British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01347.x
www.brjpharmacol.org

RESEARCH PAPER

Light modulation, not choroidal vasomotor action, is a regulator of refractive compensation to signed optical blur

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Keywords
refractive compensation; optical defocus; choroidal thickness; N^G-nitro-L-arginine methyl ester; L-arginine; low frequency flicker; ERG b/d wave ON/OFF pathway function

Received
18 March 2010
Revised
19 January 2011
Accepted
2 February 2011

BACKGROUND AND PURPOSE

The nitric oxide system has two proposed sites and mechanisms of action within the ocular growth/refractive compensation platform-neuromodulatory effects on retinal physiology, and vascular/smooth muscle effects in the choroid. The relative contribution of these mechanisms are tested here with drugs that perturb the nitric oxide system and with slow flicker modulation of the ON and OFF pathways of the retina.

EXPERIMENTAL APPROACH

Intravitreal injection of saline or 900 nmol N^G-nitro-L-arginine methyl ester or L-arginine in saline was followed by monocular defocus with ±10 D lens (or no lens), from days 5–9 under standard diurnal (SD) or daytime 1 Hz ramped flicker conditions. Biometric, electrophysiological and histological analyses were conducted.

KEY RESULTS

After 4 days of SD conditions, both drugs enhanced electroretinogram (ERG) b-wave cf. d-wave amplitudes compared with saline and reduced refractive compensation to –10 D lenses. Under flicker conditions compensation to +10 D lenses was suppressed. Choroidal thinning was observed in the drug, no lens groups under SD conditions, whereas choroidal thickening was seen in most groups under flicker conditions, irrespective of refractive outcomes.

CONCLUSIONS AND IMPLICATIONS

As choroidal thickness was not predictive of final refractive compensation across any of the variables of drug, defocus sign or light condition, it is unlikely that choroidal thickness is a primary mechanism underlying refractive compensation across the range of parameters of this study. Rather, the changes in refractive compensation observed under these particular drug and light conditions are more likely due to a neuromodulatory action on retinal ON and OFF pathways.

Abbreviations

AC, anterior chamber; AL, axial length; Ampl, amplitude; b/d ratio, ratio of the amplitudes of the ERG b- and d-waves; D, dioptres; ERG, electroretinogram; FD, form deprivation; L-Arg, L-arginine; Lat, latency; LFRF, low frequency ramped flicker; L-NAME, N^G-nitro-L-arginine methyl ester; NFL, nerve fibre layer; NL, no lens; NOS, nitric oxide synthase; PBS, phosphate buffered saline; RE, refractive error; RPE, retinal pigment epithelium; SD, standard diurnal; VC, vitreous chamber depth

Introduction

Although myopia (short-sightedness) affects 30–50% of young adults, and is rapidly increasing in prevalence and severity in many urban environments worldwide (Seet *et al.*, 2001), its aetiology both in humans and animal models remains unclear. Refractive myopia occurs when an eye is too long for the optical components to focus the image on the

retina. However, surprisingly few laboratories have investigated how optical blur on the retina might directly initiate the changes in physiological fluid flow control mechanisms that result in the excessive ocular volume and abnormal axial elongation in myopia (Crewther, 2000; Liang *et al.*, 2004; Rymer and Wildsoet, 2005; Crewther *et al.*, 2006a). Rather, it is more generally assumed that inner retinal neurones are the only neural cell types capable of detecting the sign of optical defocus (Morgan, 2003; Wallman and Winawer, 2004), and of signalling such information back to the choroid and scleral coats to initiate refractive compensation. The signalling for appropriate choroidal thickness changes has usually been assumed to be communication via paracrine molecular messengers (Morgan, 2003; Wallman and Winawer, 2004; Rymer and Wildsoet, 2005) such as nitric oxide (NO) (Fujikado *et al.*, 1997; 2001; Fischer and Stell, 1999; Nickla and Wildsoet, 2004; Nickla *et al.*, 2006), although to date the source of this NO has not been described. However, Nickla and colleagues have also hypothesized that NO may play a 'modulatory' role in controlling compensatory choroidal expansion in experimentally induced myopic defocus (Nickla and Wallman, 2010). An alternative model of refractive compensation to optical defocus (Crewther, 2000) suggests that the light stimulus driven changes in the ionic (potassium, sodium chloride and calcium) environment around the photoreceptors will change the distribution of ions across the retina, choroid and sclera, and hence both directly control the rate and direction of transretinal fluid flow and thus effect appropriate vitreal growth (Crewther *et al.*, 2006b; Goodyear *et al.*, 2008; 2009; 2010). Thus, this study was designed to examine/compare the effects on refractive compensation and choroidal thickness of pharmacological modulation of the NO system and its interaction with physical modulation of outer retinal ON and OFF pathways by flickering light.

Nitric oxide is a gaseous signalling molecule synthesized from the enzyme nitric oxide synthase (NOS), which converts L-arginine (L-Arg) to NO and citrulline. NO is an important molecular messenger in many physiological and pathological processes, particularly in functions associated with vascular, immune and/or neurotransmitter and neural activities (Bredt *et al.*, 1990; Goldstein *et al.*, 1996; Edwards *et al.*, 1998; Cudeiro and Rivadulla, 1999; Schmetterer and Polak, 2001; Toda and Nakanishi-Toda, 2007; Chai and Lin, 2008). NO is also known to be involved in the integration of neural activity with ion channels, ATP and cGMP systems, that are in turn modulated predominately by sodium and calcium activated potassium channels (Barcellos *et al.*, 2000; Chai and Lin, 2008). NOS has been found in most layers of the retina, retinal pigment epithelium (RPE), choroid and sclera of cattle, rat and chick (Yamamoto *et al.*, 1993; Goureau *et al.*, 1994; Fischer and Stell, 1999) and most recently in the salamander (Blom *et al.*, 2009). NO also affects neuronal modulation *per se* (Kurenny *et al.*, 1994; Cudeiro and Rivadulla, 1999) at the level of photoreceptors and retinal ganglion cells (Wang *et al.*, 2003), and is known to be up-regulated in rat retina by flickering light of 3 Hz (Neal *et al.*, 1998), and to cause light-evoked release of acetylcholine in rat amacrine cells (Neal *et al.*, 1997) and bipolar cells (Neal *et al.*, 1998).

The first investigations of a possible role of the NO pathway in refractive compensation to form deprivation (FD) (Fujikado *et al.*, 1996; 1997) were based on the known modulatory effects of NO on the retinal ON bipolar cells and ON pathway (Koistinaho and Sagar, 1995; Fujikado *et al.*, 1996; 1997; Neal *et al.*, 1997; 1998). Fujikado *et al.* (1997) found that the non-specific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (Koistinaho and Sagar, 1995) altered the balance of nitrite/nitrate in retinas for at least 6 days post low-dose single intravitreal injection, and significantly inhibited the development of myopia, but not normal ocular growth in hatchling chicks. A later study also found that single injections of L-NAME inhibited the development of –16 dioptres (D) lens-induced myopia (Fujikado *et al.*, 2001). By comparison, more recent research investigating the action of the NO system in refractive compensation has focused on the possible paracrine actions of NO on choroidal vasodilatation and on intrinsic smooth muscle cells of the choroid as suggested by Fischer and Stell (Fischer *et al.*, 1999a) and Nickla and colleagues (Nickla and Wildsoet, 2004; Nickla *et al.*, 2006; Nickla and Wallman, 2010). From this viewpoint, L-NAME would be expected to further enhance the constriction of the choroidal vascular bed in experimental myopia (Liang *et al.*, 1996; 2004; Crewther, 2000; Beresford *et al.*, 2001) and hence increase the myopic refractions of form deprived animals or those with negative lens-induced myopia but, as Fujikado *et al.* (1997; 2001) showed, this does not happen.

In retrospect, Fujikado's experiments also supported earlier (Gottlieb *et al.*, 1987; Barrington *et al.*, 1989; Ehrlich *et al.*, 2007; Schwahn and Schaeffel, 1997), and later (Crewther and Crewther, 2002) evidence for a relationship between refractive compensation, flicker-induced modulation of the photoreceptors and the normal physiological environment of the outer retina in the chick. More recently, it has been shown that at lower temporal frequencies, ramped flicker (LFRF) with slowON/fastOFF characteristics at 1, 2 and 4 Hz induces a myopic shift in refractive compensation to positive lenses (Crewther *et al.*, 2006a).

Thus, in this study we used electrophysiological, biometric and histological analyses to investigate the prolonged (4 days) effect of a single intravitreal injection of an NOS inhibitor or NO enhancing drug on the compensation induced by optical defocus during standard diurnal (SD) light conditions, and under LFRF light conditions with 1 Hz temporal flicker. LFRF without drugs was expected to inhibit refractive compensation to positive lenses, but not affect refractive compensation to negative lenses or normal controls without optical defocus (Crewther *et al.*, 2006a). L-NAME was selected as the inhibitor of NOS (and hence NO production), as demonstrated previously (Fujikado *et al.*, 1997; 2001; Nickla and Wildsoet, 2004; Nickla *et al.*, 2006), and L-Arg was used to increase the production of NO and associated expansion of the choroid. The long-term effects of L-Arg and L-NAME on retinal function, 4 days post-injection, are unknown, although it is well established that acute application of such drugs induces transient effects, which can be either neuroprotective or toxic (Schmetterer and Polak, 2001) and can act transiently to dilate or constrict retinal blood vessels in rat depending on concentration (Metea and Newman, 2006).

We hypothesized that if the acute effects of L-NAME (Fujikado *et al.*, 1997) and L-Arg (Wang *et al.*, 2003), which selectively suppress retinal ON and OFF pathways, respectively, persist in the eye for the 4 day experimental period,

and, if NO-induced expansion of the choroid plays an important causal role in refractive compensation to optical defocus then, L-Arg should significantly increase refractive compensation to plus lens rearing under SD and LFRF light conditions. Furthermore, if the acute action of L-NAME is primarily exerted via inhibition of choroidal vasodilatation, the myopic shift in refraction seen with negative lenses for both light conditions would be expected to be enhanced. Alternatively, if as Fujikado hypothesized, the neuromodulatory effect of NO on the retinal ON response is more important to refractive compensation than the transient vasomotor effects of NO (Ostwald *et al.*, 1995; Goldstein *et al.*, 1996; Metea and Newman, 2006), then neither L-NAME nor L-Arg should affect the myopic shift in refractions seen with LFRF in the presence of optical defocus.

Indeed, the results demonstrated the greater importance of light modulation and relative unimportance of choroidal thickness for predicting final refraction and the direction of ocular growth in the presence of optical defocus.

Methods

Animals: rearing and drug information

A total of 207 male hatchling chicks (Leghorn/New Hampshire) obtained from a local hatchery were raised for 5 days under a 12/12 h day/night cycle in a light and temperature controlled (30 ± 0.5°C) enclosure and then randomly assigned to one of three lens [±10 D or no lens (NL)] conditions and two light conditions for Experiment 2 and 3. From days 5–9, ambient luminance was either maintained constantly at 186 Lux (SD) via a 25 W halogen light globe in the roof of the enclosure, or modulated (using the same lamp) at a frequency of 1 Hz. The minimum and maximum luminance levels were 1.83 Lux and 186 Lux respectively. This LFRF luminance profile is shown in Figure 1 and adheres to the same specifications described by Crewther *et al.* (2006a).

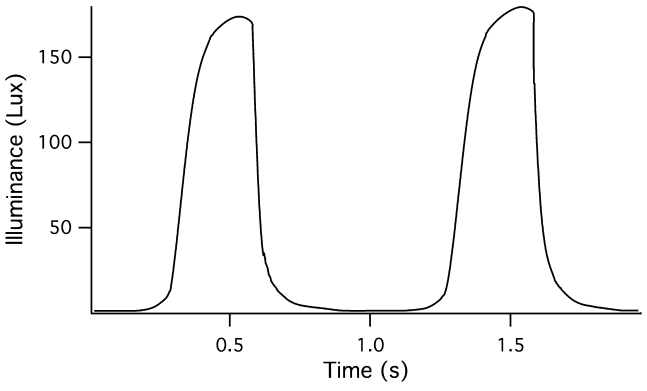

Figure 1

Illuminance profile experienced by chicks during the daytime phase of low frequency ramped flicker 1 Hz conditions. Illuminance levels (in Lux) were measured using a phototransistor calibrated with a luminance probe. The onset of illumination was characteristically slower than the offset, with approximately two-thirds of the period being near darkness.

Illuminance levels were measured using a phototransistor calibrated with a luminance probe (Tektronix, J6523 narrow-angle photometer).

On day 5, chicks were anaesthetized (in the middle of the day cycle) with a ketamine 45 mg·kg^{-1}: xylazine 4.5 mg·kg^{-1} mixture i.m. and the right eyes were intravitreally injected with either 5 µL of the carrier solution phosphate buffered saline (PBS) or 900 nmol (effective concentration) of L-NAME (Sigma-Aldrich, St. Louis, MO, USA) or 900 nmol (effective concentration) L-Arg (Sigma-Aldrich, St. Louis, MO, USA). Thus, the intravitreal dose of L-NAME used here (900 nmol), lies at the low end of the range of single doses (600–16 200 nmol) used by Fujikado *et al.* (1997) and considered by these authors to be unlikely to be toxic on the basis of changes observed following 6 days of FD. By comparison, more recent investigations have used daily injections of higher concentrations and greater volumes (Nickla *et al.*, 2006) than those demonstrated to be damaging to the retina (Fujikado *et al.*, 1997).

The dose of L-Arg chosen was also 900 nmol and was based on that used by Wang *et al.* (2003) in an *in vitro* study of retinal ganglion cells. Control intravitreal injections of 5 µL of PBS were made into the left comparative eyes. The injection volume of 5 µL (much less than the 30 µL used in previous studies (Fujikado *et al.*, 1997; 2001; Nickla and Wildsoet, 2004; Nickla *et al.*, 2006) was utilized here to reduce the acute effects of the volume of injection on intra-ocular pressure (Fujikado *et al.*, 1997). Evidence for toxicity following intravitreal injection of the concentration used here was sought both electrophysiologically and histologically (see discussion of signs of toxicity in the histology section).

Monocular defocusing goggles (±10 D) were made from modified human poly methyl methacrylate contact lenses (8.1 mm in diameter) and were attached to Velcro© and affixed to the periocular feathers for the 4 day experimental period. A NL control group was also utilized rather than plano lenses as the distance of the goggle from the eye leaves some residual defocus. The chicks and the state of cleanliness of lenses were monitored twice daily.

Biometric analysis

On day 9, chicks were anaesthetized and both eyes were refracted by retinoscopy (Reister, Jungingen, Germany) and axial dimensions were obtained from the average of at least three good A-Scan ultrasonography traces (A-Scan III, Ophthalsonic, Mentor/Teknar, Inc. St Louis, MO, USA, 7 MHz probe). Axial length was defined as the distance between the front of cornea to front of the retina.

Electrophysiology

Electroretinograms (ERGs) were recorded from NL chicks at 2 and 4 days post-injection to confirm that L-NAME (2 days $n = 3$, 4 days $n = 11$) and L-Arg (2 days $n = 3$, 4 days $n = 13$) continued to affect function of the outer retinal ON and OFF responses to light. These ERGs were compared with recordings from 12 NL chicks injected with PBS and raised in the same conditions for the same time, that is, 4 days.

Under surgical anaesthesia induced with intramuscular ketamine/xylazine (ketamine 45 mg·kg^{-1}: xylazine 4.5 mg·kg^{-1}) an intravitreal electrode (Ag/AgCl) was inserted into the superior section of the eye via a catheter placement

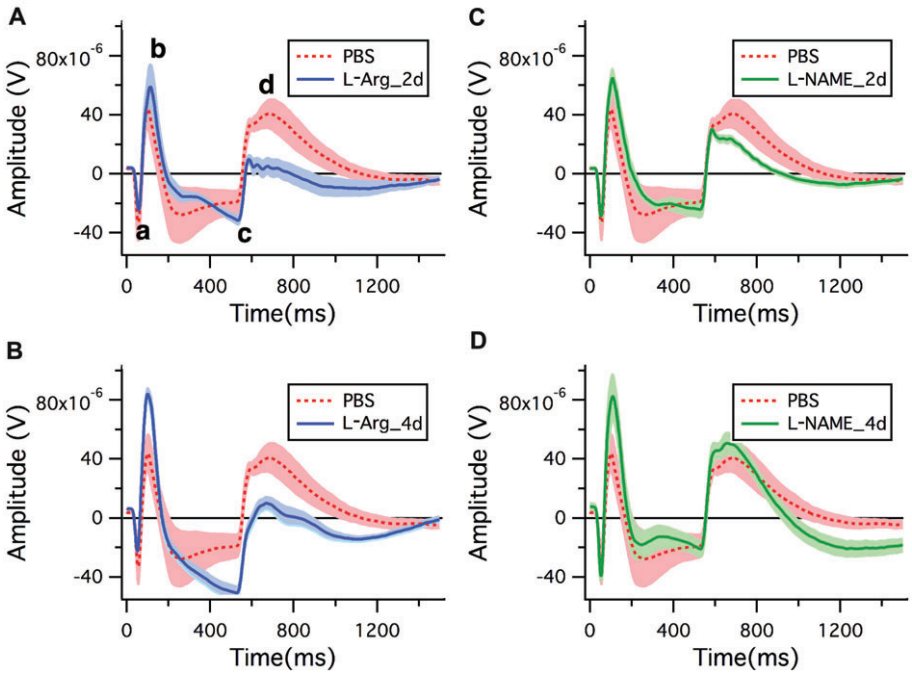

Figure 2

Electroretinogram recordings at day 9 from the experimental eyes of chicks injected with L-arginine (L-Arg), N^G-nitro-L-arginine methyl ester (L-NAME) 2 or 4 days previously, or phosphate buffered saline (PBS). Each recording is the average of 20 potentials recorded with 500 ms light on followed by light off. 'a', 'b' and 'd' waves are conventionally defined while the 'c' wave amplitude is here defined as the amplitude from baseline at light offset (N500). (A) Recordings from three chicks injected with arginine for 2 days (L-Arg 2d) compared with PBS (12 chicks, dotted line). (B) Recordings from 13 chicks injected with arginine for 4 days (L-Arg 4d) compared with PBS. (C) Recordings from three chicks injected with L-NAME for 2 days (L-NAME 2d) compared with the PBS. (D) Recordings from 11 chicks injected with L-NAME for 4 days (L-NAME 4d) compared with the PBS. The 95% confidence intervals of the waves are indicated by the shaded regions surrounding the mean waves.

unit, 5 mm temporally to midline, with scleral (nasal to midline) reference. Consistent placement of electrodes was achieved via the use of guiding arms to hold the electrode at consistent height, depth and angle, which was also verified by ophthalmoscopy. Signals were recorded (under maintained anaesthesia) via a Powerlab amplifier (ADI, Sydney, Australia) and band-pass filtered (0.3–1000 Hz). Twenty potentials were measured in each run, and five such runs were recorded for each eye. Care was taken to screen recordings for reproducibility and reliability over the 100 runs per eye.

Prior to commencement of assessable recordings, chicks were first dark adapted and then adapted to the ONset/OFFset ERG stimulus. A square wave 500 ms onset 500 ms offset light stimulation protocol [150 mm Ganzfeld stimulator with peak luminance 50 cd·m^{-2}, produced by a white light emitting diode (Luxeon III Star3W – colour temperature 5500°K), and measured using a Tektronix J6523 narrow-angle luminance probe) was employed so that ON and OFF responses could be observed separately. ERG measures included the a (photoreceptoral response to light onset), b (driven by ON bipolar activity), c (reflecting RPE conductance changes) and d (response at light offset) wave amplitudes and latencies (see Figure 2). Averages of 100 responses were taken.

Contributions to the various waves of the flash ERG were considered to be predominately drawn from outer retinal elements (Stockton and Slaughter, 1989; Sieving *et al.*, 1994; Mojumder *et al.*, 2008), and horizontal, amacrine and retinal ganglion cells do not make substantial direct contributions to the flash ERG under the light conditions used.

Histology

After the chicks had been killed on day 9, experimental and comparative left (control) eyes from each group were removed and posterior eye cups were fixed in 4% paraformaldehyde for 30 min, then washed for 5 min in PBS three times, preserved in 30% sucrose to prevent crystal formation during freezing and to inhibit cell shrinkage. Samples were cryo-sectioned prior to staining with cresyl violet and analysis under light microscopy. At least 10 slides of three sections each were concurrently prepared from each retina of both eyes of three animals from each group as a means of controlling for unwanted artefacts associated with the tissue fixation process. Thus, comparisons of pairs of eyes from at least three animals in each experimental group were made and 60 thickness measures were taken from each slide. Images were obtained using a Spot camera (Spot Flex, FX1500, Diagnostic Instruments, Inc., MI, USA) and analysed with NIH Image J software (version 10.2, US National Institutes of Health).

Prior to choroidal thickness measurements, all sections were examined for evidence of any anatomical abnormalities likely to be indicative of L-NAME- or L-Arg-induced 'tissue or cellular toxicity'. Layer thickness, including approximate transverse number and spatial distribution of cells per layer of

retina was assessed. Higher magnification inspection was made of retinal neurones and photoreceptor outer segments and individual RPE cells to check for evidence of hyper or hypo-osmotic cell profiles, nuclei with irregular profiles, or apparent holes or vacuoles, indicative of cellular dehydration or oedema as these are the classically accepted signs of generalized toxicity and cell damage (see Liang *et al.*, 1996; 2004 for examples of these signs).

All procedures were conducted in strict accordance with La Trobe University Animal Ethics Committee guidelines and adhere to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research. Drug/molecular target nomenclature conforms to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2009).

Statistical analysis

Data were first analysed to ensure no statistical differences existed due to the effect of experimental drug manipulation on the left (control) eyes. Biometric data are presented as the difference between experimental (right) and left (control) eyes (i.e. R-L). Difference measures were analysed via a series of three-way (2 light × 2 drug × 3 lens) ANOVA with an alpha level set at 0.05. Significant effects were further explored through simple main effects analysis, followed by either Student–Newman–Keuls or Games–Howell *post hoc* testing when appropriate.

Choroidal thickness data were obtained using Image J analysis software (U. S. National Institutes of Health, Bethesda, MA). ANOVA was then utilized to determine whether any lens, light or drug effects existed (alpha = 0.05) for choroidal thickness measures.

Results

Experiment 1: duration of action of L-NAME and L-Arg as indicated by electroretinography

Grand mean average waves (with 95% confidence intervals) from the ERG recordings were obtained by averaging across the multiple recordings from the chicks in each of the PBS, L-NAME and L-Arg groups 2 and 4 days post-injection (Figure 2).

The results demonstrate the persistence of altered outer retinal function 2 and 4 days post L-NAME and L-Arg injection (see Figure 2) as deviations in the grand mean averages across runs for L-NAME compared with PBS and for L-Arg compared with PBS. The main features of the ERGs analysed were the N50 (a-wave peak), the P105 (b-wave peak), the response at light off (N500 – a surrogate measure of c-wave amplitude) and the d-wave peak (P670) and are shown in Table 2. The most obvious departure from PBS controls is the behaviour of the ERG at light offset for L-Arg ($P = 0.0002$) at both 2 and 4 days, with the L-Arg N520 amplitude being significantly more negative than either PBS or L-NAME on both days. ANOVA of the latencies and amplitudes of the peaks of the ERG also show significant differences in the a- and b-wave amplitudes ($P < 0.05$). Both L-Arg and L-NAME eyes showed increased mean b-wave amplitudes in *post hoc* comparison with PBS-injected eyes at both time points. While the d-wave amplitude for L-NAME was similar to that of PBS, this amplitude was reduced for L-Arg. In order to better control for stimulus and electrode position generated variation in amplitude between recordings, a within-recording ratio of b-wave to d-wave amplitude was calculated. The mean b/d ratios found on days 2 and 4 were L-Arg: 1.95 and 1.76; L-NAME: 1.95 and 1.67; and PBS: 1.37 respectively. A significant main effect for drug condition was found ($P = 0.015$) in the b/d wave ratio, while *post hoc* testing indicated that there was no significant difference in the b/d wave ratios between L-Arg and L-NAME, although both were significantly different from that for PBS (Fisher's protected least significant difference, L-Arg, PBS: $P = 0.009$; L-NAME, PBS: $P = 0.019$). Although the temporal characteristics of our testing regime were insufficient to allow full development of the RPE c-wave it is obvious from Table 1 that there is a c-wave amplitude difference at 4 days between L-Arg and PBS. Lastly, peak latencies also showed small but significant differences (see Table 1, Figure 2). *Post hoc* tests indicate that the latency of the b-wave peaked for L-Arg at 2 days and L-NAME at 4 days and differed from the PBS controls

Experiment 2: interaction between refractive compensation to optical defocus and varied light conditions following treatment with L-NAME

As expected, eyes injected with 5 µL PBS showed good refractive compensation to applied optical defocus under 12 h day/12 h night SD conditions. Also, as previously reported (Crewther *et al.*, 2006a), 1 Hz slow ON/fast OFF ramped flicker LFRF during the day period inhibited refractive compensation to positive lens defocus while there was little effect on refraction of the negative and NL groups.

Examination of Table 2 and Figure 3A indicates that under SD light conditions a single injection of 5 µL 900 nmol L-NAME in PBS resulted in no unexpected changes in refractive compensation in the positive lens or NL groups, but in a significant reduction in the response to negative lenses. By contrast, under LFRF conditions, negative defocus showed a 5 D myopic shift while refractive compensation to positive lenses was suppressed by ~6.5 D in comparison to L-NAME + SD. No significant change in refraction was observed in the NL conditions. Axial length (cornea to proximal retina) measures revealed that the typical reduction of elongation did not occur under LFRF conditions in the presence of either L-NAME or PBS. This is consistent with the less hyperopic refractions obtained and depicted in Figure 3A,B. Chicks reared under LFRF wearing negative lenses and injected with L-NAME showed the same degree of elongation as those with PBS under SD conditions. For vitreous chamber depth the pattern of the growth for positive lens wearing groups, closely reflected the refractive changes measured (Figure 3C). With the exception of positive lens-treated chicks, L-NAME generally led to shallower anterior chamber depths in comparison to PBS groups in SD conditions (Figure 3D).

Significant effects for measures of refractive state, axial length, vitreous chamber depth and anterior chamber depth are depicted in Table 3. For all measures except anterior chamber depth, significant main effects were found for lens condition and light, with interaction effects observed

Table 1

Mean electroretinogram wave Lat and Ampl for NL L-NAME (2 and 4 days), L-Arg (2 and 4 days) and PBS (4 days)

Treatment	Days	a Wave Lat (ms)	Ampl (μV)	b Wave Lat (ms)	Ampl (μV)	c Wave Lat (ms)	Ampl (μV)	d Wave Lat (ms)	Ampl (μV)	b/d Ratio
L-NAME NL	2	54.8 (±0.98)	−34.2 (±0.30)	106.4 (±2.10)	99.6 (±0.70)	526.3 (±1.27)	−24.4 (±0.53)	596.4 (±6.33)	56.6 (±0.56)	1.90 (±0.13)
L-NAME NL	4	54.4 (±1.23)	−51.9 (±0.43)	111.0$^{\#1}$ (±2.06)	129.0*^{1} (±1.63)	528.0 (±1.45)	−21.3 (±0.52)	625.2 (±10.63)	75.7 (±0.62)	1.67 (±0.16)
PBS	4	53.7 (±0.60)	−40.7 (±0.60)	100.6$^{\#3}$ (±1.22)	87.1*^{3} (±1.01)	523.2 (±1.08)	−20.0 (±0.36)	651.1 (±10.76)	66.7 (±0.83)	1.37$^{@}$ (±0.12)
L-Arg NL	2	56.9 (±0.66)	−29.1 (±0.30)	111.0$^{\#2}$ (±1.07)	84.7 (±1.44)	532.6 (±1.15)	−31.7 (±0.29)	617.7 (±15.21)	44.9$^{\wedge}$ (±0.25)	1.95 (±0.36)
L-Arg NL	4	50.7 (±0.55)	−30.5 (±0.22)	103.9 (±2.72)	106.3*^{2} (±1.24)	525.9 (±0.92)	−51.3 (±0.41)	671.1 (±7.42)	66.0 (±0.25)	1.76 (±0.11)

Note: *^{1} and *^{2}significantly different to *^{3}; $^{\#1}$ and $^{\#2}$significantly different to $^{\#3}$; $^{\wedge}$significantly different to all other d-wave Ampls; $^{@}$significantly different to all other b/d ratios.
Ampl, amplitude; L-Arg, L-arginine; Lat, latency; L-NAME, N^{G}-nitro-L-arginine methyl ester; NL, no lens; PBS, phosphate buffered saline.

Table 2

Experimental group numbers and mean refraction (D) and axial length (mm) of right and left eyes for each lens, drug and light condition

		SD					LFRF				
		n	Exptl eye RE	Control eye RE	Exptl eye axial length (mm)	Control eye axial length (mm)	*n*	Exptl eye RE	Control eye RE	Exptl eye axial length (mm)	Control eye axial length (mm)
−10 D	L-NAME	12	−5.98*^{1} (±1.46)	−0.20 (±0.35)	8.98 (±0.12)	8.69 (±0.08)	10	−11.08*^{1} (±1.63)	0.15 (±0.55)	9.05 (±0.07)	8.74 (±0.06)
	PBS	14	−8.31 (±0.70)	0.81 (±0.42)	8.97 (±0.09)	8.77 (±0.06)	9	−8.71 (±1.11)	−0.90 (±0.46)	8.96 (±0.11)	8.69 (±0.00)
	L-Arg	11	−6.07$^{\wedge 1}$ (±1.75)	−0.32 (±0.33)	8.91 (±0.11)	8.57 (±0.11)	11	−8.77$^{\wedge 1}$ (±1.65)	0.34 (±0.29)	9.09 (±0.07)	8.68 (±0.08)
No lens	L-NAME	12	−0.04 (±0.21)	0.23 (±0.15)	8.97 (±0.08)	8.92 (±0.08)	10	−0.18 (±0.52)	0.73 (±0.23)	8.69 (±0.07)	8.65 (±0.10)
	PBS	13	0.04 (±0.42)	0.48 (±0.30)	8.83 (±0.06)	8.92 (±0.06)	11	0.59 (±0.32)	0.70 (±0.29)	8.52 (±0.12)	8.49 (±0.08)
	L-Arg	10	0.08 (±0.44)	0.10 (±0.41)	8.80 (±0.04)	8.77 (±0.03)	10	−0.30 (±0.69)	0.33 (±0.53)	8.71 (±0.03)	8.68 (±0.04)
+10 D	L-NAME	12	6.58*^{2} (±0.86)	0.25 (±0.30)	8.47*^{3} (±0.07)	8.71 (±0.06)	10	0.08*^{2} (±1.00)	0.15 (±0.37)	8.66*^{3} (±0.09)	8.74 (±0.09)
	PBS	17	6.25$^{\#1}$ (±0.60)	0.51 (±0.34)	8.55$^{\wedge 2}$ (±0.08)	8.78 (±0.06)	14	−1.52$^{\wedge 1}$ (±1.72)	−0.54 (±0.34)	8.58$^{\wedge 2}$ (±0.08)	8.58 (±0.06)
	L-Arg	11	6.43$^{\wedge 2}$ (±0.95)	−0.39 (±0.36)	8.22 (±0.08)	8.54 (±0.06)	10	−1.53$^{\wedge 2}$ (±1.64)	0.23 (±0.52)	8.84 (±0.11)	8.87 (±0.06)

Results shown as mean with standard error of the mean in parentheses. Note: *(L-NAME), $^{\wedge}$(L-Arg), $^{\#}$(PBS) and the associated numerical values indicate pairs of significant *post hoc* differences for refractive state and axial length obtained in experimental-control ANOVAs.
L-Arg, L-arginine; LFRF, low frequency ramped flicker; L-NAME, N^{G}-nitro-L-arginine methyl ester; PBS, phosphate buffered saline; RE, refractive error; SD, standard diurnal.

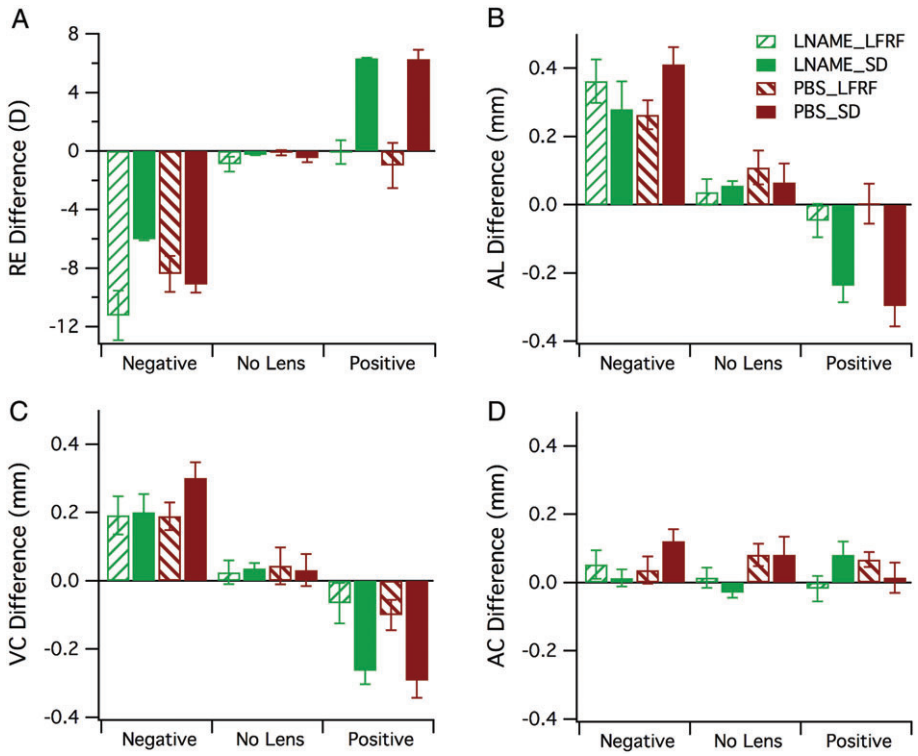

Figure 3

Comparison of biometric data for eyes of chicks injected with N^G-nitro-L-arginine methyl ester (L-NAME) and phosphate buffered saline (PBS). (A) Mean refractive difference (RE experimental eye – RE control eye) in dioptres for the L-NAME and PBS groups under standard diurnal (SD) conditions and with low frequency ramped flicker (LFRF). L-NAME reduced compensation to –10 D lens defocus under SD but not under LFRF conditions, while L-NAME under SD conditions did not alter refractive compensation to +10 D lenses, although under LFRF conditions, refractive compensation to positive lens defocus was completely suppressed. (B) Axial length difference (AL experimental eye – AL control eye) in mm for the L-NAME and PBS groups under SD and LFRF light conditions. (C) Vitreous chamber depth difference (VC experimental eye – VC control eye) in mm for the L-NAME and PBS groups under SD and LFRF light conditions. (D) Anterior chamber depth difference (AC experimental eye – AC control eye) in mm for the L-NAME and PBS groups under SD and LFRF conditions.

Table 3

ANOVA results for eyes of chicks injected with L-NAME and reared with ±10 D or no lens under standard diurnal or low frequency ramped flicker light conditions

	RE	AL	VC	AC
Lens	$P < 0.001$	$P < 0.001$	$P < 0.001$	NS
Light	NS	NS	NS	NS
Drug (L-NAME vs. PBS)	NS	NS	NS	$P = 0.024$
Lens × light	$P < 0.001$	$P < 0.001$	$P < 0.001$	NS
Lens × drug	NS	NS	NS	$P = 0.022$
Light × drug	NS	NS	NS	NS
Lens × light × drug	$P = 0.035$	$P = 0.044$	NS	NS

AC, anterior chamber depth; AL, axial length; L-NAME, N^G-nitro-L-arginine methyl ester; PBS, phosphate buffered saline; RE, refractive error; VC, vitreous chamber depth.

between lens and light, and between lens and light and drug condition.

Post hoc testing for refractive state revealed that L-NAME-injected eyes wearing negative lenses under SD conditions were significantly less myopic than those under LFRF ($P = 0.019$) and SD PBS controls ($P = 0.028$). Positive lens-treated eyes in both the L-NAME and PBS conditions were significantly less hyperopic under LFRF conditions in comparison

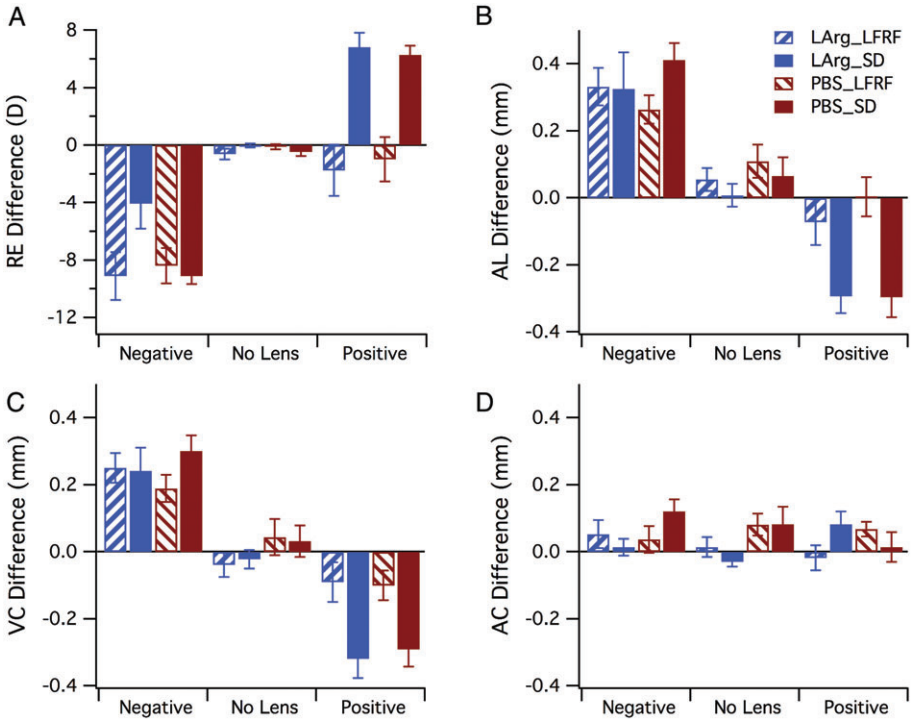

Figure 4

Comparison of biometric data for eyes of chicks injected with L-arginine (L-Arg) and phosphate buffered saline (PBS). (A) Mean refractive difference (RE experimental eye – RE control eye) in dioptres for the three lens groups after intravitreal injection of L-Arg or PBS and 4 days rearing under standard diurnal (SD) conditions or with low frequency ramped flicker (LFRF) lighting conditions. L-Arg reduced compensation to –10 D lens defocus under SD but not under LFRF conditions or in no lens chicks. LFRF resulted in elimination of any refractive compensation in +10 D rearing conditions. (B) Axial length difference (AL experimental eye – AL control eye) in mm for the L-Arg and PBS groups under SD conditions and with LFRF. (C) Vitreous chamber depth difference (VC experimental eye – VC control eye) in mm for the L-Arg and PBS groups under SD conditions and with LFRF. The major influence observed is for LFRF, particularly on positive lens rearing compared with SD conditions. (D) Anterior chamber depth difference (AC experimental eye – AC control eye) in mm for the L-Arg and PBS groups under SD conditions and with LFRF.

to SD controls ($P < 0.001$, $P = 0.001$ respectively). The refractive state of chicks in both the L-NAME and PBS groups differed significantly between lens conditions under SD light ($P < 0.001$). Under LFRF conditions, for all drug treatments the negative lens groups were significantly more myopic ($P < 0.001$) than the positive and NL groups, which did not differ from each other.

Significant differences in axial length were obtained between all lens groups under SD light in the L-NAME and PBS conditions, while under LFRF for both drug groups the axial length of the NL and positive lens reared chicks were not different from each other. Within the positive lens conditions the axial length of chicks reared under SD were shorter than those reared under LFRF for both L-NAME ($P = 0.011$) and PBS ($P = 0.001$) groups. *Post hoc* tests for vitreous chamber depth showed significant differences between drug conditions and between light conditions within the positive lens group, as vitreous chamber depth was shallower in eyes under SD light compared with LFRF conditions (L-NAME $P = 0.010$; PBS $P = 0.009$). As with axial length, no difference was found between the vitreous chamber depths of eyes in the NL compared with the positive lens condition under LFRF for L-NAME ($P = 0.004$) and PBS ($P < 0.001$).

Significant main effects for drug and an interaction effect between lens, light and drug conditions were observed for anterior chamber depth. Further analysis revealed significantly shallower anterior chamber depths in negative lens wearing eyes injected with L-NAME compared with those injected with PBS in the SD condition ($P = 0.019$). Significantly deeper anterior chambers were seen in positive lens wearing groups in comparison to negative lens L-NAME groups under SD ($P = 0.021$).

Experiment 3: interaction between refractive compensation to optical defocus and varied light conditions following injection of L-Arg

A single 5 μL injection of 900 nmol·L-Arg followed by 4 days under SD or LFRF conditions resulted in different growth patterns when compared with eyes injected with 5 μL PBS and raised under the same conditions (Figure 4A). Reduced refractive compensation to negative lenses under SD light was observed for L-Arg-injected eyes, but not under LFRF conditions where the negative lens group L-Arg + LFRF showed a similar degree of myopia to PBS control conditions. LFRF with positive lenses again led to a negative shift in refraction for both L-Arg and PBS groups, while good refractive compensation was observed in the two SD conditions.

Post hoc analysis following ANOVA (Table 4) revealed that L-Arg-injected eyes with negative lenses were significantly less myopic than PBS eyes under SD light ($P = 0.015$).

Table 4

ANOVA results for eyes of chicks injected with L-Arg and reared with ±10 D or no lens under standard diurnal or low frequency ramped flicker light conditions

	RE	AL	VC	AC
Lens	$P < 0.001$	$P = 0.023$	$P < 0.001$	NS
Light	NS	NS	NS	NS
Drug (L-Arg vs. PBS)	NS	NS	NS	NS
Lens × light	$P < 0.001$	$P < 0.001$	$P < 0.001$	NS
Lens × drug	NS	NS	NS	NS
Light × drug	NS	NS	NS	NS
Lens × light × drug	NS	NS	NS	NS

AC, anterior chamber depth; AL, axial length; L-Arg, L-arginine; PBS, phosphate buffered saline; RE, refractive error; VC, vitreous chamber depth.

Although the difference between negative L-Arg SD and LFRF treated eyes looks significant it was not, $P < 0.1$ but greater than 0.05, partly due to the degree of variability in the L-Arg groups. The refractive state of L-Arg + negative lens eyes under SD light was also not different from that of NL chicks. On comparing the change in refraction across the remaining lens conditions, no difference was observed between the NL and positive lens-treated eyes injected with L-Arg or PBS reared within LFRF or SD conditions. By comparison, an analysis of light conditions showed that SD reared chicks wearing positive lenses were significantly more hyperopic than LFRF chicks in L-Arg ($P < 0.001$) and PBS conditions ($P = 0.001$).

Similar extents of axial elongation were seen under both light conditions for eyes with negative lens, while again, a light-dependent effect was apparent in the positive lens condition. Here, LFRF resulted in a suppression of the growth changes typically associated with positive lens wear as LFRF led to significantly longer ocular dimensions than SD in positive lens chicks for L-Arg ($P = 0.015$) and PBS ($P = 0.001$) (Figure 4B). The axial length of all lens groups for L-Arg ($P < 0.001$) and PBS ($P = 0.001$) were significantly different under SD light conditions.

Changes in vitreous chamber depth closely followed alterations in the degree of axial elongation, with lens condition contributing notably to this effect (Figure 4C). Again *post hoc* tests showed that the vitreous chamber depth of all lens groups for L-Arg ($P < 0.001$) and PBS ($P = 0.001$) conditions were significantly different under SD light, while under LFRF there was no significant difference between NL and positive L-Arg-injected eyes. Further, SD light led to significantly shorter vitreous chamber depth than LFRF in positive lens chicks for L-Arg ($P = 0.011$) and PBS ($P = 0.001$). A small, non-significant, mean increase in anterior chamber depth was observed in experimental compared with fellow eyes in all conditions except L-Arg-injected eyes under LFRF and SD conditions reared with NL, and the positive L-Arg LFRF condition (Figure 4D).

A comparison of the effects on refractive compensation and vitreous chamber depth exerted by L-Arg and L-NAME revealed a surprising degree of similarity. While ANOVA showed significant main effects for lens and light (both $P < 0.001$), and a significant interaction between lens and light ($P < 0.001$), no significant effect of drug was observed for any of these measures.

Histology

Examination of microscopic data showing the vitreal surface of the nerve fibre layer (NFL) to the back of choroid, photoreceptor outer segments to NFL, and choroid (Bruch's membrane to membrane adjacent to the cartilaginous sclera), did not demonstrate any abnormal changes or features indicative of cell toxicity or death 4 days post intravitreal injections of either L-NAME or L-Arg. However, as often seen elsewhere (Bohlen *et al.*, 2009), increased dilatation of the lymphatic vessels in the choroids of the L-NAME-injected eyes in all lens groups was noted, although this did not increase the degree of variability as seen by the small standard errors.

Mean percentage difference in the choroidal thicknesses [(experimental – control eye)/control eye] for each lens and drug group under both SD and LFRF light condition are shown in Figure 5. What is immediately obvious is that 4 days post-injection under SD conditions, both L-NAME and L-Arg induced choroidal thinning. This significant thinning of the choroid is especially noteworthy as in the NL conditions and positive lens eye groups final refractions of plano and ~6.5 D, respectively, were seen following the drug treatment. By comparison, under LFRF flicker conditions all groups except the negative lens wearing L-NAME group show greater choroidal thickness in the experimental eye than its control despite showing variable refractions. Under 4 days of LFRF conditions L-Arg in particular, induced a significant increase in choroidal thickness, irrespective of refractive outcome. L-NAME also showed a choroidal thickness increase in control NL groups and +10 D lens groups. On the other hand, both SD and LFRF –10 D lens groups with L-NAME showed similar degrees of choroidal thinning, although a 6 D difference in refraction was observed between the groups.

Consistent with the above observations, ANOVA of relative % choroidal thickness revealed significant main effects for light ($P < 0.001$), but not for lens and drug conditions ($P = 0.36$, $P = 0.08$ respectively). A significant interaction between lens and drug conditions ($P = 0.03$) was also observed. Further analysis revealed that such an effect was primarily driven by

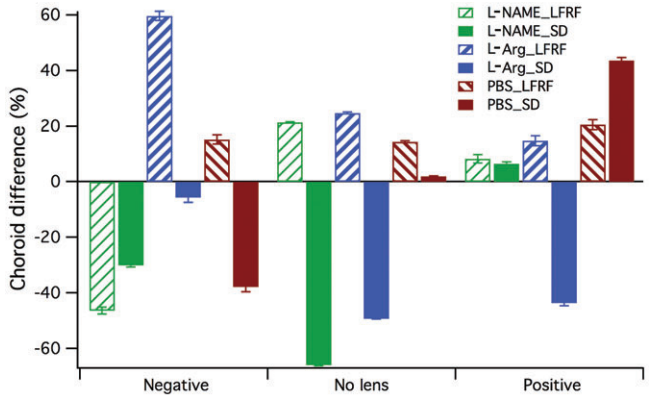

Figure 5

Mean (standard error) percentage change in choroidal thickness relative to the control eye. While the expected effect on choroidal thickness (thinning for −10 D lens, expanding for +10 D lens) was found for the saline-injected eyes, L-arginine (L-Arg)-injected eyes showed shrinkage for no lens and +10 D lens defocus, while N^G-nitro-L-arginine methyl ester (L-NAME)-injected eyes showed extreme thinning for the no lens condition. There was a small positive effect of low frequency ramped flicker (LFRF), compared with the standard diurnal (SD) reared eyes, across all defocus groups, irrespective of the refractive outcomes. Phosphate buffered saline (PBS) injected eyes showed the expected choroidal thinning under −10D SD rearing and thickening under +10D SD rearing. Under LFRF rearing all PBS lens groups showed some choroidal thickening.

lens or light condition, rather than by prolonged exposure to the NO perturbing drugs *per se*. It is also apparent that in groups of animals with little difference in refraction (i.e. all NL eyes injected with L-NAME, L-Arg or PBS) the L-NAME- and L-Arg-injected eyes had a significantly thinner choroid than that of PBS-injected eyes under SD conditions but not under LFRF conditions. Choroidal thickness of the three different negative lens and drug groups was also very variable, although these groups had similar final refractions. By comparison, under LFRF conditions, choroidal thickness of the three different drug groups was fairly similar under the NL, defocus condition.

Discussion

In this study, the competing influences of physical modulation of the retinal ON and OFF pathways by LFRF flicker and drugs that perturb the NO system were examined on refraction, axial dimensions and choroidal thickness. Initially we used ERGs to show that single low-dose injections of L-NAME or L-Arg continue to modulate outer retinal function and the balance of the ON and OFF responses to light for the 4 days of this experiment. The ERGs also showed that although L-NAME and L-Arg are known to have opposite acute effects on the responses to light ONset and OFFset, within 2 days of intravitreal injection their functional responses were similar, with both ERGs showing an increase in the ON/OFF b/d wave amplitude ratio. Under SD light conditions, these NO perturbing drugs inhibited refractive compensation only under conditions of negative lens defocus. The LFRF flicker without drug administration only affected compensation to positive lens defocus. However, when NO perturbing drugs were coupled with LFRF, the effect on refractive compensation was more complicated than a simple summation of effects, for example, for the negative lens defocus condition, LFRF caused a myopic shift (of considerable magnitude with L-NAME). Of even greater theoretical interest is that, while the expected negative association between final refraction and choroidal thickness was seen for the different defocus groups following saline injection and under SD light conditions (Wallman *et al.*, 1995; Wildsoet and Wallman, 1995), these relations vanished under the wider parametric range of drug condition and LFRF lighting condition. Under SD light conditions both L-NAME and L-Arg induced significant choroidal thinning in normal NL chicks even though there was no change in refraction. By comparison, under the LFRF light condition all groups except the L-NAME negative lens group showed choroidal thickening, irrespective of defocus condition or final refractive status. These two examples of dissociation of refraction and choroidal thickness are strong evidence that refractive status and choroidal thickness are unrelated and challenge the choroidal thickness model of refractive error (Wallman *et al.*, 1995), recently revised by Nickla and Wallman (2010).

Implications for the current literature

As a non-specific inhibitor of NOS, L-NAME has long been known to acutely inhibit the retinal bipolar response to light onset (Koistinaho and Sagar, 1995; Fujikado *et al.*, 1997) while L-Arg, the NO substrate, has been known to preferentially inhibit the OFF response to light compared with ON responses of retinal ganglion cells (Wang *et al.*, 2003). However, within 2 days of injection into normal NL eyes, the effects of both drugs on the ERG b/d wave amplitudes ratio were quite similar and resembled that expected acutely for L-Arg. The mean b/d ratios found on days 2 and 4 were: 1.95 and 1.67 and 1.76 and 1.67 for L-Arg and L-NAME, respectively, while the b/d ratio for those previously injected with PBS was 1.37, indicating that 4 days after pharmaceutical perturbation of the NO system there was a shift towards a relatively stronger ON (cf. OFF) response to light and the expected interference with refractive compensation to negative but not positive lens blur. This conforms with biometric results from previous studies where the ON and OFF responses were manipulated with sawtooth illumination and pharmacological agents (Crewther and Crewther, 2003).

Furthermore our ERG recordings give credence to the earlier observations of Fujikado *et al*. that the effect of a single low-dose of L-NAME on the ERG was reversed within 24 h while the metabolic changes lasted for at least 6 days (Fujikado *et al.*, 1997; 2001) Similarly, the effects of the NO-perturbing drugs in the rat are reported to be transitory and variable depending on concentration (Riva *et al.*, 2005; Hoffpauir *et al.*, 2006; Metea and Newman, 2006; 2007). Thus, such observations reinforce the notion that the NO system is highly important for retinal function and that non-toxic NO perturbations lead to up-regulation of cellular mechanisms, such as gene and protein expression pathways (Thiersch *et al.*, 2008), needed to maintain physiological homeostasis. Such changes have also been described recently in ischaemic retina of rat (Rehak *et al.*, 2009).

Our biometric results under SD conditions also concur with those of Fujikado *et al*. (1997; 2001) obtained after single injections of a similar concentration L-NAME during negative lens wear. Comparison of our L-NAME biometric results on positive lens compensation to those reported by Nickla and colleagues is difficult, due to their use of a more complicated protocol, higher intravitreally injected volumes (Nickla and Wildsoet, 2004) more frequent doses (Nickla, 2006) and presumably more frequent anaesthetization. Whereas we found no change in expected refractive compensation to positive lenses in SD light over the 4 day experiment, Nickla and Wildsoet (2004) reported 'no change in axial length measures' but some 'inhibition of the expected inhibition of axial growth and refractive compensation' to +15 D lenses for up to 32 h when using a much larger dose and a scleral cup depth that excludes anterior chamber measures.

The dramatic myopic shift produced by LFRF under positive lens defocus conditions in comparison to SD NL and negative lens groups replicates that seen earlier (Crewther *et al*., 2006a). However, the unexpected biometric effect of enhanced refractive compensation to negative lenses when either L-NAME or L-Arg, but not PBS, was combined with LFRF light conditions is exciting, especially when viewed in terms of the neuromodulatory effects of the NO perturbing drugs evidenced by alteration in the ERG. Further interpretation will come with tests of LFRF rearing conditions on chicks reared with direct pharmacological perturbation of the ON and OFF responses (M.J. Murphy, D.P. Crewther and S.G. Crewther, unpubl. obs.).

Perhaps the most important result from this study is the compelling evidence of dissociation of refractive outcomes and choroidal thickness. To date, the relationship between choroidal thickness and final refraction following compensation to both positive and negative optical defocus in the presence of single injection of NO perturbing drugs, such as L-NAME and L-Arg, has not been tested. Our expectation, as suggested by Nickla and Wallman (2010), would be that if NO is to play a significant or 'causal role' in signalling defocus and directing appropriate choroidal change and final refractions, its response to signed defocus must be reliably different whenever refractive error is induced. However, our anatomical results showed an increase in choroidal thickness in the normal NL groups injected with either L-NAME or L-Arg under LFRF conditions and significant thinning in the NL groups injected with either of the drugs under SD conditions, in a way that bears no relation to the final refractive differences. The main effect for light condition (LFRF, SD) supports the large body of literature relating choroidal thickening (Roy and Sherrington, 1890) and choroidal blood flow (Roy and Sherrington, 1890; Shih *et al*., 1993; Fitzgerald *et al*., 1996; Huemer *et al*., 2007) to dark to light transitions or flicker alone, irrespective of the presence of optical defocus.

A dissociation of choroidal thinning and refractive compensation has been reported previously when the presence of photoreceptor specific toxic agents tunicamycin (Ehrlich *et al*., 2007) and formoguanamine (Westbrook *et al*., 1995) resulted in choroidal thinning without abnormal ocular growth or refractive change. Most notably the dissociation of refractive status and choroidal thickness was highlighted in an ultrastructural study of the temporal sequence of retinal and choroidal changes occurring during recovery from FD myopia (Liang *et al*., 2004). Liang *et al*. demonstrated a nearly linear normalization of refraction over 5 days while choroidal thickness went from 20% of normal to ~300% of normal over the first 3 days after which a reduction in thickness to 30% greater than normal was seen by day 5 post-occlusion.

The validity of use of only one measure of choroidal thickness is recognized as not being optimal, although this has been the general practice in this area (Wallman *et al*., 1995; Beresford *et al*., 2001; Liang *et al*., 2004; Nickla and Wildsoet, 2004). The use of *post-mortem* tissues for absolute measures of choroidal thickness is also potentially subject to tissue shrinkage. However, given that all measures were relative between pairs of eyes from the same animals, enucleated and processed in the same way at the same time and with the results expressed as a percentage change in thickness between experimental and control eyes, any common shrinkage and within subject variance should be controlled.

The possibility of significant degrees of drug-induced toxicity affecting our measurements is considered low, given the well-formed ERG responses 4 days post-injection and the accurate refractive compensation to +10 D and NL defocus conditions in the presence of both drugs under SD conditions. Histological analyses of retina and choroid, also confirmed that there was no evidence of gross cell damage or oedema or change in layer thickness 4 days post intravitreal injections. Rather we expect that if the dose of L-NAME and L-Arg used did not cause immediate cell death, then the manipulation of the NO system is likely to result in rapid up-regulation of gene pathways associated with maintaining tissue homeostasis (Li *et al*., 2007). Furthermore, Ahmad *et al*. (2008) demonstrated that oxidative stress is the mechanism of toxicity for known poisons, and that non-specific NOS inhibitors such as L-NAME actually play a neuroprotective role against deltamethrin-induced apoptosis and paraquat-induced neurotoxicity in rats.

In conclusion, on the basis of the evidence presented here, it is unlikely that choroidal thickness directly, or as a result of any currently undescribed paracrine action of NO on the choroid, could be considered a primary mechanism underlying refractive compensation across the range of parameters of this study. Indeed, choroidal thickness was not predictive of final refractive compensation across any of the variables of drug, defocus sign or light condition. Rather, the changes observed in refractive compensation under these particular drug and light conditions are more likely to be related to a neuromodulatory action on retinal ON and OFF pathways.

Acknowledgements

Thanks to Sarah Scott for assistance with biometric and histological data collection and Sarah Kiely for assistance with ERG data collection. The research was supported by Internal grants from La Trobe University plus an National Health and Medical Research Council Development grant (ID448606).

Conflicts of interest

All authors declare that there are no conflicts of interest in the information and data presented in this manuscript.

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