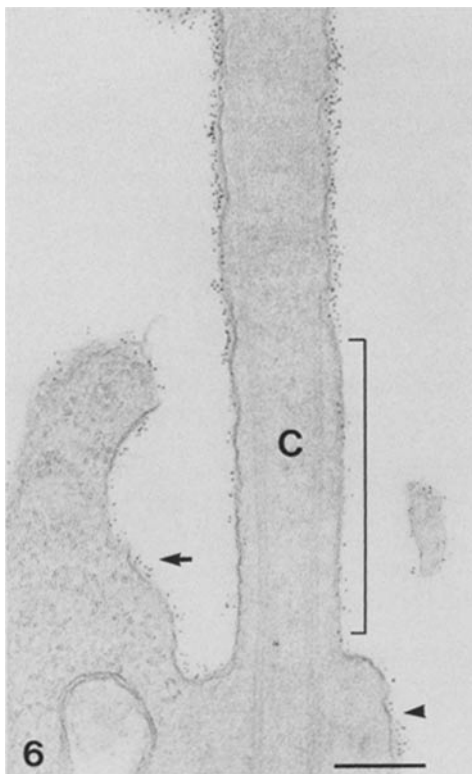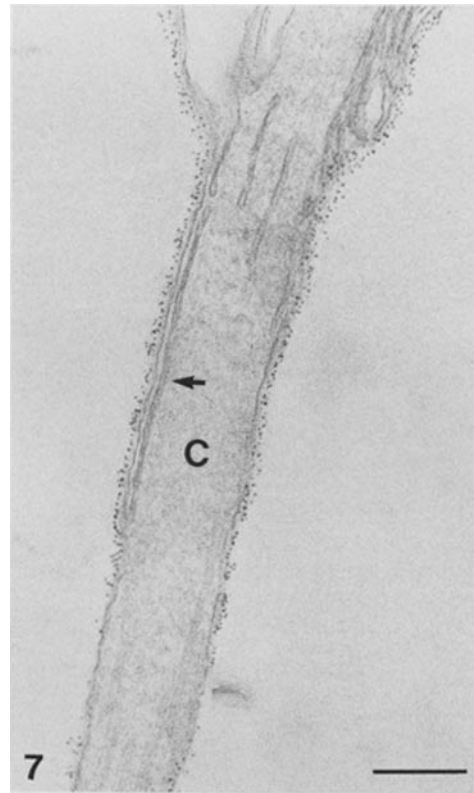

Figure 6   5-d-old retina. The differential labeling density along the connecting cilium is clearly demonstrated. The proximal connecting cilium (C) is relatively unlabeled (bracket) yet the inner segment plasma membrane is heavily labeled by the antiopsin along the wall of the invagination from which the cilium projects (arrow) and along the lateral plasma membrane (arrowhead). Bar, 0.2 μm. × 57,300.

of most cilia and is referred to as the ciliary necklace (9). It was previously proposed by Satir, Sale, and Satir (23) "that a selective barrier to membrane flow in the plane of the lipid bilayer such as the ciliary necklace would be relatively common feature of membrane design, since otherwise one might anticipate random mixing of integral components due to membrane fluidity." The molecular nature of the ciliary necklace IMPs and their role in restriction of opsin backflow in rods is not yet known. However, the rings of IMPs extend above the base of the connecting cilium in rats and often are observed in the distal portion, a region heavily labeled by antiopsin. The broad extent of these rings led Rohlich (21) to propose that the rat rod connecting cilium resembled an extended "transitional zone" over a greater portion of the cilium than the usual distribution of transitional zones at the base of motile cilia. Since the distal cilium is also labeled by antiopsin, the rings of IMPs in the rat connecting cilium may not exclusively reflect the components of the ciliary necklace. In *Xenopus laevis* retinas, the size of the ciliary IMPs (10 nm) resembled those of the rod outer segment more than the smaller (8 nm) particles of the inner segment plasma membrane, a result that led Besharse and Pfenninger (3) to conclude that some of these particles also were opsin in transit in this species. Correlation of these observations by surface immunocytochemistry and diverse ultrastructural techniques may resolve this question.

Redistribution of opsin from the inner segment of the immature rod to the outer segment of the mature rod raises the question of the mechanism for generating the polarized
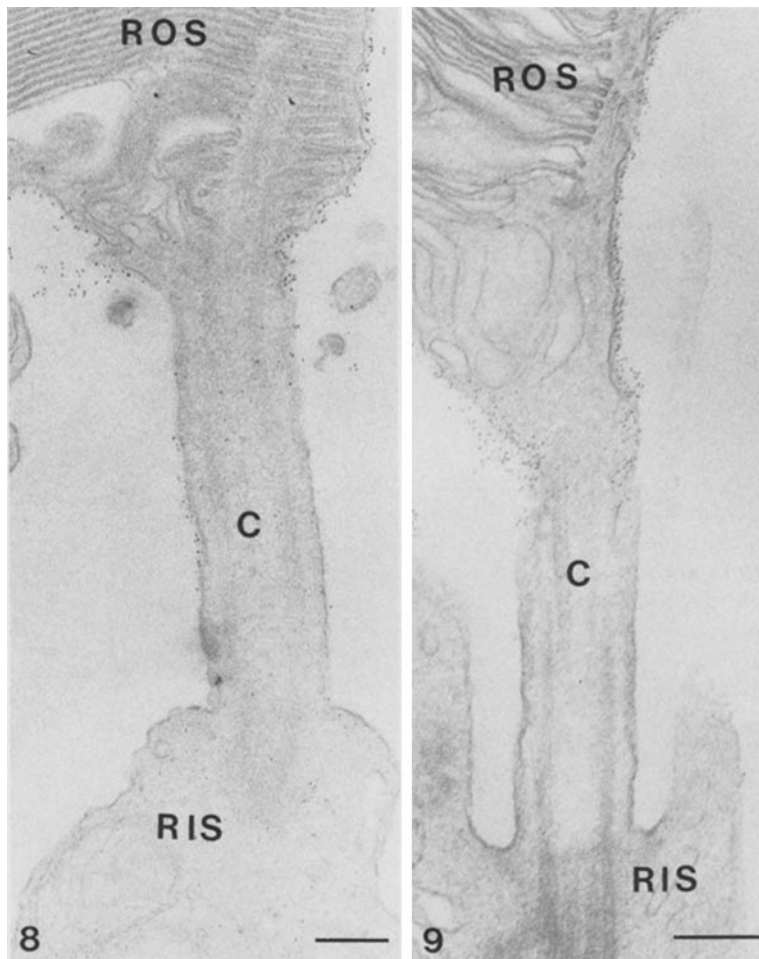
FIGURES 8 and 9   Fig. 8: 21-d-old retina. Opsin is localized on the plasma membranes of the rod outer segment (*ROS*) and distal connecting cilium (*C*). Proximal cilium and rod inner segment (*RIS*) plasma membranes are nearly unlabeled. Bar, 0.2 μm. × 46,000. Fig. 9: Human adult retina labeled with biotinyl-anti-bovine opsin and AvF. The labeling pattern of the human rod photoreceptor is comparable to that observed in the mature rat retina (cf. Fig. 8). Labeling is limited to the rod outer segment (*ROS*) and the distal connecting cilium (*C*) only. The proximal connecting cilium and the rod inner segment (*RIS*) plasma membrane are virtually unlabeled. Bar, 0.2 μm. × 60,700.

distribution of opsin. The site of opsin insertion in the inner segment of the photoreceptor may change during maturation. In the neonatal rat, opsin is detected on the entire surface of the inner segment of most photoreceptors down to the level of the outer limiting membrane—a band of *maculae adherentes* at the middle of the inner segment. Either opsin is inserted into the entire surface of the apical plasma membrane of the inner segment or it is locally inserted near the future site of the connecting cilium or some other plasma membrane site and redistributes over the inner segment surface. In the mature retina, opsin is apparently inserted into the apical plasma membrane near the base of the connecting cilium since it is not found on the lateral plasma membrane of the inner segment. The absence of opsin on the lateral plasma membrane of the mature photoreceptor may result from the rapid clearance of opsin into the outer segment which acts as a sink for opsin. Alternatively, a barrier to back diffusion of opsin may exist in the apical plasma membrane which restricts opsin redistribution once it is inserted near the connecting cilium. Finally, the low level of opsin in the inner segment of the adult rod may simply result from relatively slow rates of redistribution compared with rates of synthesis and insertion

and removal into the outer segment. If the rate of redistribution to the outer segment is faster than the rate of insertion, the steady-state density of opsin in the lateral plasma membrane of the adult rod may be held at a low level.

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