Age-related decrease in rod bipolar cell density of the human retina: an immunohistochemical study

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During normal ageing, the rods (and other neurones) undergo a significant decrease in density in the human retina from the fourth decade of life onward. Since the rods synapse with the rod bipolar cells in the outer plexiform layer, a decline in rod density (mainly due to death) may ultimately cause an associated decline of the neurones which, like the rod bipolar cells, are connected to them. The rod bipolar cells are selectively stained with antibodies to protein kinase C-α. This study examined if rod bipolar cell density changes with ageing of the retina, utilizing donor human eyes (age: 6–91 years). The retinas were fixed and their temporal parts from the macula to the mid-periphery sectioned and processed for protein kinase C-α immunohistochemistry. The density of the immunopositive rod bipolar cells was estimated in the mid-peripheral retina (eccentricity: 3–5 mm) along the horizontal temporal axis. The results show that while there is little change in the density of the rod bipolar cells from 6 to 35 years (2.2%), the decline during the period from 35 to 62 years is about 21% and between seventh and tenth decades, it is approximately 27%.

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1. Introduction

Retinal bipolar cells situated in the inner nuclear layer are predominantly involved in mediating the transfer of signals from the photoreceptors to the ganglion cells. Their synapses are located both at the level of the outer and inner plexiform layers. In the former, their dendritic processes synapse with photoreceptors in the synaptic triads, whereas in the inner plexiform layer, their axon terminals synapse with amacrine cells and/or ganglion cells (Dowling and Boycott 1966; Boycott and Dowling 1969; Kolb and Famiglietti 1976; Wässle and Boycott 1991). There are two morphological classes of bipolar cells, called rod bipolar and cone bipolar cells, each being separately connected to the rods and cones, respectively (Polyak 1941; Rodieck 1988; Wässle and Boycott 1991). In the human retina, a rod bipolar cell is connected to about 30–35 rods in the central retina and 40–45 rods in the peripheral retina (eccentricity: 12 mm; Kolb *et al* 1992).

Research studies on normal ageing of the human retina have shown an apparent reduction in thickness of the outer nuclear layer (Gartner and Henkind 1981) and decline in the density of rods (and also other neurones); changes that begin in the early fourth decade (Gao and Hollyfield 1992). The decline in density is more marked with the rods than the cones (Gao and Hollyfield, 1992; Curcio *et al* 1993). Since the rods synapse with the rod bipolar cells in the outer plexiform layer, a decrease in rod density may ultimately trigger the associated decline of the neurones connected to them.

Using a Golgi impregnation technique, Kolb *et al* (1992) characterized eight types of cone bipolar cells and one type of rod bipolar cell in the human retina. Rod bipolar cells possess large somata, which are located in the outer half of the inner nuclear layer. In the outer plexiform layer, their fine dendritic processes receive synaptic inputs in the rod spherules, whereas their axons terminate in the inner half of the inner plexiform layer (Famiglietti and Kolb 1975; Kolb and Famiglietti 1976). As shown in other vertebrates (Dacheux and Raviola 1986; Saito 1987; Strettoi *et al* 1990; Wässle *et al* 1991), these are considered to be physiological ON-type cells. Immunohistochemically, they are identified

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by their selective expression of protein kinase C-α (Negishi *et al* 1988; Graeferath *et al* 1990; Grünert and Martin 1991; Zhang and Yeh 1991; Young and Vaney 1991; Martin and Grünert 1992; Kolb *et al* 1993; Caminos *et al* 2000; Haverkamp *et al* 2003). All the rod bipolar cells are labelled by antibodies to protein kinase C-α (Graeferath *et al* 1990) in the rat retina, and throughout the retina in humans (Kolb *et al* 1993).

This study examined whether there were any changes in the density of rod bipolar cells with normal ageing, by immunohistochemistry and quantification of protein kinase C-α-positive rod bipolar cells in donor human retinas at different ages.

2. Materials and methods

2.1 *Tissue collection*

The experiment was performed on normal, residual eyeballs of human donors aged 6 years (male), 35 years (male), 62 years (female) and 91 years (male). The donors had no history of ocular disease. The cause of death, as retrieved from the case registry, was atrial valve defect (6 years), road-traffic accident (35 years) and cardiac arrest (62- and 91-year-old donors). The eyeballs were obtained from the National Eye Bank, Dr Rajendra Prasad Centre for Ophthalmic Sciences, AIIMS. Permission to use the eyeballs for research was obtained from the Institute Ethics Committee.

2.2 *Tissue fixation and processing*

The eyeballs were fixed (postmortem delay: 30–90 min) in chilled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for five days at 4°C. The retinas were separated from the adherent choroid and washed in phosphate buffer. A fragment of the horizontal temporal retina, measuring about 4 mm (width) x 1 cm (length) was dissected out from the optic disc to the periphery. All retinal samples were cryoprotected in 15–30% sucrose overnight. Transverse frozen sections (16 mm thick) were cut, serially mounted on gelatin-coated slides and kept at –20°C until use.

2.3 *Immunohistochemistry*

Sections were incubated in a primary antibody against protein kinase C-α (rabbit polyclonal, dilution: 1:50,000; Sigma Chemicals Co., St Louis, Mo, USA) for 36 h at 4°C. After washing, the sections were treated with biotinylated antirabbit secondary antibody at a dilution 1:200 for 3 h at 4°C and finally with preformed avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The antigen–antibody binding sites were visualized by treating the sections with 0.06% 3,3-diaminobenzidene tetrahydrochloride as a chromogen, using 0.5% nickel sulphate as an enhancer.

2.4 *Quantification of immunoreactive cells*

The immunolabelled sections were examined under a light microscope using a 40X objective lens. At this magnification, protein kinase C-α immunopositive somata as well as the axonal processes of the rod bipolar cells can be clearly seen in the inner nuclear layer, allowing them to be quantified reliably. The macular region was fixed and utilized as a reference point to determine the distance of the mid-peripheral retina, i.e. 3–5 mm from the macular border, where the cell numbers were counted. The macula was identified as possessing thin, elongated cones, few rods, a thick fibre layer of Henle beneath the outer plexiform layer, and thick inner nuclear- and ganglion cell layers. On immunohistochemical examination, no protein kinase C-α-positive rod bipolar cells were seen in the foveal centre, but a few positive cells, which were smaller, were seen from the slope towards the periphery (1 mm) of the fovea. The number and size of immunopositive cells increased outside the fovea in the mid-peripheral to far-peripheral retinal regions. These observations guided us in determining the retinal region of interest in cell counting. Retinal images were captured via a digital camera and viewed on the computer monitor. The counting started at a point that was 3 mm distant from the foveal border and proceeded to 5.1 mm towards the mid-peripheral retina along the horizontal temporal axis. At 40X magnification, only cells present along a distance of 350 μm could be counted at a time. Therefore, this process of counting was repeated consecutively six times over the entire counting length (350 x 6 = 2,100 μm) to reach a distance of 5.1 mm in the mid-peripheral retina. Ten alternate sections from each age group were used to determine the mean number of cells present in an area of 2,100 μm x 16 μm= 33,600 μm^2, where 2,100 μm was the length of retinal tissue examined for counting the immunopositive cells and 16 μm was the thickness of the retinal sections. From these values the cell density per unit area in sq.mm was determined. For measurement of the soma area and horizontal diameter of the rod bipolar cells, those cells that had the maximum dimensions were considered to avoid fragmented cells. The outline of those cells (N=100 cells) was drawn with a digitized system to determine the soma area (μm^2) and a vector drawn to measure the horizontal diametre of the rod bipolar cell soma. Statistical analysis was done to see the significance of changes in the number of the rod bipolar cells with ageing (non-parametric Spearman Rho correlation test).

3. Results

In the human retina (figure 1), the bipolar cells are located in the inner nuclear layer. Immunohistochemically, the somata of the protein kinase C-α-positive rod bipolar cells were found in the middle of the inner nuclear layer. Their immunoreactive dendritic processes reached the outer plexiform layer to form synaptic connections with the photoreceptor and horizontal cells, while their axons ran through and terminated in the telodendria in the sublamina b of the inner plexiform layer (figure 2). Immunoreactivity was strong in the 35-year-old donor retina (figure 2b) as compared to that in the retinas of the 6 year-old child (figure 2a) and the aged donors (62 and 91 years old; figure 2c, d).

As shown in figure 3, at any given age, the distribution of protein kinase C-α-positive rod bipolar cells was appreciably low within the macula, which increased towards the mid-peripheral part of the retina (figure 2). At the macula, the cells were small and measured about 3–4 μm in horizontal diametre (figure 3). Outside the macula, their size appeared to increase gradually with eccentricity. In the mid-peripheral part of the retina, the horizontal diametre of the immunopositive cells was about 8–9 μm in the 62- and 91-year-old donor retinas. The rod bipolar cell soma area was greater in the 62-year-old retina (60.08 μm^2, table 1) than in the other samples. Quantitative assessment showed that their density per 33,600 μm^2 of retina was 219 and 224 in the 6- and 35-year-old donors, respectively. In the 62-year-old donor retina, the rod bipolar cell density was 176, while it was 128 cells in the 91-year-old donor retina. Thus, our results showed that there was an almost stable rod bipolar cell density from the first till the fourth decade of life; following which it reduced by 21% in the seventh decade and by a further 27% in the tenth decade (table 1).

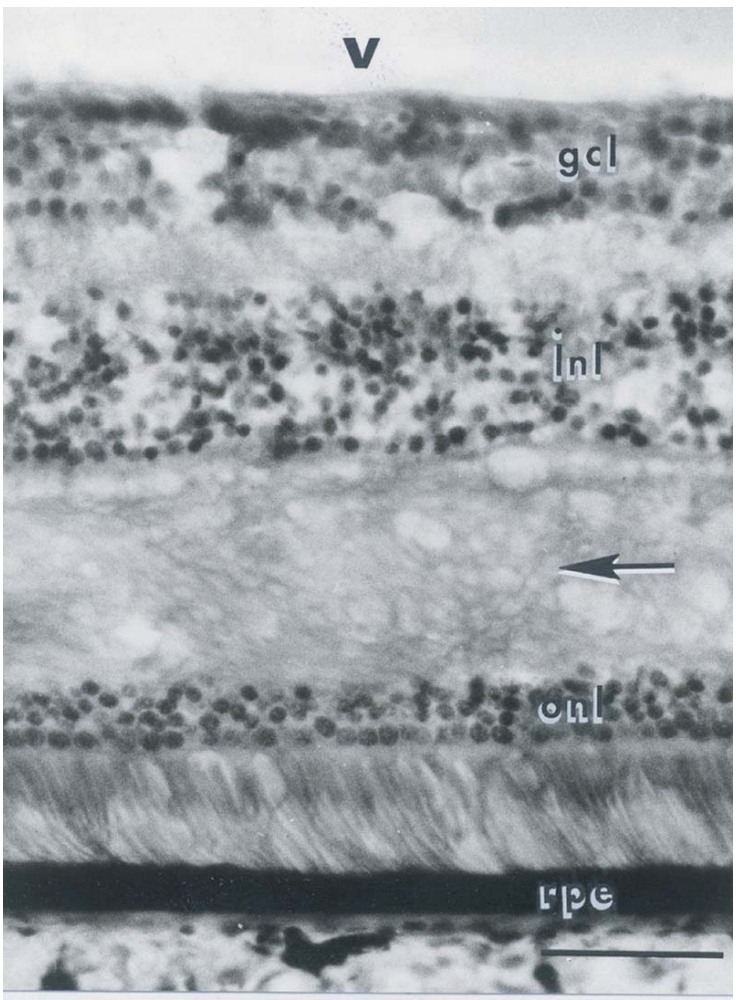

Figure 1. Light micrograph of the human retina at the macula (from 91-year-old donor). Bipolar cells are located in the inner nuclear layer (inl) and are indistinguishable from other resident cells in the haematoxylin and eosin stained preparation. (gcl, ganglion cell layer; arrow, fibre layer of Henle; onl, outer nuclear layer; rpe, retinal pigment epithelium). Scale bar: 50 μm..

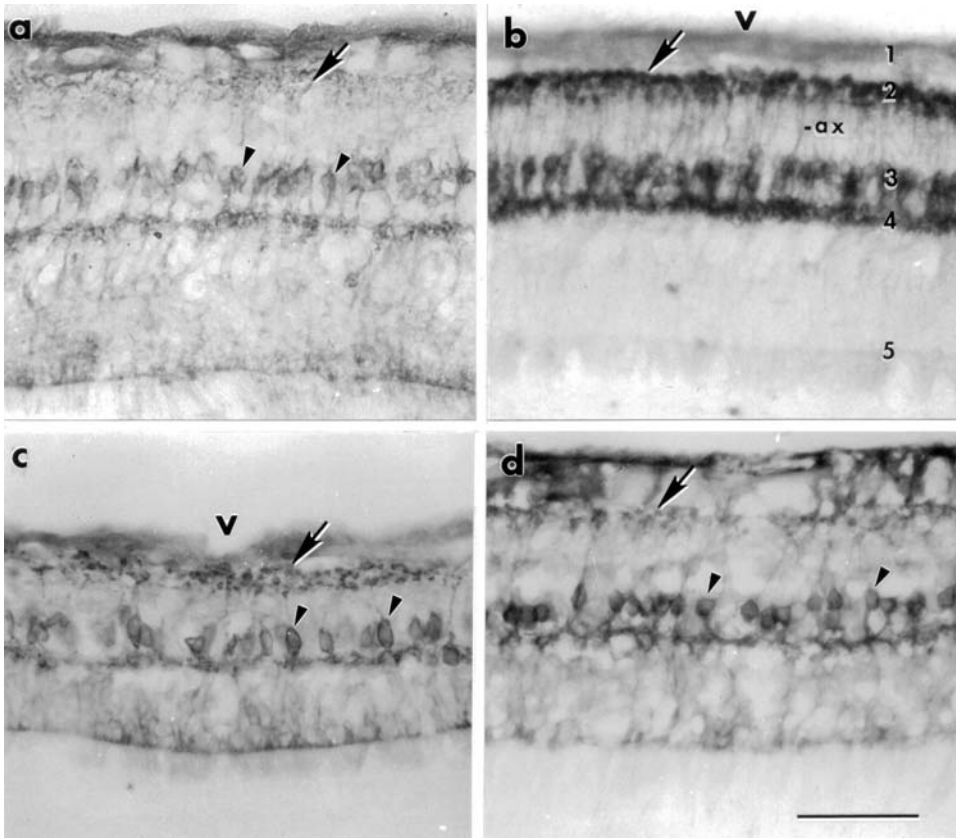

Figure 2. **(a–d)** Protein kinase C-α immunolabelling in the human retina at four different ages examined (6-, 35-, 62- and 91-year-old donors, respectively). The immunopositive rod bipolar cell somata (arrowheads, in **a**, **c**, **d**) are located in the middle of the inner nuclear layer. Their axons (ax, **b**) run through and terminate in the distal part of the inner plexiform layer (arrow). The different retinal layers are indicated in (**2b**). (1, nerve fibre layer; 2, inner plexiform layer; 3, inner nuclear layer; 4, outer plexiform layer and 5, photoreceptor layer). v, vitreal side (in **b**, **c**). Scale bar: 50 μm..

4. Discussion

The purpose of this study was to find out whether there is any change with ageing in the number of rod bipolar cells of the human retina. The density of rod bipolar cells, as identified by immunolabelling for protein kinase C-α, was almost unchanged from the first till the fourth decade of life, but declined subsequently in the seventh and tenth decades.

The rod bipolar cells were found to have their somata lying in the middle of the inner nuclear layer, and their axons terminated in sublamina b. Following the interpretations of Dacheux and Raviola (1986), Wässle *et al* (1991) and Euler and Wässle (1995), these cells could be considered ON-type rod bipolar cells.

Quantitative studies on rod bipolar cell density are limited. Among the primates, reports on the adult macaque monkey retina estimate the density of protein kinase C-α-positive rod bipolar cells to be approximately 9,000 cells per mm^2 at 5 mm eccentricity along the horizontal temporal axis (Martin and Grünert 1992). Our results in the human retina show that the density of the rod bipolar cells between 6 and 35 years of age remains almost stable, being 6,518 and 6,667 per mm^2 of retina, respectively, in the mid-periphery (eccentricity: 3–5 mm) along the horizontal temporal axis (table 1). From the fourth decade of life onwards, an appreciable decline in their density was recorded in the retinal samples. The decline was about 21% between the fourth and seventh decades, and 27% between the seventh and tenth decades. Electron microscopic examination of the ageing human retina occasionally shows profiles of pyknotic nuclei in the outer part of the inner nuclear layer (containing mostly the horizontal cells and, to a lesser extent, bipolar cells), some of which could be the rod bipolar cells (unpublished observations).

Gao and Hollyfield (1992) showed a decrease of 15% in the rod photoreceptor population of the human peripheral retina during the second decade of life, which fell to 32% by the ninth decade. Their report showed that, in the human retina, a decrease of approximately 54% of the total rod photoreceptors occurred between the fourth and ninth

Table 1. Soma area and density of rod bipolar cells in the four donor retinal samples.

Donor age (in years)	Mean soma area (μm^2)	Cell density (per 33,600 μm^2)	Estimated cell density (per mm^2)	Density change (%)*
6	35.44 ± 5.53	219	6518	–
35	35.69 ± 4.29	224	6667	2.29
62	60.08 ± 4.37	176	5238	21.43
91	52.72 ± 3.19	128	3839	26.71

* Indicates change from the preceding age studied.

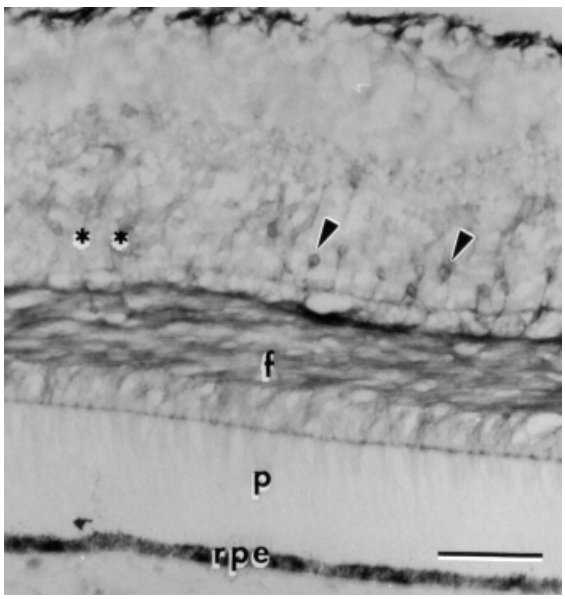

Figure 3. Immunohistochemical demonstration of protein kinase C-α labelling in the macula of the 91-year-old donor. The immunoreactive cell bodies (arrowheads) are small and scarce at the foveal border. Their number decreases towards the foveal centre (indicated by asterisks). f, fibre layer of Henle; p, photoreceptor layer; rpe, retinal pigment epithelium. Scale bar: 50 μm.

decades. Curcio *et al* (1993) reported that in midlife, rod density decreases by about 30%; beginning inferior to the fovea with a greater decrease at 0.3–3 mm eccentricity by the ninth decade. Thus, although loss of rods, as reported by earlier workers, begins from the second decade of life, the rod bipolar cells do not show a decline during this period. It is likely that the connectivity of the rod bipolar cells, even with the lesser number of rods, is still capable of maintaining their integrity. Initiation of decline in the rod photoreceptor density in the second decade (Gao and Hollyfield 1992) precedes the rod bipolar cell loss seen after the fourth decade in the present study. With advancing age, the reported significant decrease of the rods coincides with considerable reduction in the density of the rod bipolar cells. This may be due to loss of their connectivity beyond a threshold in the outer retina following the loss of rods or due to stress generated within these cells after rod photoreceptor death, which makes them vulnerable as well. The reduction of rod bipolar cell numbers with ageing is likely to contribute towards an overall decline of scotopic sensitivity in the retina of elderly persons.

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