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Research Report

The locations of mitochondria in mammalian photoreceptors: Relation to retinal vasculature

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ABSTRACT

Adult mammalian photoreceptors are elongated cells, and their mitochondria are sequestered to the ends of the cell, to the inner segments and (in some species) to axon terminals in the outer plexiform layer (OPL). We hypothesised that mitochondria migrate to these locations towards sources of oxygen, from the choroid and (in some species) from the deep capillaries of the retinal circulation. Six mammalian species were surveyed, using electron and light microscopy, including immunohistochemistry for the mitochondrial enzyme cytochrome oxidase (CO). In all 6 species, mitochondria were absent from photoreceptor somas and were numerous in inner segments. Mitochondria were prominent in axon terminals in 3 species (mouse, rat, human) with a retinal circulation and were absent from those terminals in 3 species (wallaby, rat, guinea pig) with avascular retinas. Further, in a human developmental series, it was evident that mitochondria migrate within rods and cones, towards and eventually past the outer limiting membrane (OLM), into the inner segment. In Müller and RPE cells also, mitochondria concentrated at the external surface of the cells. Neurones located in the inner layers of avascular retinas have mitochondria, but their expression of CO is low. Mitochondrial locations in photoreceptors, Müller and RPE cells are economically explained as the result of migration within the cell towards sources of oxygen. In photoreceptors, this migration results in a separation of mitochondria from the nuclear genome; this separation may be a factor in the vulnerability of photoreceptors to mutations, toxins and environmental stresses, which other retinal neurones survive.

1. Introduction

It is a feature of mammalian retina that photoreceptor metabolism, oxidative and non-oxidative, is high (Cohen and Noell, 1965), yet the photoreceptor layer of the retina lacks intrinsic blood vessels and is supplied with oxygen by diffusion from the choroid and (where present) the retinal circulation. The choroidal circulation is not responsive to the

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Abbreviations: OPL, outer plexiform layer; CO, cytochrome oxidase; OLM, outer limiting membrane; RPE, retinal pigment epithelium; ONL, outer nuclear layer; SD, Sprague–Dawley; BM, Bruch's membrane

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metabolic state of photoreceptors (Chan-Ling and Stone, 1993), with the result that the photoreceptor layer can become chronically hyperoxic (in the photoreceptor degenerations) or hypoxic (after detachment) (Stone et al., 1999). Moreover, photoreceptors are selectively vulnerable to both hypoxia and hyperoxia (Wellard et al., 2005) and depletion-induced hyperoxia of outer retina may play a role in all photoreceptor degenerations (Stone et al., 1999). Finally,

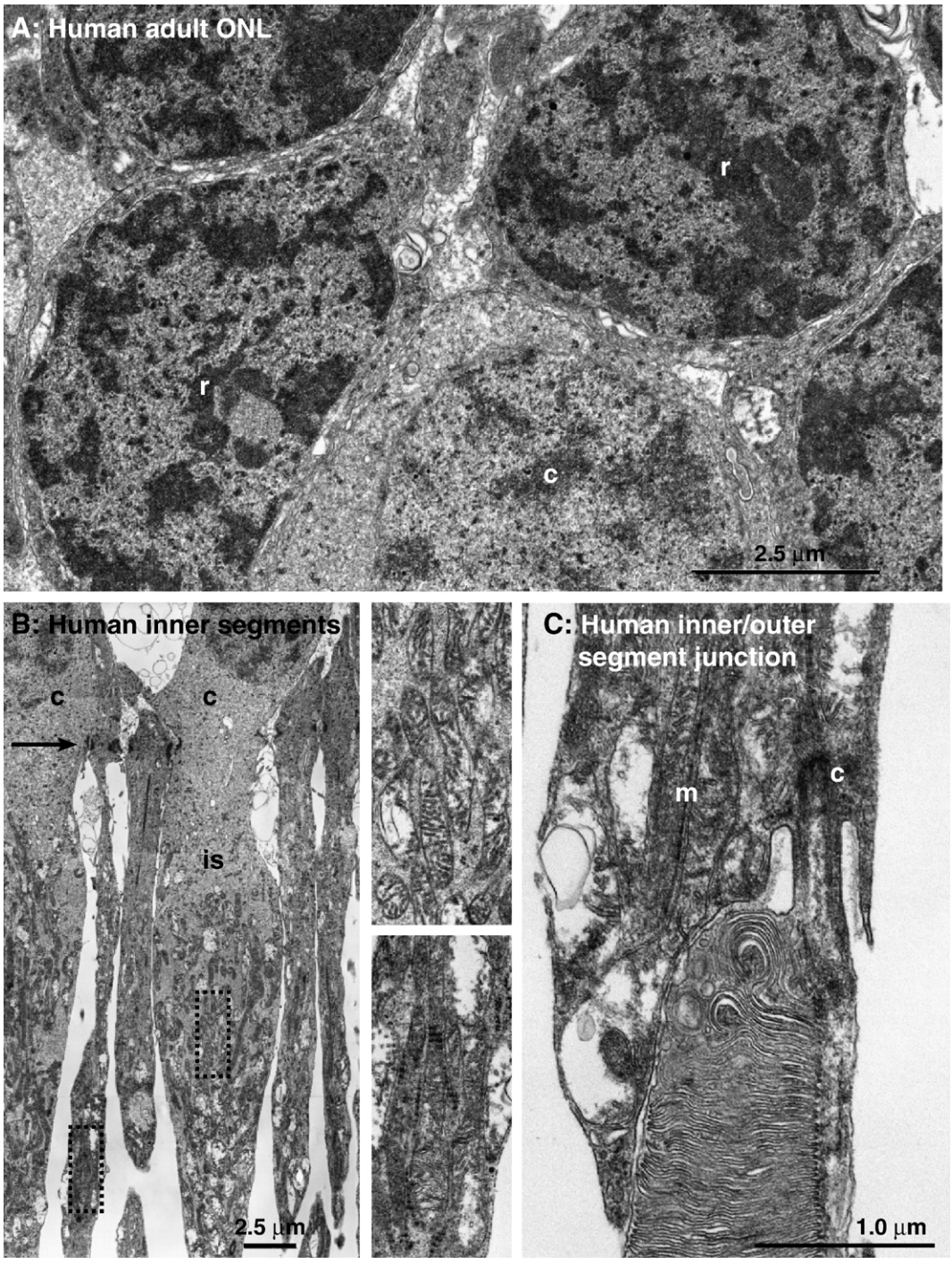

Fig. 1 – Electron microscopy of ONL and inner segments in adult retina. (A) The ONL of human retina, showing nuclei of rods (r) and a cone (c). Note the thinness of cytoplasm, particularly of rods, and the absence of large organelles from the cytoplasm. (B) The OLM/inner segment region of human retina. The arrow points to the row of adherent junctions which comprise the OLM. The nuclei of two cones (c) are apparent; the inner segments of cones (is) and the thinner inner segments of rods extend externally (downwards). The inner part of the inner segments is free of mitochondria, but the outer parts are rich in mitochondria in both cones (upper inset and right and upper rectangle in the main panel) and rods (lower inset and rectangle). (C) At the junction of inner and outer segments, the connecting cilium (c) is apparent. Mitochondria (m) in the inner segment extend to and past the base of the cilium.

oxidative metabolism occurs in specific cell organelles, the mitochondria, which are also major sources of signals that regulate apoptosis, and thus link the metabolic state of photoreceptors to their stability.

This study tests the working hypothesis that the location of mitochondria in retinal cells is determined by their migration towards sources of oxygen. We have examined whether the distribution of mitochondria at the inner end of the photoreceptors varies with the vascularity of the retina; whether mitochondria are polarised in other cells in outer retina, in Müller cells, as reported for the rabbit (Germer et al., 1998) and in retinal pigment epithelial (RPE) cells; and whether the proposed migration of mitochondria can be traced developmentally. Finally, we have tested whether mitochondria are present in neurones in inner layers of avascular retinas. Results support a recent report (Bentmann et al., 2005) that the expression of mitochondrial enzyme cytochrome oxidase (CO) is less prominent in the inner layers of avascular retinas, but not Bentmann et al.'s (2005) conclusion that mitochondria are absent from these layers. The results suggest that mitochondria migrate towards the sources of oxygen to the retina, and that in photoreceptors this migration creates a separation of the mitochondrial genome from the nuclear genome. The role of this separation in the fragility of photoreceptors is discussed.

2. Results

2.1. *Mitochondria in photoreceptors are polarised to the ends of the cell*

2.1.1. *Mitochondria are rare in somas, densely packed in inner segments*

The outer nuclear layer (ONL) in mammalian retina is a tightly packed layer, in which the somas of rods (r in Fig. 1A) and cones (c) are crowded together, with relatively little cytoplasm. Larger organelles, such as mitochondria, are sparse in these somas (human, Fig. 1A). Most of the mitochondria of photoreceptors are located in the outer part of the inner segment (the ellipsoid), where they are typically elongated along the long axis of the inner segment (Fig. 1B). The mitochondria extend externally to, or even past, the base of the cilium (example m in Fig. 1C). These mitochondria are thus located as far as possible to the external end of the photoreceptor cell, except that they do not traverse the cilium to the outer segment.

2.1.2. *Mitochondria are found in axon terminals, in vascularised retinas only*

In 3 of the 6 species studied (mouse, rat, human), mitochondria were also found at the inner end of the photoreceptor, in

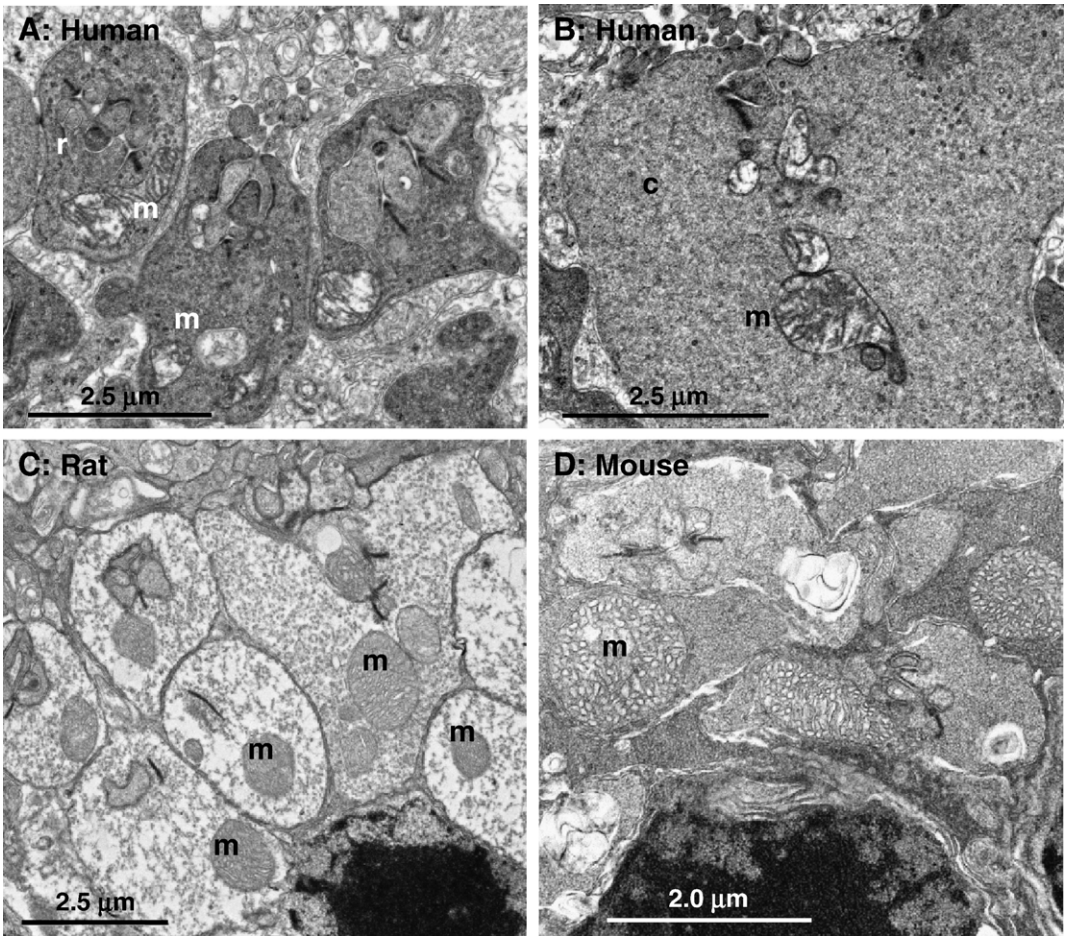

Fig. 2 – Electron microscopy of OPL in adult retinas with a retinal vasculature. Each panel shows axons terminals of rods or cones. Rods—r; cones—c; mitochondria—m. (A) Rod terminals (spherules) from human retina. Each contains a mitochondrion. (B) Cone terminal (pedicle), from human retina. (C) Rod spherules and one cone pedicle, from rat retina. (D) Rod spherules, from mouse retina.

the axon terminal in the OPL. Mitochondria (m in Fig. 2) could be recognised in rod spherules (r in A) and cone pedicles (c in B) of the human retina, and in both forms of terminal in rat (C) and mouse (D). Mitochondria were evident in a majority of terminals seen in each section. In the other 3 species (rabbit, guinea pig, wallaby), by contrast, mitochondria were absent from photoreceptor axon terminals (Figs. 3A–D). Several hundred terminals were scanned in several sections of each of the latter 3 species, without a mitochondrion being observed within a terminal. The difference between vascular and avascular retinas was very marked; our observations suggest that a mitochondrion is present in every terminal in the vascularised retinas, and in no terminals in the avascular retinas. We have previously described the presence of mitochondria in photoreceptor axon terminals in the cat (Holländer and Stone, 1972), another species with a retinal vasculature.

2.2. *Development of mitochondrial polarisation in photoreceptors*

To understand how the distribution of mitochondria in photoreceptors develops, we examined human retinas from 11 to 22.5 weeks gestational age (wa). The material examined was from the macular and perimacular region, which remains avascular during this period (Provis, 2001). At 11 wa (Fig. 4), photoreceptor nuclei were recognisable. Adherent junctions between adjacent cells were evident, marking the level of the OLM (indicated by the arrow). Cells of the RPE directly contacted the OLM. Mitochondria (shown schematically in orange at right) are recognisable and show some tendency to concentrate towards the OLM.

By 19–20 wa (Fig. 5), the nuclei of cones and rods (c and r) and the processes of Müller cells (m), could be distinguished in the macular region. The adherent junctions forming the OLM were apparent (arrow in panel at left) and cells of the RPE still abutted the OLM. Mitochondria (orange in the diagram at right) showed a strong tendency to congregate towards outer ends of rods, cones and Müller cells, at the level of the OLM.

At 22.5 wa, the cones had elongated; the examples (c) in Figs. 6A and B span the ONL from the OLM to the synaptic region of the OPL, at the top of the panels. Mitochondria showed a strong tendency to congregate at the outer ends of the cones and, less consistently, of Müller cells. At this age, the outer ends of the cones bulge through the OLM, beginning the growth of the inner segment. As they bulge, mitochondria follow the growth (Fig. 6C), flowing past the OLM towards their adult position in the inner segment (Fig. 1).

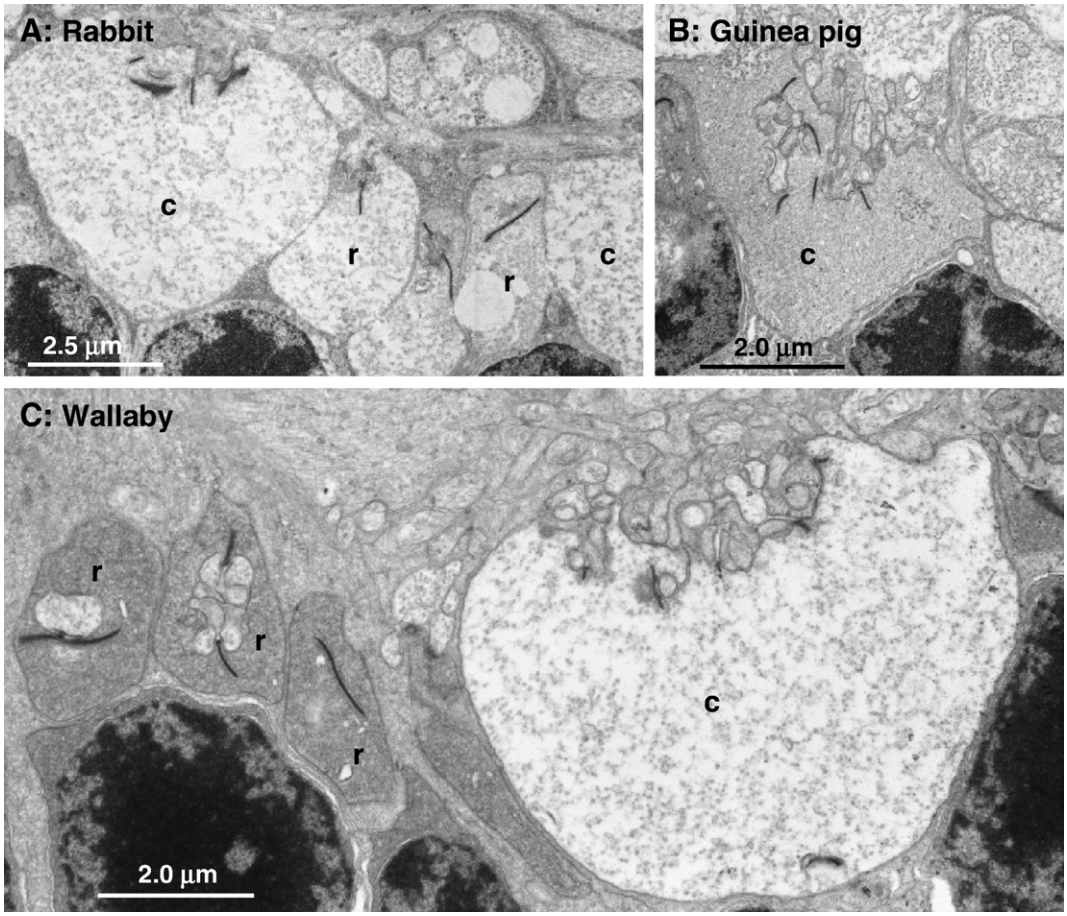

Fig. 3 – Electron microscopy of OPL in adult retinas without a retinal vasculature. Each panel shows axon terminals of rods or cones. Rod spherules—r; cone pedicles—c. No mitochondria were observed in axon terminals, in hundreds of fields scanned. (A) Rabbit retina. (B) Guinea pig. (C) Wallaby.

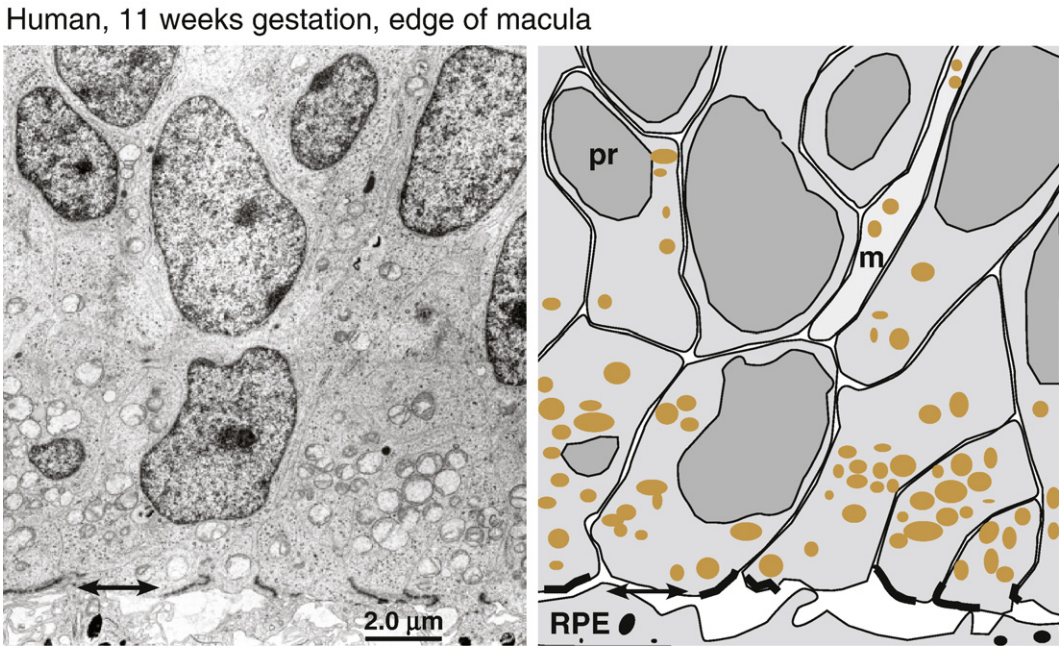

Fig. 4 – Developing human retina at 11wa, from the edge of the macular region. Dark adherent junctions mark the level of the OLM (arrows). Processes of cells of the RPE abut the OLM, at the bottom of the panels. Internal to the OLM the nuclei and somas of photoreceptors (pr) can be seen, but rods and cones are not easily distinguished. The panel at right shows the cellular structures present. A Müller cell process (m) could be identified. Mitochondria, shown in orange in the diagram, show some tendency to congregate towards the OLM.

At higher power (Fig. 6C) of the OLM region of Fig. 6B, the outer end of the cone can be identified, with Müller cell processes flanking it. Short villi extend from the Müller cell processes into the subretinal space. The cone process, at the centre of the panel, has grown outwards past the dark adherent junctions between the cone and Müller cell processes, and mitochondria are prominent in this area of growth.

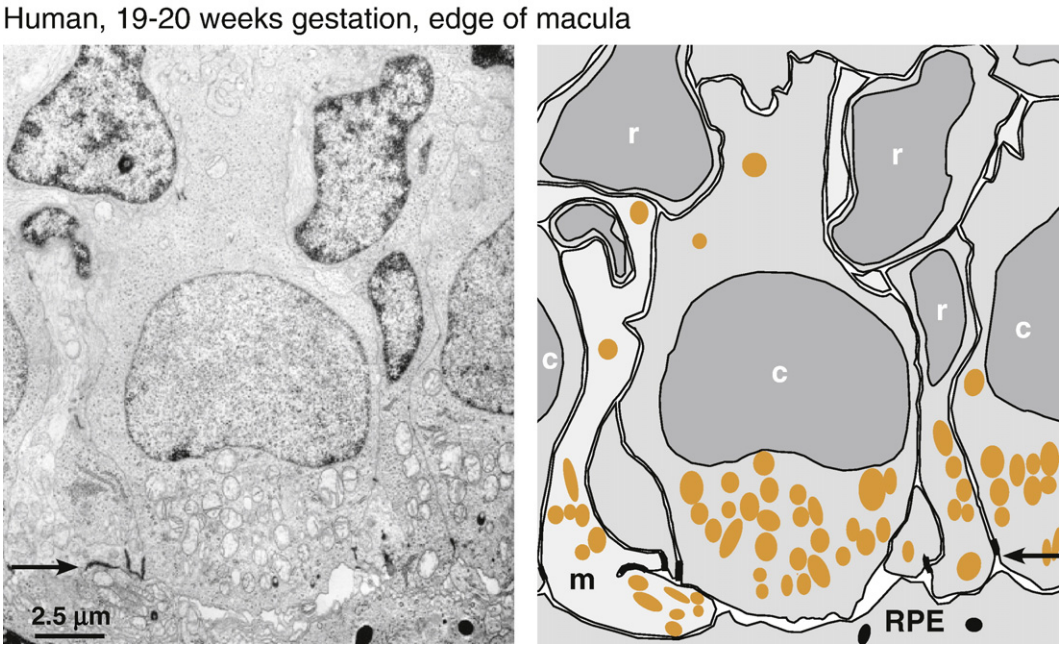

Fig. 5 – Continued development of human retina: At 19–20 weeks gestational age, cones (c) and rods (r) could be distinguished, near the edge of the macular region, and Müller cell processes (m) were increasingly distinct. Cells of the RPE still abut the OLM (indicated by the arrows). In rods, cones and Müller cell processes, mitochondria, shown in orange in the diagram, show a strong tendency to congregate at the OLM.

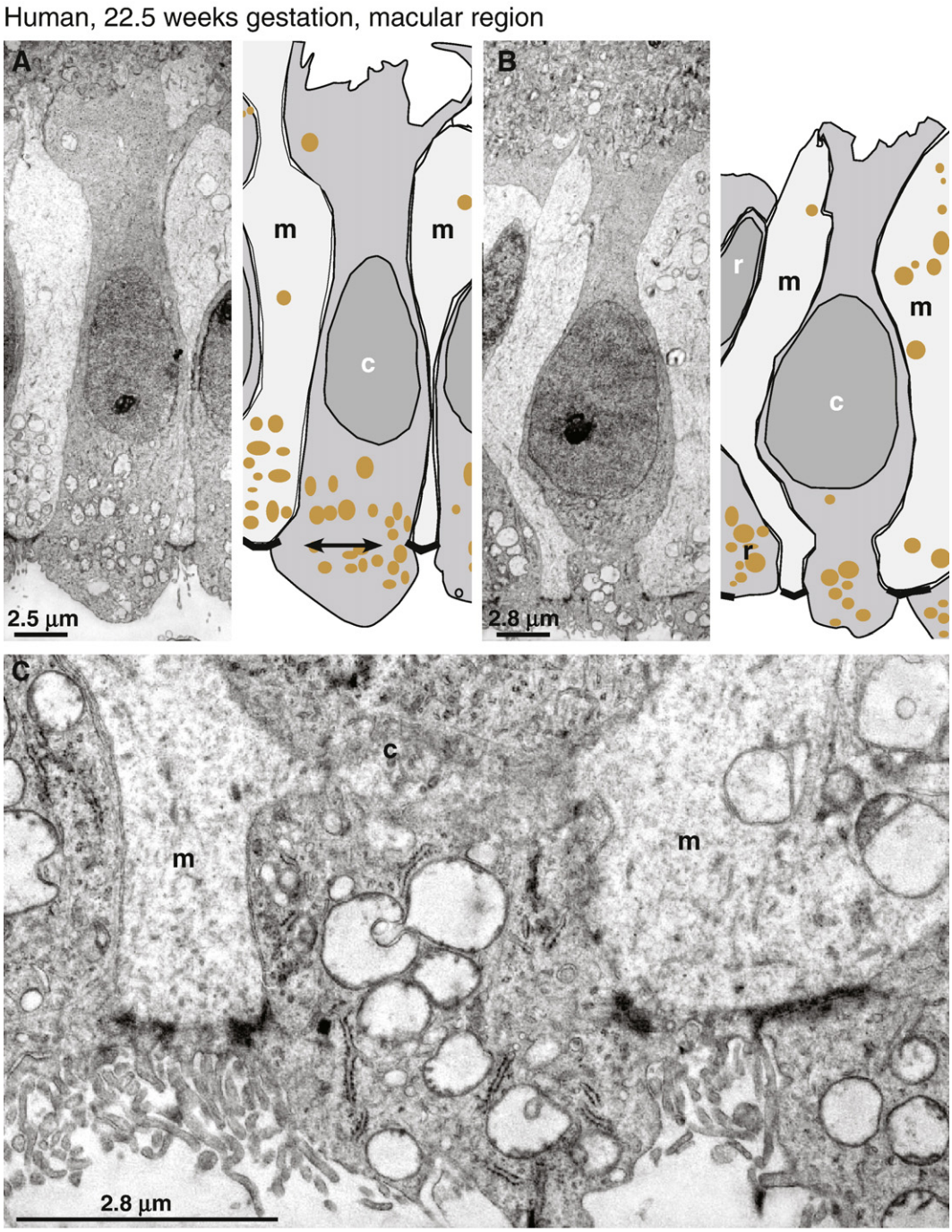

Fig. 6 – Continued development of human retina: At 22.5 weeks gestational age, cones (c) were elongated; the examples in A and B extend from the OLM (arrows) to a region of synapse formation at the top of the panel. The cones have begun the growth of their inner segments, outward past the OLM, and mitochondria (orange in the panels to the right of A and B) concentrate at the outer end of the cones. Many mitochondria are now external to the OLM, following the outward growth of the inner segment. The outer-end concentration of mitochondria is very marked in cones and rods; a rod (r) can be identified at the left side of B. This outer-end concentration of mitochondria is also present in Müller cells as well as photoreceptors but is less consistent at this age. No attempt was made in the diagrams to represent the complex synaptic region of the OPL. (C) The lower region of the photomicrograph in panel B, at higher power. This shows evidence of the flow of mitochondria (m) externally, past the OLM, as the cell forms its inner segment.

2.3. *Mitochondrial polarisation in Müller cells and RPE*

Confirming previous authors (Germer et al., 1998; Uga and Smelser, 1973a,b), mitochondria concentrated in the outer feet of Müller cells, adjacent to the OLM; examples are shown in developing retina in Figs. 5 and 6 and in adult retina in Fig. 9 (upper right panel). In two species, one avascular (wallaby, Figs. 7A, B) and one vascular (human, Figs. 7C, D), the RPE was

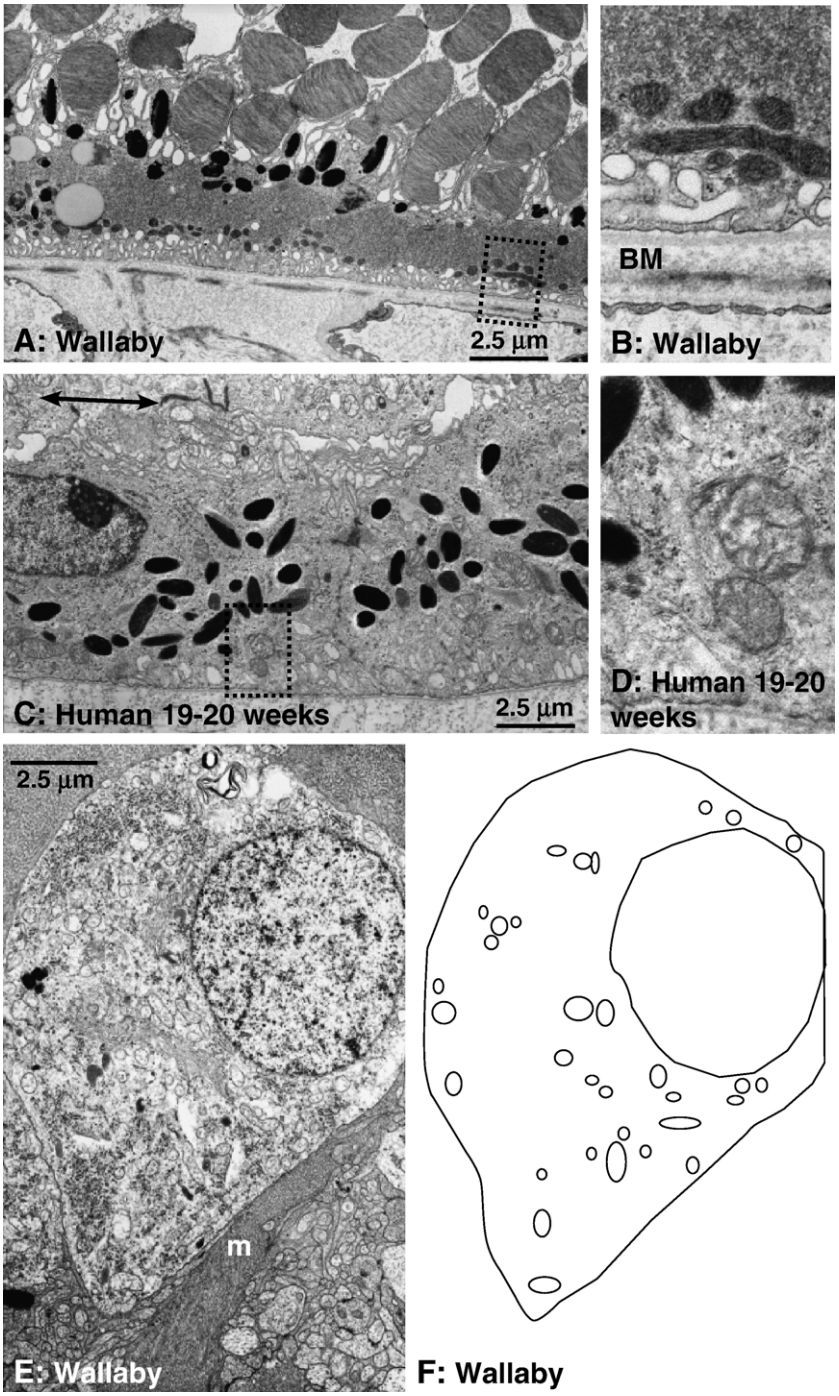

Fig. 7 – Mitochondria in the RPE, and in a ganglion cell. (A) In the adult wallaby retina, mitochondria in the RPE concentrate at the outer aspect of the cell. (B) The area in the rectangle in panel A, at 4× higher power, showing the thickness of Bruch's membrane (BM) and the apposition of mitochondria to the basal aspect of the cell. (C) In developing (19–20 wa) human retina, mitochondria concentrate at the basal side of RPE cells. (D) The area marked by a rectangle in panel C, at 4× higher power. Again the mitochondria lie at the external side of the cell. (E) The soma of a ganglion cell, from a wallaby retina. Mitochondria are numerous in the ganglion cell, but not in the cytoplasm of a neighbouring Müller cell (m). (F) Schematic diagram outlining the nucleus and soma of the ganglion cell in panel E. The small ellipses indicate the locations and approximate sizes of mitochondria.

retained in the block, and it was possible to demonstrate the distribution of mitochondria within RPE cells. In both species, mitochondria concentrated against the outer, basal side of the cell. In the wallaby and, to a lesser extent, in the developing human retina, the RPE cells had formed processes against their basement membrane (Bruch's membrane); the mitochondria concentrated just internal to the basal processes.

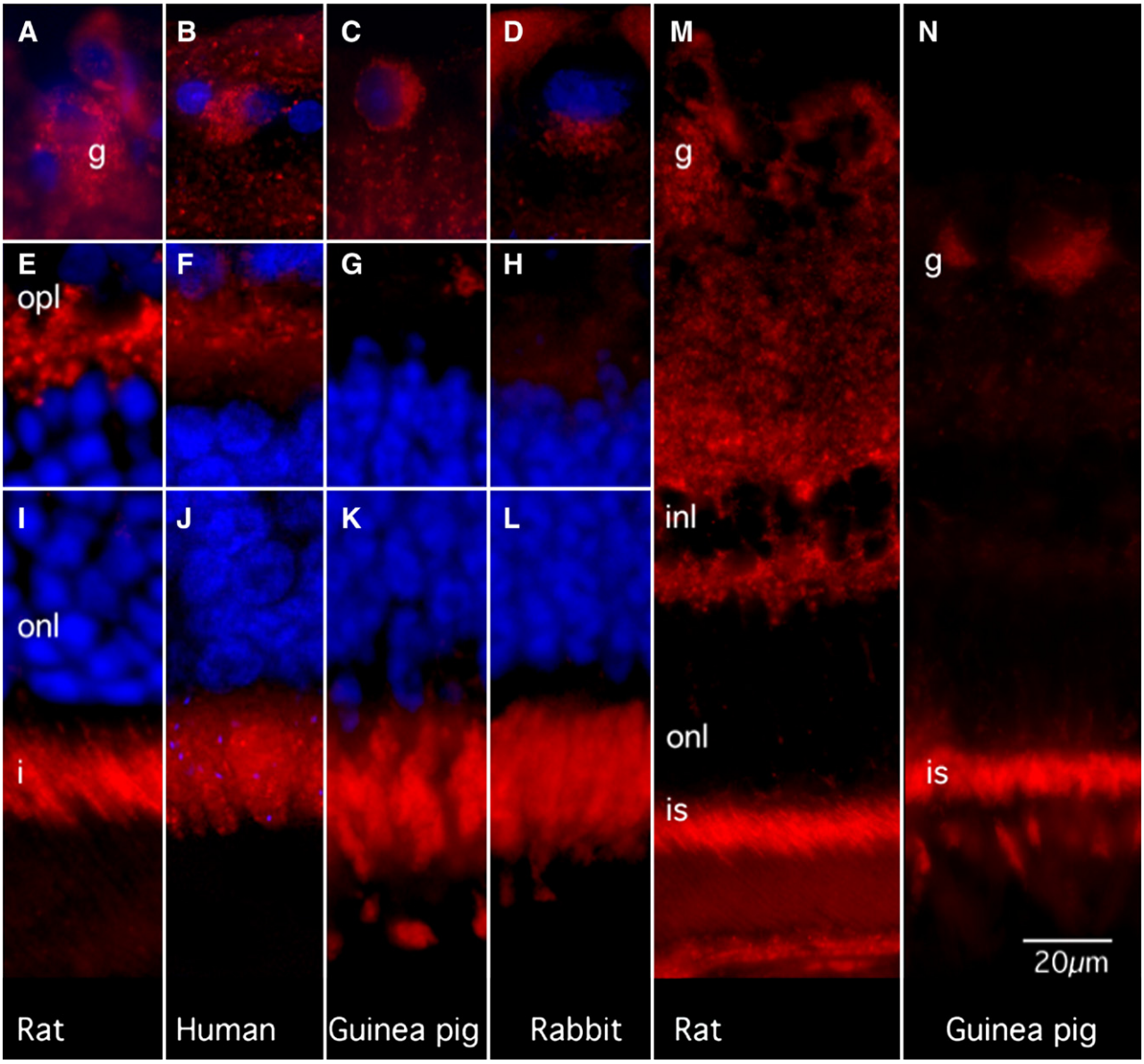

Fig. 8 – Immunohistochemical labelling of CO (red), in the retinas of the rat, human, guinea pig and rabbit. The blue is the labelling of nuclear DNA with bisbenzamide. The scale in N refers to M and N. The scale bar in N represents 10 μm in A–L. (A–D) CO labelling of neurones in the ganglion cell layer (g). (E–H) CO labelling in the OPL (opl). (I–L) CO labelling of photoreceptor inner segments (is). The nuclei are those of photoreceptors in the ONL (onl). (M) CO labelling of normal SD rat retina; g—ganglion cell layer; inl—inner nuclear layer; onl—outer nuclear layer; is—inner segments. (N) CO labelling of guinea pig retina.

2.4. *Adult retina: Are there mitochondria in avascular layers of retina?*

In all species studies studied, immunolabelling for CO decorated mitochondria in the outer part of the inner segment (Figs. 8I–L). In the Sprague–Dawley (SD) rat and human, there was evidence of punctate CO labelling in the OPL (Figs. 8E, F), presumably labelling of the mitochondria in axon terminals (Fig. 2). This punctate pattern was not apparent in the OPL of the avascular species (guinea pig, rabbit, Figs. 8G, H), consistent with the absence of mitochondria from axon terminals in these retinas.

Viewed at lower power (Figs. 8M, N), it was evident that CO labelling in the inner nuclear, inner plexiform and ganglion cell layers is much stronger in the vascular retina of the rat than in the avascular retina of the guinea pig, confirming an earlier report (Bentmann et al., 2005). However, it was possible to detect CO labelling of ganglion cells in rabbit and guinea pig (Figs. 8C, D) as well as the rat and human (Figs. 8A, B). In the electron microscope, moreover, mitochondria were readily apparent in inner nuclear layer and ganglion cells of avascular species (ganglion cell example from wallaby in Figs. 7E, F). These observations go against the conclusion (Bentmann et al., 2005) that mitochondria are absent from the inner layers of avascular retinas such as the

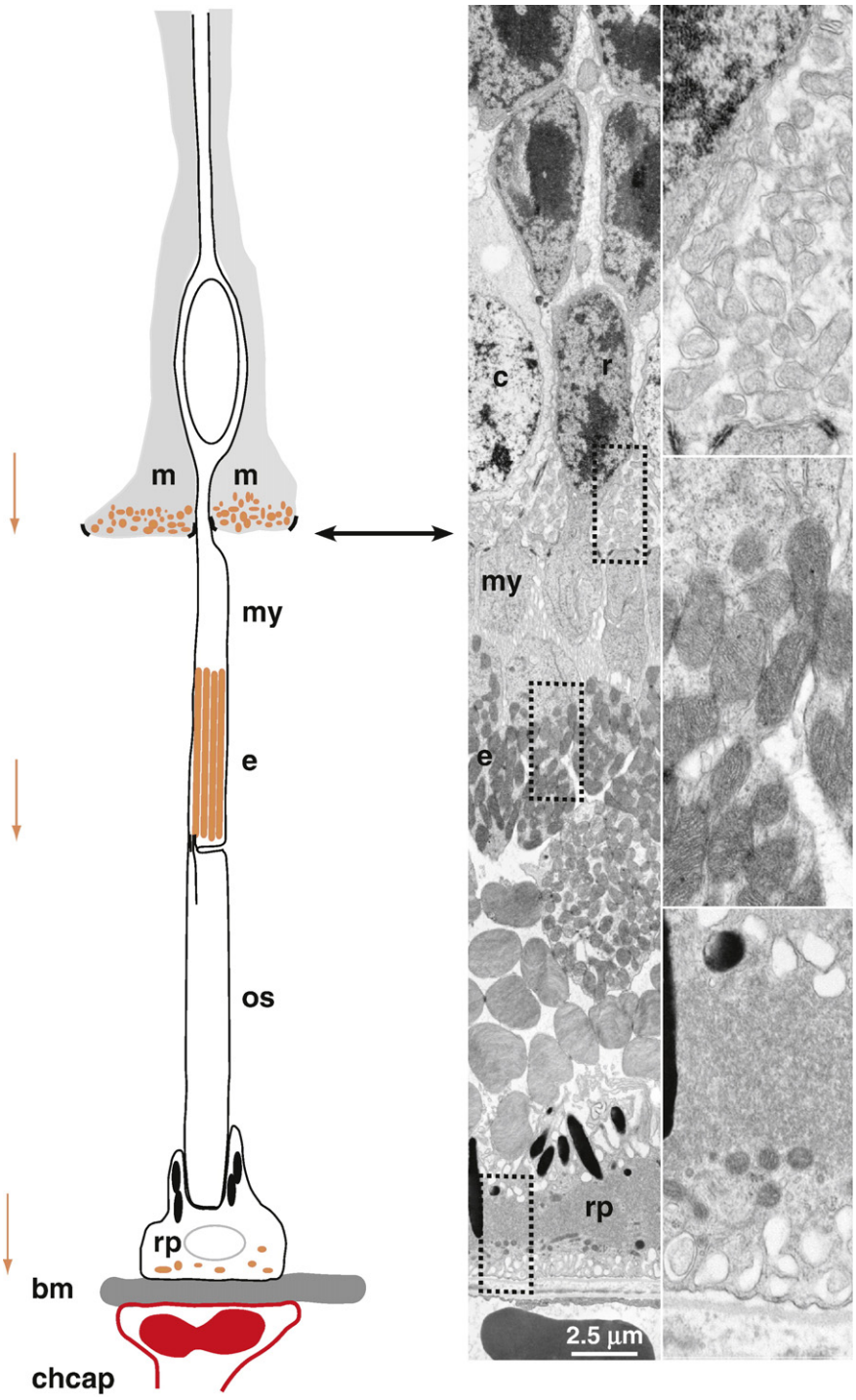

Fig. 9 – The schematic diagram at left shows the locations of mitochondrial in the outer layers of adult mammalian retina. Mitochondria are shown in orange. In Müller cell processes (m), photoreceptors and RPE cells (rp) mitochondria concentrate at the external end of the cell (orange arrows); i.e. against the OLM, against the cilium of photoreceptors and against the basal surface of RPE cells. Mitochondria concentrate in the outer ellipsoid (e) length of the inner segment; the inner myoid (m) part of the inner segment is largely free of mitochondria. The outer segment (os) of the photoreceptor is embraced by processes of and RPE cell (rp), which is separated by a thick basement membrane (Bruch's membrane, bm) from the choriocapillaris (chcap). The EM panel at right shows the appearance of these layers of retina in the wallaby. The 3 rectangles enclose (from above) the external end of a Müller cell, inner segments of photoreceptors and an RPE cell. Note the red blood cell in the choriocapillaris at bottom. These cells and the serum are the major source of oxygen for photoreceptors. The areas outlined by these rectangles are shown at higher power at right.

guinea pig. Mitochondria are present in considerable numbers but do not express CO strongly, presumably because there is little oxygen in these layers (Yu and Cringle, 2001).

Finally, we note two contrasts in mitochondrial distribution. One contrast is between ganglion cell somas in avascular retinas, which are mitochondria-rich (Figs. 7E, F), and adjacent processes of Müller cells (m in Fig. 7E), which are mitochondria-free (confirming Uga and Smelser, 1973a,b). The second is between mitochondrial distribution in cells of the outer layers of retina (photoreceptors, Müller cells, RPE cells), which is strongly polarised (Fig. 9), and the distribution within ganglion cells, which shows no polarisation (Fig. 7F).

3. Discussion

3.1. *Summary of findings*

The present observations on mitochondrial distribution within retinal cells can be explained as the result of mitochondrial migration towards sources of oxygen:

- In photoreceptors, mitochondria are found at the inner and outer ends of the cell (inner segment, axon terminal) because they migrate towards the choriocapillaris and, where they are present, to the deep capillaries of the retinal vasculature.
- Developmentally, the migration begins as early as 11 wa in human, as the choriocapillaris develops (Allende et al., 2006) but before inner segments develop, and mitochondria can migrate only as far as the OLM. As the inner segment forms, mitochondria flow across the OLM, following the formation of the inner segment.
- In Müller cells, mitochondria also migrate towards the choriocapillaris, congregating at the extreme outer end of this cell class, at the OLM.
- In RPE cells, mitochondria congregate at the basal aspect of the surface of the cell, again as close as possible to the choriocapillaris.
- By contrast, mitochondria remain in the somas of neurones of the ganglion cell layer, where oxygen gradients are low (Yu and Cringle, 2001).

3.2. *Mitochondria in anoxia*

Three prior studies have addressed the question of what determines the distribution of mitochondria in retinal cells (Bentmann et al., 2005; Germer et al., 1998; Uga and Smelser, 1973a,b). The two earlier studies considered only Müller cells. In a wide comparative survey, Uga and Smelser (1973a,b) concluded that mitochondrial distribution is determined by the vascularisation of the retina. Germer and colleagues (1998) examined mitochondrial distribution in Müller cells of rabbit retina, both *in vitro* and *in vivo*, providing evidence that mitochondria migrate within the cells towards sources of oxygen. The present results confirm both studies and extend the observations to other retinal cells, and in particular to photoreceptors. We also confirm the report (Bentmann et al., 2005) that CO expression is relatively low in the inner layers of avascular retinas but show that mitochondria, identified by electron microscopy, are present in neurones of these inner layers. It seems likely that in the absence of significant levels of oxygen, the expression of CO in these mitochondria is down regulated.

3.3. *Functional advantages of mitochondrial polarisation*

What forces other than migration-to-oxygen contribute to the pattern of mitochondrial distribution in retinal cells? Two can be proposed, crowding and demand for ATP; both could act in addition to oxygen-driven migration.

The ONL is a densely crowded layer of cell somas, up to 10 cells thick. The cytoplasm around each nucleus is narrow and larger organelles usually found near the soma (mitochondria, Golgi apparatus) are typically sequestered to the inner segment or axon. This crowding of somas is, arguably, required for the formation of the finely pixelated layer of outer segments, essential for spatial resolution. The minimum size of a soma is determined principally by the size of its nucleus (Fig. 1). Since the nucleus (typically 5 μm in diameter) is much fatter than an outer segment (1–2 μm), the somas must be layered and crowded. Some evidence that crowding is a factor in excluding mitochondria from the ONL comes from the report of some mitochondria in the ONL in the relatively stout processes of cones in mouse retina (Carter-Dawson et al., 1979). Also, the ganglion cell layer, in many areas of the retina of most species, is uncrowded and the neurones have ample cytoplasm with numerous mitochondria. Thus, the sequestration of mitochondria away from photoreceptor somas could be understood as a factor enabling the concentration of photoreceptors and the formation of a finely grained photoreceptor array. Conversely, the absence of mitochondria from axon terminals in avascular retinas (Fig. 3) cannot be explained by crowding since these terminals are as large in avascular as in vascular retina. Moreover, the concentration of mitochondria at the outer aspects of developing photoreceptors forms (Figs. 4–6) before the ONL becomes crowded and the cytoplasmic volume is reduced. The concentration of mitochondria at the basal side of RPE cells also cannot be explained by crowding.

The location of mitochondria in inner segments places them close to the K^+/Na^+ ATPases located in the inner segment membrane; these energy-intensive ion pumps are essential for maintaining the cells' dark current, the basis for neural signalling of light absorption. Similarly, the mitochondria in the axon terminal seem to be ideally located to meet the ATP demand for the processes of synaptic transmission. It was unexpected, but unambiguous, that in avascular retinas these synapses are free of mitochondria. Without a supply of oxygen, mitochondria cannot contribute to the energy requirements of these synapses, which must rely on glycolytic mechanisms. In long-axon cells such as retinal ganglions cells, the transport of mitochondria along the axons is critical to their function and stability. Blockage of the transport of mitochondria, at lamellae of the lamina cribrosa, may contribute to the death of ganglion cells in glaucoma (Hollander et al., 1995) and mutations in mitochondrial genes cause atrophy of the optic nerve (Carelli et al., 2004), emphasising the importance of the function of the transported mitochondria. The movement of mitochondria within long axons is bi-directional, however, and cannot be explained in terms of oxygen gradients. An optical advantage has also been deduced for mitochondria in the inner segments of photoreceptors; the suggestion is (Hoang et al., 2002) that the high density of mitochondria in cone inner segments enhances their waveguide properties.

3.4. *Functional disadvantages of mitochondrial polarisation: the separation of mitochondrial and nuclear genomes*

The polarised distribution of mitochondria within photoreceptors separates the mitochondrial genome from the nuclear genome. The separation, which is also present in Müller cells (see Fig. 22 in Uga and Smelser, 1973a,b), is not large, in the order of 50–100 μm, but it is unusual that mitochondria are absent from the region of the nucleus. The separation is benign in that photoreceptors can survive the full human lifetime. Nevertheless, photoreceptors are the most vulnerable of retinal cells to a range of genetic and environmental stresses. Although hypoxic stress in the foetal retina affects many aspects of retinal structure (Loeliger et al., 2004, 2005; Roufail et al., 1999), hyperoxic or hypoxic stress to the adult retina causes an increase in cell death which is specific to photoreceptors (Wellard et al., 2005). Metabolic toxins, such as iodoacetate, are toxic to all neurones. In the retina, at limited doses, the toxicity is specific to photoreceptors (Graymore and Tansley, 1959). Hundreds of mutations in scores of genes have been identified which cause photoreceptor death (Dryja and Berson, 1995); no mutations have been identified which cause the degeneration of other classes of retinal neurone.

Could the fragility of photoreceptors result from the separation of mitochondria from the nuclear genome? Damage to the mitochondrial genome has been identified as a factor in the aging of tissue and the death of cells, including retinal cells (Arnheim and Cortopassi, 1992; Barja and Herrero, 2000; Droge, 2002; Liang and Godley, 2003; Ozawa, 1995). This damage can be repaired, by mechanisms similar to those which repair nuclear DNA (Croteau et al., 1999; Shadel and Clayton et al., 1997), but the genes which express and control mtDNA repair enzymes are part of the nuclear genome. The separation of the two genomes may delay the repair of mtDNA in cells under stress, hasten their aging and death and make them relatively vulnerable to prolonged stress.

Again, the mitochondrion is the site of sequestration of oxygen into oxidative phosphorylation pathways, which produce high-energy phosphorylated nucleosides, especially ATP. This sequestration involves the production of free radicals near the inner membrane of the mitochondrion (Droge, 2002; Simonian and Coyle, 1996), and a range of anti-oxidant mechanisms has been identified which protect the mitochondria from free radical damage (Cai et al., 2000; Elliott and Volkert, 2004; Paasche et al., 2000; Sastre et al., 2000). If this protection fails, damage to mtDNA increases and cell death accelerates. The genes that produce and regulate anti-oxidant enzymes are part of the nuclear genome, and the separation of the mitochondrion from the nuclear genome may slow their function.

More generally, work on the proteome of yeast (Kumar et al., 2002; Sickmann et al., 2003) has identified 700–800 proteins which are found in, or carry the signal sequence for entrance into, mitochondria and are generated by the nuclear genome. These include enzymes for DNA repair, oxidative metabolism and anti-oxidation, and many (25%) whose functions remain unknown. The number of these mitochondrial proteins (comprising >10% of the yeast proteome) suggests a multiple interdependence of nuclear and mitochondrial genomes for cell function and survival. These suggestions are speculative but provide a testable explanation for the relative fragility of photoreceptors.

4. Experimental procedures

4.1. *Species studied*

Human retinas, foetal and adult, were obtained through the Lions NSW Eye Bank. Eyes from other species were obtained from SD albino rats, C57BL/6 mice, the tammar wallaby, New Zealand white rabbits and pigmented guinea pigs.

4.2. *Preparation of tissue for electron microscopy*

The normal adult human eye was immersion fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Delay to fixation was 2 h 10 min, during which time the eye was enucleated and then refrigerated for 1 h 25 min. Human foetal eyes were immersion fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4, with delays to fixation ranging from 5 min to 10 h. Whole animal eyes were immersion-fixed in 2% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. For all eyes, immediately after immersion, anterior structures were removed and selected retinal pieces dissected out. In the rabbit, the pieces selected were from the avascular peripheral region of the retina, well away from the vascularised/myelinated band that extends nasally and temporally from the optic disc. After washing and postfixation in 2% osmium tetroxide, tissue was stained en bloc with uranyl acetate, dehydrated and embedded in Epon-Araldite. Sections were examined in a Hitachi H7100FA electron microscope.

4.3. *Immunohistochemistry*

Eyes were immersion-fixed in 4% paraformaldehyde for 2 h and then cryoprotected by immersion in 15% sucrose overnight. Cryosections (20 μm) were cut and labelled with an antibody for cytochrome oxidase, as described previously (Mervin et al., 1999). To demonstrate the cellular structure of the retina, the sections were counterstained with a DNA-specific dye, bisbenzamide, as described previously (Bravo-Nuevo et al., 2004). Sections were examined by fluorescence microscopy, in a Zeiss Axiovision 4.5 system able to capture stacks of images spaced through the retina, and then to reduce stray light by a deconvolution algorithm.

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