

Rod Photoreceptor Neurite Sprouting in Retinitis Pigmentosa

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In animal models for retinitis pigmentosa (RP), rod photoreceptors show abnormal distribution of rhodopsin prior to undergoing cell death. To elucidate the steps in degeneration of human photoreceptors, immunocytochemistry was performed on donor retinas from 15 RP patients and five normal subjects. Rhodopsin immunolabeling in the normal retinas was restricted to the rod outer segments. In the RP retinas, rhodopsin was present in shortened rod outer segments and in the surface membranes of the rod inner segments and somata. In regions of photoreceptor death, the surviving rods had sprouted rhodopsin-positive neurites that were closely associated with gliotic Müller cell processes and extended to the inner limiting membrane. Rods and cones in the RP maculas did not form neurites, but the axons of peripheral cones were abnormally elongated and branched. Double immunofluorescence labeling showed that the rod neurites bypassed the horizontal and rod bipolar cells that are normally postsynaptic to rod axons. To our knowledge, this is the first report of rod neurite sprouting *in vivo*. We were unable to find neurites on degenerate rods in old *rds* mice, an animal model for RP. The rod neurites in the human RP retinas resemble the long, branched processes formed by rods cultured on Müller cells or purified N-CAM. Neurite growth by surviving rods in the RP retinas may be a response to neurotrophic factor upregulation, loss of inhibitory factors, or changes in molecules associated with reactive Müller cells. Such changes in the retinal microenvironment may impede functional integration of transplanted photoreceptors. The contributions of the rhodopsin-positive rod neurites and abnormal cone axons to the functional abnormalities observed in RP are unknown.

[Key words: retina, rod, cone, neurites, Müller glia, retinitis pigmentosa]

Retinitis pigmentosa (RP) is a group of inherited diseases that cause degeneration of rod and cone photoreceptors, reactive changes in the retinal pigment epithelium and Müller glia, and atrophy of blood vessels and neurons in the inner retina (Stone et al., 1992; Li et al., 1995). RP is associated with mutations in several photoreceptor-specific genes, including rhodopsin (Dryja, 1992; Humphries et al., 1993), but many gaps remain in our understanding of the mechanisms of photoreceptor dysfunction and death. Recent studies of mice carrying mutant rhodopsin transgenes demonstrated that their rods show abnormal localization of rhodopsin, transducin, and phosphodiesterase prior to undergoing cell death (Roof et al., 1994; Sung et al., 1994). To elucidate the process of photoreceptor cell death in humans, we have screened postmortem RP retinas by immunocytochemistry, using antibody markers that are specific for rods and cones, inner retinal neurons, and Müller cells. We present evidence that rod photoreceptors in these retinas form long, rhodopsin-positive neurites that extend for considerable distances into the inner retina. Although rods are known to form long neurites in culture (Araki et al., 1987; Kljavin and Reh, 1991; Gaur et al., 1992; Mandell et al., 1993; Hicks et al., 1994; Kljavin et al., 1994), to our knowledge this is the first report of rod neurite sprouting *in vivo*. While rod neurite sprouting is common in the human RP retinas, this phenomenon is not seen in several animal models of RP, including older *rds* mice examined in the present study. We demonstrate that the rod neurites extend past the neurons that normally receive rod synaptic input and are closely associated with gliotic Müller cells in the RP retinas. Finally, we discuss the possibilities that the rod neurites are formed in response to neurotrophic factor upregulation, lack of inhibitory factors in the diseased retinas, or alterations in molecules associated with the reactive Müller glia.

Materials and Methods

Tissue preparation. Postmortem eyes were obtained through the donor program of the Foundation Fighting Blindness, Baltimore, MD, from 15 RP patients who ranged in age from 24 to 89 years (Table 1). Eyes from five normal subjects, aged 51–91 years, were obtained through the Foundation and the University of Washington Lions' Eye Bank. The eyes had been fixed for 4 weeks to 4 years in 0.13 M phosphate-buffered 4% paraformaldehyde and 0.5% glutaraldehyde, or in phosphate-buffered 4% paraformaldehyde alone.

Eyes from 10- and 16-month-old retinal degeneration slow (*rds*) mice were obtained from Dr. James McGinnis, University of California Los Angeles. The eyes had been fixed in Perfix (Fisher, Santa Clara, CA) for 4 hr at 4°C and stored in 70% ethanol. The retinas were processed through a descending ethanol series into phosphate buffer and treated as below for indirect immunofluorescence.

Electron microscopy. Tissues were postfixed in 1% phosphate-buffered osmium tetroxide and embedded in Medcast (Ted Pella, Inc., Redding, CA). Ultrathin sections were stained with uranyl acetate and lead citrate.

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Table 1. Characteristics of donor retinas used in study

Reference no.	Age/gender	PM time (hr)	Diagnosis
FFB-310	24M	11.5	Usher syndrome
FFB-342	29M	16.0	Simplex RP
FFB-114	39M	5.5	XL RP
FFB-311	44M	12.0	Simplex RP
FFB-215	46M	1.0	XL RP
FFB-424	50F	6.5	AD RP ^a
FFB-231	56M	3.3	Simplex RP
FFB-335	58F	5.5	AD RP
FFB-303	68M	2.2	AD RP
FFB-316	68M	8.5	AD RP ^b
FFB-271	72F	4.0	XL RP carrier
FFB-340	73F	9.0	Simplex RP
FFB-184	76M	3.1	Multiplex RP
FFB-356	80M	5.0	AD RP
FFB-371	87M	5.0	Simplex RP
UW-403-93	51F	3.0	Normal
UW-805-92	53F	3.0	Normal
FFB-343	66M	2.5	Normal
UW-780-91	85F	3.0	Normal
FFB-363	91F	3.3	Normal

FFB, Foundation Fighting Blindness; PM, postmortem; XL, X linked; AD, autosomal dominant; UW, University of Washington.

^a Rhodopsin glutamine-64-ter mutation.

^b Rhodopsin threonine-17-methionine mutation (Li et al., 1994).

Immunocytochemistry. Tissue samples were held in 30% phosphate-buffered sucrose overnight, cryosectioned at 12 μ m, and processed for indirect immunofluorescence according to published methods (Milam and Jacobson, 1990). Some aldehyde-fixed tissues were treated with sodium borohydride, embedded in LR-White resin (Ted Pella, Inc.), and processed by the immunogold technique with silver enhancement for light microscopy (1 μ m sections) and electron microscopy (90 nm sections) (Milam and Jacobson, 1990).

Antibodies. The following antibodies against rhodopsin were used: monoclonal antibodies 1D4 (against the C-terminus, 1:20) and 4D2 (against the N-terminus, 1:20) from Dr. R. Molday, University of British Columbia, Vancouver, Canada; polyclonal anti-rhodopsin (1:200) from Dr. T. Shuster, California State University at Long Beach, CA; polyclonal anti-rhodopsin (1:5000) from Dr. E. Kean, Case Western Reserve University, Cleveland, OH; and monoclonal antibody P-Rho (1:5) against phosphorylated rhodopsin from Dr. P. Hargrave, University of Florida, Gainesville, FL. Antibodies against other rod proteins were polyclonal anti-arrestin (1:50) from Dr. H. Shichi, Wayne State University, Detroit, MI; monoclonal anti-cGMP channel protein (PMc 1D1, undiluted) and monoclonal anti-*rd*s/peripherin (3B6 and 5H2, undiluted) from Dr. R. S. Molday; polyclonal anti-recoverin (1:100) from Dr. A. Dizhoor, University of Washington, Seattle, WA; monoclonal anti-ROM-1 (1:1000) from

Dr. R. McInnes, Hospital for Sick Children, Toronto, Canada; polyclonal anti-rhodopsin kinase (1:100) from Dr. K. Palczewski, University of Washington; monoclonal anti-transducin α -rod (undiluted) from Dr. J. Saari, University of Washington; and polyclonal anti-transducin α -cone (1:10), polyclonal anti-red/green cone opsin (1:100), and polyclonal anti-blue cone opsin (1:10) from Drs. C. and K. Lerea, New York Medical College, Valhalla, NY. Antibodies against synaptic vesicle proteins were monoclonal anti-synaptophysin (1:200; Sigma, St. Louis, MO) and monoclonal anti-SV2 protein (1:400) from Drs. K. Buckley and R. B. Kelly, University of California, San Francisco, CA. Antibody markers for inner retinal neurons were polyclonal anti-L7 protein (1:200) from Dr. J. Morgan, Hoffmann LaRoche, Inc., Nutley, NJ; and monoclonal anti-calbindin (1:200; Sigma). The marker for reactive Müller cells was polyclonal anti-glial fibrillary acidic protein (GFAP; 1:200; Dako Corporation, Carpinteria, CA).

Results

Light microscopic immunocytochemistry of human retinas

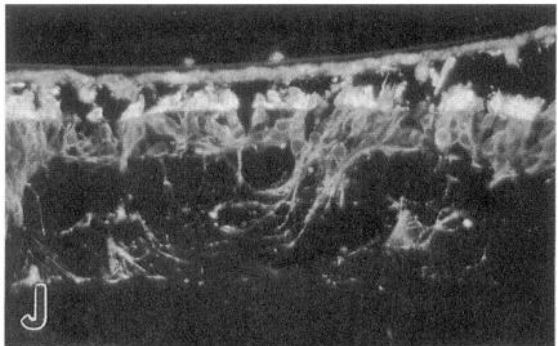
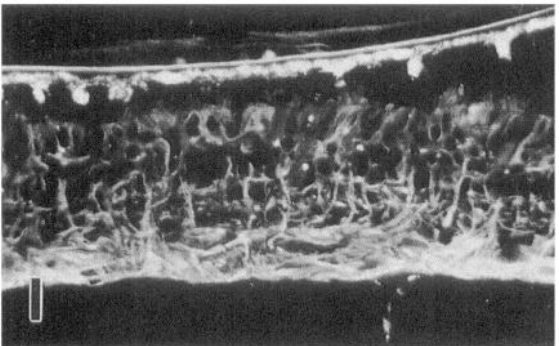
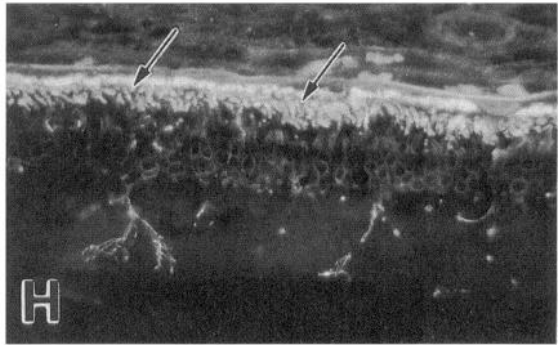
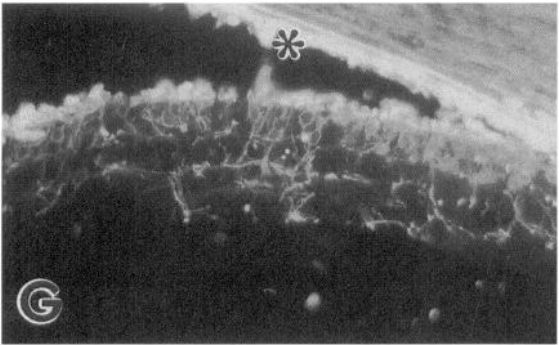
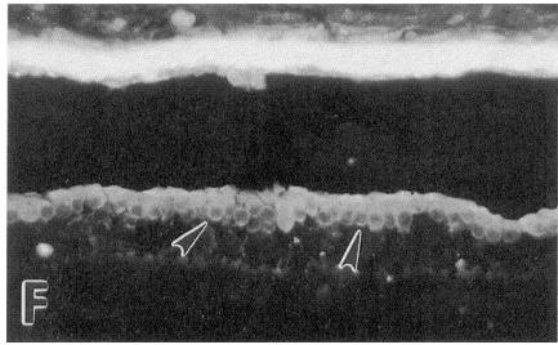
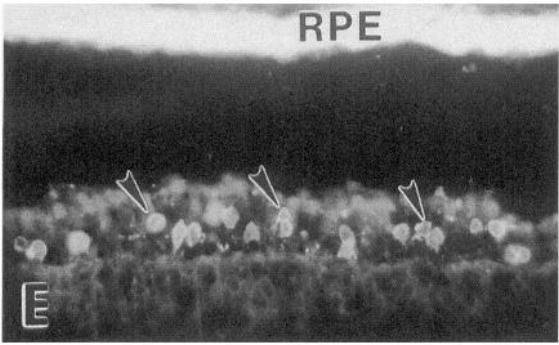
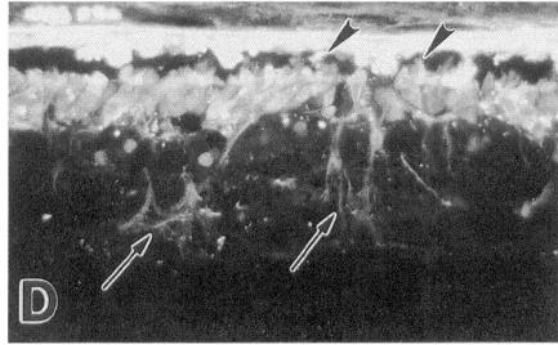
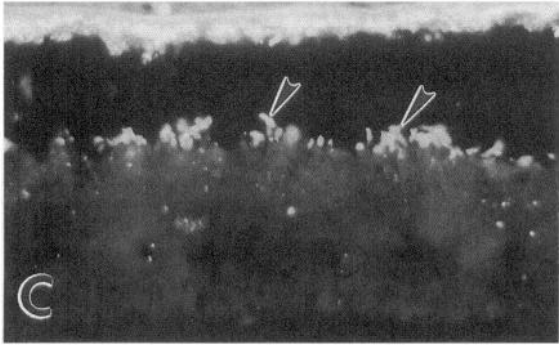
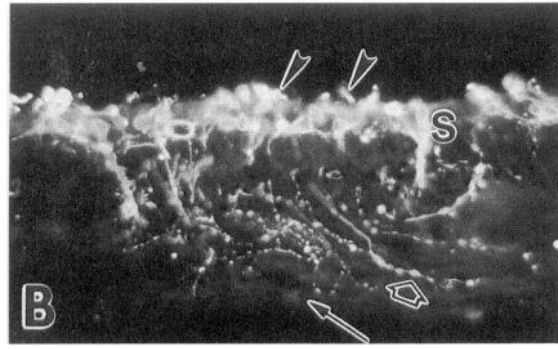
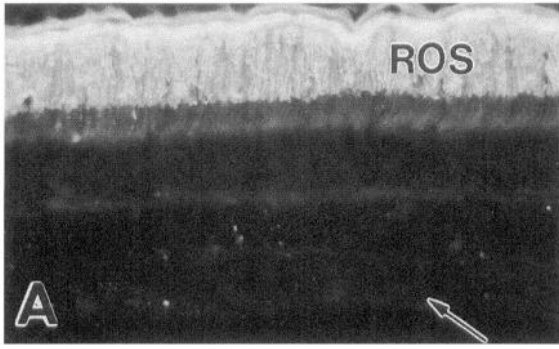
Normal retinas processed for immunofluorescence showed heavy labeling of the rod outer segments with each of the antibodies against rhodopsin (Fig. 1A). Rods in the normal retinas also showed outer segment labeling with antibodies against the cGMP channel, *rd*s/peripherin, rhodopsin kinase, ROM-1, and transducin α -rod. The rod outer segments, inner segments, and somata were reactive with anti-arrestin and -recoverin. Cone outer segments were labeled with the antibody against *rd*s/peripherin and the red/green and blue cone opsins; cone outer segments, inner segments, and cell bodies were reactive with anti-recoverin and -transducin α -cone. The outer and inner plexiform layers were labeled with anti-SV-2 (Fig. 2A), and anti-synaptophysin labeled the cone inner segments and somata, in addition to the two plexiform layers. Anti-calbindin labeled cones, horizontal cells, and some neurons in the inner nuclear layer, and anti-L7 specifically labeled the rod bipolar cells.

In all RP retinas, the rod outer segments were markedly shortened and were reactive with the antibodies against rhodopsin (Fig. 1B), phosphorylated rhodopsin, the cGMP channel, *rd*s/peripherin (Fig. 1C), and ROM-1. Rod inner segments and somata were reduced in number and were labeled with the antibodies against rhodopsin, phosphorylated rhodopsin, arrestin, recoverin (Fig. 1D), rhodopsin kinase, and transducin α -rod. Cone outer segments were also shortened and were labeled with the antibodies against the red/green and blue cone opsins, *rd*s/peripherin, and transducin α -cone (Fig. 2C). The cone inner segments and cell bodies were reduced in number and were labeled with the antibodies against calbindin, recoverin, transducin α -cone (Fig. 2C), and synaptophysin (Fig. 2D).

A striking abnormality was evident in all of the RP retinas. In the peripheral regions where the photoreceptors were reduced to one to three rows of cells, the rods gave rise to long neurites that

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Figure 1. Normal and RP human retinas labeled by immunofluorescence. *A*, In the normal retina, anti-rhodopsin labels the rod outer segments (ROS). *Arrow*, inner limiting membrane. *B*, In an RP retina, rhodopsin is localized to the short rod outer segments (*arrowheads*), inner segments, and somata (*S*). Rhodopsin is also localized in the beaded rod neurites (*open arrow*) that extend from the bases of the rods to the inner limiting membrane (*closed arrow*). *C*, In an area of an RP retina containing rod neurites, anti-*rd*s/peripherin labels the short photoreceptor outer segments (*arrowheads*) but not the rod neurites. *D*, In an RP retina, anti-recoverin labels the photoreceptor outer (*arrowheads*) and inner segments and somata, as well as the rod neurites (*arrows*). *E*, The macula of an RP retina immunolabeled for the demonstration of rhodopsin. The remaining rods show surface membrane labeling of their somata (*arrowheads*) but no rod outer segments or neurites are present. *F*, The macula of an RP retina immunolabeled for the demonstration of transducin α -cone. The cone somata (*arrowheads*) are labeled but there is no evidence of cone neurite sprouting. *G*, The midperipheral retina from a man with X-linked RP (FFB-215) immunolabeled with anti-rhodopsin. Considerable loss of rods has occurred and the remaining rods have sprouted neurites. The retina is partially detached from the retinal pigment epithelium (*). *H*, The far peripheral region of the retina shown in *G*, also immunolabeled with anti-rhodopsin. More rods are retained in this part of the retina and rod neurite sprouting is less extensive than in *G*. The rods have short outer segments (*arrows*). *I* and *J*, Double immunolabeling to demonstrate GFAP (*I*, FITC labeling) and rhodopsin (*J*, rhodamine labeling) in the same micrographic field. *I*, The Müller cells are hypertrophic and their processes are GFAP positive. *J*, The rhodopsin-positive rod neurites course along the Müller cell processes. Magnification, 230 \times .



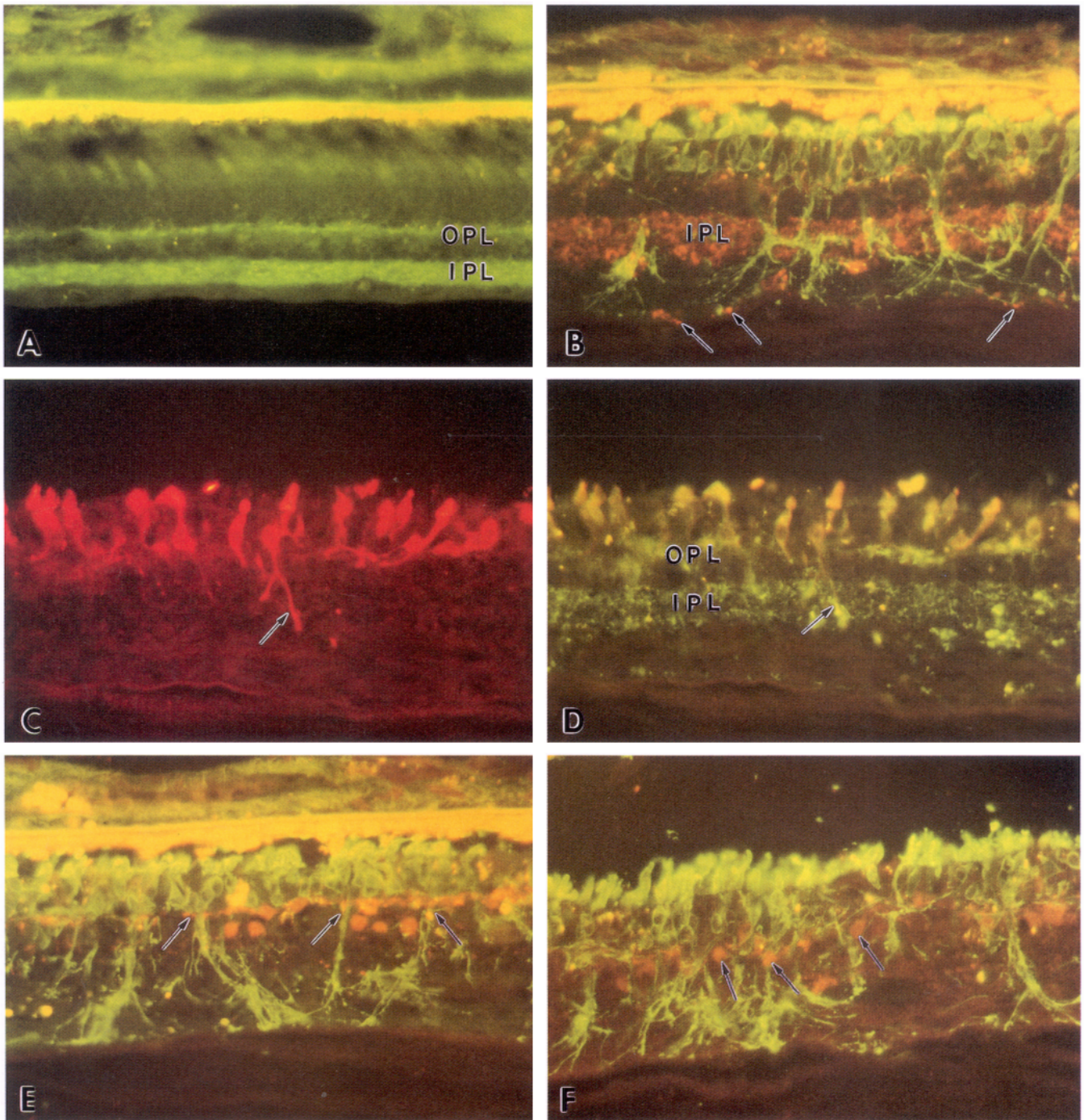


Figure 2. Normal and RP human retinas after immunofluorescence labeling. *A*, Distribution of SV2 synaptic vesicle protein in the inner (IPL) and outer (OPL) plexiform layers of a normal retina. The yellow band corresponds to autofluorescent lipofuscin in the retinal pigment epithelium. *B*, Double labeling for SV2 (rhodamine, red) and rhodopsin (FITC, green) in an RP retina. The anti-SV2 labels the outer and inner (IPL) plexiform layers, plus bulb-shaped varicosities (arrows) on the rhodopsin-positive rod neurites. *C*, Cones in the peripheral region of an RP retina labeled for the demonstration of transducin α -cone, which fills the cone cytoplasm. Some cone axons (arrow) are abnormally long and branched, extending into the inner plexiform layer (see *D*). *D*, Section in *C* double labeled for the demonstration of transducin α -cone (rhodamine) and synaptophysin (FITC) in the inner (IPL) and outer (OPL) plexiform layers. The cones are labeled with both antibodies, and the cone axon (arrow) extends into the inner plexiform layer. *E*, Double labeling for calbindin (rhodamine) and rhodopsin (FITC) in an RP retina. The rhodopsin-positive rod neurites pass through the band of calbindin-positive horizontal cell processes (arrows). Five amacrine cells are also labeled with anti-calbindin. *F*, Double labeling of L-7 (rhodamine) and rhodopsin (FITC) in the peripheral region of an RP retina. The rhodopsin-positive rod neurites course between and past the labeled rod bipolar cells (arrows) and reach the inner limiting membrane. Magnification, 280 \times .

were labeled with each of the antibodies against rhodopsin and phosphorylated rhodopsin (Fig. 1B). The neurites often had beaded varicosities and clusters of bulb-shaped terminals that were labeled with antibodies against synaptophysin and SV-2 (Fig. 2B). Some of the labeled terminals were found in the innermost part of the retina, between the nerve fiber layer and the inner limiting membrane (Fig. 2B). The rod neurites were also labeled with antibodies against the rod cytoplasmic proteins arrestin, recoverin (Fig. 1D), rhodopsin kinase, and transducin α -rod. The short rod outer segments were well labeled with antibodies against *rds*/peripherin, ROM-1, and the cGMP channel, but the rod neurites were not labeled with these antibodies (Fig. 1C).

The maculas of the RP retinas contained scattered rod somata that lacked outer segments but were labeled with antibodies against rhodopsin (Fig. 1E) and the rod cytoplasmic proteins. These rods did not form prominent neurites as found in the peripheral regions of the same retinas. Cones in each RP macula were reduced to a monolayer of somata with absent or very short outer segments that were reactive for the cone opsins, recoverin, and transducin- α cone. The cone somata and axons were well labeled with the antibodies against recoverin and transducin α -cone (Fig. 1F), but there was no indication of cone neurite formation in the RP maculas.

Cones in the peripheral parts of the RP retinas showed the same patterns of immunolabeling as found in the maculas. Labeling with the antibody against transducin- α cone revealed that many of the cone axons were abnormally branched and elongated (Fig. 2C). Double labeling with the antibodies against transducin- α cone and synaptophysin revealed that some cone axons passed through the inner nuclear layer and terminated in the inner plexiform layer (Fig. 2D).

Rod neurite sprouting was present in the retinas of all of the RP patients, including those with known rhodopsin mutations (Table 1) and those with X-linked RP, who presumably had normal rhodopsin because the gene for this protein is located on chromosome 3 (Dryja, 1992). Within a given retina, rod neurite sprouting was most pronounced in regions that had undergone significant photoreceptor death. For example, within the retina of the 46-year-old man with X-linked RP (FFB-215, Table 1), neurite sprouting was extensive in the midperipheral retina (Fig. 1G), where significant rod cell death had already occurred, while in the far peripheral retina (Fig. 1H) most rods were still viable and only a few had formed neurites.

Because ganglion cells, the third-order retinal neurons, can be lost in RP (Stone et al., 1992), it seemed possible that the prominent rod neurites had formed in response to transneuronal degeneration of second-order postsynaptic neurons, for example, the horizontal and rod bipolar cells. To test this, double labeling was performed with anti-rhodopsin and specific markers for the horizontal cells (anti-calbindin) and the rod bipolar cells (anti-L7 protein). Anti-calbindin labeled a continuous band of horizontal cell processes in the outer plexiform layer in the normal retinas. In the RP retinas, a similar calbindin-labeled band was present and a few of the labeled horizontal cell processes gave rise to abnormal apical sprouts. The rhodopsin-positive rod neurites did not terminate on the horizontal cell processes but passed directly through the labeled band into the inner retina (Fig. 2E). A single row of L7-labeled rod bipolar cells was present in the peripheral regions of both the normal and RP retinas (Fig. 2F), indicating that this cell type had not undergone significant transneuronal degeneration in response to death of rods. The rhodopsin-positive neurites coursed between and past the labeled rod

bipolar somata and terminated against the inner limiting membrane (Fig. 2F).

GFAP immunolabeling in normal retinas is limited to the astrocytes near the inner limiting membrane (Eisenfeld et al., 1984). As shown previously in other RP retinas (Milam and Jacobson, 1990; Li et al., 1994, 1995), Müller cells undergo reactive gliosis following death of photoreceptors. The Müller processes in the maculas and peripheral regions of the RP retinas were hypertrophic and reactive throughout with anti-GFAP (Fig. 1I), and some Müller nuclei were enlarged and had migrated from the inner nuclear layer into the photoreceptor layer (not shown). Double labeling of the same photographic field revealed that the rhodopsin-positive neurites coursed along the GFAP-positive radial Müller processes (Fig. 1J), but there was no indication by light or electron microscopy of Müller cell labeling with anti-rhodopsin.

Electron microscopy of human retinas

The fine structure of the rods and cones in the normal retinas was as described previously for human photoreceptors (Hogan et al., 1971). The rod and cone axons terminated in the outer plexiform layer as ribbon synapses with numerous small, clear synaptic vesicles ~ 50 nm in diameter. Immunogold labeling of the normal rods with anti-rhodopsin was limited to their outer segments (Fig. 3A). Rods in the peripheral regions of the RP retinas had very short outer segments that were well labeled with anti-rhodopsin (Fig. 3B). In addition, rhodopsin labeling was prominent on the surface membranes of the rod inner segments (Fig. 3B), somata, and synapses (Fig. 3C), and in the Golgi regions of the inner segments. No rhodopsin immunoreactivity was found in Müller glia or any other retinal cells. The rod neurites were recognizable in the inner retina by virtue of their surface labeling with anti-rhodopsin (Fig. 4A). By conventional EM, the rod neurites contained numerous longitudinally oriented microtubules, and expanded regions of the neurites corresponding to the beaded varicosities observed by light microscopy were filled with numerous small vesicles that were ~ 50 nm in diameter, as well as prominent, dense multivesicular bodies that measured up to ~ 400 nm in diameter (Fig. 4B). The regions of the neurites that contained the small vesicles were immunolabeled with antibodies against synaptophysin and SV-2 synaptic vesicle protein (not shown). No synaptic ribbons were identified in the rod neurites. The neurites were closely apposed to enlarged Müller cell processes filled with intermediate filaments (Fig. 4C) that were immunopositive for GFAP (not shown). The rod neurites terminated next to the inner limiting membrane basal lamina and did not extend past this layer into the vitreous humor (Fig. 4A,C).

*Light microscopic immunocytochemistry of *rds* mouse retinas*

There were no published reports of rod neurite sprouting in the retinas of the animal models for RP, although rhodopsin had been localized in these retinas by immunocytochemistry (Jansen et al., 1987; Nir et al., 1989; Cantera et al., 1990; Nir et al., 1990; Roof et al., 1994; Sung et al., 1994). It seemed that rod degeneration might occur too rapidly in most of the animal models for detection of rod neurites, so we examined the retinas of 10- and 16-month-old *rds* mice, which have a very slow course of rod degeneration. Immunolabeling with anti-rhodopsin (monoclonal antibodies 4D2 and 1D4) and -recoverin revealed that an incomplete monolayer of rod somata persisted at 10 months but that all rods had degenerated by 16 months. The

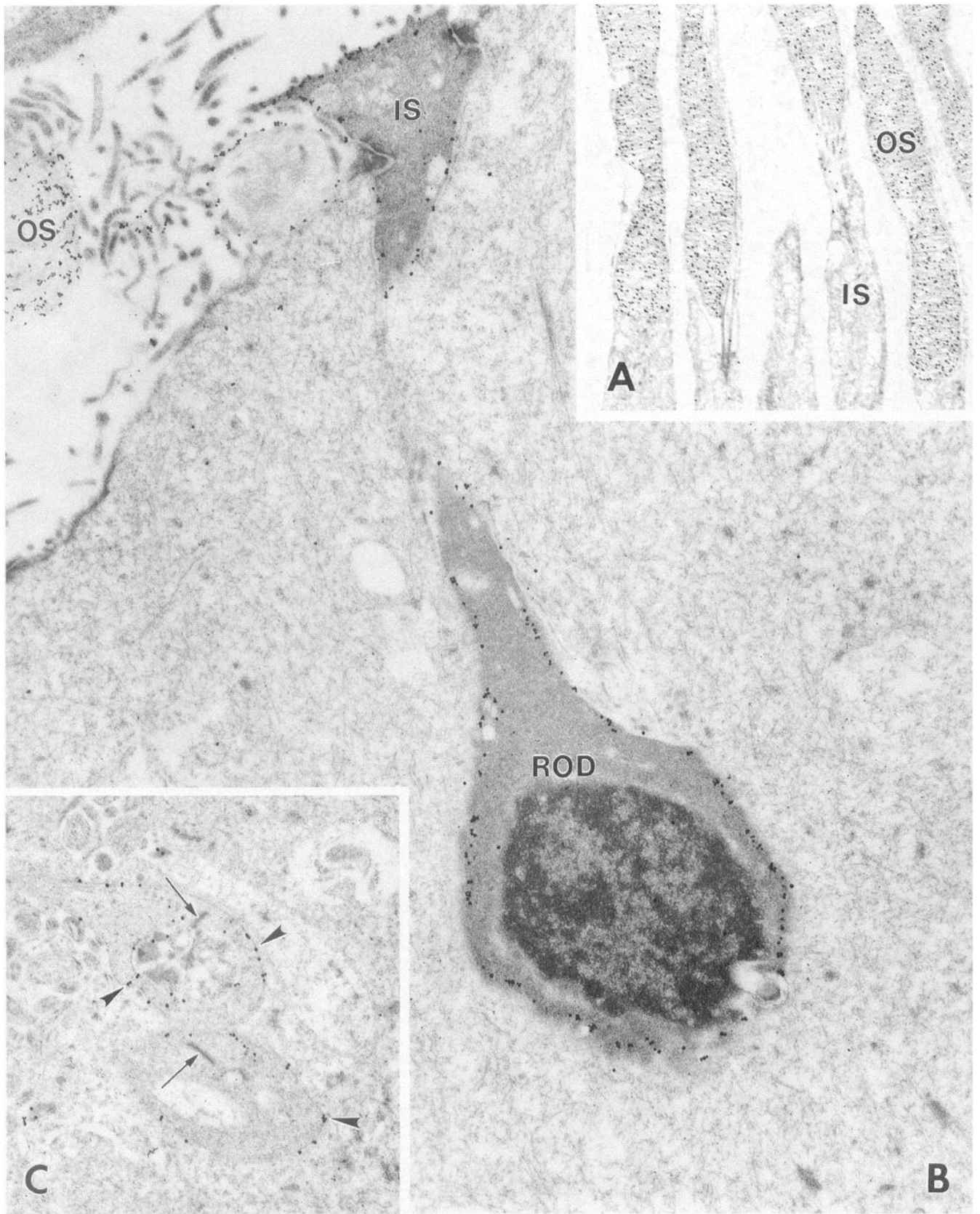


Figure 3. Electron micrographs of normal and RP retinas labeled with anti-rhodopsin. **A**, Normal retina shows labeling with anti-rhodopsin of the rod outer segments (*OS*). The inner segments (*IS*) are free of label. **B**, The peripheral region of an RP retina shows labeling with anti-rhodopsin of the short outer segment (*OS*) and the surface membrane of the inner segment (*IS*) and rod soma (*ROD*). **C**, The peripheral region of an RP retina shows labeling with anti-rhodopsin of the surface membranes of the rod synapses (*arrowheads*). *Arrows*, synaptic ribbons. Magnification: **A**, 7,380 \times ; **B**, 16,530 \times ; **C**, 13,350 \times .

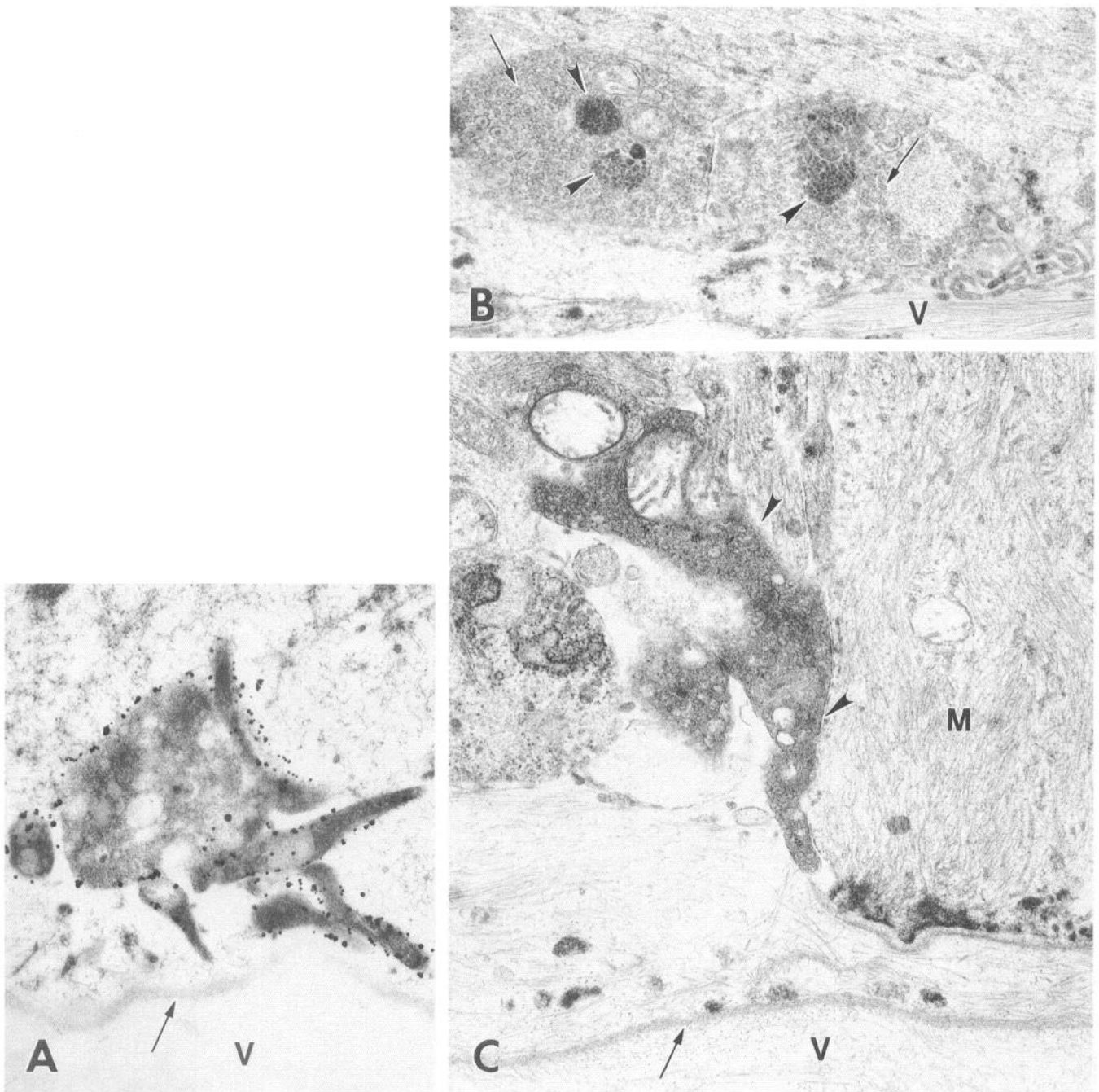


Figure 4. Electron micrographs of rod neurites in RP retinas. **A**, Immunogold labeling demonstrates rhodopsin in the surface membranes of rod neurites near the inner limiting membrane (*arrow*). **V**, vitreous. **B**, The rod neurites contain 50 nm synaptic vesicles (*arrows*) and dense multivesicular bodies (*arrowheads*). **V**, vitreous. **C**, The rod neurites (*arrowheads*) are closely apposed to a Müller cell process (**M**), which is filled with intermediate filaments. *Arrow*, inner limiting membrane; **V**, vitreous. Magnification, 23,250 \times .

rods at 10 months lacked outer segments, but the surface membranes and cytoplasm of their somata were well labeled with anti-rhodopsin (Fig. 5) and anti-recoverin, respectively. There was no evidence of neurite sprouting by these cells.

Discussion

This appears to represent the first demonstration of rod neurite sprouting *in vivo*. The neurites are formed by human rods that have survived significant death of neighboring photoreceptors. In rodent models of RP, the surviving rods show enlargement of

their spherules in the outer plexiform layer (Jansen and Sanyal, 1992; Sanyal, 1993) and delocalization of rhodopsin and phototransduction proteins to the rod somata and synapses (Jansen et al., 1987; Nir et al., 1989, 1990; Cantera et al., 1990; Roof et al., 1994; Sung et al., 1994), yet these rods have not been noted to form neurites. We looked for neurites on surviving rods in old *rds* mice, using the same immunolabeling methods used on the human retinas, but also found no evidence of neurite sprouting. It is possible that rod neurites are detected in the human RP retinas because the process of rod cell death occurs

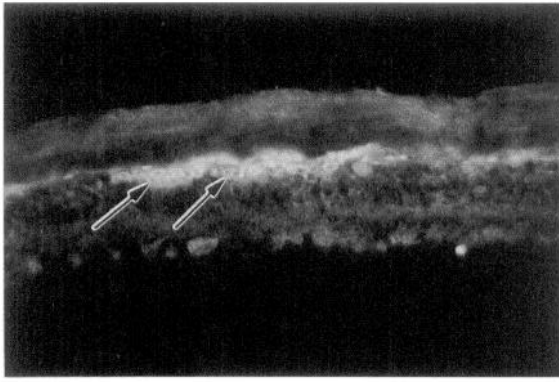


Figure 5. Retina of a 10-month-old *rds* mouse immunolabeled with anti-rhodopsin. The remaining rods lack outer segments, and their somata (arrows) show surface labeling for rhodopsin. No neurite sprouting is noted. Magnification, 230 \times .

over years to decades rather than weeks to months, as found in rodents. Use of similar immunocytochemical methods on dystrophic retinas from 4-year-old dogs, the longest-lived animal models for human RP, also provided no evidence for neurite sprouting by the surviving rods (G. Aguirre, personal communication). Because rod neurite sprouting appears not to occur in nonhuman retinas, it may be due to properties peculiar to the human rods or to changes in the microenvironment unique to the RP retinas.

Neurite outgrowth is usually associated with the process of terminal differentiation of neurons and depends on interactions with neurotrophic factors, cell adhesion molecules, and the extracellular matrix. Mature neurons can respond to injury by sprouting neurites, possibly involving reactivation of a developmental mechanism, and their survival is promoted by various neurotrophic factors (Jelsma and Aguayo, 1994). Rod neurite sprouting in the RP retinas may result from survival factor upregulation in response to ongoing photoreceptor cell degeneration. In culture conditions, basic fibroblast growth factor (bFGF) promotes survival and differentiation of rods (Hicks and Courtois, 1992), and neurite outgrowth from other types of neurons (Hatten et al., 1988). Intravitreally injected bFGF rescues rat photoreceptors from light damage (Faktorovich et al., 1992), and the level of bFGF in retinal glia and photoreceptors is increased following optic nerve injury (Kostyk et al., 1994), consistent with the observation that optic nerve sectioning protects photoreceptors from light damage (Bush and Williams, 1991). Injected bFGF also delays photoreceptor degeneration in RCS rats (Faktorovich et al., 1990) and levels of photoreceptor bFGF are increased in degenerate *rd* mouse retinas (Gao and Hollyfield, 1995). However, elevations in bFGF or in its receptor were not found in degenerate retinas of RCS rats (Connolly et al., 1992; Rakoczy et al., 1993), and the role of bFGF and other survival factors in the human retinal degenerations is at present unknown.

Rod axons *in vivo* are normally short and terminate in the outer plexiform layer. In culture, rods can form long, beaded neurites as found in the RP retinas (Araki et al., 1987; Kljavin and Reh, 1991; Gaur et al., 1992; Mandell et al., 1993; Hicks et al., 1994; Kljavin et al., 1994). Inhibitory factors that normally restrict rod axons to the outer plexiform layer may be missing in the culture conditions and in the diseased RP retinas. Alternatively, rod neurites may form in the RP retinas in response to changes in substrate factors, including cell adhesion molecules

and components of the extracellular matrix (Bixby et al., 1994; Martini, 1994; Schachner, 1994). The rod neurites in the RP retinas were intimately associated with the surfaces of the hypertrophic Müller cell processes, and Müller glia are also the preferred substrate for neurite extension by mammalian rods *in vitro* (Kljavin and Reh, 1991; Gaur et al., 1992; Hicks and Courtois, 1992; Hicks et al., 1994). Following death of photoreceptors, Müller cells undergo reactive gliosis, including increased expression of GFAP (Eisenfeld et al., 1984; Milam and Jacobson, 1990; Li et al., 1994, 1995). The reactive Müller cells in the RP retinas have significantly increased surface membrane areas and associated molecules, including N-CAM (Z.-Y. Li, unpublished observations). Rods also show robust neurite outgrowth in culture on an N-CAM substrate (Kljavin et al., 1994). Thus, rod neurite formation in the RP retinas may be influenced by changes in the neighboring reactive Müller glia and their associated surface molecules.

In addition to rhodopsin, the rod neurites in the RP retinas contained several rod cytoplasmic proteins but not the outer segment membrane proteins, *rds/peripherin*, ROM-1, and the cGMP channel. Of the four proteins, rhodopsin is present in both the disk and plasma membranes of the outer segment, *rds/peripherin* and ROM-1 are found only in disk membranes, and the cGMP channel is restricted to the plasmalemma (Molday and Molday, 1993). The absence of *rds/peripherin* and ROM-1 in the neurites is consistent with the normal separation of rod disc membranes from the plasmalemma of the outer segment. The absence of delocalized cGMP channel in the surface membranes of the rod somata and neurites, if not due to masking, indicates that this outer segment plasmalemmal protein does not reach the neurites by simple backflow. It is possible that these rod proteins are sorted by different mechanisms in the inner segment, where separate post-Golgi vesicles are thought to carry rhodopsin to the outer segment and synaptophysin to the synapse region (Deretic and Papermaster, 1991; Holtzman, 1992; St. Jules et al., 1993). In the pathologic rods studied here, both rhodopsin and synaptophysin, as well as SV-2 synaptic vesicle protein, were routed into the neurites, suggesting that rhodopsin trafficking was abnormal and/or the mechanism that normally restricts rhodopsin to the outer segment had been lost (Spencer et al., 1988; Mandell et al., 1993).

Differential trafficking of rod outer segment membrane proteins is also found in experimentally detached cat retinas. These rods show delocalization of rhodopsin to the surface membranes of their inner segments, somata, and synapses (Lewis et al., 1991), while *rds/peripherin* is restricted to the truncated outer segments and to cytoplasmic vesicles in the inner segments (Fariss et al., 1993). The presence of rhodopsin in abnormal sites in the surface membrane does not lead to neurite formation in this model, however, and the axons of detached rods actually retract toward the somata (Erickson et al., 1983). It may be relevant that most photoreceptors die within several months after retinal detachment and by several weeks to months in the rodent models for RP, while the time scale for photoreceptor death in human RP spans a period of years to decades. The rod neurites may also persist for extended periods in the RP retinas, as they were present in retinas from donors ranging in age from 24 to 87 years (Table 1).

Because some loss of retinal ganglion cells occurs in RP (Stone et al., 1992), it seemed possible that the rod neurites had formed after transneuronal degeneration of cells that normally receive direct synaptic input from rod axons. Using specific im-

munomarkers (Röhrenbeck et al., 1989; Grunert and Martin, 1991), we found that horizontal and rod bipolar cells were qualitatively normal, with no indication of transneuronal degeneration in regions of rod neurite sprouting, although some calbindin-positive horizontal cells had developed apical sprouts as found in the degenerate retinas of RCS rats (Chu et al., 1993). Double-labeling studies revealed that the rhodopsin-positive rod neurites extended through the calbindin-labeled band of horizontal cell processes and past the L-7-labeled rod bipolar cells to terminate at the inner limiting membrane. We also found no evidence by EM that the rod neurites formed specialized junctions with neurons, suggesting that the rod neurites lacked the ability to recognize normal signals for termination on horizontal and bipolar cell processes, or that these signals were missing from the postsynaptic cells.

Rods in the maculas of the RP retinas showed pathologic changes, including loss of their outer segments and rhodopsin delocalization to their somata. However, these rods did not form neurites, in spite of significant death of rods and cones in the macular regions. Cones throughout the RP retinas had truncated outer segments but they did not show opsin delocalization to their somata or neurite sprouting. In the peripheral parts of the RP retinas, some cone axons were abnormally elongated, reaching the inner plexiform layer. The cone axonal changes were much less extensive than the long, beaded neurites formed by the rods, but may similarly represent a response to growth/survival factors, as suggested for horizontal cell sprouts in RCS rat retinas (Chu et al., 1993).

Rhodopsin levels in RP retinas, as determined by fundus reflectometry, can be significantly higher than indicated by the reduced rod outer segment function (Perlman and Auerbach, 1981; Kemp et al., 1988). Thought originally to reflect loss of other phototransduction proteins in the rod outer segments, this observation may also reflect greatly increased amounts of rhodopsin present in the rod neurites in the inner retina. Transmission from rods and cones to inner retinal neurons can also be abnormal in RP, as assessed by electroretinographic and psychophysical techniques (Berson et al., 1968; Hood and Greenstein, 1990; Greenstein and Hood, 1992; Cideciyan and Jacobson, 1993; Falsini et al., 1994). It is not known if the rhodopsin-positive rod neurites and abnormal cone axons contribute to these functional abnormalities in RP.

Our findings have relevance for therapies aimed at restoring vision to RP patients by means of photoreceptor transplantation (Schuschereba and Silverman, 1992; Bok et al., 1993; Milam, 1993; Gouras et al., 1994). This approach is based on the assumption that transplanted normal photoreceptors can form synapses on surviving inner retinal neurons and reestablish a functional visual pathway. However, if changes in the retinal microenvironment lead to robust neurite sprouting by rods as shown here, the transplanted rods may not receive the signals for axon termination and synapse formation needed for their functional integration into the RP retinas. Perhaps the macular region should be considered for photoreceptor transplantation because rods and cones in this region do not appear to undergo axonal changes. Additional information is required on the pathologic alterations in RP retinas because at least one feature, rod neurite sprouting, does not appear to occur in the animal models.

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