Changing topography of the RPE resulting from experimentally induced rapid eye growth

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Abstract

The retinal pigment epithelium (RPE) of the quokka wallaby, *Setonix brachyurus*, grows and changes throughout life. To investigate factors that determine changes in the quokka RPE, we have examined topography of this tissue in experimentally enlarged eyes. Unilateral eyelid suture was conducted at the time of normal eye opening, postnatal day (P) 110, and animals were examined at 1 or $1\frac{1}{2}$ years of age. The numbers and densities of RPE cells and the extent of multinucleation were compared with those in normal animals. Eyelid suture resulted in a 9.8% and 17.4% increase in retinal area at 1 and $1\frac{1}{2}$ years, respectively; a significant degree of myopia was associated with this enlargement. Cell density topography in experimental eyes was not the same as in controls. Cells from central retina were disproportionately larger in the experimental than control eyes. However, the RPE cell topography in sutured eyes was not the same as that of aged retinae of a similar size. Notably, in sutured eyes there was no development of the high or highest cell densities seen in equatorial and temporal central RPE in aged retinae, respectively. Furthermore, the degree of cell enlargement in peripheral regions was slight compared with that observed in similar-sized, aged retinae. There was no increase in RPE cell number; rather, average cell area increased accompanied by no change or a slight decrease in RPE thickness. Consequently, overall volume of cells did not change significantly. The large number of multinucleate cells normally seen in aged animals was not observed in experimentally enlarged eyes, implying that an increase in cell volume may be the trigger for multinucleation.

Keywords: Aging, Cell size, Mammal, Multinucleate, Retinal growth, Development

Introduction

Our previous studies of the RPE of the marsupial wallaby, the quokka (*Setonix brachyurus*), have demonstrated a distinctive cell density topography and distribution of multinucleate cells (Fleming et al., 1996*b*,*c*). These features in adults change gradually with age and may be determined either by variable retinal expansion during the continued slow growth of the adult quokka eye or by other factors which arise as part of the aging process, such as differences in metabolic activity between retinal regions (Fleming et al., 1996*c*).

The RPE is a complete and continuous sheet of tissue across the retina and closely linked with the neural retina. Therefore, if uneven retinal expansion or contraction takes place, RPE cells will increase or decrease in area most in regions of greatest expansion or contraction, respectively. These changes, when mapped, provide an accurate picture of retinal expansion or contraction and are presumably mirrored in the adjacent neural retina. We have shown recently that, in the aging quokka, the average cell area of RPE cells in midtemporal retina (adjacent to the *area centralis*) de-

creases. This region of the retina is contracting as the animal ages and we have suggested that this contraction may compensate for loss of neural cells (Fleming et al., 1996*c*).

The factors which control eye growth may be manipulated experimentally by depriving the eye of form vision at a critical period during development. Abnormal eye enlargement can be induced by suturing the eyelids closed at the time of eye opening, the application of refractive lenses, or the administration of selective neurotoxins (Wiesel & Raviola, 1977; Wildsoet & Pettigrew, 1988; Christensen & Wallman, 1991). The effects of eyelid suture upon eye growth were first described by Wiesel & Raviola (1977) for the primate eye. Features of this model in primates include an overall increase in retinal surface area, a thinning of the sclera, often a thickening of the choroidal tissue, an increase in axial length disproportionate to the other eye dimensions, and significant myopia. Although form deprivation myopia has since been demonstrated for species as varied as chicken (Wallman et al., 1978; Yinon et al., 1980; Hodos et al., 1985), guinea pig (Lodge et al., 1994), cat (Wilson & Sherman, 1977), tree shrew (McBrien & Norton, 1992), and man (Robb, 1977; O'Leary & Millodot, 1979; Hoyt et al., 1981; Rabin et al., 1981), other features of the condition appear to vary between species. There are few studies of the effects of deprivation myopia upon topography of retinal cells, the RPE being the focus for one (Lin et al., 1993), and amacrine cells for another (Teakle et al., 1993).

In the present study, we have examined the effects of eyelid suture on topography of the RPE in the quokka. Individuals of this species demonstrate a protracted development (Harman & Beazley, 1987), more closely resembling that of primates (Wiesel & Raviola, 1977) than other species examined such as birds and small eutherian mammals (Yinon et al., 1980; Lodge et al., 1994; McBrien & Norton, 1992). Development of and cell generation in the visual system in the quokka takes place from around the time of birth until about 100 days after birth (Harman, 1991; Harman & Beazley, 1986, 1987, 1989; Harman & Jeffery, 1995; Harman et al., 1989, 1995; Fleming et al., 1996a), and eye opening takes place 110 days after birth. Young animals leave the pouch permanently at approximately 250 days after birth and are sexually mature by 1–1.5 years.

The first aim of this study was to determine whether experimentally induced eye enlargement would produce, albeit more rapidly, the topography observed in the normal, slowly growing eyes of this species. A difference in patterns would enable us to distinguish between topographic changes which are a response simply to retinal enlargement as distinct from those which are a result specifically of features of the aging process.

A second aim of this study was to determine whether experimentally inducing additional growth of the eye would stimulate further cell or nulear proliferation. If there were no further cell proliferation in the RPE, individual cell size would increase. There is a strong correlation between RPE cell size and number of nuclei (Swanson, 1969; Marshak et al., 1976; Bodenstein & Sidman, 1987; Fleming et al., 1996b); therefore, it would be expected that any increase in mean cell size would be accompanied by an increase in the number of multinucleate cells. Alternatively, if cells become multinucleate as a result of ontogenetic factors (Stroeva & Panova, 1983) and irrespective of cell size, then the number of these cells would not rise in the experimental condition.

Methods

Animals

This project has the approval of the Animal Welfare Committee of The University of Western Australia and complies with National Health and Medical Research Council (Australia) guidelines for the use of animals in research.

Animals were obtained from a colony maintained in large outdoor yards under natural conditions by the Department of Zoology, The University of Western Australia. Experimental eye enlargement was produced in seven quokkas. One eye of each animal was deprived of normal vision by suturing the eyelid closed at the time of normal eye opening, at postnatal day (P) 110. At this stage the total retinal area is less than half that of the adult (Beazley & Dunlop, 1983; Fleming et al., 1996c). To do so, the animal was anesthetized by inhalation of Halothane and oxygen (3:1 ratio). Eyelid rims were removed and the edges sutured together; the stitches were removed under general anesthetic 4 weeks later. Five animals were sacrificed (Zoletil i.m. followed by Valabarb i.p., 0.5 ml/kg body weight for each) at 1 year of age and an analysis of the dimensions of the eye was undertaken. Two animals were sacrificed at $1\frac{1}{2}$ years in order to determine whether there was further eye enlargement beyond 1 year, and if there were, to determine the effect of this growth upon the RPE. Both age groups have been included in the analysis of RPE cell densities and the examination of the number and distribution of multinucleate cells. Experimentally enlarged eyes were compared with similar-sized control eyes

from animals aged 4–6 years (n = 5) and 7–9 years (n = 3) which have been analyzed in detail as part of separate studies (Fleming et al., 1996b,c). Unoperated partner eyes of experimental animals were also used as paired controls.

Measurement of refractive error

Two experienced orthoptists measured refractive errors for experimental animals at 1 year of age (n = 5) and control animals ranging in age from 1 year to 12–15 years (n = 8). Refractive errors, expressed in dioptres (D), were corrected for working distance and astigmatism. A number of normal control animals as well as the unoperated partner eyes of experimental animals were tested on two separate occasions, and it was determined that there was no pupillary reflex in this species. Similar findings are reported for a wide range of mammalian species (Walls, 1942; Hughes, 1977). Results from the separate occasions were within 0.25 D of each other (n = 6). For experimental animals, the open eye was measured before and after anesthetic (Zoletil i.m., 0.5 ml/kg body weight), the sutured eye was surgically opened and measured under anesthetic, prior to sacrifice. There was no difference in refractive error for the open eye before and after anesthetic, suggesting that values in the sutured eye before anesthetic would have matched those afterwards.

The measurement of eye dimensions and the preparation of wholemounts

Following sacrifice, animals were intracardially perfused with 1% paraformaldehyde/2% glutaraldehyde in phosphate buffer. Eyecups were oriented with a knick on dorsal cornea. Following enucleation and removal of the extraocular muscles, callipers were used to measure the equatorial naso-temporal and dorso-ventral extents as well as the axial length of the eye (from the back of the eye, adjacent to the optic nerve head, to the front of the cornea). Approximate eye volume was calculated mathematically using $\frac{4}{3}\pi r^2$ with r being an average of naso-temporal, dorso-ventral, and axial lengths. The eyes were then opened, and the maximum thickness and diameter of the lens measured. The lens, sclera, cornea, iris, and choroid combined, as well as the RPE and neural retina, were dissected and weighed.

The RPE with neural retina attached were fixed for a further 48 h in 10% buffered formalin. Six radial incisions were made in order to flatten the retinae, RPE uppermost, onto freshly double-subbed glass slides (5% gelatine/0.5% chrome alum). Tracings of wholemounted retinae were made immediately and the area described by them calculated with the aid of a digitizing tablet connected to an IBM-compatible computer. The approximate densities of retinal and choroidal tissues (g/mm2) were calculated in order to determine whether thinning of these tissues had occurred as a result of eye suturing. Retinae were then dried, bleached with potassium permanganate/oxalic acid, stained with cresyl violet, dehydrated, and coverslipped (Fleming et al., 1996b). Areas of wholemounts were measured after these procedures in order to determine the extent of retinal shrinkage; cell area and density measurements were corrected accordingly.

Analysis of wholemounted material

Cell area/density

Since RPE cells form a continuous sheet, cell area in this tissue is the reciprocal of cell density. We measured RPE cell area from

a systematic 1% sample of the entire surface of retinal whole-mounts as routinely performed for ganglion cells and RPE cells previously (Beazley & Dunlop, 1983; Fleming et al., 1996b,c). For each retina, approximately 250 to 300 evenly spaced sites were sampled. Analyses were conducted at 500× magnification with a light microscope using a *camera lucida*, digitizing tablet and IBM-compatible computer. Cells within a square 100 μm × 100 μm were measured, including those bordering the upper and left sides but not those at the right and lower sides. From these data it was possible to calculate and map cell density. Cell densities are represented as the number of cells per $(0.1 \text{ mm})^2$. The number of cells in 0.1 mm^2 for each of the sample sites were then summed resulting in a 1% count from sample sites evenly spaced across the retina. Total cell numbers were then calculated from the 1% sample by multiplying by 100.

Multinucleate cells

Assessment of the frequency and distribution of multinucleate RPE cells was made by systematically scanning the entire surface of wholemounted retinae. The analysis was conducted at 400× magnification with a light microscope connected to an MD-1 digitizer, IBM-compatible computer and Hewlett-Packard plotter. The location of each multinucleate cell was plotted and a total count obtained.

The areas of uninuclate and binucleate cells were determined for both sutured and open eyes of a single experimental animal. To do so, every binucleate cell encountered (distributed over the entire retinal surface) was measured, along with two immediately adjacent uninucleate cells. Adjacent cells were chosen since, in sectioned material, "cell height" (thickness of the RPE) is consistent between neighboring cells, and therefore cell area is indicative of cell volume. Cell areas were measured using a light microscope, *camera lucida*, digitizing tablet and IBM-compatible computer.

Retinal sections

Radial retinal sections were prepared from two experimental animals which demonstrated marked differences in retinal area between open and sutured eyes. Patches of tissue, approximately 2 mm × 2 mm, from the geometric retinal center (close to the optic nerve head) and midway between the retinal center and periphery in each quadrant were removed from the retinal wholemounts at the time of dissection, prior to being processed for histological examination. These patches of tissue were dehydrated in alcohol, embedded in Historesin, and radially sectioned at 6 μm. RPE thickness or "cell height" was analyzed for every tenth field of view (10% sample) for two sections from each region. An estimate of cell volume was obtained by multiplying measurements of "cell height" by the average cell area in the corresponding retinal regions.

Statistical analyses

Sutured and partner control eyes of experimental animals were compared directly by paired t-test $(t_{p(df)})$. Comparison between sutured eyes and those of control animals was conducted by independent t-test $(t_{(df)})$.

As in our previous studies of the adult and developing RPE (Fleming et al., 1996a,b), for statistical analysis of cell density topography, maps of cell densities were divided geometrically into four quadrants (dorsal, ventral, temporal, and nasal) and five con-

centric rings of equal width. The inner three rings were considered to represent central retina, the fourth ring the equatorial zone, and the fifth the periphery. In this species, the optic nerve head lies close to the geometric center of the retina and the *area centralis* in the ganglion cell layer of the neural retina could be considered to lie in the outermost of the three central rings in temporal retina. Average RPE cell densities were calculated for each of the 20 designated areas and are represented as the mean ± 1 S.D., averaged between all animals within a group.

The RPE cell density topography in experimentally enlarged eyes was compared with that in eyes from animals of the same age and also with topography in retinae from older animals which had attained the same average cell density as a result of normal aging. The density of RPE cells from sutured eyes was compared with that from open partner eyes and from eyes of age-matched and older control animals (ANOVA, $F_{(df)}$). An assessment of specific topographic changes was also made by comparing the RPE cell density in each of the 20 designated retinal regions in sutured eyes either with that in open partner eyes or in control animals which demonstrated a similar average RPE cell density (4–6 year-olds, $n = 6$ and 7–9 year-olds, $n = 3$). Comparison of cell density in retinal regions between age groups was conducted by independent t-test $(t_{(df)})$. Levels of significance are indicated as * $(P = 0.5)$, ** $(P = 0.01)$, and *** $(P = 0.001)$.

Results

There was a significant effect of eye suturing upon retinal growth and optics in the quokka. Eyelid suture resulted in enlargement of the eye accompanied by markedly myopic refractive errors (Table 1A). The extent of the response varied between individuals. For the purposes of the present study, the primary focus has been on the effect of experimentally induced eye enlargement upon topography of the RPE.

Refractive state

Sutured eyes were myopic (-2.15 ± 2.75 D), there was a significant effect on refractive error $(t_{p(4)} = 5.61^{**})$. The refractive error of the unoperated eyes of 1-year-old experimental animals ($+4.55 \pm 0.11$ D) was not significantly different from that of eyes from age-matched control animals ($+4.50 \pm 0.48$ D). Form deprivation, therefore, produced a refractive difference of 6.70 ± 2.39 D between open and sutured eyes (Fig. 1A). The myopic changes observed in sutured eyes of experimental animals were far in excess of those observed as part of normal aging between 1 and 12–15 years of age (Fig. 1B).

Eye dimensions and shape

In accord with the change seen in refractive error, all sutured eyes were significantly enlarged when compared with controls of the same age (Fig. 2). Sutured eyes were larger than open contralateral eyes along the naso-temporal dimension $(t_{p(4)} = -2.92^*)$ and axial length of eyes was significantly increased $(t_{p(4)} = 2.47^*)$. The increase in axial length (4.8%) was greater than that in the other dimensions (naso-temporal 3.0%, dorso-ventral 1.8%). The average diameter of experimental eyes was 0.35 mm larger than open eyes, equivalent to a 15.8% increase in volume $(t_{p(4)} = -3.06^*)$. The surface area of the RPE and neural retina was 9.8 ± 3.6% larger than controls in 1-year-old animals $(t_{p(4)} = -5.40^{**}$, Fig. 3A) and 17.4 ± 3.8% larger for the two 1½-year-old animals

Table 1A. *Ocular dimensions for experimental animals*

Individual	Retinal area (mm²)		Volume (mm³)		Axial length (mm)		Refractive error (Dioptres)		
	Open	Sutured	Open	Sutured	Open	Sutured	Open	Sutured	Difference
1 year old									
E1	212	224	529	729	10.8	10.8	4.5	0	−4.5
E2	210	242	636	796	10.0	11.1	4.8	−1.5	−3.3
E3	213	232	697	742	10.3	11.0	4.5	−3.3	−7.8
E4	202	215	666	697	10.5	10.6	4.5	−4.3	−8.8
E5	191	215	710	783	10.6	11.2	4.5	−4.8	−9.3
Mean ± 1 s.d.	206 ± 9	226 ± 12	647 ± 72	749 ± 4	10.4 ± 0.3	10.9 ± 0.2	4.6 ± 0.1	−2.2 ± 2.75	−6.7 ± 2.4
Difference	20 mm²** (9%)		102 mm³*		0.5 mm*		6.7**		
1.5 year old									
E6	224	254							
E7	228	227							
Difference	39 mm² (17%)								

*Denotes significant difference at $P \leq 0.05$.
**Denotes significant difference at $P \leq 0.01$.

Table 1B. *Total RPE and multinucleate cells number*

Control animal		Experimental animal			Multinucleate cells	
Open eye		Open eye	Sutured eye		Open eye	Sutured eye
1 year old						
C1	402,603	E1	432,716	413,063	101	121
C2	430,881	E2	352,207	349,384	310	371
C3	442,241	E3	390,642	384,113	—	—
		E4	402,912	377,453	—	—
		E5	394,209	380,017	270	216
Mean	425,200 ± 20,400		394,500 ± 28,900	380,700 ± 22,800	227	236
1.5 year old						
C4	422,022	E6	363,421	364,068	492	536
C5	407,199	E7	364,458	376,671	253	320
C6	401,127					
Mean	410,100 ± 10,700		364,400 ± 1,400	370,400 ± 8,900	285 ± 140	313 ± 158

4–6 year old		7–9 year old	
Age (years)	RPE cell number	Age (years)	RPE cell number
4	434,157	7.8	415,136
5.2	400,969	8.8	422,278
5.3	440,694	9.0	428,478
5.5	423,826		
6.0	463,168		
Mean[a]	432,562 ± 22,802		421,964 ± 6,676

[a]No significant difference between the groups.

(statistics not possible on two animals). The open eyes were not different in area to retinae of age-matched control animals (Table 1A).

Despite the marked enlargement of sutured eyes at 1 year, the tissue density (mg/mm²) of the sclera, cornea, iris, and choroid combined, or RPE and neural retina, was not significantly changed (Table 2A). However, suturing had a significant effect upon lens shape at 1 year of age. Lenses f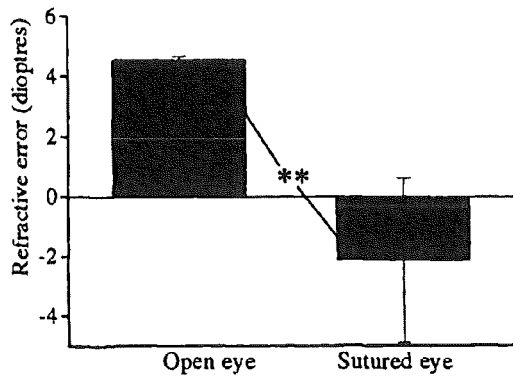rom sutured eyes were more spher-ical than those in the open eyes (Table 2B), with a significantly lower lens diameter to depth ratio ($t_{p(4)}$ = 3.03*).

Response of the RPE to changes in eye size

RPE cell number

There was no difference in RPE cell number between retinae from sutured eyes and those from animals aged P110, the age of

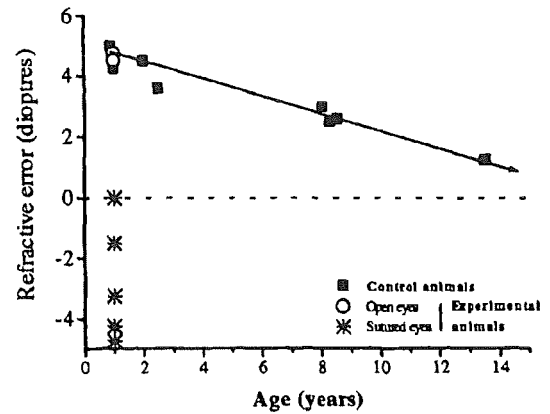## A 1-year-old experimental animals B

Fig. 1. Refractive errors for (A) open and sutured eyes of 1-year-old experimental animals and (B) experimental and control animals of a range of ages. Eyelid suture produced a dramatic shift towards myopic refractive error which is in excess of the refractive error of either the open contralateral eyes or the normal aging animal. Two-tailed, paired t-test; error bars are s.d.

eye opening and at which eye suturing was performed. These results suggest that the final RPE cell number had been reached at the time of eye opening and experimental enlargement did not increase this value. Not surprisingly, therefore, there was no increase in cell number in sutured eyes when compared with open contralateral eyes at 1 year of age (Table 1B). In summary,

although retinae grew larger in the experimental condition, there was no evidence for compensatory cell proliferation.

Comparable with this finding, the mean area of an RPE cell at 1 year of age was an average of 13.7% larger in the retinae of sutured eyes than in controls ($t_{p(4)}$ = -9.12***, Fig. 3B). There was no difference in average cell area between retinae from open

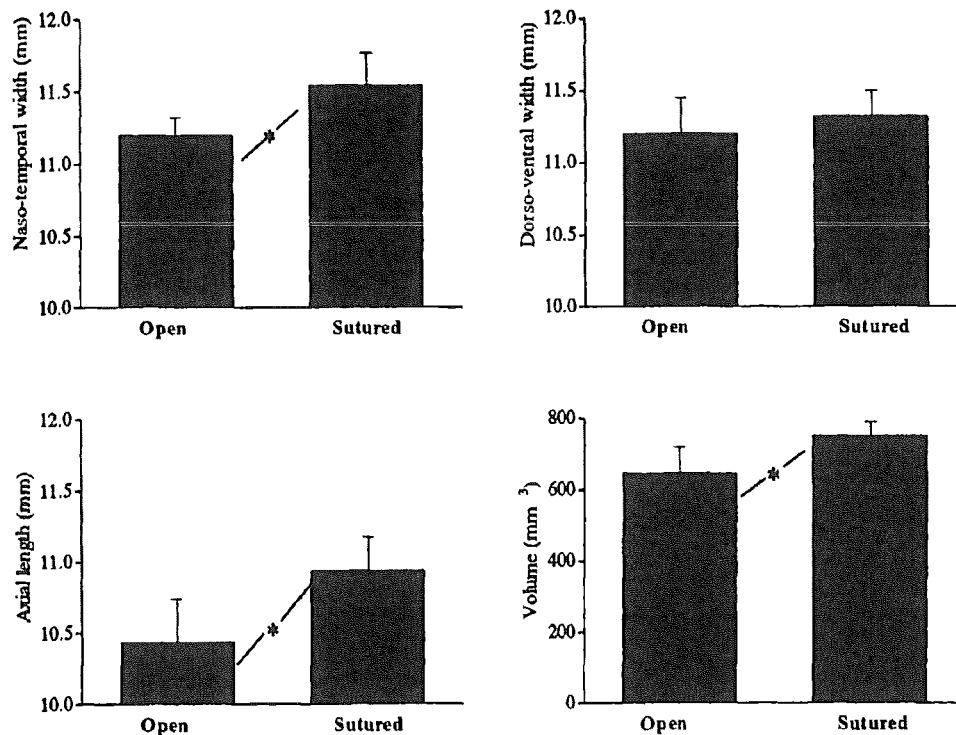## 1-year-old experimental animals

Fig. 2. Eye dimensions for open and sutured eyes for 1-year-old experimental animals. Sutured eyes are larger than the open contralateral eye for most dimensions resulting in a significantly greater volume (n = 5). Two-tailed, paired t-test; error bars are s.d.
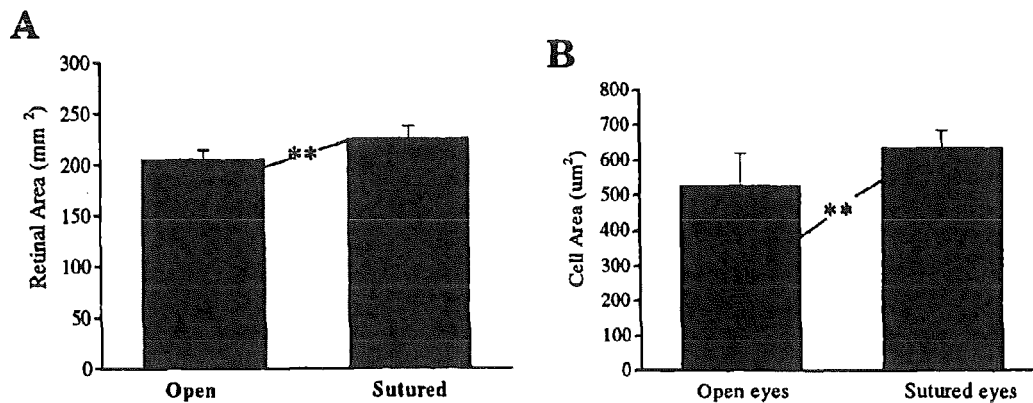
A

B

Fig. 3. (A) Retinal area for control and experimental animals aged 1 year. The retinal area of sutured eyes is significantly larger than that of the open contralateral eye. A similar pattern was observed for 1½-year-old animals. (B) Average RPE cell area for control and experimental animals aged 1 year. Average RPE cell area (μm^2) is larger for sutured than open eyes. A similar pattern was observed for 1½-year-old animals. Two-tailed, paired t-test; error bars are s.d.

contralateral eyes of experimental animals and age-matched control animals.

Cell density topography

Comparison with same-aged retinae. A similar topography of RPE cell density was observed in both the 1- and 1½-year-old experimental animals, therefore both age groups have been grouped together in the representation of cell densities. Not surprisingly,

average RPE cell density was significantly lower in sutured than in open eyes (11.9%, $t_{p(4)}$ = 12.3***). Nevertheless, the normal basic topographic pattern for these ages, characterized by highest cell density in peripheral retina and lowest density in central, nasal retina was also seen in the sutured eyes (Fig. 4A).

It appears, therefore, that cells over the entire retina increased in size to some extent, contributing to the general fall in cell density in the experimental condition. A closer examination, however, of specific regions in sutured eyes revealed that cell enlarge-
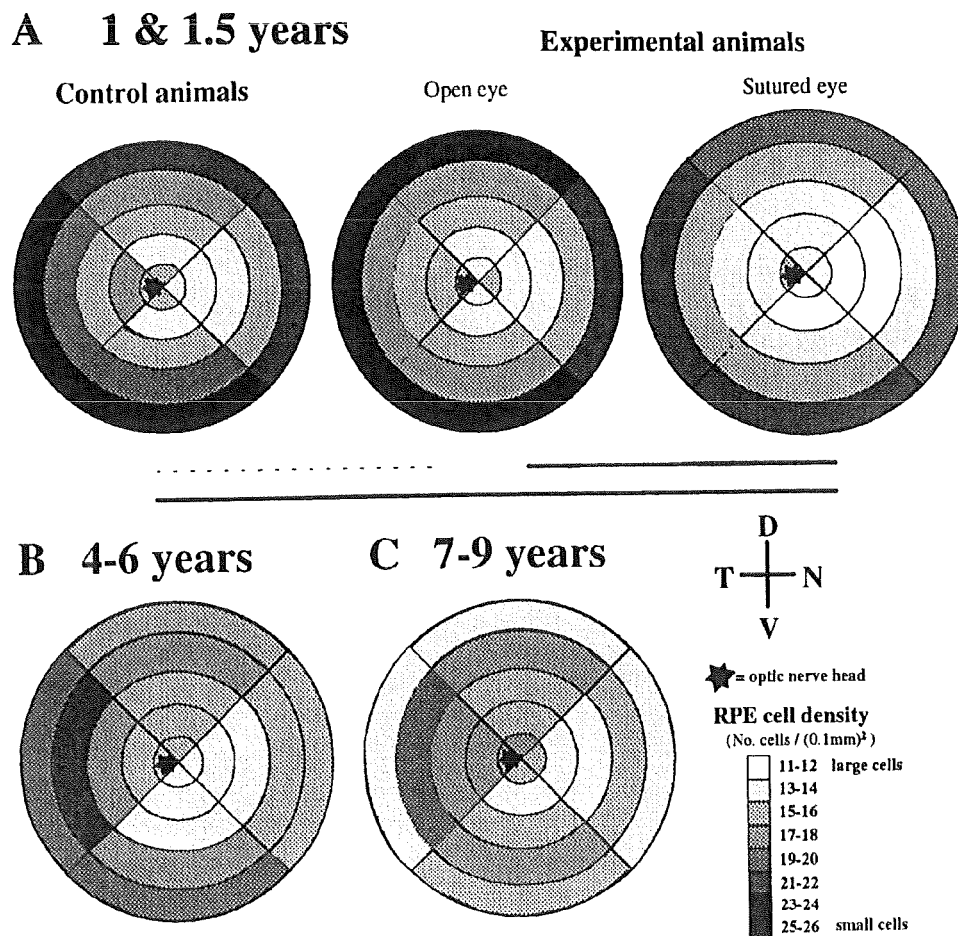
Table 2A. *Differences in tissue density for 1-year-old experimental animals*

Individual	Sclera (mg/mm^2)		Iris and choroid (mg/mm^2)		RPE and neural retina (mg/mm^2)	
	Open	Sutured	Open	Sutured	Open	Sutured
1	0.36	0.41	0.23	0.26	0.26	0.11
2	0.32	0.30	0.14	0.15	0.28	0.20
3	0.37	0.31	0.20	0.17	0.17	0.21
4	0.35	0.30	0.15	0.14	0.23	0.23
5	0.39	0.35	0.21	0.18	0.19	0.20
Mean ± 1 s.d.	0.36 ± 0.02	0.34 ± 0.05	0.19 ± 0.04	0.18 ± 0.05	0.22 ± 0.05	0.19 ± 0.05
Difference	NS		NS		NS	

Table 2B. *Differences in lens shape for 1-year-old experimental animals*

Individual	Lens depth (mm)		Lens diameter (mm)		Lens shape (ratio depth/diameter)	
	Open	Sutured	Open	Sutured	Open	Sutured
1	4.7	5	6.5	6.4	1.38	1.28
2	4.6	4.7	6.5	6.4	1.41	1.36
3	4.8	4.8	6.4	6.3	1.33	1.31
4	4.5	4.6	6.5	6.3	1.44	1.36
5	4.7	4.9	6.5	6.7	1.38	1.37
Mean ± 1 s.d.	4.7 ± 0.1	4.8 ± 1.2	6.5 ± 0.05	6.4 ± 0.2	1.388 ± 0.001	1.336 ± 0.001
Significance	NS		NS		$t_{(p)4}$ = 3.03 (significant at P = 0.01)*	

*Denotes significant difference at $P \leq 0.05$.

A 1 & 1.5 years

Experimental animals

Control animals

Open eye

Sutured eye

B 4-6 years

C 7-9 years

$$T \underset{V}{\overset{D}{+}} N$$

★ = optic nerve head

RPE cell density
(No. cells / (0.1mm)²)

| | 11-12 | large cells |
| 13-14 |
| 15-16 |
| 17-18 |
| 19-20 |
| 21-22 |
| 23-24 |
| 25-26 | small cells |

Fig. 4. Diagram of average RPE cell density for (A) 1- and 1½-year-old control and experimental animals, (B) 4–6 years, and (C) 7–9 years. In the 1- and 1½-year-old groups, the juvenile pattern of high cell density peripherally compared with central regions is evident. Cell densities are significantly lower in retinae from the sutured eyes of experimental animals than those of the open eye or eyes of age-matched control animals (ANOVA indicates a significant effect of eye upon cell density, but little relationship with retinal quadrant or eccentricity, $F_{(1,160)} = 44.25$, $P = 0.001$ and $F_{(1,120)} = 10.99$, $P = 0.001$, respectively, solid lines). Cell densities in the retinae from sutured eyes are not different from those in 4–6 or 7–9 year old animals (ANOVA, $P > 0.05$); however, as is evident from these maps, the topography of cell densities is different, with the older age groups demonstrating distinctly higher cell density in equatorial and central temporal regions and lowest cell density at the retinal periphery. There is no significant difference in average cell density between open eyes of experimental animals and age-matched control animals (dotted line). 1- and 1½-year-old groups: control animals ($n = 3$), experimental animals ($n = 5$); 4–6 years ($n = 6$), and 7–9 years ($n = 3$). Ventral is down, temporal is to the left.

ment was not even across the retina. Cells in central regions of sutured eyes were significantly enlarged compared to those in control eyes (Fig. 5A).

Comparison with same-sized, older retinae. The major change in RPE cell density between normal young (1 and 1.5 years) and old (7–9 years) retinae is a considerable decrease in peripheral regions (Fig 5B). Therefore, retinal expansion in normal aging is most marked in the periphery, whilst enlargement as a result of eye suture is greatest in central retina (compare Figs. 5A and 5B). In line with this finding, cell density in retinae from sutured eyes was significantly lower centrally, particularly in temporal and dorsal regions, and higher peripherally than in aged eyes. Cells in the nasal and ventral portions of central retina in the sutured eyes were of a size similar to those in the aged adults (Figs. 5C and 5D). The distinct peripheral region of low cell density and the equatorial and particularly temporal regions of higher cell density seen in both
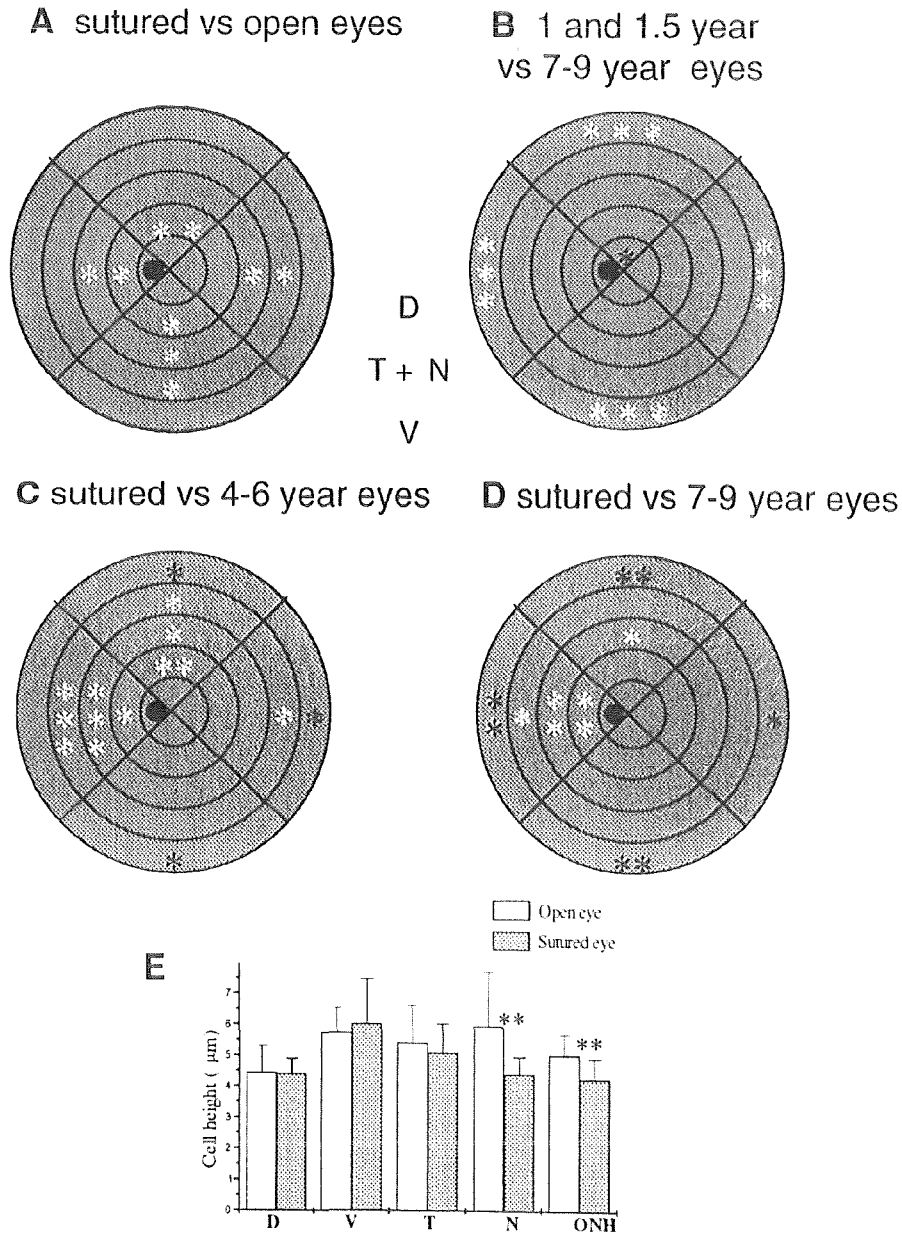
4–6 and 7–9 year-old age groups (Fig. 4) were not evident in the experimentally enlarged retinae.

In summary, the cell density topography of the RPE in sutured eyes does not closely resemble that in either age-matched controls or in older eyes with a similar average cell density. These data indicate that the excessive growth of sutured eyes is not uniform across the retina and that this uneven pattern of growth is not the same as that seen in normally aging and enlarging eyes.

There was no significant change in cell height (RPE thickness) in most retinal region (Fig. 5E).

Multinucleate cells

The number of multinucleate RPE cells in retinae of sutured eyes was not significantly different to that in the open partner eyes. There was an up to threefold difference in multinucleate cell number between individuals of the same age, while the number of cells was

A sutured vs open eyes

B 1 and 1.5 year vs 7-9 year eyes

D

T + N

V

C sutured vs 4-6 year eyes

D sutured vs 7-9 year eyes

☐ Open eye
▨ Sutured eye

E

Fig. 5. (A–D) are diagrams showing the retinal regions displaying significant differences (using independent t-tests) between sutured eyes and eyes of various ages, with * indicating $P = 0.05$, ** indicating $P = 0.005$, and *** indicating $P = 0.001$. In (A) is shown sutured eyes *versus* control eyes at 1 and $1\frac{1}{2}$ years of age; in (B) for comparison, normal 1- and 1.5-year old eyes *versus* normal 7–9 year old eyes ($n = 3$). In (C) and (D) are shown sutured eyes *versus* 4–6 year old ($n = 6$) and 7–9 year old ($n = 3$) eyes, respectively. In (A), (C), and (D), white * indicates lower and black * indicates higher density in sutured retinae than in comparison retinae. In (B), white * indicates lower and black * higher density, respectively, in 7–9 year old than in younger retinae. Most expansion in sutured eyes [white * in (A)] is seen in central retina whereas expansion resulting from normal aging [white * in (B)] is most marked in the periphery. In (E) is shown the average RPE cell height at five retinal regions sampled from sutured and control eyes from two 1-year-old animals which demonstrated the greatest increase in retinal area in the sutured eye. Only RPE cells in nasal and optic nerve head locations appear to be significantly shorter in the sutured eye than in the open eye. Average cell height was measured from radial sections. ONH: optic nerve head; D: dorsal; T: temporal; V: ventral; and N: nasal.

very similar between left and right eyes of individuals, despite the fact that one eye was sutured (Table 1B). The number of multinucleate cells in either eye of experimental animals was not different from that expected for animals of a similar age (Fig. 6A). In comparison with older animals which have a similar retinal area, the number of multinucleate cells in the sutured retinae was low (Fig. 6B). For ex-

ample, the retinae of sutured eyes in two experimental animals (the largest of the sutured retinae), had 320 and 536 multinucleate cells, whilst the retinae of 2- and 6-year-old animals, in a similar range for retinal area, had 631 and 3271 multinucleate cells, respectively.

The number of multinucleate cells is correlated with average cell area both in the sutured ($r_{(8)} = 0.72^*$) and the open eyes
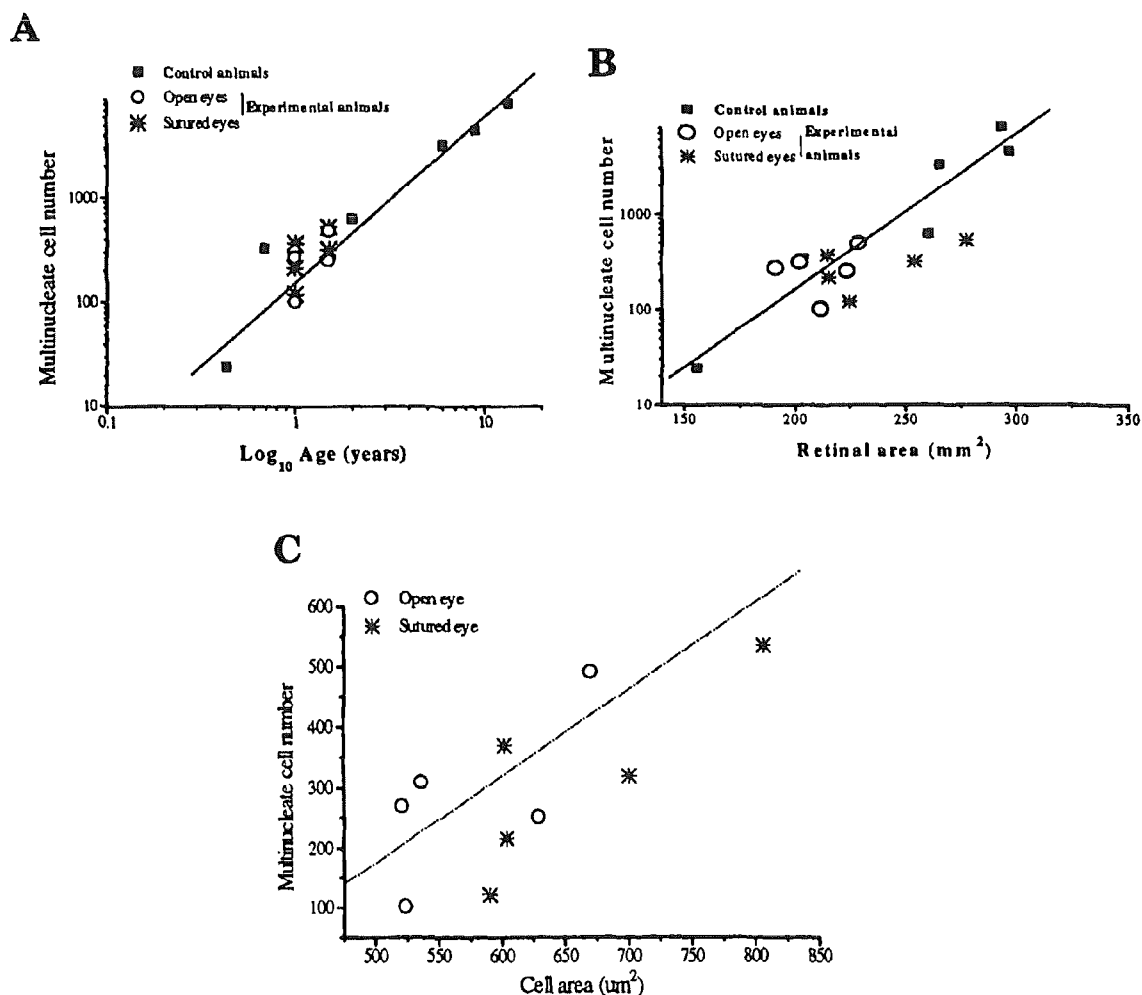
Fig. 6. Graphs of the number of multinucleate cells compared with (A) age, (B) retinal area, and (C) cell area. The number of multinucleate cells in the sutured eyes is strongly correlated with the age of the animal, whilst there is less correlation with retinal area. Within the group of experimental animals, the number of multinucleate cells is correlated with average cell area ($r_{(8)}$ = 0.72*).

(Fig. 6C). Binucleate cells in the sutured eye were approximately twice the area of uninucleate cells, as they are in the open contralateral eye (Fig. 7, Table 3). However, the average areas of uninucleate and binucleate cells in the sutured eyes were around 27.0% and 11.6% larger, respectively, than those in the open eye (Fig. 7).

The distribution of multinucleate RPE cells in sutured eyes did not differ from that observed in open contralateral eyes (Fig. 8) or that of control animals of a similar age (Fleming et al., 1996c). Multinucleate cells were present panretinally in both the open and sutured eyes; there was no disproportionate accumulation of these cells in peripheral retina as is observed in retinae of older animals (Fleming et al., 1996b,c).

Discussion

General description of experimentally enlarged eyes

The experimentally enlarged quokka eye demonstrates many of the features of previously described models of form-deprivation myopia (Wiesel & Raviola, 1977). There is an overall increase in

retinal surface area and eye dimensions, accompanied by a change from 4 D to around −2 to −4 D, $8\frac{1}{2}$ months after eyelid suture. There is a great deal of individual variation in the response to eyelid suture as has been found in other species: monkey (Wiesel & Raviola, 1977), chick (Troilo, 1990), and marmoset (Troilo & Judge, 1993). The degree of retinal enlargement observed at $1\frac{1}{2}$ years of age is greater than that in the 1-year-old animals, suggesting that the effect of eye suture may be ongoing in the quokka.

An increase in axial length and the indication of a rounder lens shape in the sutured condition supports the observation of a shift towards more myopic refractive error. The rounder lens shape may be a result of local factors, such as differences in lens growth or rigidity, whilst the increase in axial length reflects the overall growth of the eye. The degree of retinal expansion in the experimental condition is equivalent to the eye growth which normally takes place over 6–8 years in the quokka. However, the resulting change in optics in the sutured eye is much greater than that seen in the aging eye of similar size. Therefore, axial length alone cannot be responsible for the 6.70 ± 2.39 D change. Lens refraction appears also to play a part. However, changes in corneal

1½-year-old experimental animal
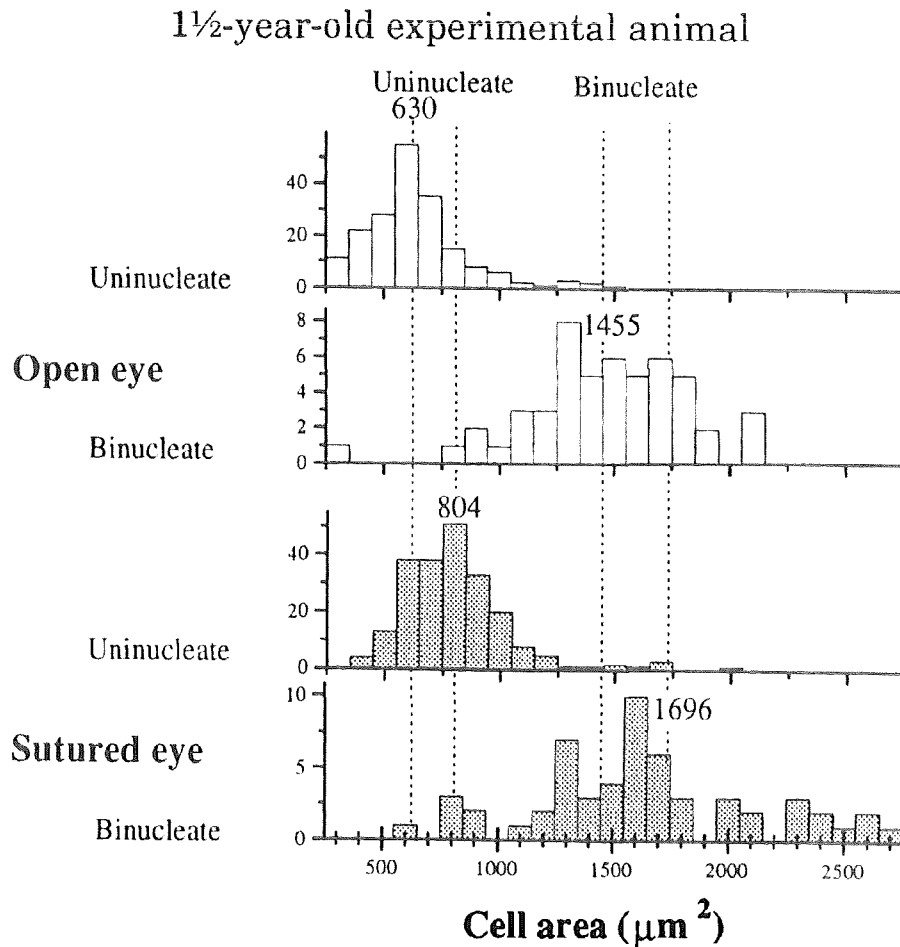
Fig. 7. Frequency histograms of RPE cell area for uninucleate and binucleate cells in the open and sutured eyes of an experimental animal aged 1½ years. Binucleate cells are approximately twice the size of uninucleate cells for both retinae, while the average cell size (values and dotted lines on graph) is larger in the sutured eye than those in the open eye.

curvature may also be involved and were not able to be measured in the present study.

RPE cell number and the thickness of other tissues

Eye enlargement induced by form deprivation is not accompanied by an increase in RPE cell number in the quokka. Similarly, Lin et al. (1993) found no evidence for proliferation of RPE cells in the chick model of form-deprivation myopia in response to increased retinal size; a similar lack of change in dopaminergic amacrine cell number has been observed in the chick (Teakle et al., 1993). In the quokka, the induced eye enlargement after the completion of cell generation in the RPE (Fleming et al., 1996a) did not stimulate further cell division. Therefore, proliferation of adult RPE cells remains apparently restricted to pathologic conditions or retinal disturbances (Bell & Stenstrom, 1983; Kirchof et al., 1989; Stroeva & Panova, 1983) which experimentally induced eye enlargement apparently does not simulate.

We observed a slight thinning of scleral, retinal, and choroidal tissues, without significant change in their weight, in the quokka. Thinning of the sclera has similarly been demonstrated for myopic

eyes in the tree shrew (Kang & Norton, 1993) and monkey (Wiesel & Raviola, 1977), although only at the posterior pole. In chick, thinning was observed in the choroid in the deprived condition accompanied by the increased synthesis of proteoglycans in the sclera but the choroid became thicker again during the recovery period accompanied by a reduction of proteoglycans synthesis in the sclera (Gottlieb et al., 1991, 1993). In line with this observation, a significant increase has been noted in scleral cellular proliferation in the visually deprived chick eye and an increase in the overall dry weight (Christensen & Wallman, 1991). Similarly, for the cornea, cell proliferation and growth, rather than stretching or swelling, occurs in deprivation myopia in the chick (Rada et al., 1993).

The effect of experimentally induced eye enlargement upon RPE cell topography

Cells of the RPE enlarge in area to accommodate the areal increase in the sutured eye and concurrently become, to some extent, thinner (i.e. the RPE sheet is thinner). The juvenile topographic pattern of higher cell density peripherally than centrally is retained in the sutured retinae. However, although all retinal regions contributed

Table 3. *Mononucleate and binucleate cell sizes and t-test comparisons*

	Cell area (μm^2) (mean ± 1 s.d.)	N
Sutured eye		
mononucleate cells	804.2 ± 243.0	220
binucleate cells	1695.5 ± 603.4	58
Open eye		
mononucleate cells	630.5 ± 218.2	191
binucleate cells	1455.4 ± 346.0	51

Comparison between groups of cells		
Cell group	*t* value	Probability
Sutured eye mononucleate and sutured eye binucleate cells	17.3	7.1 E−46***
Open eye mononucleate cells and open eye binucleate cells	20.9	5.3 E−56***
Open eye binucleate cells and sutured eye binucleate cells	2.5	0.014*
Open eye mononucleate cells and sutured eye mononucleate cells	7.6	2.4 E−13***

*Denotes significant difference at $P \leq 0.5$.
***Denotes significant difference at $P \leq 0.001$.

to the retinal enlargement, there is a disproportionate increase in cell size in the central retina. Presumably these regions are most expandable.

In the chick model of form-deprivation myopia, RPE cell expansion was similarly found in all retinal areas sampled, although the expansion was "less pronounced in the temporal region" (Lin et al., 1993). By contrast, in normal chick development, considerable enlargement takes place in the periphery (Lin et al., 1993). This was a similar finding to ours in the quokka; during normal aging, the retinal enlargement is most pronounced in the periphery and as a result of eye suture, enlargement is most pronounced in the central regions.

It seems then that the experimentally enlarged retina demonstrates changes which produce, basically, an enlarged version of the unsutured eye, with more increase in retinal area centrally than

1.5 year old experimental animal

Fig. 8. Maps of the location of multinucleate cells in open and sutured eyes of a 1½-year-old animal. Each small dot represents a single multinucleate cell. Star represents the optic nerve head.

peripherally. In the quokka, the retina expands continuously at a low rate throughout adult life. One of our aims was to determine whether the enlargement of the sutured eye simply and rapidly produced the equivalent of an "old" eye. It did not. There are major differences in RPE cell topography in sutured eyes from those in the normal aging animal. In particular, retinae from sutured eyes do not display higher RPE cell densities in temporal and dorsal central regions as has been observed in aging adult retinae (of similar average cell density). In addition, although cell density in the peripheral regions of sutured retinae falls slightly, the change is slight by comparison with aging eyes and is not accompanied by the emergence of a population of heterogeneous, often multinucleate cells as it is in the mature adult.

Evidence from the present study of the quokka RPE, and that of the chick RPE (Lin et al., 1993), suggest that topographic changes within the retina may be aided by differential tissue elasticity or growth potential. For example, temporal central regions of neural retina, in the presumptive *area centralis*, are described as being less elastic than areas outside this region (Robinson, 1991; Kelling et al., 1989; Mastronarde et al., 1984). However, here we have shown that more central RPE cells, including those in the temporal quadrant, in which is located the *area centralis* (Beazley & Dunlop, 1983), are significantly larger in the sutured eye than its partner eye whereas peripheral cells are not. Therefore, it seems that in the younger retina, central regions are, in fact, most elastic, thus arguing against this idea. By contrast, we have previously shown (Fleming et al., 1996c) that retinal expansion in adult quokkas over 2 years of age takes place primarily in the peripheral retina, which therefore may be one of the most elastic regions of the eye during later life. Therefore, the RPE cell density increase in the temporal, central retina and the marked decrease in density in the periphery seen in aged animals appear not to be triggered simply by eye enlargement, but to reflect a slower, aging processes of this tissue.

The differences in topography seen in experimentally enlarged and normally aging eyes may be a result of the speed of retinal expansion. The experimentally enlarged eyes expand very rapidly when compared with the normal aging expansion in this species. A degree of enlargement similar to that induced experimentally in 9 months would normally take 4–8 years. The slow growth seen in normal aging results in RPE cells increasing their volume for which peripheral cells show the greatest tendency. By contrast, rapid, induced growth results in cells becoming larger in area but with unchanged or slightly reduced tissue thickness, a tendency which appears to be shown more by central than peripheral cells. These differences may be produced simply by variations across the retina in elasticity in young and aging RPE with the most elastic retinal region being central retina in the younger eye and peripheral retina in the aging eye.

The effect of eye enlargement upon the total number of multinucleate RPE cells

Evidence from the present study suggests that the number of multinucleate cells in the RPE may be affected by genetic predisposition, age, and to some degree cell size. In support of an intrinsic or genetic control over the formation of these cells, there is a large amount of variation in the number of multinucleate cells between individuals, whilst within individuals, left and right eyes, despite the experimental enlargement of one, have a very similar number.

It has been suggested that some multinucleate cells within the RPE may form as a response to being "stretched" (Stroeva & Panova, 1983; Fleming et al., 1996c). The resulting enlarged cell
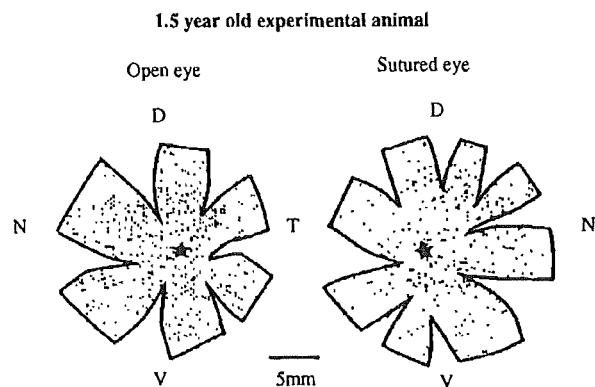
size induces a slightly greater number of cells to exceed a hypothetical "threshold" cytoplasm volume to nucleus ratio (Fankhauer, 1952; Swanson, 1969; Alberts et al., 1989) and therefore is induced to undergo nuclear division.

In our experimental model of retinal growth, enlargement of RPE cells is accompanied by no change or a slight thinning of the tissue, so that the volume of cells does not increase significantly. This consistent volume may account for the small differences in multinucleate RPE cell number between open and sutured eyes. This finding contrasts with those for the RPE in the aged adult quokka, in which the regions of large cell size at the retinal periphery are not thinner than in other parts of the eye and may even be slightly thicker (Fleming et al., 1996c). Therefore, the areal expansion of RPE cells at the retinal periphery in aged adults is accompanied by a significant increase in cell volume which provides added evidence that it is cell volume, not area, which stimulates the multinucleation process. However, other factors present during the aging process may also play a part in producing the widespread multinuclation seen in the periphery of aged adult retinae.

It seems likely therefore that eye suturing does not reproduce the pattern of eye growth seen in normal aging of the RPE for two reasons. The first is that the retinal regions more susceptible to expansion appear to be different in the slowly growing, aging eye from those in the more rapidly expanding, younger, sutured eye (Fleming et al., 1996c). The reason for this is not yet clear. However, it is possible that, for example, the relative position of the insertion of extraocular muscles alters between young and aged adulthood, or that there are regional differences in scleral thickness in the two groups of animal. Eyes from aged animals do indeed have noticeably coarser, tougher scleral coats than do young adults, as was evident to me when performing eye injections as part of other studies in this species. Possibly, this toughening of the sclera as the animal ages renders the eye capable of further expansion only in the periphery. The second is that the expansion during form deprivation is not accompanied by a volumetric increase of cells, the process which may be the trigger for multinucleation. It is also possible that the phenotype of RPE cells is altered in some way by lid suture in a manner not seen in normal conditions.

Examination of experimentally enlarged eyes will be conducted at a later age (4 years) to determine whether cell volume is in fact the trigger for multinucleation. Cells in the RPE will have had sufficient time to increase their volume as well as being stimulated to increase their area. There are two possible findings; one is that the enlarged eye will have volumetrically larger cells and will be similar to its partner eye in topography and have the same number of multinucleate cells. The other possibility is that the enlarged eye will have volumetrically larger cells but will contain considerably more multinucleated cells. This second finding would suggest that number of nuclei is linked to cell volume. The volumetrically enlarged cells may have more multiple nuclei also. We have previously shown that the number of nuclei in the RPE of individual retinae is linked to cell size (Fleming et al., 1996b). In addition, it would be interesting to determine whether changes that occur as a feature of the aged retina are enhanced, diminished, or not affected in the sutured condition. For example, it would be informative to ascertain whether the increase in cell density that is observed in temporal and dorsal central regions of aged retinae will take place in the form-deprived condition. Such experiments are in progress.

Acknowledgments

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