Published in final edited form as:

Exp Eye Res. 2009 June ; 88(6): 1092–1099. doi:10.1016/j.exer.2009.01.012.

Inhibiting the neuronal isoform of nitric oxide synthase has similar effects on the compensatory choroidal and axial responses to myopic defocus in chicks as does the non-specific inhibitor L-NAME

Debora L. Nickla, Petya Damyanova, and Grace Lytle

The New England College of Optometry, Bioscience Department, 424 Beacon St., Boston MA, 02115

Abstract

In birds, the choroid plays a role in the visual regulation of eye growth, thickening in response to myopic defocus, and thinning in response to hyperopic defocus, in both cases moving the retina towards the image plane. This response is rapid, occurring within hours of the defocus stimulus. These changes are consistently associated with slower changes in the sclera, that result in the appropriate changes in axial elongation, decreasing growth in response to myopic defocus and increasing it in response to hyperopic defocus. The molecular mechanisms underlying the scleral response involve changes in the synthesis of extracellular matrix molecules, however, those underlying the changes in choroidal thickness are not known. However, evidence suggests that it may involve the gaseous signal molecule nitric oxide, as nitric oxide is a potent smooth muscle relaxant, and injections of the non-specific nitric oxide synthase inhibitor L-NAME transiently inhibits the thickening response. Interestingly, it also dis-inhibits ocular growth, in accordance with a mechanistic link between the two responses. If nitric oxide is part of the signal cascade underlying the visual regulation of eye growth, it would be important to ascertain the source of the molecule. As a first step towards doing so, we used various more specific NOS inhibitors and studied their effects on the choroidal and growth responses. Birds (7–12 days old) were fitted with +10 D lenses on one eye. On that day, single intravitreal injections (30 μ l) of the following inhibitors were used: nNOS inhibitor N^ω-propyl-L-arginine (n=12), iNOS inhibitor L-NIL (n=16), eNOS/iNOS inhibitor L-NIO (n=15), non-specific inhibitor L-NMMA (n=30) or physiological saline (n=18). Ocular dimensions were measured using high-frequency A-scan ultrasonography at the start of the experiment, and at 7, 24 and 48 hours after. We found that the nNOS inhibitor N^ω-propyl-L-arginine had the same inhibitory effects on the choroidal response, and dis-inhibition of the growth response, as did L-NAME; neither of the other inhibitors had any effect except L-NMMA. We conclude that the choroidal compensatory response is influenced by nNOS, possibly from the intrinsic choroidal neurons, or the parasympathetic innervation from the ciliary and/or pterygopalatine ganglia.

Keywords

Myopic defocus; nitric oxide; choroid; NOS; emmetropization; N^ω-propyl-L-arginine; L-NAME

INTRODUCTION

It is generally accepted that the growing eye uses visual feedback to coordinate its growth rate to match the front optics (cornea and lens) in order to achieve emmetropia, whereby the image is focused on the retina (review: Wallman, 1995). The most compelling evidence for this process of emmetropization is demonstrated by the finding that when spectacle lenses are worn, all species tested show compensatory responses that result in the image being focused on the retina with the lens in place (Schaeffel et al., 1988; Schaeffel et al., 1990; Schaeffel and Howland, 1991; Smith, 1991; Irving et al., 1992; Wildsoet and Wallman, 1995; Shaikh et al., 1999). When the lenses are removed, the eyes respond in the opposite direction to compensate for the defocus that was induced by the wearing of the lens. This process is bidirectional: both myopic (image focused in front of the retina) and hyperopic (image focused behind the retina) defocus are compensated for, and are magnitude-dependent (i.e. show a linear response to the magnitude of the defocus) (Irving et al., 1992; Wildsoet and Wallman, 1995). In birds, the mechanisms underlying these adjustments involve two components: a slower scleral one, in which changes in the synthesis of extracellular matrix molecules result in the appropriate changes in axial elongation (Rada et al., 1994; Nickla et al., 1997), and a faster choroidal one, in which changes in the thickness of the choroid move the retina towards the image plane (Wallman et al., 1995; Wildsoet and Wallman, 1995). The molecular events that form the basis of these responses are better understood for the scleral process, in which alterations in the rates of synthesis of the proteoglycan aggrecan are strongly correlated with the changes in axial growth rate (Rada et al., 1992; Rada et al., 1994; Nickla et al., 1997). By contrast, those underlying the changes in choroidal thickness are not known. Some possibilities include changes in fluid flux between the vasculature and the stromal extracellular matrix (Wallman et al., 1995; Junghans et al., 1999), changes in the large hydrophilic matrix proteoglycans (Wallman et al., 1995; Nickla et al., 1997) or changes in the tonus of the non-vascular smooth muscle that spans the choroidal stroma (Wallman et al., 1995; Poukens et al., 1998). With respect to this latter possibility, evidence supporting a role for the gaseous neurotransmitter nitric oxide (NO) is particularly relevant because nitric oxide is a potent smooth muscle relaxant (review: (Bredt and Snyder, 1992), and NO-positive axon terminals are found closely apposed to these smooth muscle fibers (Poukens et al., 1998).

Nitric oxide is ubiquitous in the eyes of all vertebrates (Koistinaho and Sagar, 1996), including birds (Sato, 1990; Fischer and Stell, 1999). It is present in retinal neurons, RPE (Goldstein et al., 1996), intrinsic choroidal neurons (Flugel et al., 1994; Bergua et al., 1996; Schroedl et al., 2000; Schroedl et al., 2004), and in terminals from the parasympathetic ciliary (Sun et al., 1994; Nilsson, 1996) and pterygopalatine ganglia (Yamamoto et al., 1993; Cuthbertson et al., 1997). A role for NO in the choroidal response is supported by the finding that inhibiting nitric oxide synthase (NOS) with intravitreal injections of the non-specific NOS inhibitor L-NAME inhibits the increase in thickness induced by myopic defocus. This effect is transient (it wears off in 24 hours) and dose-dependent (Nickla and Wildsoet, 2004). It also produces a disinhibition of axial growth in these eyes, in support of the hypothesis that the choroidal response is causally linked to the growth response (Nickla and Wildsoet, 2004; Nickla et al., 2006). In further support of this notion, if L-NAME is injected into form-deprived eyes immediately *prior* to the daily removal of the diffusers for 2 hours (which normally prevents the myopia), the vision-induced protection against myopia is prevented (Nickla et al., 2006). Because of the prospect that the choroidal response forms part of the signal cascade in the visual regulation of eye growth (Nickla et al., 2006; Nickla, 2007), the elucidation of the source of the NO, and its site of action, is important.

A first step towards elucidating the source of the NO is to determine which of the NOS isoforms are involved. There are three isoforms: two are constitutive (cNOS), and one is inducible (iNOS) by immunological signals such as cytokines (review: Goldstein et al., 1996). The

constitutive forms are nNOS (neuronal) and eNOS (endothelial; formerly known as endothelium-derived relaxing factor). Activation of both of these enzymes requires calcium influx and binding to calmodulin; the release of NO is short-lived (minutes to hours) and hence these isoforms are involved in short-term physiological phenomena. The inducible iNOS, which is involved in cytotoxic and immune responses, and in pathologies such as retinal ischemias. iNOS is controlled at the level of transcription, and thus is longer-lasting (days) than the constitutive forms (reviews: (Snyder and Brecht, 1992; Dawson and Snyder, 1994; Stewart et al., 1994). If the choroidal response is mediated via the non-vascular smooth muscle, then the likely isoform would be nNOS, as both the intrinsic choroidal neurons and the pterygopalatine terminals are positive for nNOS, and both contact these smooth muscle cells (Poukens et al., 1998; Schroedl et al., 1998). If the response is mediated via changes in vascular permeability, and/or changes in blood flow, then eNOS would be the likely candidate. The transient nature of the choroidal response (i.e. less than 24 hours (Nickla and Wildsoet, 2004)) makes it unlikely that iNOS is involved. The purpose of this study was to use various more specific NOS inhibitors toward the goal of clarifying the role of NO in the signal cascade mediating emmetropization. We here report that the highly selective nNOS inhibitor N^ω-propyl-L-arginine inhibits the compensatory choroidal response to myopic defocus and dis-inhibits axial growth; the choroidal effect is dose-dependent. Neither L-NIL (28-fold more specific for iNOS than cNOS (eNOS and nNOS)) (Moore et al., 1994; Moore and Handy, 1997) nor L-NIO (similar selectivity as L-NIL for iNOS but 4-fold more specific for eNOS than iNOS) (Rees et al., 1990; Moore et al., 1994) had any significant effects. Finally, the non-specific NOS inhibitor L-NMMA inhibited the choroidal response to defocus, but with a longer delay than L-NAME (24 hours as opposed to 7 hours), and with a 10-fold higher ED₅₀. Parts of this work have been presented in abstract form (Lytle and Nickla, 2005; Damyanova et al., 2007).

METHODS

Subjects

Subjects were White Leghorn chickens (*Gallus gallus domesticus*), hatched in an on-site incubator and raised in temperature-controlled brooders. The light cycle was 12L/12D (8:00 am to 8:00 pm). The average light intensity in the cage was 300 lux. Food and water were supplied ad libitum. In all experiments, the right eye was treated (lens and injections) and the left eye served as the untreated control. Care and use of the animals conformed to the ARVO Resolution for the Care and Use of Animals in Research.

Drugs and Injections

All drugs were dissolved in physiological saline, and eyes were injected intravitreally using a Hamilton syringe and a 30G needle under isoflurane inhalation anesthesia. Injections were done by puncturing the sclera about 45 degrees superior to temporal. In cases where there was obvious ocular damage from the injection (e.g. cloudy cornea or lens) the data were not used; this was very infrequent (2–3 birds). If we assume an average vitreous chamber volume of 180 µl for this age chick (Luft et al., 2003; Schmid and Wildsoet, 2004), the approximate dilution of the injected drugs would be about 10-fold (dilutions in Table 1). In accordance with much of the pharmacological literature in the field the data are presented as the dose of drug injected (Table 1). Table 1 also shows EC₅₀ for all drugs, the doses tested, and the corresponding concentrations in the syringe, including the data for L-NAME. All fellow eyes were untreated, and the data combined; these are referred to as “untreated fellow control eyes”. Saline injections were given as injection controls; these data are referred to as “saline control eyes”.

Experiment 1: Effects of specific NOS inhibitors on eyes responding to myopic defocus

At 8–12 days of age, chicks received a single intravitreal injection (30 μ l) of one of the following: the nNOS inhibitor N^ω-propyl-L-arginine (dose: 0.6 μ moles, n=12)(EC₅₀=57 nM; Zhang et al., 1997), the iNOS inhibitor L-NIL (dose: 0.6 μ moles, n=5; 6.0 μ moles, n=5; 60 μ moles, n=6)(EC₅₀=3.3 μ M; Moore et al., 1994), the eNOS/iNOS inhibitor L-NIO (dose: 0.6 μ moles, n=10; 6.0 μ moles, n=5)(EC₅₀=0.5 μ M for eNOS; Rees et al., 1990), or physiological saline as injection controls (n=18). +10 D lenses attached to Velcro rings, the matching ring of which was glued to the feathers around the eyes, were put on the eyes following the injection. Ocular dimensions were measured with high-frequency A-scan ultrasonography (for details, see (Nickla et al., 1998)) at time 0 (right before the injection), and at 7 hours, 24 hours, and 48 hours post-injection. Axial length is defined as the distance from the front of the cornea to the front of the sclera, and the change in length over time reflects the rate of elongation. Changes in choroidal thickness and anterior chamber depth were also determined. Refractive errors were measured using a Hartinger's refractometer at the end of the experiment. For both L-NIL and L-NIO, there were no significant differences between the doses of 0.6 and 6.0 μ moles, therefore these data were combined for both drugs. The data from the highest dose for L-NIL (60 μ moles) are presented separately.

To test for dose-dependence of the effect on the choroid of N^ω-propyl-L-arginine, in a separate experiment single 30 μ l injections of N^ω-propyl-L-arginine (0.3 μ moles (n=5), 0.06 μ moles (n=5) and .006 μ moles (n=5)) were given to eyes wearing +10 D lenses, as described above. Ultrasonography was done at time 0 (right before the injection) and 7 hours later. Data from the first (above) experiment were used for the highest dose (0.6 μ moles) and for saline controls.

Scleral Glycosaminoglycan synthesis—Following the final measurement, eyes were dissected out and a 7mm punch was taken from the posterior pole. The scleras were scraped free of choroid, retina and RPE, and the punches incubated in DMEM or L-15 medium containing radiolabeled Na₂SO₄ (approximately 20 μ Ci/ml) for 4 hours at 37 degrees in 5% CO₂. The tissue was then digested in proteinase-K (Sigma) and the glycosaminoglycans (GAGs) were precipitated with 0.05% cetylpyridinium chloride and collected on Whatman filters (GF/A) using a Millipore 12-port manifold. This assay measures the incorporation of labeled inorganic sulfur into the glycosaminoglycan side chains of proteoglycans and is an index of proteoglycan synthesis (for details, see Nickla et al., 1999). The N^ω-propyl-L-arginine and L-NIL experiments were done at the same time, with the same isotope, while the L-NIO one was done at a later time, and did not include a saline control group. The difference in counts per minute (CPMs) between the groups precluded graphing them together, therefore the data are presented separately (Figure 4).

Experiment 2: Effects of the non-specific NOS inhibitor L-NMMA on eyes responding to myopic defocus

At 12 days of age, chicks received a single intravitreal injection (30 μ l) of L-N monomethyl arginine (L-NMMA) (dose: 0.12 μ moles (n=5), 1.2 μ moles (n=10), 4.8 μ moles (n=9), 20 μ moles (n=6) or physiological saline (n=6)). +10 D lenses were immediately fitted to eyes following the injection. Ocular dimensions were measured with high-frequency A-scan ultrasonography at the same times as above.

Statistical Analysis—Unpaired (two-tailed) t-tests were used in all comparisons between groups of eyes. In some cases ANOVAs were used to describe between-group differences.

RESULTS

We tested three relatively specific NOS inhibitors of the different NOS isoenzymes. The effects of the specific nNOS inhibitor N^ω-propyl-L-arginine resulted in the same effects on the choroidal, refractive, and axial (growth) responses as did the non-specific inhibitor L-NAME (Nickla and Wildsoet, 2004) and L-NMMA: it transiently inhibited the choroidal response, and dis-inhibited the growth response. By contrast, it did *not* have a similar inhibitory effect on the growth of the anterior chamber. Neither of the other two inhibitors, L-NIO or L-NIL, had any significant effects.

I. The effects of NOS inhibitors on eyes responding to myopic defocus

Choroidal Thickness—Eyes injected with N^ω-propyl-L-arginine showed a significant inhibition in the choroidal response to myopic defocus induced by positive lenses at 7 hours (drug vs saline controls: 13 vs 116 μm, $p < 0.01$; Figure 1A). By 24 hours, while the difference between the drug and saline control choroids was still significant, there was a trend toward choroidal thickening in the drug-injected eyes (57 vs 127 μm, $p = 0.04$). There were no significant differences between eyes injected with N^ω-propyl-L-arginine and untreated fellow control eyes at either time point (drug vs fellow: 13 vs -12 μm at 7 hrs; -6 vs -27 μm at 24 hrs). Neither L-NIL nor L-NIO had this effect at any dose tested: choroids of these eyes showed an increase in thickness in response to the myopic defocus similar to that of saline treated eyes at both 7 and 24 hours (7 hrs: L-NIO and L-NIL vs saline controls: 75, 103 and 116 μm; figure 1A). Data for the highest dose of L-NIL (60 μmoles) is presented separately: while the choroidal response was smaller than that of the combined lower doses (7 hours), it was not significantly different, and was still significantly larger than that of N^ω-propyl-L-arginine (Figure 1A, L-NIL, white diamond). All of these effects were significantly different from untreated fellow control eyes at $p < 0.01$.

The inhibition of the choroidal thickening response at 7 hours showed a dose-dependence (Figure 1B). The ED₅₀ = 0.3 μmoles, which is similar to that of L-NAME (0.26 μmoles) (Nickla and Wildsoet, 2004).

Refractive Error and Ocular Elongation—Eyes injected with N^ω-propyl-L-arginine did *not* become hyperopic in response to the myopic defocus induced by the positive lenses (Figure 2A); at 48 hours they were not significantly different than untreated fellow controls (1.4 D vs 0.8 D) and were significantly *less* hyperopic than saline-injected eyes (drug vs saline: +1.4 D vs +5.2 D; $p < 0.01$). Neither of the other specific inhibitors showed this effect (Figure 2A); there were no significant differences between eyes injected with L-NIO, L-NIL, or saline controls at 48 hours (respectively: +4.2 D, +3.6 D, +5.2 D).

The inhibition by N^ω-propyl-L-arginine of the refractive compensation was axial in origin: these eyes grew significantly *faster* than saline controls over 24 hours (Figure 2B; 102 vs 48 μm, $p < 0.05$) and growth rate did not differ from that of untreated fellow controls (102 and 115 μm/24 hours). Growth rates of neither L-NIO nor L-NIL differed from that of saline controls (respectively: 52, 37 and 48 μm; Figure 2B). At 48 hours, there were no longer significant differences in axial elongation between drug-injected and saline controls for any of the drugs (data not shown), when the refractive effect was manifest. At this time, the choroids had thickened by approximately 200 μm (data not shown), which accounts for approximately half (2.5 D) of the hyperopic shift in refractive error (Wildsoet and Wallman, 1995) shown in figure 2A. By contrast to the effect of L-NAME (and LNMMA; below), there were no significant differences in the growth of the anterior chamber over the times measured (Figure 3).

Glycosaminoglycan synthesis—In accord with its effect on ocular growth rate, N^ω-propyl-L-arginine *prevented* the inhibition of scleral GAG synthesis in response to positive

lenses; GAG levels were significantly *higher* than those of both saline- and L-NIL-injected eyes (Figure 4A: 6571 DPM vs 4816 and 3994 DPM; $p<0.05$, $p=0.05$, respectively). They did not differ from untreated fellow controls. Similarly, L-NIO inhibited GAG synthesis relative to untreated fellow controls (there were no saline controls in this experiment; figure 4B; $p<0.05$). Therefore, the disinhibition by N^ω-propyl-L-arginine is likely mediated at the level of scleral proteoglycan synthesis.

II. The effects of the non-specific NOS inhibitor L-NMMA

Another L-arginine analog (like L-NAME) that acts as a non-specific NOS inhibitor is L-NMMA (EC₅₀ in Table 1; Kubes et al., 1991). This drug had similar effects on the choroid as did L-NAME: it inhibited the choroidal expansion in response to myopic defocus induced by positive lenses (Figure 5A). However, the effect was not as rapid or as effective as that of L-NAME: at 7 hours, when L-NAME shows complete inhibition of the choroidal response (Nickla and Wildsoet, 2004), there was no effect (Figure 5A: drug vs saline: 74 vs 82 μ m). By 24 hours, when the effect of L-NAME had worn off (Nickla & Wildsoet, 2004), L-NMMA produced a small inhibition in the response (84 vs 171 μ m; $p<0.05$). The inhibition was gone by 48 hours; choroids had thickened as much as those of saline controls (Figure 5A: 186 vs 225 μ m). The inhibitory effect at 24 hours was dose-dependent, with an ED₅₀=2.3 μ moles (Figure 5A, right), about 10-fold higher than that of L-NAME (Nickla and Wildsoet, 2004).

Similar to the effect of L-NAME, and different from that of N^ω-propyl-L-arginine (Figure 3), the growth of the anterior chamber *was* inhibited (4.8 μ moles: 25 vs 85 μ m, one-tailed t-test $p<0.05$; figure 5B). However, while the drug-injected eyes tended to grow faster over 48 hours, the difference was not significant (204 vs 142 μ m; $p=0.3$; data not shown).

DISCUSSION

Nitric oxide synthase (NOS) is the enzyme that catalyzes the synthesis of nitric oxide from L-arginine. It exists in three isoforms, all of which are found in the eye. Immunohistochemical labeling for nNOS has been found in the pterygopalatine (Cuthbertson et al., 1997) the ciliary neurons (Sun et al., 1994), and their terminals in the choroid. iNOS activity has been found in the retina, in macrophages and neutrophils (Goldstein et al., 1996) and the protein (immunohistochemistry) was found in the outer segments of the photoreceptors, the RPE and choroid (Fujii et al., 1998) and in Mueller cells (Goureau et al., 1994). eNOS is not found in the retina, but is present in the vascular endothelium of the choroid. Use of NOS inhibitors has been the single most important tool in the elucidation of the myriad roles of NO in physiology over the past 20 years (review: Moore and Handy, 1997). In the retina, it is involved in visual transduction, synaptic transmission and horizontal cell coupling (Koistinaho and Sagar, 1996). In the choroid, a role in blood flow regulation is fairly well-established (Deussen et al., 1993; Mann et al., 1995; Zagvazdin et al., 1996). However, another potential role for NO is mediating the choroidal changes in thickness that occur in response to myopic defocus (Nickla and Wildsoet, 2004), and perhaps the diurnal changes in thickness as well (Nickla et al., 1998). It is possible that these changes are mediated by changes in tonus of the non-vascular smooth muscle that spans the choroid (Wallman et al., 1995); these cells are contacted by NO-positive terminals (Poukens et al., 1998) and NO is a potent relaxant of smooth muscle. Furthermore, the non-specific NOS inhibitor L-NAME transiently inhibits the choroidal response (Nickla and Wildsoet, 2004). In this study we tested four NOS inhibitors of varying selectivities for the different isoforms to clarify which NOS system(s) might be involved.

The main finding from this study is that the nNOS inhibitor N^ω-propyl-L-arginine has similar effects as those of the non-specific inhibitor L-NAME on the choroidal and ocular growth responses to myopic defocus: it transiently inhibits the choroidal thickening and it disinhibits axial growth (Nickla and Wildsoet, 2004). Furthermore, the ED₅₀ is similar to that for L-

NAME. In accordance with the growth dis-inhibition, the synthesis of scleral extracellular matrix glycosaminoglycans is significantly higher in drug-injected eyes than in saline controls, supporting the hypothesis that the choroidal and axial responses may be mechanistically linked. Whether NO acts directly on the sclera or influences another molecule that affects scleral GAG synthesis is not known. The other two NOS inhibitors, L-NIO (more specific for eNOS and iNOS) and L-NIL (more specific for iNOS) did not have any significant effects at the doses used. L-NMMA had similar effects as L-NAME but at a higher ED₅₀ and with a longer delay.

Can we infer from these results that nNOS is the enzyme that modulates choroidal thickness and scleral GAG synthesis? On the one hand, N^ω-propyl-L-arginine is a potent (EC₅₀=57 nM) and highly selective inhibitor of nNOS, displaying a 3158-fold selectivity over iNOS and a 149-fold selectivity over eNOS (Zhang et al., 1997), well exceeding the “potency gap” of >100:1 proposed by Moore and Handy (1997) as a criterion for a selective response. On the other hand, because the effective concentration in our experiments was 0.01 M (ED₅₀=0.3 μmoles), orders of magnitude above the published EC₅₀ values, it is possible that the responses had saturated, and that the drug acted on more than one isoform, regardless of the differences in selectivity. We suspect this was not the case because the EC₅₀ values for L-NAME, L-NIO and L-NMMA in intact retinal tissues are as much as 3,000 X higher than in homogenates (Wellard et al., 1995), and would likely be even higher in an intact animal. In fact, the EC₅₀ of N^ω-propyl-L-arginine is similar to that of the non-selective inhibitor L-NAME (0.3 vs 0.26 μmoles; Nickla and Wildsoet, 2004), suggesting that our doses were not excessive. Further evidence that N^ω-propyl-L-arginine was not acting on iNOS comes from the fact that L-NIL, which is relatively more specific for iNOS, was ineffective even at 100 times the effective dose of N^ω-propyl-L-arginine. Finally, the effective dose for N^ω-propyl-L-arginine and L-NAME were similar, even though L-NAME is over a hundred-fold less effective on iNOS (Table 1), again suggesting that our treatments are not acting via iNOS.

The evidence for the involvement of eNOS is less clear because of the relative lack of specificity of L-NIO. However, because its EC₅₀ for eNOS is the same as that of L-NAME (Table 1), but only L-NAME is effective, it is unlikely that it acts via eNOS. Furthermore, if the effective dose of L-NIO on eNOS in the chick eye is similar to that of its effect on blood pressure (presumably via eNOS; EC₅₀ = 19.5 mg/kg) in the rat (Rees et al., 1990), we should have seen some effect at the highest dose we used but did not. Thus, although we cannot definitively exclude a role for eNOS, it is more parsimonious to infer that nNOS is the most important isoform in our system.

If indeed nNOS plays a role in the choroidal responses to retinal defocus, the source of the NO is open to speculation. Possible candidate sources are retinal cells, intrinsic choroidal neurons, or the parasympathetic inputs from either the ciliary ganglion, and/or the pterygopalatine ganglion. The likelihood that it is retinal is relatively small, due to the fact that although NO freely diffuses across membranes, it would have to traverse half the distance of the retina, and cross the RPE and Bruchs membrane to exert effects on the choroid. The very short half-life of NO also argues against this route of action. With respect to the parasympathetic innervation, removing the ciliary input via ciliary ganglionectomy produced an increase in choroidal thickness in normal eyes. It had no effect however, on the choroidal response to myopic defocus induced by prior form deprivation (Schmid et al., 1999), therefore it appears that this input does not strongly influence the compensatory choroidal response. Removing the pterygopalatine input via transection of nerve VII (preganglionic input) (Schroedl et al., 2006) also had no effect on the choroidal response to myopic defocus, however, when both inputs were lesioned, the choroidal response was significantly attenuated (Nickla and Schroedl, 2008), suggesting that there is overlap (redundancy) between the two parasympathetic pathways in mediating the choroidal (and perhaps growth) response. Lastly, a role for the

intrinsic neurons cannot be ruled out: there is as yet no known function for these cells. This answer awaits the technique to lesion this system, as yet unknown.

Finally, although the effects on axial elongation are similar for N^ω-propyl-L-arginine and L-NAME, there is a minor, but perhaps important difference between the two in their effects on the anterior chamber. Both L-NAME (Nickla and Wildsoet, 2004) and L-NMMA *inhibit* the growth of the anterior chamber over 24–48 hours while dis-inhibiting growth of the vitreous chamber. This indicates that the control system for growth of the front of the eye is separate from that for the back of the eye. This is supported by other studies: for instance, constant light results in corneal flattening, and elongation of the vitreous chamber (Lauber et al., 1961; Li et al., 1995). Similarly, topical application of melatonin results in a decrease in anterior chamber depth, and a (non-significant) increase in vitreous chamber depth (Summers Rada and Wiechmann, 2006). By contrast, N^ω-propyl-L-arginine does *not* have this inhibitory effect on the anterior chamber. One implication of this difference is that perhaps the arginine analogs L-NAME and L-NMMA are functioning in this part of the eye as muscarinic antagonists (Buxton et al., 1993), and hence are affecting an entirely different signaling system. The fact that they are *not* acting on muscarinic receptors at the *back* of the eye is evidenced by the finding that atropine and pirenzapine both have effects opposite those of the NOS inhibitors, both on the growth system (they *inhibit* growth) (Stone et al., 1991; McBrien et al., 1993; Leech et al., 1995; Cottrill and McBrien, 1996; Tigges et al., 1999) and on the choroidal response (they *potentiate* it; Dhillon and Nickla, 2008).

In conclusion, our results suggest that the nNOS isoform is the predominant one regulating the changes in choroidal thickness in response to myopic defocus. We speculate that the source might be the axon terminals from the parasympathetic ganglia. If, as has been suggested, NO forms part of the signal cascade in the visual regulation of eye growth, localizing this source would be a crucial step towards developing pharmacological interventions in the progression of myopia in humans.

Acknowledgments

The authors thank Kristen Totonelly and Jacqueline Harger for their care of the chickens.

Grant support: NIH-EY013636

References

- Bergua A, Mayer B, Neuhuber W. Nitroergic and VIP-ergic neurons in the choroid and ciliary ganglion of the duck *Anis carina*. *Anat Embryol* 1996;193:239–248. [PubMed: 8881473]
- Bredt DS, Snyder SH. Nitric Oxide: A novel neuronal messenger. *Neuron* 1992;8:3–11. [PubMed: 1370373]
- Buxton I, Cheek D, Eckmann D, Westfall D, Sanders K, Keef K. NG-Nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ Res* 1993;72:369–379.
- Cottrill CL, McBrien N. The m1 muscarinic antagonist pirenzepine reduces myopia and eye enlargement in the tree shrew. *Invest Ophthalmol Vis Sci* 1996;37:1368–1379. [PubMed: 8641840]
- Cuthbertson S, Jackson B, Toledo C, Fitzgerald MEC, Shih YF, Zagvazdin Y, Reiner A. Innervation of orbital and choroidal blood vessels by the pterygopalatine ganglion in pigeons. *J Comp Neurol* 1997;386:422–442. [PubMed: 9303427]
- Damyanova P, Lytle G, Dhillon B, Nickla D. The effects of various inhibitors of the nitric oxide synthase isoforms on the compensatory choroidal response and scleral proteoglycan synthesis. *Invest Ophthalmol Vis Sci*. 2007 E-Abstract 5928.
- Dawson TM, Snyder SH. Gases as biological messengers: Nitric oxide and carbon monoxide in the brain. *J Neurosci* 1994;14:5147–5159. [PubMed: 8083727]

- Deussen A, Sonntag M, Vogel R. L-arginine-derived nitric oxide: A major determinant of uveal blood flow. *Exp Eye Res* 1993;57:129–134. [PubMed: 8405178]
- Dhillon B, Nickla D. The ocular growth inhibition effected by dopamine agonists and atropine is associated with transient increases in choroidal thickness in chicks. *Invest Ophthalmol Vis Sci*. 2008 E-Abstract 1732.
- Fischer A, Stell W. Nitric oxide synthase-containing cells in the retina, pigmented epithelium, choroid, and sclera of the chick eye. *J Comp Neurol* 1999;405:1–14. [PubMed: 10022192]
- Flugel C, Tamm ER, Mayer B, Lutjen-Drecoll E. Species differences in choroid vasodilative innervation: evidence for specific intrinsic nitrergic and VIP-positive neurons in the human eye. *Invest Ophthalmol Vis Sci* 1994;35:592–599. [PubMed: 7509326]
- Fujii S, Honda S, Sekiya Y, Yamasaki M, Yamamoto M, Saijoh K. Differential expression of nitric oxide synthase isoforms in form-deprived chick eyes. *Curr Eye Res* 1998;17:586–593. [PubMed: 9663848]
- Goldstein IM, Ostwald P, Roth S. Nitric oxide: A review of its role in retinal function and disease. *Vision Res* 1996;36:2979–2994. [PubMed: 8917798]
- Goureau D, Hicks D, Courtois D, Kozak Y. Induction and regulation of nitric oxide synthase in Muller glial cells. *J Neurochem* 1994;63:310–317. [PubMed: 7515948]
- Irving EL, Sivak JG, Callender MG. Refractive plasticity of the developing chick eye. *Ophthalmic & Physiological Optics* 1992;12:448–456. [PubMed: 1293533]
- Junghans B, Crewther S, Liang H, Crewther D. A role for choroidal lymphatics during recovery from form deprivation myopia? *Optom Vis Sci* 1999;76:796–803. [PubMed: 10566864]
- Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* 1991;88:4651–4655. [PubMed: 1675786]
- Koistinaho J, Sagar S. NADPH-diaphorase-reactive neurons in the retina. *Prog Ret Res* 1996;15:69–87.
- Laubert JK, Shutze JV, McGinnis J. Effects of exposure to continuous light on the eye of the growing chick. *Proc Soc Exp Biol Med* 1961;106(4):871–872. [PubMed: 13759412]
- Leech EM, Cottriall CL, McBrien N. Pirenzapine prevents form deprivation myopia in a dose dependent manner. *Ophthalmic Physiol Opt* 1995;15:351–356. [PubMed: 8524553]
- Li T, Troilo D, Glasser A, Howland HC. Constant light produces severe corneal flattening and hyperopia in chickens. *Vision Res* 1995;35:1203–1209. [PubMed: 7610581]
- Luft W, Ming Y, Stell W. Variable effects of previously untested muscarinic receptor antagonists on experimental myopia. *Invest Ophthalmol Vis Sci* 2003;44:1330–1338. [PubMed: 12601066]
- Lytle G, Nickla D. The effect of the neuronal nitric oxide synthase inhibitor TRIM on chick eyes responding to myopic defocus. *Invest Ophthalmol Vis Sci*. 2005 E-Abstract: 3339.
- Mann R, Riva C, Stone R, Barnes G, Cranstoun S. Nitric oxide and choroidal blood flow regulation. *Invest Ophthalmol Vis Sci* 1995;36:925–930. [PubMed: 7706041]
- McBrien NA, Moghaddam HO, Reeder AP. Atropine reduces experimental myopia and eye enlargement via a nonaccommodative mechanism. *Invest Ophthalmol Vis Sci* 1993;34:205–215. [PubMed: 8425826]
- Moore PK, Handy RLC. Selective inhibitors of neuronal nitric oxide synthase -- is no NOS really good NOS for the nervous system? *TIPS* 1997;18:204–211. [PubMed: 9226999]
- Moore WM, Webber RK, Jerome GM, Tjoeng FS, Misko TP, Currie MG. L-N6-(1-Iminoethyl)lysine: A selective inhibitor of inducible nitric oxide synthase. *J Med Chem* 1994;37:3886–3888. [PubMed: 7525961]
- Nickla D. Transient increases in choroidal thickness are consistently associated with brief daily visual stimuli that inhibit ocular growth in chicks. *Exp Eye Res* 2007;84:951–959. [PubMed: 17395180]
- Nickla D, Schroedl F. Influences of the autonomic nervous system on the compensatory responses of the chick eye to defocus: Single- and double-lesion studies of the pterygopalatine ganglion. *Invest Ophthalmol Vis Sci*. 2008 E-Abstract: 3590.
- Nickla D, Wildsoet C. The effect of the nonspecific nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester on the choroidal compensatory response to myopic defocus in chickens. *Optom Vis Sci* 2004;81:111–118. [PubMed: 15127930]

- Nickla D, Wilken E, Lytle G, Yom S, Mertz J. Inhibiting the transient choroidal thickening response using the nitric oxide synthase inhibitor L-NAME prevents the ameliorative effects of visual experience on ocular growth in two different visual paradigms. *Exp Eye Res* 2006;83:456–464. [PubMed: 16635488]
- Nickla DL, Rada JA, Wallman J. Isolated chick sclera shows a circadian rhythm in proteoglycan synthesis perhaps associated with the rhythm in ocular elongation. *J Comp Physiol [A]* 1999;185:81–90.
- Nickla DL, Wildsoet C, Wallman J. Compensation for spectacle lenses involves changes in proteoglycan synthesis in both the sclera and choroid. *Curr Eye Res* 1997;16:320–326. [PubMed: 9134320]
- Nickla DL, Wildsoet C, Wallman J. Visual influences on diurnal rhythms in ocular length and choroidal thickness in chick eyes. *Exp Eye Res* 1998;66:163–181. [PubMed: 9533843]
- Nilsson SF. Nitric oxide as a mediator of parasympathetic vasodilation in ocular and extraocular tissues in the rabbit. *Invest Ophthalmol Vis Sci* 1996;37:2110–2119. [PubMed: 8814150]
- Poukens V, Glasgow BJ, Demer JL. Nonvascular contractile cells in sclera and choroid of humans and monkeys. *Invest Ophthalmol Vis Sci* 1998;39:1765–1774. [PubMed: 9727398]
- Rada JA, Matthews AL, Brenza H. Regional proteoglycan synthesis in the sclera of experimentally myopic chicks. *Experimental Eye Research* 1994;59:747–760. [PubMed: 7698268]
- Rada JA, McFarland AL, Cornuet PK, Hassell JR. Proteoglycan synthesis by scleral chondrocytes is modulated by a vision dependent mechanism. *Curr Eye Res* 1992;11:767–782. [PubMed: 1424721]
- Rees DD, Palmer R, Schulz R, Hodson H, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 1990;101:746–752. [PubMed: 1706208]
- Sato T. NADPH-diaphorase positive amacrine cells in the retinae of the frog (*Rana esculenta*) and pigeon (*Columbia livia*). *Archs Hist Cyt* 1990;53:63–69.
- Schaeffel F, Howland H. Properties of the feedback loops controlling eye growth and refractive state in the chicken. *Vision Res* 1991;31:717–734. [PubMed: 1843772]
- Schaeffel F, Troilo D, Wallman J, Howland H. Chickens without accommodation become myopic or hyperopic from wearing lenses. *Invest Ophthalmol Vis Res (ARVO Suppl)* 1988;29:445.
- Schaeffel F, Troilo D, Wallman J, Howland HC. Developing eyes that lack accommodation grow to compensate for imposed defocus. *Vis Neurosci* 1990;4:177–183. [PubMed: 2271446]
- Schmid GF, Papastergiou GI, Lin T, Riva CE, Laties AM, Stone RA. Autonomic denervations influence ocular dimensions and intraocular pressure in chicks. *Exp Eye Res* 1999;68:573–581. [PubMed: 10328971]
- Schmid KL, Wildsoet C. Inhibitory effects of apomorphine and atropine and their combination on myopia in chicks. *Optom Vis Sci* 2004;81:137–147. [PubMed: 15127933]
- Schroedl F, Bergua A, Brehmer A, Neuhuber WL. Nonvascular smooth muscle as a main target for intrinsic choroid neurons in duck. *Exp Eye Res* 1998;67:78.
- Schroedl F, Brehmer A, Neuhuber W. Intrinsic choroidal neurons in the duck eye express galanin. *J Comp Neurol* 2000;425:24–33. [PubMed: 10940939]
- Schroedl F, Brehmer A, Neuhuber W, Nickla D. The autonomic facial nerve pathway in birds: a tracing study. *Invest Ophthalmol Vis Sci* 2006;47:3225–3233. [PubMed: 16877385]
- Schroedl F, De Stefano M, Reese S, Brehmer A, Neuhuber W. Comparative anatomy of nitrergic intrinsic choroidal neurons (ICN) in various avian species. *Exp Eye Res* 2004;78:187–196. [PubMed: 14729351]
- Shaikh AW, Siegwart JT, Norton T. Effect of interrupted lens wear on compensation for a minus lens in tree shrews. *Optom Vis Sci* 1999;76:308–315. [PubMed: 10375247]
- Smith, EL. Experimentally induced refractive anomalies in mammals. In: Grosvenor, T.; Flom, MC., editors. *Refractive Anomalies: Research and Clinical Applications*. Boston: Butterworth-Heinemann; 1991. p. 246–267.
- Snyder SH, Brecht D. Biological roles of nitric oxide. *Scient Amer* 1992;266:68–77.
- Stewart AG, Phan L, Grigoriadis G. Physiological and pathophysiological roles of nitric oxide. *Microsurgery* 1994;15:693–702. [PubMed: 7533876]
- Stone RA, Lin T, Laties AM. Muscarinic antagonist effects on experimental chick myopia. *Exp Eye Res* 1991;52:755–758. [PubMed: 1855549]

- Summers Rada J, Wiechmann A. Melatonin receptors in chick ocular tissues: Implications for a role of melatonin in ocular growth regulation. *Invest Ophthalmol Vis Sci* 2006;47:25–33. [PubMed: 16384940]
- Sun W, Erichsen JT, May P. NADPH-diaphorase reactivity in ciliary ganglion neurons: a comparison of distributions in the pigeon, cat and monkey. *Vis Neurosci* 1994;11:1–5. [PubMed: 8011573]
- Tigges M, Iuvone P, Fernandes A, Sugrue M, Mallorga P, Laties AM, Stone R. Effects of muscarinic cholinergic receptor antagonists on postnatal eye growth of rhesus monkeys. *Optom Vis Sci* 1999;76:397–407. [PubMed: 10416935]
- Wallman, J. How many myopias?. In: Christen, Y.; Doly, M.; Droy-Lefaix, MT., editors. *Vision et Adaptation* (vol 6 of *Les Seminaires Ophtalmologiques d'IPSEN*). Vol. 6. Paris: Elsevier; 1995. p. 140-150.
- Wallman J, Wildsoet C, Xu A, Gottlieb M, Nickla D, Marran L, Krebs W, Christensen A. Moving the retina: Choroidal modulation of refractive state. *Vision Res* 1995;35:37–50. [PubMed: 7839608]
- Wellard JW, Miethke P, Morgan I. Neural barriers affect the action of nitric oxide synthase inhibitors in the intact chicken retina. *Neuroscience Letters* 1995;201:17–20. [PubMed: 8830302]
- Wildsoet C, Wallman J. Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks. *Vision Res* 1995;35:1175–1194. [PubMed: 7610579]
- Yamamoto R, Brecht DS, Dawson TM, Snyder SH, Stone RA. Enhanced expression of nitric oxide synthase by rat retina following pterygopalatine parasympathetic denervation. *Brain Res* 1993;631:83–88. [PubMed: 7507791]
- Zagvazdin YS, Fitzgerald MEC, Sancesario G, Reiner A. Neural nitric oxide mediates Edinger-Westphal nucleus evoked increase in choroidal blood flow in the pigeon. *Invest Ophthalmol Vis Sci* 1996;37:666–672. [PubMed: 8595967]
- Zhang HQ, Fast W, Marletta MA, Martasek P, Silverman RB. Potent and selective inhibition of neuronal nitric oxide synthase by N omega-propyl-L-arginine. *J Med Chem* 1997;40:3869–3870. [PubMed: 9397167]

Figure 1.

Change in choroidal thickness in response to +10 D lens over 7 (black bars) and 24 (white bars) hours after the injections for all three inhibitors, and saline controls. There is a significant inhibition of the response compared to saline controls over both intervals for *n*^ω-propyl-L-arginine (Nw-PLA), but no effect by either of the other two inhibitors. The diamond with the L-NIL data is the highest dose tested: 60 μmoles . “Fellow” is data from all untreated fellow eyes in all graphs. Error bars are standard errors of the mean in all figures. Asterisk: $p < 0.05$ in all graphs. B. Dose response curve for *n*^ω-propyl-L-arginine. The $\text{ED}_{50} = 0.3 \mu\text{moles}$.

Figure 2.

A. Change in refractive error in response to +10 D lens after 48 hours for all three inhibitors, and saline controls. *n*^ω-propyl-L-arginine (Nw-PLA) prevents the refractive compensation to the myopic defocus; eyes do not become hyperopic. There are no significant differences for the other two inhibitors. “Fellow” is data for untreated fellow eyes. B. Change in axial length in response to +10 D lens after 24 hours for all three inhibitors, and saline controls. The eyes injected with *n*^ω-propyl-L-arginine do *not* inhibit their rate of growth in response to the lenses, and do not differ from untreated fellow control eyes. The other two inhibitors, and the saline control eyes all show axial growth inhibition.

Figure 3.

Change in anterior chamber depth over 24 hours for all three inhibitors, and saline controls. There are no significant effects of any of the drugs over this interval, or over 48 hours (data not shown).

Figure 4. Scleral glycosaminoglycan synthesis (GAG) *in vitro* for all groups. A: n^{ω} -propyl-L-arginine (Nw-PLA) does not have any effect on GAG synthesis, but L-NIL shows a significant inhibition, in accordance with the axial elongation data, as does N-NIO (B).

Figure 5.

Effects of another non-specific NOS inhibitor, L-NMMA, on the choroidal response (A) and the anterior chamber depth (B). A. Change in choroidal thickness over 7, 24 and 48 hour intervals for L-NMMA (black bars) and saline controls (white bars). There is a significant inhibition in the response for drug-injected eyes at 24 hours, but not at 7 or 48 hours. Inset: The response shows a dose-dependence with an $\text{ED}_{50} = 2.3 \mu\text{moles}$. B. Change in anterior chamber depth at various doses of L-NMMA, versus saline controls. There is a significant inhibition in the growth of the anterior chamber at all doses, similar to L-NAME.

Table 1

Summary of specificities for NOS inhibitors used in experiments, compared to those for L-NAME. Doses used, corresponding concentrations and approximate dilutions (10-fold) in the vitreous chamber are indicated. References for EC50s in text.

DRUG	EC50 (μM)	Dose (μmoles)	Concent. (M)	Dilution
L-NAME	eNOS: 0.5 iNOS: > 1000 nNOS unknown	High: 16 * ED50: 0.26	High: 0.5 ED50: 0.008	0.05 M 0.8 mM
N^w-PLA	eNOS: 180 iNOS: 8.5 nNOS: 0.057	High: 0.6 ** ED50: 0.3	High: 0.02 ED50: 0.01	2 mM 1.0 mM
L-NIL	eNOS: 92 iNOS: 3.3 nNOS: 61	High: 60 Low: 0.6	High: 2.0 Low: 0.02	0.2 M 2 mM
L-NIO	eNOS: 0.5 iNOS: 2.2 nNOS: 3.9	High: 6.0 Low: 0.6	High: 0.2 Low: 0.02	0.02 M 2 mM
L-NMMA	eNOS: 0.7 iNOS: 3.9 nNOS: 0.65	High: 20 ** ED50: 2.3	High: 0.7 ED50: 0.08	0.07 M 8 mM

* ED50 data from Nickla & Wildsoet, 2004.

** ED50 data from current experiments for comparison.