Retinoic acid signals the direction of ocular elongation in the guinea pig eye

Sally A. McFadden [a,*], Marc H.C. Howlett [a], James R. Mertz [b]

[a] School of Behavioural Sciences, Faculty of Science and IT, The University of Newcastle, Newcastle, NSW 2308, Australia
[b] Biological Sciences, New England College of Optometry, Boston, MA 02115, USA

Abstract

A growing eye becomes myopic after form deprivation (FD) or compensates for the power and sign of imposed spectacle lenses. A possible mediator of the underlying growth changes is all-*trans* retinoic acid (RA). Eye elongation and refractive error (RE) was manipulated by raising guinea pigs with FD, or a spectacle lens worn on one eye. We found retinal-RA increased in myopic eyes with accelerated elongation and was lower in eyes with inhibited elongation. RA levels in the choroid/sclera combined mirrored these directional changes. Feeding RA (25 mg/kg) repeatedly to guinea pigs, also resulted in rapid eye elongation (up to 5 times normal), and yet the RE was not effected. In conclusion, RA may act as a signal for the direction of ocular growth.
Crown Copyright © 2003 Published by Elsevier Ltd. All rights reserved.

Keywords: Retinoid; Myopia; Emmetropization; Growth; Rodent

1. Introduction

Newborn eyes are beset with the problem of matching their expanding size with their optical power to attain and maintain an emmetropic refractive state. Eyes which elongate too rapidly become myopic, which means that in a relaxed eye, images are focused in front of the photoreceptor plane. The rate of eye growth is visually mediated since a young growing eye will accelerate its growth and become highly myopic if deprived of form vision. Form deprivation myopia (FDM) has been demonstrated in many species, and rapidly develops if the eye is sutured or wears a diffuser (guinea pig: Lodge, Peto, & McFadden, 1994; tree shrew: Norton, 1990; marmoset: Troilo & Judge, 1993; chick: Wallman & Adams, 1987; monkey: Wiesel & Raviola, 1977). The error signal that guides this growth is likely to be related to visual blur, since animal models have shown that the eye will adjust its length to precisely compensate for the power of imposed spectacle lenses. Such spectacle lens compensation is sensitive to both the amount of the imposed blur and the sign of the defocus (macaque: Hung, Crawford, & Smith, 1995; guinea pig: McFadden & Wallman, 1995; chick: Schaeffel, Glasser, & Howland, 1988; marmoset: Whatham & Judge, 2001). Eyes which have worn positive or negative spectacle lenses, reduce or increase their elongation rates, respectively, and over time the imposed refractive error (RE) disappears. In the chick, the eye can almost completely compensate for between −10D and +15D lenses within one week (Irving, Sivak, & Callender, 1992), while the monkey can respond to ±3D over several months (Hung et al., 1995; Wallman & McFadden, 1995), and the guinea pig eye can respond to ±4D within six days (Howlett, 2003; Howlett & McFadden, 2002).

These visually mediated growth changes are reflected in concurrent changes in proteoglycan synthesis in the sclera. Manipulations which accelerate ocular growth (diffusers and negative lenses) result in decreases in proteoglycan synthesis in fibrous sclera while the opposite is true when the growth rate has been turned down (recovery from diffuser wear or positive lenses) (chick: Marzani & Wallman, 1997; Rada, Achen, & Rada, 1998; Rada, Perry, Slover, & Achen, 1999; tree-shrew: McBrien, Lawlor, & Gentle, 2000; Norton & Rada, 1995; marmoset: Rada, Nickla, & Troilo, 2000).

* Corresponding author. Tel.: +61-249-215634; fax: +61-249-216980.

E-mail address: sally.mcfadden@newcastle.edu.au (S.A. McFadden).

However, it is unknown how the visual error signal is translated to affect chemical and physical changes in the sclera. One possible mediator is all-*trans* retinoic acid (RA), since in the chick, visual conditions which enhance or inhibit eye growth result in elevated or reduced levels of retinal-RA respectively. For example, chicks which experience form deprivation (FD) have elevated retinal-RA levels in the occluded eye (Seko, Shimizu, & Tokoro, 1998). Furthermore, intravitreal injection of disulfiram, an inhibitor of RA synthesis, reduces the degree of myopia produced by FD (Bitzer, Feldkaemper, & Schaeffel, 2000). Retinal-RA levels are also changed in chicks which have worn spectacle lenses; becoming elevated with the enhanced growth that accompanies −15D lens wear or reduced in eyes that have axial growth slowed by +15D lenses (Mertz, Howlett, McFadden, & Wallman, 1999). Exactly the opposite changes in levels of RA occur in the chick choroid (Mertz & Wallman, 2000).

The chick differs from mammals and primates in that it has dual cartilaginous and fibrous layers within its sclera (Marzani & Wallman, 1997; Rada, Thoft, & Hassell, 1991), a pronounced choroidal response to defocus (Wallman et al., 1995), and responds to a much greater range of defocus (Irving et al., 1992) than is typical in mammals and primates. In this study we investigated the role of RA in the guinea pig model of eye growth. In particular, we asked whether the level of RA in ocular tissues was correlated with the refractive state and rate of growth of the guinea pig eye, by manipulating the RE and ocular elongation with diffusers and lenses, and also examined the effects on ocular elongation of feeding RA to young guinea pigs.

2. Methods

2.1. Animals and housing

Pigmented guinea pigs (*Cavia porcellus*, $n = 46$) were maternally reared with their mother until being weaned at three weeks of age, after which they were held in groups of 2–3. Animals were housed in plastic boxes ($65 \times 45 \times 20$ cm) with wire mesh lids. Boxes contained a small hiding shelf at one end ($32 \times 16 \times 14$ cm) and were lined with wood shavings. Water (supplemented with Vitamin C) and food (guinea pig pellets, hay and occasional fresh vegetables) were freely available. Lighting was provided by ceiling fluorescent tubes (36 W) on a 12 h light/dark cycle (lights on at 10 a.m.). The room temperature was 22 °C. Care and use of animals was in compliance with the ethical standards of the NSW Animal Research Act and as approved by the University of Newcastle Animal Care and Ethics Committee.

2.2. Experiment 1: endogenous levels of RA after visual manipulations

In order to determine if endogenous levels of RA in ocular tissues reflect the refractive state of the eye or its axial length, guinea pigs were raised under a variety of visual conditions designed to manipulate their RE and axial growth rate. In particular, we sought to accelerate the axial growth rate by raising guinea pigs with either a diffuser or a −4D spectacle lens, or inhibit it by raising animals with a +4D lens or allowing four days of normal vision after diffuser wear. In this latter "recovery" condition, eyes which have had an elevated growth rate from the FDM induced by a diffuser, rapidly decrease their elongation rate once the diffuser is removed (tree shrew: Gentle & McBrien, 1999; monkey: Smith, Hung, Kee, & Qiao, 2002; marmoset: Troilo & Nickla, 2000; chick: Wallman & Adams, 1987). The amount of axial elongation and RE was assessed in each animal at the end of the treatment period. The eyes of treated animals were then processed by HPLC to ascertain the levels of RA in the retina and the choroid/sclera combined.

2.2.1. Diffuser and lens wear

The diffusers were white translucent hemispheres (diameter 12 mm), and +4D and −4D lenses were made from polymethylmethacrylate (diameter 12 mm, optic zone, 10.5–11.5 mm, back optic radii, 8 mm, Gelflex, Perth, Australia). The diffusers and lenses were mounted onto a velcro lined ring, the matching arc of which was glued around one eye of the guinea pig. The technique is similar to that used routinely in chicks (Wallman & Adams, 1987; Wallman, LeDoux, & Friedman, 1978) and is detailed in Howlett (2003).

Group 1 ($n = 13$) were raised with FD from a diffuser worn over one eye from 5 to 21 days of age, and were measured on day 21. Group 2 ($n = 8$) wore a diffuser as for group 1, but the diffuser was removed on day 21 and the animal was allowed normal vision within the home cage for four days, and then measured at 25 days of age. Group 3 ($n = 5$) wore a −4D spectacle lens over one eye from 2 to 12 days of age, and were measured on day 12. Group 4 ($n = 4$) similarly wore a +4D lens on one eye. In all animals, the fellow eye was untreated and served as a matched control.

2.2.2. Measuring endogenous levels of RA in the retina and choroid/sclera

The level of RA in the retina was measured in all groups, while levels in the choroid combined with the sclera were obtained only after FD and recovery from FD (groups 1 and 2). Eyes were removed and hemisected on the line of the ora serrata. A whole mount was made from the posterior layers of each eye, the retinal and choroidal plus scleral layers were dissected apart and the pigment epithelium was carefully removed from both and dis-

carded. Procedures were carried out in ice and under dim red light. Levels of RA were obtained in tissue samples by normal phase HPLC. These methods have been previously detailed in Mertz and Wallman (2000). In some cases, in order to obtain large enough samples, it was necessary to combine tissues from multiple eyes. In particular, for retinal samples, each eye was able to be separately assayed for animals wearing lenses, while for the form deprived animals, two diffuser wearing eyes were combined, and two fellow control eyes were combined making a total of seven samples of each eye type. Similarly, in group 2, two eyes were combined for each sample. For the measurement of RA levels in the choroidal and scleral tissue combined, samples were combined from four eyes, giving three and two estimates of the level in diffuser wearing and fellow eyes in each of groups 1 and 2, respectively. The number of samples is shown in Table 1.

2.3. Experiment 2: feeding guinea pigs RA

To determine if exogenous administration of RA affected eye growth, guinea pigs were fed RA ($n = 9$) or the carrier alone (peanut oil, PO, $n = 7$) at 7, 9 and again at 11 days of age. RA and PO were given by gavage at approximately 11 a.m. Ocular parameters from ultrasound biometry and REs were measured daily for each eye from 7 to 12 days of age at approximately 10 a.m. Animals were refracted first then anaesthetized for ultrasound biometry (see below). On the three days in which animals were fed RA or PO, gavage was done immediately after the ultrasound biometry while they were still sedated.

2.3.1. Preparation and administration of RA

Guinea pigs were fed 25 mg/kg of all-*trans* retinoic acid (RA, Sigma, St. Louis, MO) mixed with 0.4 ml of PO. The RA was dissolved in diethyl ether, and added to the PO and then the diethyl ether was gently blown off with nitrogen gas resulting in a suspension. Control groups were administered 0.4 ml of PO alone. Guinea pigs were administered the appropriate substance by gavage via the mouth to the stomach, while lightly anaesthetized with 1% halothane.

2.4. Measurement of ocular dimensions

In both experiments, axial ocular dimensions were measured by a-scan ultrasonography using a transducer with peak sensitivity at 20 MHz (Panametrics Model 176599), with a 100 MHz a/d sampling board (Sonix 8100). The transducer was fitted with a 17 mm standoff filled with distilled water and sealed with a thin layer of parafilm. Ultrasound transmission gel (Parker Aquasonic 100) was placed on the outer side of the parafilm (≈3 mm thick) to couple the transducer to the cornea. Clear ultrasonic peaks were obtained from the front of the cornea (C1), the front and back of the lens (L1 and L2), the front of the retina (R) and the back of the sclera (S2) (Fig. 1A). Peaks selected for the posterior layers: The vitreal/retinal (R), choroidal/RPE (Ch) and

Table 1
Refractive error, axial length and level of RA in the retina and choroidal/scleral combined in the diffuser or spectacle lens wearing eye (treated) and their untreated control eye (fellow) in four groups with different visual manipulations

Eye	n	RE (D)	Axial length (mm)	n	Retinal-RA (µg/g wet wt)	n	Choroidal/Scl-RA (µg/g wet wt)
Group 1: form deprivation—diffusers							
Treated		-5.87 ± 0.76	8.415 ± 0.043		2.524 ± 0.054		0.820 ± 0.032
Fellow		0.77 ± 0.23	8.311 ± 0.027		1.491 ± 0.092		0.317 ± 0.015
	13	$p < 0.01$	$p < 0.01$	7	$p < 0.001$	3	$p < 0.01$
Group 2: recovery from form deprivation							
Treated		-0.26 ± 0.96	8.442 ± 0.027		0.998 ± 0.021		0.250 ± 0.020
Fellow		1.24 ± 0.34	8.435 ± 0.026		1.422 ± 0.023		0.335 ± 0.025
	8	$p = 0.134$	$p = 0.779$	4	$p < 0.001$	2	$p = 0.310$
Group 3: −4D lenses							
Treated		-2.98 ± 0.39	8.018 ± 0.085		2.502 ± 0.066		–
Fellow		2.80 ± 0.50	7.914 ± 0.066		1.550 ± 0.091		
	5	$p < 0.001$	$p < 0.05$	5	$p < 0.01$		
Group 4: +4D lenses							
Treated		1.65 ± 0.49	7.995 ± 0.111		1.012 ± 0.049		–
Fellow		2.19 ± 0.27	7.986 ± 0.127		1.480 ± 0.099		
	4	$p = 0.367$	$p = 0.765$	5	$p < 0.01$		

n, number of animals (left) or samples (right). For example, $n = 13$ means that there were 13 treated and 13 fellow eyes. The smaller n for the samples was due to the necessity to pool tissue from a number of eyes.
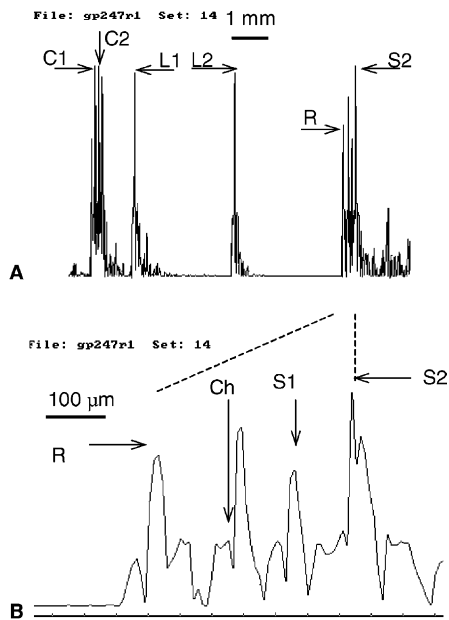
Fig. 1. Example ultrasound traces. A. Peaks corresponding to the front and back of the cornea (C1 and C2), the front and back of the lens (L1 and L2), the retinal/vitreous interface (R) and the back of the sclera (S2) are shown. B. An expanded view of the peaks arising from the posterior ocular layers. The peaks selected for the front of the retina, choroid (Ch) and the front and back of the sclera (S1 and S2) are indicated.

scleral/choroidal interfaces are shown in Fig. 1B. The validation of these peaks is described in Howlett (2003). The peaks for the posterior layers were highly consistent. Repeated measurements on 10 normal guinea pigs gave a mean standard deviation of ±1.84, ±3.95 and ±4.95 μm for the thickness of the retina, choroid and sclera, respectively and ±24.54 μm for the summed components which give rise to axial length. Thus the resolution of the ultrasound allowed us to track very small changes in the depth of the various ocular components. The axial length of the eye was designated as the anterior corneal surface to the back of the sclera. The term "anterior segment" refers to the thickness of the cornea and anterior chamber combined (C1 to L1).

Ultrasound measurements were taken while guinea pigs were lightly anaesthetized with 1% halothane in oxygen. Guinea pigs were lightly restrained while anaesthetized to allow alignment of the transducer probe centrally to the pupil along the optic axis. Positioning was aided by resting the guinea pig at the centre of a spherical device (Fig. 2). Approximately 12 measures were taken from each eye sequentially, with the probe realigned several times.

2.5. Measurement of refractive error

RE measurements were obtained to the nearest 0.25 D using streak retinoscopy in both the horizontal and vertical directions. All refractive data is presented as the
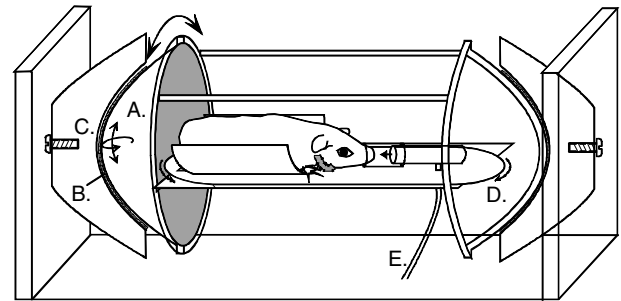

Fig. 2. Apparatus used to position the guinea pig eye at the centre of a rotating sphere. The two disk ends (A) form part of a complete sphere (the distance between the two ends is exaggerated for clarity) and could be rotated with the aid of a vaseline seal (B) between each disk and its seating mould (C). The platform under the guinea pig could also be rotated by 180° to allow access to each eye (D). The guinea pig was supported by a soft support around the neck and by the nose guard which carried the gaseous anaesthetic (E).

mean of both measures. Streak retinoscopy occurred approximately 15 min after the eyes of awake animals had been cyclopleged with two drops of 1% cyclopentolate. RE measures were taken along the pupillary axis, perpendicular to the plane of limbus. Particular care was taken to ensure that the centre of the reflex was used.

2.6. Data analysis

Data are presented as the mean ± SEM. In guinea pigs which wore lenses or diffusers, the data is often expressed as the difference between the treated and fellow eye. In order to compare RA and PO-fed guinea pigs, in the case where the initial measure on day 6 for a particular component were disparate by more than 15%, the ultrasound values for that component were standardised by calculating normalised values by dividing each daily measurement for an individual animal by the mean initial value. In the cases where standardised values are presented, raw values are also shown. The amount of daily change was calculated from the raw value for each day minus the value for the preceding day.

Statistical analyses used repeated measures mixed General Linear Model procedures (SPSS® V10.0 for Windows). Post hoc analysis was performed using Tukey's least significant difference (LSD) or Dunnett's pairwise multiple comparisons. Differences between the amounts of change that occurred between 24 h periods were analysed by two-tailed *t*-tests, and between treated and fellow eyes by paired *t*-tests.

3. Results

3.1. Experiment 1: endogenous levels of RA after visual manipulations

In summary, we found that our visual manipulations were effective in changing the RE and the amount of

ocular elongation. Of greater interest, was that the level of RA in both the retina and the choroid/sclera was tightly coupled to the sign of defocus of these visual manipulations. The direction of change in levels of RA was the same in both the retina and the choroidal/scleral fractions.

3.1.1. The effect of the visual manipulations

FD for 16 days created a difference of −6.64 D of myopia between the diffuser wearing eye and its fellow eye with a corresponding significant increase in axial length of 104 μm (Table 1). This myopia was reduced to only a −1.5 D difference between the eyes after four days of normal vision. The axial elongation in the recovering eye was sharply inhibited, since the eyes of guinea pigs in group 2 that had recovered for four days were only 27 μm greater in axial length than the form deprived eyes, yet the fellow eyes grew by 124 μm over this same 4-day period ($F = 6.824$, $p < 0.01$).

Spectacle lens wear shifted the RE and axial elongation in the direction of the imposed lens power worn. In the guinea pig, a zero powered lens worn for 10 days creates a small degree of myopia (difference between lens wearing and fellow eye is $−2.17 \pm 0.3$ D) (Howlett, 2003). In the current study, where an eye had worn a −4D lens for 10 days, it became on average $−5.8 \pm 0.6$ D significantly more myopic than its fellow eye (Table 1) which is −3.6 D more than one would expect for a zero powered lens. This 90% refractive compensation for the −4D lens was also accompanied by a significantly longer axial length (Table 1), some 104 ± 28 μm greater in the −4D lens wearing eye. In contrast, a +4D lens resulted in only a $−0.50 \pm 0.50$ D difference between the eyes, some +1.67 D more than for a zero powered lens. This hyperopic relative shift was accompanied by a significantly shorter vitreous chamber (difference of $−48 \pm 18$ μm, $p < 0.05$). Thus, compensation was almost complete for −4D lens (−3.6 D), and partial for a +4D lens (+1.7 D) and these compensatory refractive changes were accompanied by acceleration or inhibition, respectively, in the elongation of the major posterior component of axial length. These results are similar to other results on guinea pig lens compensation (Howlett, 2003; Howlett & McFadden, 2002; Wallman & McFadden, 1995).

3.1.2. The relationship between the level of RA and the direction of eye elongation

Eyes which had experienced FD or hyperopic defocus (from minus lens wear) developed a myopic RE and a longer axial length (groups 1 and 3) and had nearly double the level of retinal-RA relative to their fellow eye (+165%), while those eyes which had experienced myopic defocus (groups 2 and 4) had significantly lower levels of RA in the experimental eye relative to the fellow eye (−69%, see Table 1). The level of retinal-RA in the experimental eyes which had worn diffusers or minus lenses was also significantly higher than those
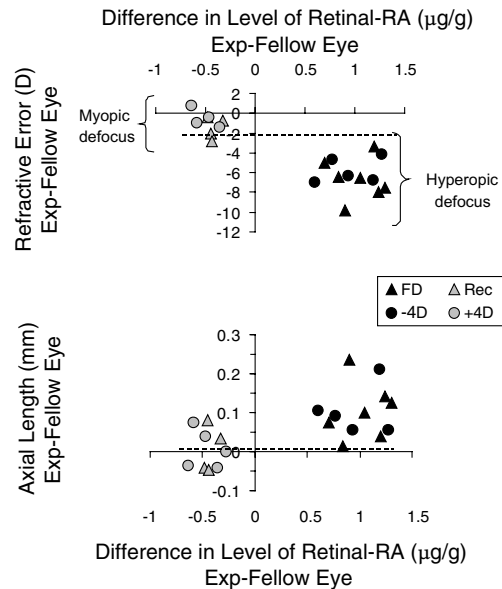

Fig. 3. Endogenous levels of retinal-RA after various visual manipulations. The correlation between the difference in RA between individual samples from treated and fellow eyes and (A) the corresponding mean RE differences and (B) the mean axial length differences between the eyes. Groups 1–4 are shown. FD, form deprivation; Rec, Recovery from FD; −4D and +4D are the spectacle lens wearing groups. The dashed lines show the RE and axial length offset for a zero powered lens (see text). A value for a particular sample consisted of the mean of the individual eyes pooled within each sample (there was no pooling required for the lens wearing eyes, see Table 1 for numbers pooled).

eyes which had worn plus lenses or which were recovering from FD ($F = 192.6$, $p < 0.001$), while the fellow eyes did not differ ($p = 0.5$). The retina appeared to be able to up-regulate the level of RA more readily than it could down regulate it. This indicates an asymmetric bi-directional difference in the level of retinal-RA that is related to the type of blur experienced (Fig. 3).

The levels of RA in the choroid/sclera were approximately 1/5 of those in the retina in the normal fellow eyes in both groups 1 and 2 (FD, 21.3%; recovery, 23% of retinal-RA level), and required greater pooling of samples to be able to discern the amount of RA above baseline. This reduced the power of our choroidal/scleral analysis. Nevertheless, it was clear that the choroidal/scleral levels of RA were significantly enhanced after FD (2.6 times greater than in the fellow eye) and were inhibited, although not significantly so with only two samples, after recovery from FD (reduced by 25%, Table 1). Like for retinal-RA, the increases were greater than the decreases. Thus, in the guinea pig, it is possible that choroidal/scleral-RA levels change consensually with the retinal-RA bi-directional changes.

3.1.3. The relationship between the level of RA and the degree of eye elongation

If one only looks at the changes on one side of the sign response, it does not appear that retinal-RA levels are

related to the degree of myopia or eye elongation. In particular, for groups 1 and 3 in which RA levels had been elevated, there was no correlation between the amount of myopia (which ranges over 6 D) or relative increase in axial length and the level of retinal-RA (Fig. 3, black symbols, $r^2 = 0.002$ and 0.02, respectively). Similarly, although severely limited by sample pooling, there is no tendency for greater levels of imposed myopic defocus or degree of inhibition in ocular elongation to induce greater increases in retinal-RA (Fig. 3, grey symbols, $r^2 = 0.24$ and 0.005, respectively). The lack of a magnitude response for a particular form of blur, reinforces the idea that the extreme difference in the direction of change in levels of RA for the opposing visual manipulations is indeed a sign response rather than some artifact of a magnitude response.

3.2. Experiment 2: feeding guinea pigs RA

Although endogenous levels of retinal-RA and choroidal/scleral-RA were clearly increased by those visual manipulations which induced both myopia and ocular elongation, given the co-dependency of these two parameters, it is unclear whether the increase in retinal-RA arises from the visual error signal, which then independently of RA, initiates ocular elongation, or whether RA might be involved in directly signalling the direction of eye growth. Thus we were curious to determine whether RA fed to guinea pigs would cause their eyes to either become myopic and/or elongate. We found that feeding RA dramatically increased ocular elongation, but did not substantially change the RE.
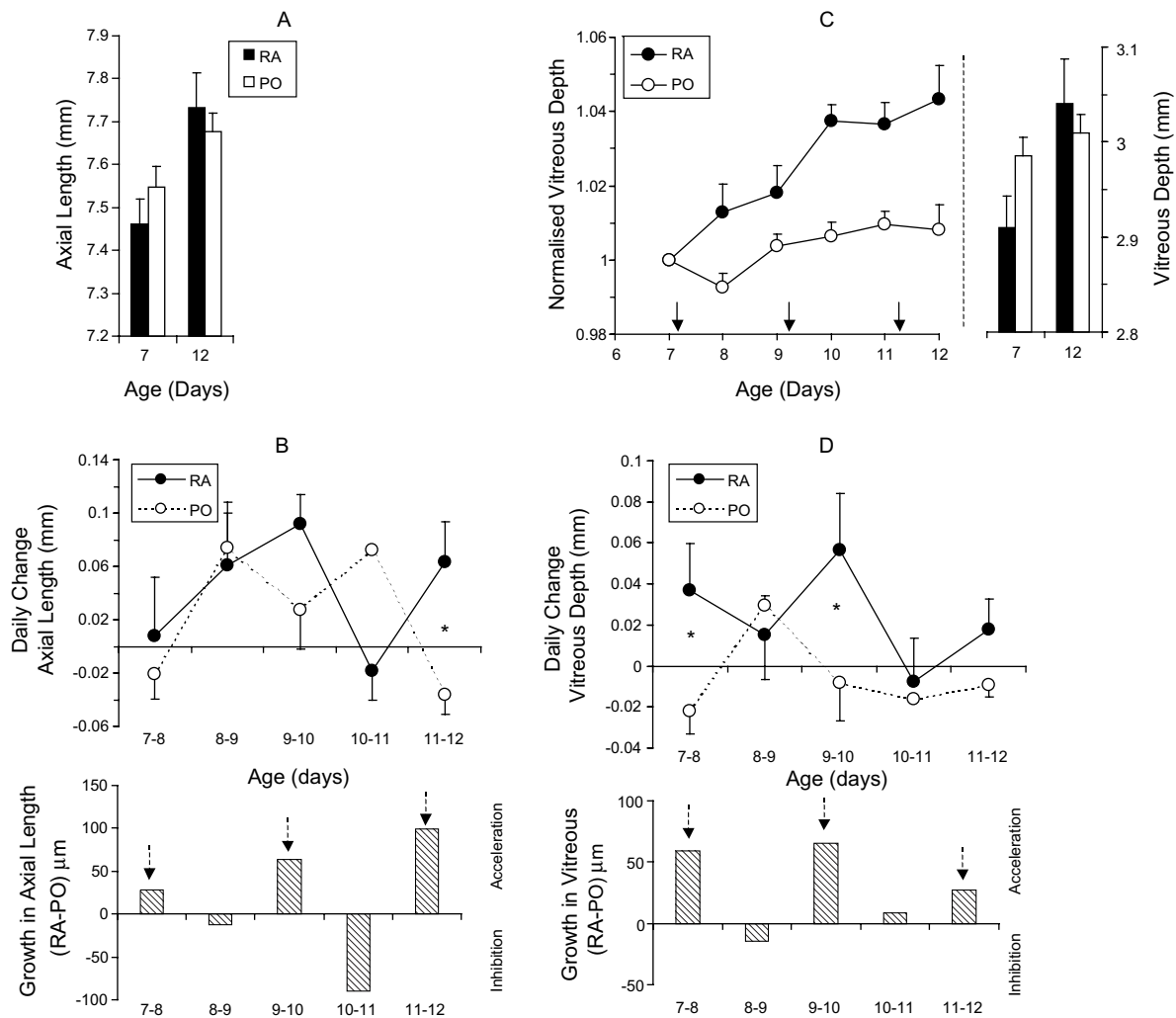

Fig. 4. Ocular elongation after feeding guinea pigs RA or PO. (A) Axial length at the beginning and end of the experimental period. (B) The daily change in axial length calculated as the 24 h difference relative to the previous day, and the difference between the daily change means for the RA and PO-fed groups (bottom). The dashed arrows indicate the increased elongation 24 h after RA was fed. (C) The change in the depth of the vitreous chamber normalized to eliminate the starting differences between RA and PO groups. Solid arrows indicate the days on which RA or PO was fed. The corresponding raw data is shown on the right. (D) The daily change in vitreous chamber depth calculated as the 24 h difference relative to the previous day, and the difference between the daily change means for the RA and PO-fed groups (bottom). * : $p < 0.5$.

3.2.1. Ocular elongation

Feeding guinea pigs RA every second day resulted in a significant increase in their axial length that was greater than in the animals fed PO (241 ± 40 and 129 ± 14 μm, respectively, $F = 6.85$, $p < 0.05$, Fig. 4A). This elongation was reflected in a significant expansion of the vitreous chamber which over the course of the six days, elongated by 139 ± 35 μm compared to the PO fed animals which only grew by 24 ± 20 μm ($F = 9.52$, $p < 0.01$, Fig. 4C). The amount of vitreous expansion was greater 24 h after each dose of RA (Fig. 4C, days 7–8, 9–10 and 11–12), and the daily change was significantly higher after each of the first two doses of RA compared to the animals fed PO (Fig. 4D). In contrast, the daily vitreous expansion was slowed to match that of the control animals, two days after RA was fed (days 9 and 11, Fig. 4D). A similar pattern also occurred in the rate of ocular enlargement, where the daily change in axial length was greater 24 h after each dose of RA on days 8, 10 and 12 and was equal to or below that of PO on the intervening days (RA–PO: at 24 h, $+62 \pm 20$ μm; at 48 h, -51 ± 39 μm, Fig. 4B, bottom). Thus a single dose of RA increased the rate of eye elongation for the first 24 h. This enhanced elongation was maintained and the growth rate returned to normal until another dose of RA was given, wherein, the rate again increased relative to the control animals. As a result, over six days, the axial length in RA-fed animals elongated nearly twice as much as the control animals, and became 112 μm longer than expected, while the vitreous chamber increased by over five times the normal rate.

3.2.2. Refractive error

Guinea pigs, like other species, are normally hyperopic at birth (maximum of +8 D), and emmetropise over the first 30 days of life (Howlett, 2003; Lodge et al., 1994). Surprisingly, despite the doubled rate of ocular elongation, the animals fed RA had an orderly and normal progression in RE reduction (Fig. 5), and did not differ from the PO controls between 8 and 12 days of 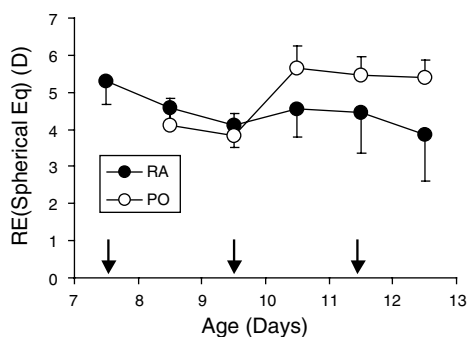age (RA–PO RE, 0.55 D, $p = 0.13$). In Experiment 1, an increase of 104 μm in axial length, resulted in a myopic refractive shift of over -6 D. In contrast, our RA-fed guinea pigs had increased their axial lengths by 112 μm, without any accompanying change in RE.

3.2.3. Changes in the other ocular components

Changes in ocular elongation without corresponding changes in RE could be achieved if (1) the choroid and sclera thickened by the same amount as the eye elongated and pushed the retina forward, (2) the eye simply grew proportionally or (3) the optical components decreased their power. We did not find any evidence for choroidal or scleral expansion, instead these structures were thinner. We have no evidence bearing on whether RA caused a overall increase in eye size. For the optical components to lose power, the curvature of the cornea would need to flatten or the lens would need to thin or reduce its refractive index. We find limited evidence of changes in the anterior segment, but do not know whether these changes were associated with a reduced optical power.

3.2.4. Lens and anterior segment

Feeding RA caused an initial shrinking in the crystalline lens by 15 μm, but this was not significant and was not sustained over the six days (Fig. 6A). Over the course of the experiment, the lens in the PO-fed animals increased in thickness by 24 μm, while that in the RA-fed animals grew by 63 μm. On the other hand, the anterior segment, appeared to be effected by RA, increasing in depth 24 h after a dose of RA, and expanding less than control animals 48 h after RA (Fig. 6B). An expanded anterior segment could be associated with a flatter cornea, but corneal curvature would need to be measured to confirm such a possibility.

3.2.5. Retina and sclera

Eyes which elongate rapidly such as in FD, have a thinner fibrous sclera (Gottlieb, Joshi, & Nickla, 1990; Phillips & McBrien, 1995). We found that both the sclera and the retina became thinner after six days of RA dosing compared to the PO-fed animals (retina: -10.1 vs. -3.1 μm; sclera: -14.1 vs. $+6.1$ μm, respectively, SEM $= 0.1$ μm in all cases, Fig. 6C and D). Over the course of the six days, these changes were significant (standardized retina: $F = 7.26$, $p < 0.05$; standardized sclera: $F = 13.87$, $p < 0.05$). It is interesting to note that the thinning of the sclera was delayed until two days after the first dose of RA ($p < 0.05$).

4. Discussion

We found that visual manipulations which caused myopia and axial elongation (FD and minus lenses) also resulted in nearly a doubling (+165%) in the levels of

Fig. 5. Refractive error in guinea pigs fed either RA or PO. Solid arrows indicate the days on which RA or PO was fed.
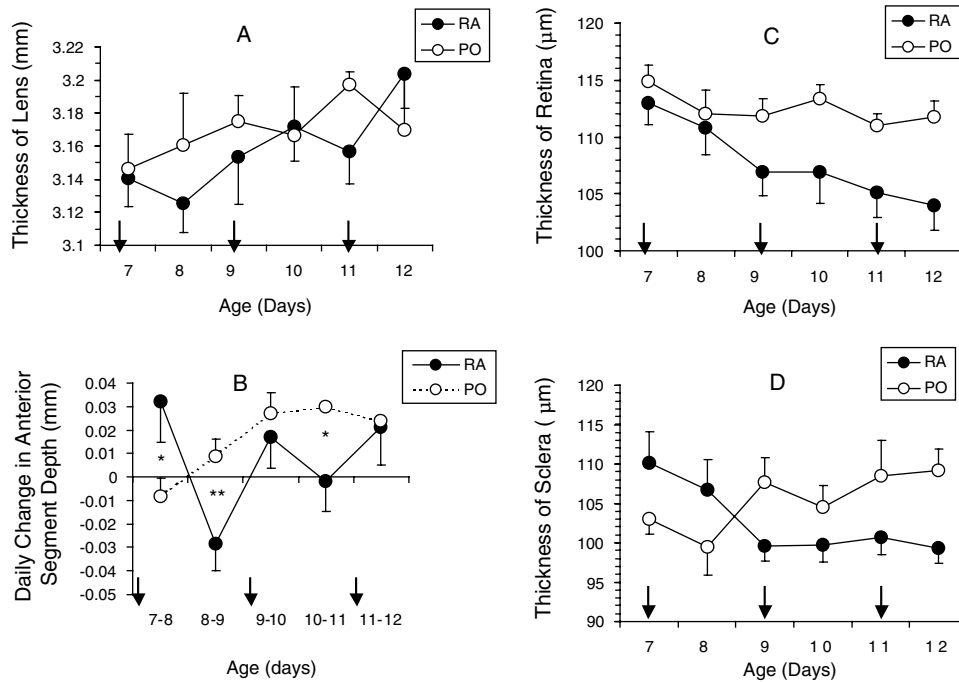
Fig. 6. Changes in the thickness of various ocular components after feeding guinea pigs RA or PO: (A) crystalline lens; (B) daily change in the anterior segment calculated as the 24-h difference relative to the previous day; (C) retina; and (D) sclera. Solid arrows indicate the days on which RA or PO was fed.

retinal-RA, while those that imposed myopic defocus (recovery from FD and positive lens wear) almost halved the retinal-RA (−69%). Our results show that there is a bi-directional change in retinal-RA which is sensitive to the sign of imposed defocus and the direction of ocular elongation in a mammal. Interestingly, although the level of retinal-RA is highly sensitive to the direction of ocular change, we argue that it is not modulated as a function of the degree of elongation or myopia.

Although the endogenous levels of RA in choroid/sclera fractions were 1/5 that of the retina, we also found that FD was associated with significant increases in the level of RA in the choroid/sclera while in eyes which were recovering from FD, the levels of RA were decreased, suggesting that unlike the chick (Mertz et al., 1999), the direction of change of RA in the choroid/sclera is identical to that in the retina.

These results pose two problems: Firstly, although endogenous levels of retinal-RA and choroidal/scleral-RA were clearly increased by those visual manipulations which induced both myopia and ocular elongation, given the co-dependency of these two parameters, it is unclear whether the increase in retinal-RA arises from the visual error signal, which then independently of RA, initiates ocular elongation, or whether RA might be involved in directly signalling the direction of eye growth. Secondly, we need to distinguish between whether the retinal-RA or the choroidal/scleral-RA is

the primary signal. We will first discuss the functional significance of the RA-signal, before turning to each of these two issues.

4.1. The functional significance and characteristics of a bi-directional RA signal

The endogenous level of RA could be either up- or down-regulated relative to the baseline level. Thus the response is clearly bi-directional. We argue that the stimulus for that response is also bi-directional. The two candidates are either the sign of defocus or the direction of change in the rate of elongation. We have argued below that it is more likely to be the latter. It is clear that both the FD and −4D lens groups significantly accelerated their growth (Table 1). Conversely, the eyes which had recovered from FD must have sharply inhibited their elongation rate to achieve an identical axial length between the eyes. More complex, is the response of the eyes which wore a +4D lens. There are two issues related to their response. First, in guinea pigs, a zero powered lens causes a small acceleration in ocular elongation (10 ± 3 μm, Howlett, 2003), and the +4D lens response is inhibited relative to this background elongation rate. Second, the vitreous expansion is significantly reduced in the lens wearing eye, but when measuring axial length we see little or no change because this inhibition in posterior elongation is offset by an increase in the anterior segment (Howlett, 2003;

McFadden & Wallman, 1995). This suggests that there is also a bi-directional change in the rate of elongation, which matches that in the RA signal.

In contrast, we found no evidence that the amount of increase in the level of RA after FD or negative lens wear was related to the amount of increase in axial length ($r^2 = 0.01$, $p = 0.67$, Fig. 3B, black symbols) or increase in vitreous chamber depth ($r^2 = 0.01$, $p = 0.69$). Similarly, this is true for the inhibited eyes (difference between the experimental and fellow eyes for axial length and vitreous depth was not significantly correlated with the retinal-RA changes, $p = 0.6$ for both). However, we cannot comment on whether the choroidal and/or scleral-RA levels are modulated by the magnitude of either defocus or ocular elongation rate.

If our retinal-RA signal is sign, but not magnitude dependent, it is similar to the another feasible candidate that may signal defocus. In the chick, imposed myopic and hyperopic defocus have opposing rapid effects upon glucagon amacrine cell activity, as indicated by ZENK expression (Bitzer & Schaeffel, 2002; Fischer, McGuire, Schaeffel, & Stell, 1999) or glucagon mRNA levels (Feldkaemper, Wang, & Schaeffel, 2000). Within 2 h, ZENK expression is enhanced by positive lenses or recovery from FD and inhibited by FD and minus lenses. These glucagon containing amacrine cells are sensitive to the direction of change in ocular growth but not its magnitude (Bitzer & Schaeffel, 2002). These results are complicated in that, regardless of the sign of defocus, ZENK expression is also elevated in the untreated fellow eye (Bitzer & Schaeffel, 2002; Fischer et al., 1999). However, it is interesting to note that, like the glucagon immunoreactive amacrine cells, amacrine cell populations immunoreactive for retinoic acid binding proteins also survive treatment with quisqualic acid (Fischer, Seltner, Poon, & Stell, 1998), as does spectacle lens compensation (Diether & Schaeffel, 1999).

4.2. Is the RA signal a by-product of blur or does it initiate ocular elongation?

It is unlikely that the bi-directional change in endogenous levels of retinal-RA was only a by-product of the direction of the retinal error signal associated with the type of blur imposed on an eye, independent of the ocular consequences of this blur, since when we fed RA to normal guinea pigs, the eyes rapidly elongated. It is possible that boosting the availability of retinal-RA through feeding, imitated the visual error signal associated with FD or hyperopic defocus, and indirectly accelerated eye elongation. However, it would be surprising if the visual error signal was not magnitude dependent. Further, the speed of the changes from fed-RA were too rapid to support this idea. Eyes on average elongated over 60 μm within 24 h, while the average rate

of change for an imposed visual error signal was only 1/6 as fast (FD: 6.5 μm/day; −4D lens: 10.4 μm/day).

Furthermore, the visual error signal is unlikely to be the same for a diffuser and a −4D lens. Visual degradation from a diffuser will reduce contrast and filter high spatial frequencies, while a minus lens will primarily modify the magnification of the point spread function. In the chick, the response of the eye to these two error signals has different temporal properties, being more rapid for a negative lens than a diffuser (Kee, Marzani, & Wallman, 2001). Also, some treatments which stop or attenuate FDM do not effect the compensatory response of the eye to negative lenses, for example, constant light, 6-hydroxy-dopamine and stroboscopic illumination (Bartmann, Schaeffel, Hagel, & Zrenner, 1994; Kee et al., 2001; Schaeffel, Hagel, Bartmann, Kohler, & Zrenner, 1994). Optic nerve section also severely attenuates the myopia induced by negative lenses (Wildsoet & Wallman, 1995) but has less influence on the development of FDM (Troilo & Wallman, 1991). If similar disparities were true for the guinea pig, it may be simplistic to assume that there is a single retinal error signal based on one biochemical parameter for both FD and negative lenses. Yet, the endogenous changes we found in the level of retinal-RA was remarkably similar for FD and negative lenses.

In contrast, if RA is pertinent at a later stage of the biochemical cascade involved in the modification of eye growth, and RA-levels signal whether the eye should stimulate or suppress eye elongation, then we might expect that artificially boosting the level of RA in ocular tissues by feeding guinea pigs RA, would imitate some component of the cascade involved in stimulating eye elongation (rather than suppressing it) as indeed we found. We might have expected this rapid elongation (115 μm increase in vitreous chamber depth) to have resulted in a myopic refractive shift. Both FD and −4D lens wear increased axial length by over 104 μm, and the RE changed by between 5 and 6 D (Table 1). We did not find such a myopic shift after RA-initiated eye elongation. Fed-RA also causes substantial eye elongation in the chick, without a change in RE (Mertz et al., 1999). It is possible that the fed-RA influenced the refractive measures through some unknown effect, such as by changing the refractive index of the vitreous or the reflectivity of the fundus, although we did not see any difference in the strength of the reflex. The fact that the RE remained unchanged, suggests that other changes, possibly optical, offset this elongation, and that the retina does not treat it as a normal blur signal. If it did, the eye would have become myopic.

4.3. The effect of fed-RA on the levels of RA in ocular tissues

RA is delivered by serum albumin in normal tissues and when we fed guinea pigs RA, we would expect to find

elevated levels in both the retina and choroid. When a single dose of 24 mg/kg of RA was fed to chicks by gavage, the levels of RA were doubled within the retina and increase by over 70 times the endogenous levels in the choroid (Mertz et al., 1999). It is extremely likely that feeding RA to guinea pigs, also elevated its levels in the ocular tissues of this species. Furthermore, RA must have reached the ocular tissues, since we found impressive consequences on eye elongation, and this stimulation was as one would predict based on our finding that visual manipulations that enhanced eye growth, also elevated naturally occurring RA-levels. Nevertheless, it would be useful to extend our results by studying the dose-response and temporal characteristics of endogenous levels of RA after feeding guinea pigs RA.

In our guinea pigs, artificial enhancement of RA through feeding, thus by-passing the natural retinal error signal, led to a doubling in the rate of ocular elongation, primarily by expansion of the vitreous chamber. Thus, RA is able to directly initiate changes in eye size. Whether these changes are the same as the physiological changes in the sclera that accompany visual perturbations, remains to be seen.

4.4. RA may affect eye growth through the choroid, rather than directly acting on the sclera in mammals

We have shown that RA is elevated in both the retina and sclera/choroid of the guinea pig eye during visually mediated ocular growth. We know that visually induced RE changes, in both chicks and mammals, involves active remodeling of the scleral extracellular matrix. In chicks the sclera consists of two layers, an outer fibrous layer and an inner cartilaginous layer (Marzani & Wallman, 1997; Rada et al., 1991). Unlike the chick, the sclera in eutherian mammals typically consists only of a fibrous layer (Rada, Matthews, & Brenda, 1994). The visually induced changes seen in the chick's fibrous layer is thought to be analogous to the alterations that occur in the mammalian sclera. As occurs in the fibrous layer of the chick sclera, proteoglycan synthesis of the mammalian sclera decreases during conditions that accelerate the rate of ocular elongation and increases when the rate of elongation is slowed (tree shrew: McBrien et al., 2000; Norton & Rada, 1995; marmosets: Rada et al., 2000). Increased eye growth also reduces the rate of collagen accumulation and increased scleral levels of matrix metalloproteinase-2 (MMP-2). During recovery from induced myopia, proteoglycan synthesis increases, the level of active gelatinase A is lower and mRNA levels of tissue inhibitors of metalloproteinases-1 (TIMP-1) are elevated (Guggenheim & McBrien, 1996; McBrien et al., 2000).

There is evidence to suggest that RA may play a role in the visual regulation and remodeling of the chick sclera. In particular, the choroid synthesizes and secretes large amounts of RA; this choroidal-RA secretion is inversely related to the rate of ocular elongation; and it is capable of being translocated to the nuclei of scleral cells (Mertz & Wallman, 2000). Extracellular matrix synthesis and ECM degradation are to some degree regulated by RA, as RA alters the expression of collagen, laminin, metalloproteinases and their inhibitors. In general expression of metalloproteinases is repressed by RA whereas RA increases the steady-state levels of the mRNA for TIMPs (see Gudas, Sporn, & Roberts, 1994, for review).

Given that increased eye growth in the tree shrew is associated with increased scleral levels of MMP-2 whereas during recovery from induced myopia, proteoglycan synthesis increases, the level of active gelatinase A is lower and inhibitors of TIMP-1 are elevated, if the same were true for the guinea pig, elevated levels of scleral-RA should down-regulate scleral growth. We found exactly the opposite effect in our choroidal/scleral samples, whereby FD elevated and recovery from FD down-regulated the levels of choroidal/scleral-RA. Thus it seems unlikely that RA is acting directly as a growth modulator at the level of the sclera in the guinea pig. It is possible that the changes in our choroidal/scleral fractions were dominated by the RA-levels in the choroidal component, and choroidal-RA may produce more complex changes in the phenotype of the choroid (which are possibly different to that in the chick) which then indirectly alter scleral gene expression.

Although the mechanisms of our observations regarding the influence of RA in modulating ocular elongation remain obscure, we can conclude that in the guinea pig, ocular levels of RA in the retina and the choroid, are stimulated by visual conditions which enhance ocular elongation and inhibited by those which reduce the rate of ocular growth in a bi-directional sign dependent manner. Furthermore, feeding RA to a young growing mammalian eye rapidly enhances ocular elongation, but in a way which incorporates other ocular compensatory changes so that the RE remains unchanged.

References

Bartmann, M., Schaeffel, F., Hagel, G., & Zrenner, E. (1994). Constant light affects retinal dopamine levels and blocks deprivation myopia but not lens-induced refractive errors in chickens. *Visual Neuroscience, 11*, 199–208.

Bitzer, M., Feldkaemper, M., & Schaeffel, F. (2000). Visually induced changes in components of the retinoic acid system in fundal layers of the chick. *Experimental Eye Research, 70*, 97–106.

Bitzer, M., & Schaeffel, F. (2002). Defocus-induced changes in ZENK expression in the chicken retina. *Investigative Ophthalmology & Visual Science, 43*, 246–252.

Diether, S., & Schaeffel, F. (1999). Long-term changes in retinal contrast sensitivity in chicks from frosted occluders and drugs: Relations to myopia? *Vision Research, 39*, 2499–2510.

Feldkaemper, M. P., Wang, H. Y., & Schaeffel, F. (2000). Changes in retinal and choroidal gene expression during development of refractive errors in chicks. *Investigative Ophthalmology & Visual Science, 41*, 1623–1628.

Fischer, A. J., McGuire, J. J., Schaeffel, F., & Stell, W. K. (1999). Light- and focus-dependent expression of the transcription factor ZENK in the chick retina. *Nature Neuroscience, 2*, 706–712.

Fischer, A. J., Seltner, R. L., Poon, J., & Stell, W. K. (1998). Immunocytochemical characterization of quisqualic acid- and N-methyl-D-aspartate-induced excitotoxicity in the retina of chicks. *Journal of Comparative Neurology, 393*, 1–15.

Gentle, A., & McBrien, N. A. (1999). Modulation of scleral DNA synthesis in development of and recovery from induced axial myopia in the tree shrew. *Experimental Eye Research, 68*, 155–163.

Gottlieb, M. D., Joshi, H. B., & Nickla, D. L. (1990). Scleral changes in chicks with form-deprivation myopia. *Current Eye Research, 9*, 1157–1165.

Gudas, L. J., Sporn, M. B., & Roberts, A. B. (1994). Cellular biology and biochemistry of the retinoids. In M. B. Sporn, A. B. Roberts, & D. S. Goodman (Eds.), *The retinoids* (pp. 443–520). New York: Raven Press.

Guggenheim, J. A., & McBrien, N. A. (1996). Form-deprivation myopia induces activation of scleral matrix metalloproteinase-2 in tree shrew. *Investigative Ophthalmology & Visual Science, 37*, 1380–1395.

Howlett, M. H. C. (2003). *The visual regulation of eye growth and refractive error in the guinea pig* (pp. 1–249). PhD thesis. Australia: The University of Newcastle.

Howlett, M. C., & McFadden, S. A. (2002). A fast and effective mammalian model to study the visual regulation of growth. *Investigative Ophthalmology & Visual Science, 43*, 2928 [Abstract].

Hung, L. F., Crawford, M. L., & Smith, E. L. (1995). Spectacle lenses alter eye growth and the refractive status of young monkeys. *Nature Medicine, 1*, 761–765.

Irving, E. L., Sivak, J. G., & Callender, M. G. (1992). Refractive plasticity of the developing chick eye. *Ophthalmic & Physiological Optics, 12*, 448–456.

Kee, C. S., Marzani, D., & Wallman, J. (2001). Differences in time course and visual requirements of ocular responses to lenses and diffusers. *Investigative Ophthalmology & Visual Science, 42*, 575–583.

Lodge, A., Peto, T., & McFadden, S. (1994). Form deprivation myopia and emmetropization in the guinea pig. *Proceedings Australian Neuroscience Society, 5*, 123 [Abstract].

Marzani, D., & Wallman, J. (1997). Growth of the two layers of the chick sclera is modulated reciprocally by visual conditions. *Investigative Ophthalmology & Visual Science, 38*, 1726–1739.

McBrien, N. A., Lawlor, P., & Gentle, A. (2000). Scleral remodeling during the development of and recovery from axial myopia in the tree shrew. *Investigative Ophthalmology & Visual Science, 41*, 3713–3719.

McFadden, S., & Wallman, J. (1995). Guinea pig eye growth compensates for spectacle lenses. *Investigative Ophthalmology & Visual Science, 36*, 758 [Abstract].

Mertz, J. R., Howlett, M. H. C., McFadden, S., & Wallman, J. (1999). Retinoic acid from both the retina and choroid influences eye growth. *Investigative Ophthalmology & Visual Science, 40*, 849 [Abstract].

Mertz, J. R., & Wallman, J. (2000). Choroidal retinoic acid synthesis: A possible mediator between refractive error and compensatory eye growth. *Experimental Eye Research, 70*, 519–527.

Norton, T. T. (1990). Experimental myopia in tree shrews. In G. R. Bock & K. Widdows (Eds.), *Myopia and the control of eye growth* (Vol. 155, pp. 178–194). West Sussex: John Wiley & Sons.

Norton, T. T., & Rada, J. A. (1995). Reduced extracellular matrix in mammalian sclera with induced myopia. *Vision Research, 35*, 1271–1281.

Phillips, J. R., & McBrien, N. A. (1995). Form deprivation myopia: Elastic properties of sclera. *Ophthalmic & Physiological Optics, 15*, 357–362.

Rada, J. A., Achen, V. R., & Rada, K. G. (1998). Proteoglycan turnover in the sclera of normal and experimentally myopic chick eyes. *Investigative Ophthalmology & Visual Science, 39*, 1990–2002.

Rada, J. A., Matthews, A. L., & Brenda, H. (1994). Regional proteoglycan synthesis in the sclera of experimentally myopic chicks. *Experimental Eye Research, 59*, 747–760.

Rada, J. A., Nickla, D. L., & Troilo, D. (2000). Decreased proteoglycan synthesis associated with form deprivation myopia in mature primate eyes. *Investigative Ophthalmology & Visual Science, 41*, 2050–2058.

Rada, J. A., Perry, C. A., Slover, M. L., & Achen, V. R. (1999). Gelatinase A and TIMP-2 expression in the fibrous sclera of myopic and recovering chick eyes. *Investigative Ophthalmology & Visual Science, 40*, 3091–3099.

Rada, J. A., Thoft, R. A., & Hassell, J. R. (1991). Increased aggrecan (cartilage proteoglycan) production in the sclera of myopic chicks. *Developmental Biology, 147*, 303–312.

Schaeffel, F., Glasser, A., & Howland, H. C. (1988). Accommodation, refractive error and eye growth in chickens. *Vision Research, 28*, 639–657.

Schaeffel, F., Hagel, G., Bartmann, M., Kohler, K., & Zrenner, E. (1994). 6-Hydroxy dopamine does not affect lens-induced refractive errors but suppresses deprivation myopia. *Vision Research, 34*, 143–149.

Seko, Y., Shimizu, M., & Tokoro, T. (1998). Retinoic acid increases in the retina of the chick with form deprivation myopia. *Ophthalmic Research, 30*, 361–367.

Smith, E. L., Hung, L. F., Kee, C. S., & Qiao, Y. (2002). Effects of brief periods of unrestricted vision on the development of form-deprivation myopia in monkeys. *Investigative Ophthalmology & Visual Science, 43*, 291–299.

Troilo, D., & Judge, S. J. (1993). Ocular development and visual deprivation myopia in the common marmoset (*Callithrix jacchus*). *Vision Research, 33*, 1311–1324.

Troilo, D., & Nickla, D. (2000). The response to form deprivation by occluders differs from that by lid suture in marmosets. *Investigative Ophthalmology & Visual Science, 41*, 134 [Abstract].

Troilo, D., & Wallman, J. (1991). The regulation of eye growth and refractive state: An experimental study of emmetropization. *Vision Research, 31*, 1237–1250.

Wallman, J., & Adams, J. I. (1987). Developmental aspects of experimental myopia in chicks: Susceptibility, recovery and relation to emmetropization. *Vision Research, 27*, 1139–1163.

Wallman, J., LeDoux, C., & Friedman, M. B. (1978). Simple devices for restricting the visual fields of birds. *Behavior Research Methods and Instrumentation, 10*, 401–403.

Wallman, J., & McFadden, S. (1995). Monkey eyes grow into focus. *Nature Medicine, 1*, 737–739.

Wallman, J., Wildsoet, C., Xu, A., Gottlieb, M. D., Nickla, D. L., Marran, L., Krebs, W., & Christensen, A. M. (1995). Moving the retina: Choroidal modulation of refractive state. *Vision Research, 35*, 37–50.

Whatham, A. R., & Judge, S. J. (2001). Compensatory changes in eye growth and refraction induced by daily wear of soft contact lenses in young marmosets. *Vision Research, 41*, 267–273.

Wiesel, T. N., & Raviola, E. (1977). Myopia and eye enlargement after neonatal lid fusion in 30 monkeys. *Nature, 266*, 66–68.

Wildsoet, C., & Wallman, J. (1995). Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks. *Vision Research, 35*, 1175–1194.