**Cellular Reorganization in the Human Retina during Normal Aging**

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**PURPOSE.** To characterize the nature and extent of neuronal reorganization in the human retina during normal aging.

**METHODS.** Retinas of young (18–34 years old) and aged (68–77 years old) human donors were examined. Immunocytochemical methods and antibodies directed against Go-alpha, protein kinase C, parvalbumin, calbindin, calretinin, and choline acetyltransferase were used to stain different retinal cell types. Confocal images of retinal sections from the optic disc to the peripheral edge were taken at three eccentricities, and the density and length of cellular processes were quantified with neuroanatomical analysis software.

**RESULTS.** Dendritic fibers of rod and On-cone bipolar cells were found to extend well beyond the normal boundary of the outer plexiform layer (OPL) into the outer nuclear layer (ONL) in aged retinas. Length and density of these elongated fibers were significantly greater in aged than in young retinas. This phenomenon demonstrated a clear spatial gradient that was most prevalent in the periphery and was infrequent in the central region of the retina. Horizontal cells, which normally make triad synaptic connections with photoreceptors and bipolar cells, also had dendrites that extended into the ONL in aged retinas, and these were spatially juxtaposed with the elongated dendrites of bipolar cells.

**CONCLUSIONS.** Rod and On-cone bipolar cells, as well as horizontal cells of the human retina, undergo extensive dendritic reorganization during normal aging. Although literature on aging has tended to emphasize degenerative and regressive changes, the present findings provide evidence for a remarkable degree of cellular plasticity in the aged human retina. (Invest Ophtalmol Vis Sci. 2007;48:2824–2850) DOI:10.1167/iovs.06-1228

Many older people experience some loss of vision, even in the absence of any identifiable eye disease. Advancing age has been associated with decreases in visual acuity and reductions in scotopic sensitivity, contrast sensitivity, motion sensitivity, and poor performance on a variety of visual discrimination tasks. The neurobiological basis of decreased visual performance in older persons is unknown, but a variety of age-related cellular, neurochemical, and molecular changes have been reported at different levels of the visual system (for reviews, see Nagasubramanian and Weale, Schmolesky et al., and Spear).

Some visual impairment may be attributable to degenerative and regressive changes in the retina. Aging has been associated with a marked loss of rod and cone photoreceptors, retinal ganglion cells, and alterations in retinal pigment epithelial cells, which may disrupt their supportive role in retinal function. Although some adaptive changes may occur in the retina because of normal aging, such as expansion of rod inner segments and higher levels of glial fibrillary acidic protein in astrocytes, to our knowledge there is no evidence of neuronal plasticity in aged human retina.

Recently, we discovered that the dendrites of rod bipolar cells in aged mice extend to innervate the outer nuclear layer (ONL), where they appear to form contacts with the spheres of rod photoreceptors. This observation provided evidence for an unexpected degree of neuronal plasticity associated with normal aging and prompted us to ask whether a similar phenomenon occurs in the aged human retina. Marked differences have been observed between mouse and human with respect to normal organizational properties (for instance, the rod-cone ratio) and to the events that are known to take place during normal aging. With respect to the latter, substantial loss of photoreceptors and retinal ganglion cells occurs in the aged human retina, but this is not the case in the C57BL/6 mouse, the strain used in our previous aging study. For this reason, it seemed important to determine whether age-related elongation of bipolar cell dendrites occurs in the human retina.

In the present study, we relied on immunocytochemical staining of different retinal cell types to compare young and aged human retinas. In aged retinas, rod bipolar cells were found to send elongated dendritic processes to the ONL analogous to what was found in aged mouse retinas. Additionally, in aged human retinas, the dendrites of On-cone bipolar cells were observed to extend into the ONL, and horizontal cells were found to extend processes into the ONL that were closely juxtaposed to the elongated bipolar cell dendrites. Collectively, these observations provide evidence for a substantial degree of reorganization in the neural retina of older humans.

**MATERIALS AND METHODS**

**Human Tissue Collection**

Human eyes were obtained from donors through the National Disease Resource Interchange (NDRI) under approval of the Institutional Review Board at the University of California, Davis, and in accordance with the Declaration of Helsinki. Tissue was examined from 12 donors of both sexes who had no reported history of eye disease. Eight eyes were deemed suitable for study; three of these were from young donors between the ages of 18 and 34 years, and five others were from older donors between the ages of 68 and 77. Four eyes were deemed unsuitable for study because of a history of diabetes or degradation of the tissue resulting from inadequate or delayed fixation.

**Tissue Preparation**

All eyes were fixed in 10% formalin within 10 hours of the time of death. Retinas were removed and placed in 4% paraformaldehyde.
Comparison of retinal sections from young and aged donors stained for rod and On-cone bipolar cells with an antibody specific for Go-alpha. (A) Section of retina from the young donor group exhibiting the normal confinement of On-bipolar cell dendrites to the OPL. (B) Section from the aged donor group displaying elongated On-bipolar cell dendritic fibers extending beyond the OPL and well into the ONL. Scale bar: 50 μm. (C) Higher-magnification retinal section from a young donor. Left: strip of the same retinal section stained with DAPI to highlight the cell layers. (D–F) Higher-magnification retinal sections from aged donors displaying aberrant On-bipolar cell dendritic processes. Scale bar: 20 μm.

Immunohistochemistry
Radial sections that spanned the optic disc to the peripheral edge were taken from the temporal portion of each retina within 2 mm of the macula. Sections were cryoprotected in 25% sucrose overnight before they were embedded in OCT (Ted Pella, Torrance, CA) and were cut at a thickness of 10 μm on a cryostat (Leica, Deerfield, IL). All sections were then incubated in a blocking solution containing 10% normal donkey serum, 2% bovine serum albumin, and 0.3% Triton X-100 in PBS for 1.5 hours at room temperature. Primary antibodies were diluted in fresh blocking solution, and the sections were incubated overnight at 4°C. After washing in PBS, the sections were incubated for up to 4 hours at room temperature in secondary antibodies diluted in PBS. Sections were counterstained with DAPI (1:500; KPL, Gaithersburg, MD) and mounted with mounting medium (Vectashield; Vector Laboratories, Burlington, CA). The following primary antibodies were used: rabbit polyclonal and mouse monoclonal anti-G protein Go-alpha (1:500; Chemicon, Temecula, CA); rabbit anti-protein kinase C (PKC; 1:1000; Upstate, Charlotteville, VA); rabbit anti-calretinin (1:1000; Swant, Bellinzona, Switzerland); rabbit anti-parvalbumin (1:1000; Swant); rabbit anti-calbindin (1:500; Swant); and goat anti-choline acetyltransferase (ChAT; 1:50; Chemicon). The appropriate fluorescent secondary antibodies (all diluted to 1:500) were conjugated to CY3 (Jackson ImmunoResearch, West Grove, PA) and Alexa 488 (Molecular Probes, Eugene, OR).

Fluorescence Imaging and Analysis
Fluorescent images were acquired with the use of a confocal microscope (FV500 series; Olympus, Tokyo, Japan) equipped with 405 diode, multiline argon, and krypton lasers. Image stacks were collected through the z-axis with either a 20× or a 40× oil objective at a pixel resolution of 1024 × 1024. Zsteps were made at 0.6 and 0.3 μm, respectively. Laser intensity and photomultiplier tube levels were adjusted (Fluoview software; Olympus) to optimize the capture of elongating dendrites. Images at a magnification of 20× were taken from three eccentricities in the temporal retinal sections: 650 μm from the optic disc (central), 650 μm from the peripheral edge (peripheral), and midway between the two areas (middle). Thus, three sections at different eccentricities from eight donor eyes yielded 24 samples. Contrast and brightness were enhanced (Photoshop; Adobe, Mountain View, CA). For consistency, anti-Go-alpha was always pseudocolored red, and other stains used in double-label experiments were pseudocolored green. For analysis, fluorescent images were imported (Neurolucida; MBF Bioscience, Williston, VT) where the Go-alpha fibers were traced. Only the portions of the fibers that extended beyond the outer plexiform layer (OPL) were traced, and the border between the OPL and the ONL was determined by drawing a line along the inner edges of the photoreceptor somas in the DAPI-stained images. From the tracings, fiber length and density were calculated (Neurolucida Explorer; MBF Bioscience). Horizontal cell dendritic processes could not be traced because of the discontinuous nature of the parvalbumin labeling. Instead, the percentage of On-bipolar cell–elongated dendritic fibers that were closely juxtaposed to parvalbumin stain was determined. Photoreceptor counts in the ONL were made at all three eccentricities from the same samples of young and aged tissue using the DAPI-stained images. Results for all measures were expressed as normalized values per millimeter of retinal cross-section.

Statistical Analysis
Values are presented as mean ± SEM. Student’s t-tests were performed on data in figures to compare mean values. Significant difference between groups was set at P < 0.05.

RESULTS
Extension of Bipolar Cell Dendrites into the ONL
Comparison of aged and younger retinas revealed that bipolar cells in the aged human retina extend processes out of the normal confines of the OPL into the ONL. Figure 1 shows photomicrographs depicting this phenomenon at high and low magnifications in retinal sections stained with an antibody specific for the G-protein Go-alpha, a marker for On-bipolar cells. As may be seen, in aged retinas the dendrites of On-bipolar cells extend into the ONL, normally a fiber- and synapse-free layer (Figs. 1B, 1D–F). By contrast, the younger retinas rarely contained such extended dendrites (Figs. 1A, 1C). Elongated fibers in the aged retinas varied in length (Table 1), with some nearly reaching the outermost border of the ONL.
The G-protein Go-alpha antibody labels On-cone bipolar cells and rod bipolar cells. To differentiate cone and rod bipolar cells, we used two different antibodies: the Go-alpha shown in Figure 1 and PKC, which labels rod bipolar cells. Proximal dendrites were generally heavily labeled with PKC, but in most cases it was not possible to discern the entire length of the elongated fiber. Nevertheless, by double labeling with the two antibodies, we were able to show that the dendrites of rod and of On-cone bipolar cells extend into the ONL in the aged human retina. This is illustrated in Figure 2, which shows a section of retinal tissue from a 68-year-old donor that was double labeled with PKC (green) and Go-alpha (red). As can be seen, all extended processes labeled with PKC were also labeled with Go-alpha (Figs. 2C, 2D), indicating that these stem from rod bipolar cells. Some Go-alpha–stained fibers were not double labeled with the PKC antibody (arrowheads), which suggests that these are On-cone bipolar cells. Thus, the elongated fibers in the aged retina stem from cone and rod bipolar cells.

Quantification of On-Bipolar Cell Dendritic Extension

The degree of On-bipolar cell dendritic elongation was quantified by tracing the portions of the Go-alpha–stained dendritic fibers that extended beyond the outer border of the OPL (Figs. 3A, 3B). Aged retinas were characterized by a significantly greater number of elongated dendritic processes (Fig. 3C), and the average length of dendrites in the older retina was longer than that of younger retinas (Fig. 3D). In the aged retina, the average length of these fibers was 12.29 ± 0.81 μm (n = 34), whereas in the younger group the mean fiber length was 6.85 ± 0.57 μm (n = 8; P > 0.001). The density of these fibers per millimeter of tissue was 24.15 ± 3.81 in the older retinas (n = 45) and only 2.97 ± 1.05 fibers per millimeter in the younger retinas (n = 27; P < 0.001). Because PKC labeling was not as robust as that of the Go-alpha stain, we did not attempt to quantify separately the growth of rod and On-cone bipolar cell processes.

The extension of On-bipolar cell dendrites was compared at three different retinal eccentricities: 650 μm from the optic disc (central), 650 μm from the peripheral edge (peripheral), and midway between the two areas (middle). This comparison showed clear regional differences. Peripheral and middle eccentricities contained a significantly higher number of elongated fibers in the older group (P < 0.001), whereas the central region showed few extended fibers (Fig. 3E). Similarly, average fiber length comparisons in young and aged retinas differed across eccentricities (Fig. 3F); such processes were significantly longer in the peripheral region of aged retinas (P < 0.01). Although elongated bipolar cell fibers were consistently greater in number and length in aged retinas, significant variability was observed (Table 1).

Horizontal Cells in Aged Retinas

In the aged retinas, horizontal cell processes were also found to extend into the ONL. Figure 4 shows young and aged retinas stained with an antibody specific for parvalbumin, a marker for horizontal cells. Horizontal cells (stained green) can be seen extending dendritic processes into the ONL in the aged samples (Figs. 4B, 4C), whereas the dendrites of these interneurons were confined to the OPL in the young samples (Fig. 4A). Moreover, virtually all elongated fibers (approximately 98%) emanating from On-bipolar cells were colocalized with fibers extending from horizontal cells.

Other Cells Appear Normal in Aged Retinas

Other retinal cell populations were labeled using different antibodies and were compared in young and aged retinas. Calbindin was used to label cone photoreceptors, amacrine

### Table 1. Fiber Number and Length Normalized per Millimeter of Retina

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**Figure 2.** Go-alpha and PKC double-labeled section from a 68-year-old donor. (A) Tissue stained with anti Go-alpha, showing dendrites of rod and On-cone bipolar cells sprouting into the ONL. (B) Same tissue stained with anti–PKC showing dendrites of rod bipolar cells. (C) Double-labeled overlap of staining in (A) and (B). Arrowheads indicate processes not double labeled with PKC. (D) Same section counterstained with DAPI, demonstrating that many of the aberrant processes extend nearly to the outer border of the ONL. Scale bar: 50 μm.
cells, bipolar cells, and some cells in the ganglion cell layer. An antibody against calretinin was used to label cone photoreceptors and amacrine cells in the inner nuclear layer (INL), and one against ChAT was used to label cholinergic amacrine cells. The processes of cells stained with these three antibodies appeared normal (Fig. 5).

As described, extended fiber growth of bipolar cell dendrites into the ONL occurred most abundantly in the peripheral retina, and the least amount of such fiber growth was seen in the central retina. To address the possible role of retinal cell loss in the process of fiber growth, we counted cells in the ONL at central and peripheral eccentricities in the same sections from which fiber counts were made (Fig. 6). Photoreceptor numbers decreased in both cases; however, the loss of photoreceptors was greater in the central retina (27% decrease) than in the periphery (7% decrease). This is in contrast to the trend seen in bipolar cell fiber growth, which was greater in the periphery than in the central retina (compare Figs. 6A and 6B).

We also attempted to assess the role of synaptic connections between photoreceptors and bipolar cells by labeling postsynaptic glutamate receptors with mGluR6 and presynaptic sites with Go-alpha.

**Figure 3.** Quantification of On-bipolar cell–elongated dendritic fibers. (A) Go-alpha–labeled retinal sections such as this were used to make traces. (B) An example of a tracing made using the retinal section from (A). The outer border of the OPL was defined by tracing along the edges of the DAPI-counterstained somas on the inner edge of the ONL. Dendritic processes that extended beyond this border were traced and quantified. Scale bar: 50 μm. (C) Number of elongated dendritic fibers per millimeter of tissue in young versus aged donor retinas. (D) Average length of elongated dendritic fibers in young versus aged donor retinas. (E) Fiber number and (F) average fiber length at three distinct retinal eccentricities in young versus aged retinas. Asterisks: statistically significant differences.

**Figure 4.** Retinal sections double labeled for On-bipolar cells and horizontal cells. Horizontal cells stained with anti-parvalbumin were observed to exhibit elongated dendritic processes extending into the ONL in older retinas. (A) Section of retina from a young donor. Both On-bipolar cell dendrites (red, Go-alpha) and horizontal cell dendrites (green) are confined to the OPL. Left: strip of the same retinal section stained with DAPI to highlight the cell layers. (B, C) Sections of retina from aged donors. Dendritic processes from horizontal cells (green) extend beyond the OPL and into the ONL. Processes are closely juxtaposed to those of On-bipolar cells. Scale bar: 10 μm.
with PSD95. Although these markers showed robust labeling in rodent retinas,\textsuperscript{10} they labeled cells in the human tissue poorly (data not shown), perhaps reflecting the necessarily longer fixation times or delayed fixation of the human tissue.

**DISCUSSION**

We have found that the retinas of older people (68–77 years of age) exhibit some unexpected differences from those of younger people (18–34 years of age) with respect to the morphologic properties of certain retinal interneurons. In particular, the dendrites of On-bipolar cells were found to extend far beyond their normal confines of the OPL to innervate the ONL. Although such elongated processes were occasionally noted in young retinas, they were more frequent and significantly longer in aged retinas. By means of double labeling with two different antibodies, we showed that the extended processes stemmed from On-cone and rod bipolar cells. We also found that the processes of horizontal cells are reorganized in the aged retinas; these were seen to extend in tandem with bipolar cell processes, suggesting intact synaptic triads of a photoreceptor terminus, a bipolar cell dendrite, and a horizon-

**FIGURE 5.** Other retinal cell populations do not exhibit aberrant fiber growth in aged human retina. Several antibodies were used to stain unique populations of photoreceptors, amacrine, bipolar, and ganglion cells in young (left) and aged (right) human retinas. (A) Calbindin labels cone photoreceptors, amacrine cells, bipolar cells, and some cells in the ganglion cell layer. (B) Calretinin labels amacrine cells in the INL. (C) CHAT labels cholinergic amacrine cells. Scale bar: 50 μm.
The present study was motivated by our recent observation that dendrites of rod bipolar cells sprout in aging mice.\(^{10}\) We were curious to determine whether this phenomenon was limited to the aged mouse retina or whether equivalent growth of bipolar cell dendrites also occurs in the human. Our results indicate unequivocally that the growth of bipolar cell dendrites does occur in the human retina. Thus, bipolar cell growth may be a general feature of aged retinas; it would be worth exploring this possibility in other species.

We also noted clear differences in the morphologic reorganization observed in the aged mouse compared with the human retina. The changes observed in the mouse appear to be cell specific. We observed these changes only in rod bipolar cells, not in cone bipolar or horizontal cells. By contrast, in the present study On-cone bipolar cells and horizontal cells showed extended processes. In this respect, the cellular reorganization observed in the aged human retinas appeared to be more extensive than that previously noted in the aged mouse retina. Another obvious difference between the aged mouse and the aged human retina is that the changes observed in the human show a clear-cut central to peripheral gradient. The extension of bipolar and horizontal cell processes was most common in the periphery and was virtually absent in the central segment. By contrast, elongated On-bipolar cell dendrites were found throughout the aged mouse retina. We observed no indication that the magnitude of this phenomenon exhibited appreciable regional differences. We have no explanation for the regional differences in the dendritic growth exhibited by the aging human retina, but this is unlikely to have reflected regional loss of photoreceptors.

This work, and our previous study on the aged mouse retina, raises two related issues. What is the cause of the sprouting of retinal interneurons in aged retinas? Does this phenomenon have any functional consequences? At present, the answers to these questions are unknown.

One possibility is that the growth of retinal interneurons described here is simply an intriguing epiphenomenon. This would be the case if some gene still to be discovered triggered an intrinsic program for dendritic growth in these neurons at an old age. Such growth might be expected to have minimal functional consequences or perhaps even to contribute to visual impairment. Although we cannot rule out such a scenario, this seems unlikely. A more plausible possibility is that the reorganization we have described here reflects a reaction to an extrinsic signal and that the growth of processes in the aged retina is an attempt to compensate for some age-related loss in retinal circuitry. This notion is supported by the results of previous studies that have described changes in retinal interneurons similar to those described here in cases of certain retinal disorders. Thus, rod bipolar cell dendrites have been found to sprout in humans with retinitis pigmentosa.\(^{12}\) This has also been reported in the Royal College of Surgeons rat, the animal model of this degenerative disease in which there is progressive loss of rod photoreceptors.\(^{13}\) Rod bipolar cell dendritic extension also occurs in Bassoon\(^{14}\) and Cacna1f\(^{15}\) mutant mice, strains that have poor synaptic transmission because of defective or absent ribbon synapses between photoreceptors and second-order neurons. Moreover, animal models of retinal detachment also exhibit rod bipolar cell dendritic sprouting.\(^{16}\) In these studies, elongation of horizontal cells was also noted, a phenomenon we observed in the aged human retinas.

Our results seem to rule out the possibility that photoreceptor loss triggers the growth of bipolar cell dendrites described here. In agreement with Curcio et al.,\(^ {5}\) counts of photoreceptors revealed that the loss of these cells in the aged human retina is greater in the central retina but negligible in the periphery. Thus, the greatest photoreceptor loss occurs in the retinal region, where bipolar cell fiber growth is uncommon; conversely, the greatest elongation of bipolar cell dendrites occurs where there is insignificant loss of photoreceptors. This is consistent with our data on aging mice retina, which show photoreceptor loss is minimal throughout the retina despite extensive growth of elongated bipolar cell dendrites with age. A possibility not excluded by our data is that afferent synapses are lost or become dysfunctional with age in a region-specific manner in the aged human retina. Our attempts to label presynaptic and postsynaptic sites were unsuccessful, warranting further investigation.

It is important to stress that the human donors whose retinas we studied did not have a known history of retinal disease and that the aged mice in our earlier study had normal results on ophthalmologic examination.\(^ {10}\) Thus, the growth of fibers we documented in aged retinas does not appear to reflect a response to eye disorder. Nevertheless, some signal in the aged retina likely triggers the dendritic sprouting of certain retinal interneurons, an important point for future study.

The literature on the neurobiology of old age has primarily emphasized cell loss and degeneration,\(^ {5,18}\) but the results of the present study offer a more optimistic view of the cellular events accompanying the aging process. Although it remains to be determined what triggers the reorganization of retinal neurons described here, the fact that in aged human retinas neurons are capable of dendritic growth offers hope for future...
therapies involving the rewiring of neuronal connections in the aging nervous system.

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References