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Nitric Oxide Synthase Inhibitors Prevent the Growth-inhibiting Effects of Quinpirole

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Abstract

Purpose—Both dopamine and nitric oxide (NO) have been implicated in the signal cascade mediating ocular growth inhibition. If both are part of the same pathway, which precedes the other? We tested the hypothesis that dopamine acts upstream of NO, by using two NOS inhibitors in combination with the dopamine agonist quinpirole, and measuring the effects on ocular growth rate.

Methods—Chicks wore −10 D lenses or diffusers (FD) for 4d starting at age 13d. Experimental eyes received daily 20 μ l injections of the following: Quinpirole: lens: n=12; FD: n=20; n- ω -propyl-L-arginine (n-PLA): lens: n=6; FD: n=4; quinpirole + n-PLA: lens: n=17; FD: n=19; quinpirole + L-NIO: lens: n=12; FD: n=12. Saline injections were done as controls. High frequency ultrasonography was done at the start, and on day 5, prior to injections and 3 hours later. Refractions were measured on day5.

Results—As expected, quinpirole prevented the development of axial myopia in both paradigms. When quinpirole was combined with either NOS inhibitor, however, eyes became myopic compared to quinpirole (FD: n-PLA: −5.9D vs −3.4D; L-NIO: −5.8D vs −3.4D; LENS: n-PLA: −3.5D vs −0.4D; p<0.05 for all; L-NIO was not significant). This was the result of a dis-inhibition of vitreous chamber growth vs quinpirole (FD: n-PLA: 401 vs 275 μ m/4d; L-NIO: 440 vs 275 μ m/4d; LENS: n-PLA: 407 vs 253/4d; L-NIO: 403 vs 253 μ m/4d; p<0.05). Only n-PLA prevented the quinpirole-induced choroidal thickening in lens-wearing eyes (0 vs 31 μ m/3hr; p<0.05). Choroidal thickening was not inhibited by either drug in FD eyes.

Conclusions—Dopamine acts upstream of NO and the choroidal response in the signal cascade mediating ocular growth inhibition in both form deprivation and negative lens wear. That neither NOS inhibitor inhibits choroidal thickening in FD eyes suggests that the choroidal mechanisms differ in the two paradigms.

Keywords

chick; choroid; nitric oxide; myopia; emmetropization; form deprivation

The process of emmetropization is the regulation of ocular growth by visual input during post-natal development, to result in the plane of focus of distant objects falling on the retina. This is accomplished by the modulation of the axial elongation so that the final length of the eye matches the focal plane of the front optics (review: ¹). In animal models, when spectacle lenses are worn that focus an image in front of (myopic defocus) or behind (hyperopic defocus) the retina, ocular growth slows in the case of myopic defocus, and increases, in the

case of hyperopic defocus, resulting in an emmetropic eye ^{2,3}. A second, faster component of this compensatory process involves changes in choroidal thickness, with the choroid thickening to push the retina forward toward the image plane in response to myopic defocus, and thinning to pull it back, in response to hyperopic defocus ^{4,5}. The signal sequence involved in emmetropization almost certainly begins at the retina, and continues through the RPE and choroid to effect biosynthetic changes in the sclera resulting in changes in eye length. The molecules involved in this signal cascade are unknown, however, several promising candidates have evolved from animal models. One that is arguably the most promising is the retinal neuromodulator, dopamine (review: ⁶). Retinal dopamine levels fluctuate in a diurnal cycle in most species, being low at night and high during the day, thus mediating the physiological processes involved in light adaptation, such as horizontal cell uncoupling (reviews: ^{7,8}). Dopamine release is also stimulated by increases in light intensity ^{9,10} and by temporal transients (flashing lights ¹¹⁻¹³).

The initial evidence for the involvement of dopamine in the emmetropization pathway was the finding of lower daytime levels of retinal dopamine levels in form deprived chick eyes ¹⁴; this was subsequently found to be true in monkeys as well ¹⁵. Furthermore, intravitreal injections of apomorphine (chicks: ¹⁴; monkeys: ¹⁶) or dopamine (rabbits: ¹⁷) prevented the development of deprivation myopia. A D2 receptor-mediated process was supported by the findings that quinpirole, a D2 agonist, prevented myopia in response to form deprivation ¹⁸ and negative lens-wear ¹⁹. Together these results support a role for dopamine in the visual regulation of eye growth. If this is true, a crucial question for developing future therapies is: what is the visual stimulus that drives dopamine release? A recent study might shed light on this issue: Rose et al. ²⁰ reported that in children, the amount of time spent out of doors was a protective factor in the development of myopia, and suggested that the critical factor might be light intensity. Subsequent studies in animal models support this hypothesis. When chickens were raised under high (10,000 lux) versus low (50 lux) luminance levels for 90 days, those raised in low levels became myopic while those raised in high levels became hyperopic ²¹. A role for dopamine was supported by the result that DOPAC levels were reduced in these myopic eyes, and increased in hyperopic eyes ¹⁰. Finally, in chicks ²² and monkeys ²³, high luminance levels retarded the development of myopia in response to form deprivation, and in chicks, this inhibition was prevented by intravitreal injections of spiperone, a D2 dopamine antagonist.²²

If dopamine plays a role in ocular growth inhibition, where in the signal cascade from retina to sclera is it, and what other molecules are involved? Some evidence supports a link between dopamine and the gaseous neurotransmitter nitric oxide (NO). If NO release is inhibited by intravitreal injections of nitric oxide synthase (NOS) inhibitors, the ocular growth inhibition normally effected by myopic defocus is prevented ^{24,25}. NOS inhibitors also prevent the choroidal thickening normally induced by myopic defocus. These results suggest that NO may be involved in defocus-induced ocular growth inhibition and choroidal thickening. Dopamine as well, may cause choroidal thickening, as injections of the agonists apomorphine and quinpirole result in choroidal thickening ¹⁹, presumably similar to NO. So which molecule precedes the other? There is evidence in various systems for dopaminergic activation of NO release: First, apomorphine increased the concentration of NO end-products in an *in vitro* preparation of rat brains ^{26,27}. Second, histological studies showed a close synaptic association between TH-positive axon terminals onto NADPH-positive neurons in rat striatum, suggesting that the activity of NO-positive neurons is regulated by dopamine ²⁸. Third, exogenous dopamine applied to dark-adapted fish retinas *in vivo* enhanced the production of NO, and inhibiting dopamine synthesis suppressed light-evoked NO release.²⁹

We hypothesize that dopamine precedes NO in the signal cascade mediating ocular growth inhibition. In a pilot study in eyecups, the agonist apomorphine caused choroidal thickening, suggesting that dopamine affects choroidal thickness, and acts upstream of the choroid. These choroids also released more NO than controls in plain medium³⁰. If dopamine acts upstream of NO in the same pathway, then injections of NOS inhibitors concurrent with the dopamine agonist quinpirole should prevent both the ocular growth inhibition^{18, 19} and choroidal thickening¹⁹ normally effected by quinpirole. We combined quinpirole with either of two NOS inhibitors: n- ω -propyl-L-arginine (nNOS inhibitor) or L-NIO (more selective for eNOS), in both form deprived eyes and negative lens-wearing eyes, and asked (1) do the inhibitors prevent the quinpirole-induced ocular growth inhibition, and (2) are there differences between the two paradigms? Parts of this manuscript were presented in abstract form.³¹

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) and the light intensity was approximately 300 lux. Food and water were supplied ad libitum. In all experiments, the right eye was treated and the left eye served as untreated controls. Care and use of the animals conformed to the ARVO Resolution for the Care and Use of Animals in Research.

Experimental Design

Negative lenses (−10 D) or translucent diffusers were mounted on velcro rings and were attached to the matching ring that was glued to the feathers around one eye, in the morning, starting at age 13 days. At around noon on that day, chicks were anaesthetized with isoflurane inhalation anesthesia (0.8% in oxygen), and intravitreal injections (20 μ l) of the drug dissolved in saline, or saline (0.75%) were given at the same time, for 4 days. Drugs (Tocris) used were the relatively D2 selective³² dopamine agonist quinpirole, the potent (EC₅₀=57 nM) and selective nNOS inhibitor n- ω -propyl-L-arginine (NPA) (3158-fold selectivity over iNOS; 149-fold selectivity over eNOS³³), and the relatively non-selective eNOS/iNOS inhibitor L-NIO (EC₅₀ for eNOS, iNOS and nNOS, respectively: 0.5, 2.2, 3.9 μ M)³⁴. The groups were as follows: quinpirole alone: 10 nmoles in 20 μ l; lens: n=12; diffuser: n=20; NPA alone: 0.4 μ moles; lens: n=6; diffuser: n=4; quinpirole + NPA: 10 μ l each, at double the above concentration; lens: n=17; diffuser: n=19; and quinpirole + L-NIO: 20 μ l L-NIO, 300 nmoles; lens: n=12; diffuser: n=12. Each experiment had a number of diffuser- or lens-wearing saline controls to control for inter-experiment variability; these data were combined (n's in Table 1), as there were no significant differences between saline controls within the two experimental groups. A 30G needle was inserted (about 5 mm) through the skin of the lids over the superior temporal sclera after removing the feathers and cleaning the skin with alcohol. Care was taken to use the same injection site for subsequent injections. The needle remained in place for 30 seconds before being slowly withdrawn while the skin around the site was held tightly together using a small forceps. The lenses or diffusers were replaced immediately.

In all experiments, axial dimensions were measured using high frequency A-scan ultrasonography under isoflurane inhalation anesthesia (details in³⁵) at the start of lens or diffuser-wear, and on day 5, immediately prior to the injections, and then again 3 hours later to assess the choroidal response. Axial length is defined as the distance between the front of the cornea to the front of the sclera, and the data are given in rates (μ m per 4 days). Vitreous chamber depth is defined as the distance from the back of the lens to the front of the retina;

these data are also given as rates (μm per 4 days). Data on choroidal thickness changes are given as change per 3 hours. Refractive errors (RE) were measured using a Hartinger's refractometer (details in ³⁶) at the end of the experiment. The means, standard errors, and n's for all data are given in Table 1. The inequality in some categories for n's within experimental groups are due to the loss of a few data points due to measurement error or unusable ultrasound traces. For the saline groups, refractions only (no ultrasound) were done on a subset in each paradigm and are included in the data (FD, n=4; lens: n=9). In all graphs, "fellow" denotes data from all fellow eyes combined within the paradigm (lens wear or form deprivation) as there were no significant differences between drug groups.

Statistics

The Shapiro-Wilk normality test showed no evidence against normality for any parameter in any of the experimental groups, so we applied the classical one-way ANOVA to test significance between drug and saline-injected groups. *Post hoc* Bonferroni tests were used for comparisons between treatment groups and the saline group when the overall ANOVA showed significant differences across groups (Table 1). Tests between untreated fellow eyes (combined from all groups within either the lens- or diffuser paradigms) and experimental eyes used two-tailed Student's t-tests.

RESULTS

Refractive Error

As expected, quinpirole prevented the development of both lens-induced ¹⁹ and form deprivation-induced (FD) myopia ¹⁸ compared to saline controls (Figure 1: FD ANOVA, $p=0.001$; -3.4 vs -6.2 D; $p=0.0008$; Lens ANOVA, $p=0.0007$; -0.4 D vs -4.8 D; $p=0.0004$). However, if quinpirole is combined with either NOS inhibitor NPA or L-NIO, this inhibitory effect is eradicated in form deprived eyes (quinpirole vs combined with NPA and L-NIO respectively: -3.4 D vs -5.9 and -5.8 D; $p=0.0095$; $p=0.0481$). In form-deprived eyes, the refractive errors of neither of the NOS inhibitor-combined groups differed from those of saline controls (FD: -5.8 D and -5.7 D vs -6.2 D). In lens-wearing eyes, NPA was effective at countering the effect of quinpirole (-0.4 D vs -3.5 D; $p=0.0198$), but this effect was not significant for L-NIO (-0.4 D vs -2.4 D, $p=0.6973$).

Ocular Growth: Vitreous Chamber Depth

As shown before ^{18, 19}, quinpirole inhibits myopia by inhibiting growth of the back of the eye, as measured by the change in vitreous chamber depth compared to saline controls (Figure 2A) (FD ANOVA, $p=0.0042$; 275 μm vs 384 $\mu\text{m}/4\text{d}$; $p=0.0847$; Lens: ANOVA, $p=0.0044$; 253 μm vs 378 $\mu\text{m}/4\text{d}$; $p=0.0584$). The vitreous chamber elongation in quinpirole-injected eyes did not differ from those of untreated fellow controls in either paradigm (FD: 275 vs 211 μm ; $p>0.05$; Lens: 253 vs 224 μm ; $p>0.05$). For axial elongation (Figure 2B), while there was a tendency for the same effect, the differences did not reach statistical significance (FD ANOVA, $p=0.0064$; 330 vs 444 $\mu\text{m}/4\text{d}$; $p=0.2855$; Lens ANOVA, $p=0.0861$; 315 vs 469 μm ; $p=0.126$).

The myopia in eyes injected with the combination of quinpirole and either NOS inhibitor was the result of a dis-inhibition of vitreous chamber elongation; in both paradigms these eyes grew significantly faster than eyes injected with quinpirole alone (Figure 2A: quinpirole+NPA: FD: 401 vs 275 $\mu\text{m}/4\text{d}$; $p=0.0341$; Lens: 407 vs 253 μm ; $p=0.0056$; quinpirole + L-NIO: FD: 440 vs 275 μm ; $p=0.0071$; Lens: 403 vs 253 μm ; $p=0.0162$). A similar trend was seen for axial length (Figure 2B: quinpirole+NPA: FD: 492 vs 330 $\mu\text{m}/4\text{d}$; $p=0.0232$; Lens: 457 vs 315 μm ; $p=0.1825$; quinpirole + L-NIO: FD: 530 vs 330 μm ; $p=0.0111$; Lens: 451 vs 315 μm ; $p=0.2798$), despite not reaching statistical significance in

the lens paradigm. Within both paradigms, eye growth as measured by axial or vitreous chamber elongation did not differ from that in the respective saline-injected control eyes (Table 1). To summarize, combining quinpirole with either of the NOS inhibitors countered the refractive and ocular growth inhibition normally effected by quinpirole. The ocular growth inhibition was more apparent when measuring the growth at the back of the eye as reflected by vitreous chamber elongation, mainly because of the increased variability in the axial length data due to an “injection effect”, which commonly causes a decrease in anterior chamber growth (see Table 1; asterisks indicate groups in which anterior chamber growth was significantly decreased relative to uninjected fellow controls; note however that anterior chamber growth in all experimental groups was less than that in fellow eyes).

Transient Choroidal Thickening

It has previously been shown that quinpirole causes a transient increase in choroidal thickness within 3 hours of the injection into negative lens-wearing eyes¹⁹. We here find a similar effect in lens-wearing eyes (Figure 3; quinpirole vs saline: ANOVA, $p=0.0118$; 31 vs $-9\text{ }\mu\text{m}/3\text{ hrs}$; $p=0.0147$). Because NPA was shown to prevent the transient choroidal thickening in response to positive lens-induced myopic defocus²⁵, if it is so that quinpirole mediates the effects of myopic defocus as manifested in brief daily occluder-removal¹⁸, then it should similarly inhibit the thickening when combined with quinpirole, and in fact, there was a tendency for this, but in lens wearing eyes only ($0\text{ }\mu\text{m}$ vs $31\text{ }\mu\text{m}/3\text{ hrs}$; $p=0.0798$). L-NIO had no effect (Figure 3) on lens-wearing eyes, and neither NPA nor L-NIO inhibited thickening in form deprived eyes (ANOVA $p=0.0795$).

NPA Alone

Injections of the nNOS inhibitor *n*- ω -propyl-L-arginine alone had no effect on the development of form deprivation-induced myopia (vs saline: -4.2 D vs -6.2 ; $p=0.15$) or lens-induced myopia (-5.1 D vs -4.8 D ; $p=0.5$; Table 1). In keeping with the lack of a refractive effect, the drug-injected form-deprived eyes grew similar to saline controls (vit: 312 vs $384\text{ }\mu\text{m}$; $p=0.15$; axial: 422 vs $444\text{ }\mu\text{m}$; $p=0.53$). In the lens-wearing group, however, drug-injected eyes grew significantly faster (vit: 530 vs $378\text{ }\mu\text{m}$; $p=0.008$; axial: 604 vs $469\text{ }\mu\text{m}$; $p=0.036$), despite the absence of a refractive effect. We did not test the effects of L-NIO alone in either paradigm.

DISCUSSION

Our results show that combining either the nNOS inhibitor *n*- ω -propyl-L-arginine (NPA), or the non-specific NOS inhibitor L-NIO, with quinpirole prevents the ocular growth inhibition that normally is conferred by quinpirole. This is true for both paradigms of myopia-induction, form deprivation and negative lens wear, supporting a similar mechanism of action for these two molecules in the two paradigms, despite some evidence suggestive of different dopaminergic mechanisms (review: ^{6, 37}). These results support the hypothesis that dopamine acts upstream of nitric oxide in the signal cascade mediating ocular growth inhibition, and furthermore, that the release of NO is essential to the ocular growth inhibition mediated by quinpirole in negative lens-wearing and form-deprived eyes. NPA also prevents the transient choroidal thickening normally occurring in response to quinpirole in the lens-wear paradigm only, similar to its effect in eyes exposed to myopic defocus by positive lenses²⁵. If both dopamine and NO are indeed part of the signal cascade mediating ocular growth inhibition, two crucial questions remaining are: first, from which ocular tissues are they released, and second, on what tissues do each exert its effect?

Dopamine and NO

It is generally believed that the dopamine involved in the signal cascade in eye growth regulation is released from dopaminergic amacrine cells in the retina⁶. Both form deprivation⁹ and negative lens-wear^{38, 39} result in rapid reductions in vitreal levels of the dopamine metabolite DOPAC, considered to be the best indicator of dopamine release⁴⁰. In the retina as well, DOPAC levels are decreased in eyes wearing negative lenses and increased in eyes wearing positive lenses³⁹, or recovering from form-deprivation myopia⁴¹. Finally, there is a steep gradient in dopamine concentration from the retina in both inner and outer directions: to the vitreous, where it is reduced to 1/10, and to the choroid and sclera, where it is reduced by 1/3 and 1/20 respectively³⁸. Because the defocus-related effects of the lenses alter the DOPAC levels in both retina and vitreous, but have no effect on levels in the choroid or sclera³⁸, it is most likely that the tissue of origin of this visually-influenced molecule is in fact the retina, as opposed to the RPE or the choroid, neither of which are allegedly dopaminergic (although there is some evidence for dopaminergic innervation of the choroid⁴²). Our evidence that NPA prevented the quinpirole-induced choroidal thickening in lens-wearing eyes is also consistent with the hypothesis that dopamine is released upstream of the choroid, in either the retina or RPE.

As already discussed, there is evidence for dopaminergic modulation of NO release in other systems²⁶⁻²⁹. In chick eyes, the neuronal NOS inhibitor NPA transiently dis-inhibited ocular growth and prevented the defocus-induced transient choroidal thickening in eyes wearing positive lenses, supporting a link between NO, choroidal thickening and ocular growth inhibition²⁵. The growth-inhibiting dopamine agonists apomorphine and quinpirole also induce transient choroidal thickening¹⁹, linking dopamine to this sequence of events that lead to growth inhibition, and supporting the hypothesis that dopamine acts upstream of the choroid. Our current findings that two NOS inhibitors prevent the growth-inhibiting effect of quinpirole (and that NPA prevents the quinpirole-induced choroidal thickening in lens-wearing eyes) strengthen this association. If dopamine is released by retinal amacrine cells, is there a direct link between dopamine and NO, or is there an intermediary signal molecule?

If we assume that dopamine is released by amacrine cells in response to the relevant visual stimuli (myopic defocus?), it could act on other retinal neurons, or on the RPE, the apical aspect of which contains D2/D4 receptors⁴³. NO is ubiquitous in ocular tissues, being found in retina⁴⁴⁻⁴⁷, RPE and choroid^{44,48, 49} and in axon terminals of parasympathetic origin^{50, 51}, thus any of these could be downhill sources of NO. If we assume that NO directly acts on the choroid to mediate thickening, as has been suggested^{24, 25}, then it is unlikely that the amacrine cell-released dopamine acts on nitrergic retinal cells to release NO, as the NO would have to traverse the RPE, Bruch's and the high-volume blood flow of the choriocapillaris to exert its effect. For this same reason, it is unlikely that dopamine released from the retina crosses the RPE and choriocapillaris to act on nitrergic choroidal neurons. Alternative possibilities are that (1) dopamine binds receptors in the RPE that releases a signal molecule which effects the release of NO from a choroidal source, such as the ICNs or the axon terminals of the parasympathetic inputs to the choroid, or (2) the RPE is the source of NO, which diffuses through Bruch's and the choriocapillaris to reach the choroid. Further research is required to answer this important question.

Why Did Both NOS Inhibitors Inhibit Quinpirole, but Only One Inhibit the Effects of Lens-Induced Myopic Defocus?

In previous work, we found that of three tested NOS inhibitors, only NPA had similar effects as those of the non-specific inhibitor L-NAME on eyes exposed to myopic defocus. From this we inferred that the neuronal isoform was the one that was part of the inhibitory

signal cascade activated by myopic defocus and leading to growth inhibition²⁵. In this study we used both NPA, and L-NIO, which is relatively non-selective, in combination with quinpirole, and found that both drugs prevented the inhibitory effects of quinpirole on eye growth in both paradigms. What are the implications? If we assume that the inhibitors were acting on separate isoforms (NPA on nNOS; L-NIO on iNOS or eNOS), then (1) if quinpirole (dopamine) was effective on *both* isoforms to effect ocular growth inhibition, then neither inhibitor alone should have prevented quinpirole's effect, (2) if quinpirole acted only on nNOS, then NPA should have prevented quinpirole's effect, but L-NIO should have been *ineffective*, (3) if quinpirole acted only on eNOS (or iNOS), then L-NIO should have prevented quinpirole's effect, but NPA should have been ineffective. Because *both* inhibitors were effective at preventing the quinpirole-induced growth inhibition suggests that the L-NIO was exerting a "cross-over" effect on nNOS, perhaps due to the multiple injection protocol over the 4 day period in this paradigm, as opposed to the single-injection one over a one day period in the former study²⁵. It should be noted that NPA alone showed a significant affect on eye growth only in the lens-wearing eyes (which was not reflected in refractive error): eyes grew faster than saline controls. If this is a real effect, it would suggest that loss of nitric oxide alone might lead to ocular growth stimulation. This would have to be tested explicitly.

An alternative explanation for the lack of effect of L-NIO in preventing the positive-lens-induced ocular growth inhibition²⁵ in the prior study versus its effectiveness in preventing quinpirole's effect in the present study, is that dopamine (quinpirole) does not, in fact, mediate the effects of myopic defocus. This supposition however, is not supported by existing data: Quinpirole prevented the myopiagenic effects of form deprivation, and injecting spiperone, a D2 antagonist prior to giving these eyes brief periods of daily vision prevented the vision-related (presumably small amounts of myopic defocus) effects¹⁸. These results support the hypothesis that dopamine mediates the effects of form vision (or myopic defocus). Quinpirole also prevented the myopia-genic effects of negative lens-wear in a similar manner¹⁹, further supporting the above hypothesis.

The Choroid

It was previously shown that the growth inhibition elicited by quinpirole in eyes wearing negative lenses was associated with transient choroidal thickening¹⁹, possibly indicating that the two are in the same pathway. On the other hand, in negative lens-wearing and form deprived eyes, ocular growth stimulation was associated with choroidal thinning, possibly linking these in the same pathway⁵². We here show that co-injections of quinpirole with the nNOS inhibitor NPA inhibited the choroidal thickening response in lens-wearing eyes, as would be expected if a thinner choroid was an integral part of the growth stimulatory cascade, however, L-NIO was ineffective: choroids thickened presumably in response to the quinpirole. Because both inhibitors prevented the quinpirole-induced ocular growth inhibition, but only one prevented choroidal thickening, suggests that choroidal thinning is *not* an integral part of the growth-stimulating pathway. This idea is supported by several studies: first, double parasympathectomy inhibited (rather than stimulated) eye growth in form deprived eyes, but it did *not* prevent the choroidal thinning response⁵³. Second, muscarinic agonists that caused choroidal thinning in normal eyes had no effect on eye growth⁵⁴. Alternatively, the apparent lack of effect of L-NIO in the former study may be reflective of a difference in the time course for the effects between the two drugs, as we only measured 3 hours after the injection, or of a different efficacy of the inhibitor's effect on NO and on the choroid.

Another interesting result was that neither inhibitor was effective at preventing choroidal thickening in form-deprived eyes. This might reflect a deprivation-related difference in the

physiological state of choroids versus those in lens-wearing eyes. This idea is supported by evidence indicating pathological changes in the RPE of form deprived eyes that were indicative of hypo-osmolarity of the choroids⁵⁵. Another finding supporting physiological differences in choroids of deprived eyes versus those of lens-wearing eyes is that in double-parasympathectomized form deprived eyes, the choroids that thinned in response to the deprivation (without attendant myopia development) thickened upon removal of the diffuser, despite the absence of myopic defocus⁵³. It follows that the compensatory choroidal thinning induced by negative-lens-wear is fundamentally different from that (non-compensatory) thinning induced by form deprivation. By extension, the lack of effect of the inhibitors on the choroidal thickening in these form deprived eyes presumably implies that this thickening is mediated by a non-nitric mechanism.

In conclusion, our results suggest that dopamine acts upstream of NO and the choroidal response in the signal cascade mediating ocular growth inhibition in both form deprivation and negative lens wear. That NPA inhibited the quinpirole-induced choroidal thickening in lens-wearing eyes but not in form-deprived eyes suggests that choroidal thinning is not required for ocular growth stimulation. That neither inhibited choroidal thickening in FD eyes suggests that the choroidal mechanisms differ in the two paradigms.

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REFERENCES

1. Wallman J, Winawer J. Homeostasis of eye growth and the question of myopia. *Neuron*. 2004; 43:447–68. [PubMed: 15312645]
2. Irving EL, Sivak JG, Callender MG. Refractive plasticity of the developing chick eye. *Ophthalmic Physiol Opt*. 1992; 12:448–56. [PubMed: 1293533]
3. Schaeffel F, Glasser A, Howland HC. Accommodation, refractive error and eye growth in chickens. *Vision Res*. 1988; 28:639–57. [PubMed: 3195068]
4. Wildsoet C, Wallman J. Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks. *Vision Res*. 1995; 35:1175–94. [PubMed: 7610579]
5. Wallman J, Wildsoet C, Xu A, Gottlieb MD, Nickla DL, Marran L, Krebs W, Christensen AM. Moving the retina: choroidal modulation of refractive state. *Vision Res*. 1995; 35:37–50. [PubMed: 7839608]
6. Feldkaemper M, Schaeffel F. An updated view on the role of dopamine in myopia. *Exp Eye Res*. 2013 In press.
7. Witkovsky P. Dopamine and retinal function. *Doc Ophthalmol*. 2004; 108:17–40. [PubMed: 15104164]
8. Witkovsky P, Deary A. Functional roles of dopamine in the vertebrate retina. *Prog Ret Res*. 1991; 11:247–92.
9. Megaw PL, Morgan IG, Boelen MK. Dopaminergic behaviour in chicken retina and the effect of form deprivation. *Aust N Z J Ophthalmol*. 1997; 25(Suppl 1):S76–8. [PubMed: 9267633]
10. Cohen Y, Peleg E, Belkin M, Polat U, Solomon AS. Ambient illuminance, retinal dopamine release and refractive development in chicks. *Exp Eye Res*. 2012; 103:33–40. [PubMed: 22960317]
11. Weiler R, Baldridge WH, Mangel SC, Dowling JE. Modulation of endogenous dopamine release in the fish retina by light and prolonged darkness. *Vis Neurosci*. 1997; 14:351–6. [PubMed: 9147486]
12. Umino O, Lee Y, Dowling JE. Effects of light stimuli on the release of dopamine from interplexiform cells in the white perch retina. *Vis Neurosci*. 1991; 7:451–8. [PubMed: 1764415]

13. Boelen MK, Boelen MG, Marshak DW. Light-stimulated release of dopamine from the primate retina is blocked by 1-2-amino-4-phosphonobutyric acid (APB). *Vis Neurosci.* 1998; 15:97–103. [PubMed: 9456509]
14. Stone RA, Lin T, Laties AM, Iuvone PM. Retinal dopamine and form-deprivation myopia. *Proc Natl Acad Sci U S A.* 1989; 86:704–6. [PubMed: 2911600]
15. Iuvone PM, Tigges M, Fernandes A, Tigges J. Dopamine synthesis and metabolism in rhesus monkey retina: development, aging, and the effects of monocular visual deprivation. *Vis Neurosci.* 1989; 2:465–71. [PubMed: 2577263]
16. Iuvone PM, Tigges M, Stone RA, Lambert S, Laties AM. Effects of apomorphine, a dopamine receptor agonist, on ocular refraction and axial elongation in a primate model of myopia. *Invest Ophthalmol Vis Sci.* 1991; 32:1674–7. [PubMed: 2016144]
17. Gao Q, Liu Q, Ma P, Zhong X, Wu J, Ge J. Effects of direct intravitreal dopamine injections on the development of lid-suture induced myopia in rabbits. *Graefes Arch Clin Exp Ophthalmol.* 2006; 244:1329–35. [PubMed: 16550409]
18. McCarthy CS, Megaw P, Devadas M, Morgan IG. Dopaminergic agents affect the ability of brief periods of normal vision to prevent form-deprivation myopia. *Exp Eye Res.* 2007; 84:100–7. [PubMed: 17094962]
19. Nickla DL, Totonelly K, Dhillon B. Dopaminergic agonists that result in ocular growth inhibition also elicit transient increases in choroidal thickness in chicks. *Exp Eye Res.* 2010; 91:715–20. [PubMed: 20801115]
20. Rose KA, Morgan IG, Ip J, Kifley A, Huynh S, Smith W, Mitchell P. Outdoor activity reduces the prevalence of myopia in children. *Ophthalmology.* 2008; 115:1279–85. [PubMed: 18294691]
21. Cohen Y, Belkin M, Yehezkel O, Solomon AS, Polat U. Dependency between light intensity and refractive development under light-dark cycles. *Exp Eye Res.* 2011; 92:40–6. [PubMed: 21055401]
22. Ashby R, Ohlendorf A, Schaeffel F. The effect of ambient illuminance on the development of deprivation myopia in chicks. *Invest Ophthalmol Vis Sci.* 2009; 50:5348–54. [PubMed: 19516016]
23. Smith EL 3rd, Hung LF, Huang J. Protective effects of high ambient lighting on the development of form-deprivation myopia in rhesus monkeys. *Invest Ophthalmol Vis Sci.* 2012; 53:421–8. [PubMed: 22169102]
24. Nickla DL, Wildsoet CF. 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; 81:111–8. [PubMed: 15127930]
25. Nickla DL, Damyanova P, Lytle G. 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. *Exp Eye Res.* 2009; 88:1092–9. [PubMed: 19450449]
26. Melis MR, Succu S, Argiolas A. Dopamine agonists increase nitric oxide production in the paraventricular nucleus of the hypothalamus: correlation with penile erection and yawning. *Eur J Neurosci.* 1996; 8:2056–63. [PubMed: 8921295]
27. Morris BJ, Simpson CS, Mundell S, Maceachern K, Johnston HM, Nolan AM. Dynamic changes in NADPH-diaphorase staining reflect activity of nitric oxide synthase: evidence for a dopaminergic regulation of striatal nitric oxide release. *Neuropharmacology.* 1997; 36:1589–99. [PubMed: 9517430]
28. Fujiyama F, Masuko S. Association of dopaminergic terminals and neurons releasing nitric oxide in the rat striatum: an electron microscopic study using NADPH-diaphorase histochemistry and tyrosine hydroxylase immunohistochemistry. *Brain Res Bull.* 1996; 40:121–7. [PubMed: 8724430]
29. Sekaran S, Cunningham J, Neal MJ, Hartell NA, Djamgoz MB. Nitric oxide release is induced by dopamine during illumination of the carp retina: serial neurochemical control of light adaptation. *Eur J Neurosci.* 2005; 21:2199–208. [PubMed: 15869516]
30. Nickla, D. Evidence that changes in choroidal thickness play a causal role in emmetropization; Presentation at ARVO meeting, Ft; Lauderdale, FL. Apr 30. 2006 Available at: http://files.abstractsonline.com/SUPT/163/1601/Presentation_Title_PDF_wlinks.htm

31. Totonelly K, Lee L, Cheng A, Nickla DL. Evidence that dopamine acts upstream of dopamine in the signal cascade mediating ocular growth inhibition. *Invest Ophthalmol Vis Sci*. 2012; 53 E-abstract 4660.
32. Sullivan RM, Talangbayan H, Einat H, Szechtman H. Effects of quinpirole on central dopamine systems in sensitized and non-sensitized rats. *Neuroscience*. 1998; 83:781–9. [PubMed: 9483561]
33. 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; 40:3869–70. [PubMed: 9397167]
34. 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; 37:3886–8. [PubMed: 7525961]
35. Nickla DL, Wildsoet C, Wallman J. Visual influences on diurnal rhythms in ocular length and choroidal thickness in chick eyes. *Exp Eye Res*. 1998; 66:163–81. [PubMed: 9533843]
36. Wallman J, Adams JL. Developmental aspects of experimental myopia in chicks: susceptibility, recovery and relation to emmetropization. *Vision Res*. 1987; 27:1139–63. [PubMed: 3660666]
37. Nickla DL, Totonelly K. Dopamine antagonists and brief vision distinguish lens-induced-and form-deprivation-induced myopia. *Exp Eye Res*. 2011; 93:782–5. [PubMed: 21872586]
38. Ohngemach S, Hagel G, Schaeffel F. Concentrations of biogenic amines in fundal layers in chickens with normal visual experience, deprivation, and after reserpine application. *Vis Neurosci*. 1997; 14:493–505. [PubMed: 9194316]
39. Guo SS, Sivak JG, Callender MG, Diehl-Jones B. Retinal dopamine and lens-induced refractive errors in chicks. *Curr Eye Res*. 1995; 14:385–9. [PubMed: 7648864]
40. Megaw P, Morgan I, Boelen M. Vitreal dihydroxyphenylacetic acid (DOPAC) as an index of retinal dopamine release. *J Neurochem*. 2001; 76:1636–44. [PubMed: 11259481]
41. Pendrak K, Nguyen T, Lin T, Capehart C, Zhu X, Stone RA. Retinal dopamine in the recovery from experimental myopia. *Curr Eye Res*. 1997; 16:152–7. [PubMed: 9068946]
42. Schrodl F, Tines R, Brehmer A, Neuhuber WL. Intrinsic choroidal neurons in the duck eye receive sympathetic input: anatomical evidence for adrenergic modulation of nitrergic functions in the choroid. *Cell Tissue Res*. 2001; 304:175–84. [PubMed: 11396712]
43. Rymer J, Wildsoet CF. The role of the retinal pigment epithelium in eye growth regulation and myopia: a review. *Vis Neurosci*. 2005; 22:251–61. [PubMed: 16079001]
44. Fischer AJ, Stell WK. 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]
45. Koistinaho J, Sagar S. NADPH-diaphorase-reactive neurons in the retina. *Prog Ret Eye Res*. 1995; 15:69–87.
46. Goldstein IM, Ostwald P, Roth S. Nitric oxide: a review of its role in retinal function and disease. *Vision Res*. 1996; 36:2979–94. [PubMed: 8917798]
47. Sato T. NADPH-diaphorase positive amacrine cells in the retinae of the frog (*Rana esculenta*) and pigeon (*Columbia livia*). *Arch Histol Cytol*. 1990; 53:63–9. [PubMed: 2364012]
48. Schrodl, F. Intrinsic choroidal neurons. In: Troger, J.; Kieselbach, G.; Bechrakis, N., editors. *Neuropeptides in the Eye*. Research Signpost; Trivandrum; 2009. p. 169–97.
49. Bergua A, Mayer B, Neuhuber WL. Nitrergic and VIPergic neurons in the choroid and ciliary ganglion of the duck *Anis carina*. *Anat Embryol (Berl)*. 1996; 193:239–48. [PubMed: 8881473]
50. Nilsson SF. Nitric oxide as a mediator of parasympathetic vasodilation in ocular and extraocular tissues in the rabbit. *Invest Ophthalmol Vis Sci*. 1996; 37:2110–9. [PubMed: 8814150]
51. Sun W, Erichsen JT, May PJ. NADPH-diaphorase reactivity in ciliary ganglion neurons: a comparison of distributions in the pigeon, cat, and monkey. *Vis Neurosci*. 1994; 11:1027–31. [PubMed: 7947396]
52. Nickla DL, Wallman J. The multifunctional choroid. *Prog Retin Eye Res*. 2010; 29:144–68. [PubMed: 20044062]
53. Nickla DL, Schrodl F. Parasympathetic influences on emmetropization in chicks: evidence for different mechanisms in form deprivation vs negative lens-induced myopia. *Exp Eye Res*. 2012; 102:93–103. [PubMed: 22828050]

54. Nickla DL, Zhu X, Wallman J. Effects of muscarinic agents on chick choroids in intact eyes and eyecups: evidence for a muscarinic mechanism in choroidal thinning. *Ophthalmic Physiol Opt.* 2013; 33:245–56. [PubMed: 23662958]
55. Junghans BM, Crewther SG, Liang H, Crewther DP. A role for choroidal lymphatics during recovery from form deprivation myopia? *Optom Vis Sci.* 1999; 76:796–803. [PubMed: 10566864]

Figure 1.

Refractive error in diopters (D) at the end of the 4 days in form deprived eyes (FD: black bars) and lens-wearing eyes (Lenses: white bars), injected with drugs or saline, and untreated fellow controls. Quin: quinpirole; Quin+NPA: quinpirole +NPA; Quin+NIO: quinpirole + L-NIO; Sal: saline controls combined from all experimental groups; Fellow: untreated fellow eyes from all experiments. * $p<0.05$; ** $p<0.001$

Figure 2.

Change in eye length (μm) over 4 days in form deprived eyes (FD: black bars) and lens-wearing eyes (Lenses: white bars), injected with drugs or saline, and untreated fellow controls. A. Change in vitreous chamber depth (lens to sclera). B. Change in axial length (front of cornea to sclera). Abbreviations are the same as above. * $p < 0.0584$; ** $p < 0.01$

Figure 3. Change in choroidal thickness (μm) over 3 hours after the injection in lens-wearing eyes. Abbreviations are the same as above. * $p < 0.05$

Table 1

Means, standard errors (in parentheses), and p values for experimental groups in both paradigms.

	QUIN		Q+NPA		NPA		Q+L-NIO		SALINE		ANOVA		Post-hoc pair comparison p values *			
	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	P Value	Q vs sal	Q vs NPA	Q vs NIO	NPA vs sal	L-NIO vs sal
FD																
REF	18	-3.4 (0.4)	19	-5.9 (0.6)		-4.2 (1.0)	12	-5.8 (0.7)	26	-6.2 (0.4)	0.001	0.0008	0.0095	0.0481	1.0	1.0
VIT	20	275 (29)	18	401 (31)		312 (31)	12	440 (49)	22	384 (26)	0.0042	0.0847	0.0341	0.0071	1.0	1.0
AXI	20	330 (34)	18	492 (38)		422 (18)	12	530 (47)	22	444 (24)	0.0064	0.2855	0.0232	0.0111	1.0	0.5601
CHO	20	21 (4.6)	19	19 (6.0)		32 (18)	6	-5.0 (11)	22	3.0 (7)	0.0795	0.2386	1.0	0.5463	0.4385	1.0
AC	18	129* (14)	15	153 (20)		136 (21)	12	155 (32)	22	148 (16)	0.7614	1.0	1.0	1.0	1.0	1.0
LENS																
REF	12	-0.4 (0.9)	17	-3.5 (0.6)		-5.1 (0.8)	12	-2.4 (0.8)	23	-4.8 (0.5)	0.0007	0.0004	0.0198	0.6973	1.0	0.3686
VIT	12	253 (37)	17	407 (32)		530 (40)	12	403 (33)	14	378 (29)	0.0044	0.0584	0.0056	0.0162	1.0	1.0
AXI	12	315 (26)	15	457 (57)		604 (47)	12	451 (41)	14	469 (43)	0.0861	0.126	0.1824	0.2798	1.0	1.0
CHO	12	31 (5.0)	17	0.0 (9.0)		19 (9.0)	12	17 (8)	14	-9.0 (9)	0.0118	0.0147	0.0798	1.0	1.0	0.3448
AC	11	144* (15)	15	152 (14)		98 (22)	12	121* (16)	20	184 (25)	0.1212	0.3557	1.0	1.0	1.0	0.1708

* For AC only, denotes significant difference from combined fellow (untreated) controls; for FD=238 µm; Lens=206 µm. This is a reflection of a vitreal "injection effect" on anterior chamber depth.

Bold denotes statistical significance

Note that ANOVA does not include NPA alone