# MECHANISMS OF EMMETROPIZATION: EVIDENCE THAT DOPAMINE ACTS UPSTREAM OF NITRIC OXIDE IN THE SIGNALING CASCADE MEDIATING OCULAR GROWTH INHIBITION

A thesis presented to the graduate faculty of
New England College of Optometry in partial fulfillment
of the requirements for the degree of Master of Science

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## Abstract

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New England College of Optometry, 2014

***Purpose:*** Both dopamine (DA) and nitric oxide (NO) have been implicated in the signaling cascade regulating ocular growth inhibition. It is unclear where in the signaling cascade DA and NO exert their effects and whether these two molecules act in the same pathway. If they are indeed involved together, this study seeked to understand the relationship between the two molecules. Specifically, it will determine which molecule acts upstream of the other. This current study tested the hypothesis that dopamine acts upstream of nitric oxide by using two nitric oxide synthase (NOS) inhibitors in combination with the DA agonist quinpirole and measured the effects on ocular growth.

***Methods:*** 12-day-old chicks were fitted with either translucent diffusers or -10D lenses on the right eye for 5 days to induce form deprivation- or lens induced-myopia, respectively. Daily intra-vitreal injection of 20µl of either (a) quinpirole (n=20, diffusers; n=12, lenses), (b) $N^{\omega}$-propyl-L-arginine (NPLA) (n=4, diffusers; n=6, lenses), (c) NPLA and qunipirole (10µl ea) (n=19, diffusers; n=17, lenses), (d) L-$N^5$-(1-Iminoethyl)-ornithine (L-NIO) and quinpirole (10µl ea) (n=12, diffusers; n=12, lenses), or (d) saline (n=26, diffusers; n=23, lenses) were given in the experimental eye. Ocular measurements with the A-scan

ultrasonography were made on day 0, pre-injection and 3 hours post-injection, and on day 5. Refractive errors were measured on the final day.

***Results:*** As expected, quinpirole prevented the development of axial myopia in both form deprivation (FD) and negative lens-wearing eyes. The combination of either NOS inhibitor with quinpirole resulted in myopic eyes compared to quinpirole alone (FD: NPLA: -5.9 D vs. -3.4 D; L-NIO: -5.8 D vs. -3.4 D; negative lens: NPLA: -3.5 D vs. -0.4 D; $p < 0.05$ for all; L-NIO was not significant). This was the result of a dis-inhibition of vitreous chamber growth (FD: NPLA: 401 vs. 275 μm/4 d; L-NIO: 440 vs. 275 μm/4 d; negative lens: NPLA: 407 vs. 253μm/4 d; L-NIO: 403 vs. 253 μm/4 d; $p < 0.05$). Interestingly, NPLA prevented the quinpirole-induced choroidal thickening only in lens wearing eyes (0 vs. 31 μm/3 h; $p < 0.05$). Neither NOS inhibitor prevented choroidal thickening in form deprived eyes.

***Conclusions:*** Dopamine acts upstream of nitric oxide and the choroidal response in the signaling cascade mediating ocular growth inhibition in both myopia paradigms. The lack of inhibition of the choroidal thickening with either NPLA or L-NIO in the form deprivation paradigm suggests that perhaps the mechanism regulating the choroidal response may in fact differ between the two.

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## 1. Introduction

Myopia, or nearsightedness, is a highly prevalent ocular condition affecting about 30% of the population in the United States and as high as 80% in East Asia (review: Pan, Ramamurthy, & Saw, 2012). Along with this increased prevalence, myopia also poses a public health challenge as it involves a substantial risk for multiple associated ocular pathologies and complications (review: Saw, Gazzard, Shih-Yen, & Chua, 2005). Although it is well understood that myopia is an optical misalignment resulting in the plane of the image falling in front of the retina, its etiology is still unclear. Due to the complexity of myopia development and progression, there is a lack of consensus on whether myopia is the product of inheritance or the influence of environmental experiences.

Factors such as genetics have been shown to increase the risk of myopia development. A strong association has been shown between the number of myopic parents and the increased prevalence of myopia in children (Ip, Huynh, Robaei, Rose, Morgan, Smith, Kifley, & Mitchell, 2007). For example, children with two myopic parents were more likely to develop myopia than those with one or neither parent being myopic (Mutti, Mitchell, Moeschberger, Jones, & Zadnik, 2002; Pacella, McLellan, Grice, Del Bono, Wiggs, & Gwiazda, 1999). Classical twins studies further support the theory that myopia is inheritable (Hammond, Snieder, Gilbert, & Spector, 2001). Recent research has reported a genetic correlation between refraction and axial length among twins, suggesting that perhaps there is a component of genetics in myopia (Dirani, Shekar, & Baird, 2008).

On the other hand, environmental factors have also been thought to contribute to the development of myopia. It has been hypothesized that a relationship existed between near

work and the onset of myopia, with an increased amount of near work to be associated with an increased risk of myopia development (Saw, Chua, Hong, Wu, Chan, Chia, Stone, & Tan, 2002). More recently, research has highlighted the role of the environment in the prevention of myopia development and progression. Specifically, research has shown that outdoor activity is protective against myopia (Jones, Sinnott, Mutti, Mitchell, Moeschberger, & Zadnik, 2007; Rose, Morgan, Ip, Kifley, Huynh, Smith, & Mitchell, 2008). In fact, less time spent outdoors has been associated with an increase in myopia (French, Morgan, Mitchell, & Rose, 2013; Guggenheim, Northstone, McMahon, Ness, Deere, Mattocks, Pourcain, & Williams, 2012).

Other evidence also suggests the role of peripheral refractive error in the onset of myopia (Mutti, Hayes, Mitchell, Jones, Moeschberger, Cotter, Kleinstein, Manny, Twelker, Zadnik, & CLEERE Study Group, 2007), thus, highlighting the complex nature of this ocular condition. The debate continues as to whether myopia is influenced by genetic or environmental factors, but current literature suggests that it may be a multifactorial process. It is highly likely that some individuals are predisposed to myopia development but environmental signals may provide the cues to either retard or trigger myopia progression. This interaction between both genetic and environmental factors may be the main contributors. However, the question still remains: what is the underlying mechanism regulating myopia development and progression?

### *1.1. Animal Models of Myopia—Chick Model*

The establishment of animal models of myopia has provided the greatest insight in

understanding the normal development and regulation of eye growth and how disruptions to the regulatory systems can result in refractive error states. The ability to induce refractive error in animal models and experimentally manipulate environmental settings and conditions has provided a framework to study ametropia that parallels the natural occurrences in humans. Form deprivation, for example, initially with lid suturing but now with the use of translucent diffusers in animal models has been an established and accepted method of inducing myopia. This paradigm of myopia mirrors the myopic development in human infants as a result of eyelid closure or hemangiomas (Hoyt, Stone, Fromer, & Billson, 1981; Robb, 1977). In addition, the use of lenses in animal studies to mimic the defocus that humans experience has further shown the relevance and applicability of animal models in the study of the etiology of human myopia.

A number of animal species has been used in the study of myopia including mammals (cats, rabbits, rodents, monkeys), birds, fishes, and reptiles (review: Norton, 1999). Perhaps the most commonly used model in the study of myopia has been that of the chick. The chick has been an ideal model due to its high availability, low cost, rapid development, and quick response to experimental manipulations. Form deprivation, as previously mentioned, when used in chicks has resulted in axial elongation and myopia development (Wallman, Turkel, & Trachtman, 1978). Myopia studies with chicks have also shown structural and chemical changes within the eyes associated with myopia development (Gottlieb, Fugate-Wentzek, & Wallman, 1987; Stone, Lin, Laties, & Iuvone, 1989). The chick model, like other animal models, is not without its limitations. First, chick eyes are laterally displaced compared to human eyes. In addition, the chick eye lacks an intraretinal blood supply or a fovea. It is

arguable that due to these differences in the anatomical structure and design of the chick eye, they may not serve as the appropriate model for human myopia development; however, results from studies with chick models have translated well to humans and other animal models. For instance, results showing a significant decrease in the variability of refractive error patterns in chicks from hatching to about 8 weeks (Wallman, Adams, & Trachtman, 1981) parallels the decreased variability in the astigmatic refractive error of human infants (Gwiazda, Scheiman, Mohindra, & Held, 1984). The chick model has also been invaluable in elucidating that accommodation is not the underlying mechanism of atropine drops in its prevention of myopia development (McBrien, Moghaddam, & Reeder, 1993). Moreover, results from chick studies have suggested that ocular growth is mediated locally, with similar findings reported in monkeys.

### *1.2. Local Regulation of Ocular Growth*

It is generally accepted that the signaling cascade regulating ocular growth inhibition begins at the level of the retina. Optic nerve section studies showed that control of eye growth appears to be independent of higher order processes. Specifically, optic nerve sections in chicks wearing partial occluders did not prevent axial elongation or myopia development, supporting a localized retinal mechanism in regulating eye growth (Troilo, Gottlieb, & Wallman, 1987). In addition, animal studies inducing unilateral myopia in the experimental eye subjected either to form deprivation or lens wear did not subsequently induce myopia in the fellow control eye, suggesting local regional regulation of eye growth. This hypothesis has been supported by studies showing that partial occlusion in chicks with

translucent occluders resulted in myopia correlating only to the deprived sections of the retina, with the non-deprived regions remaining relatively emmetropic (Wallman, Gottlieb, Rajaram, & Fugate-Wentzek, 1987). Similar results were reported in monkeys subjected to hemi-field form deprivation (Smith III, Huang, Hung, Blasdel, Humbird, & Bockhorst, 2009). Results from lens wear in chicks inducing local retinal defocus further support the hypothesis of a local regulation in eye growth. In these studies, hemi-field lenses creating defocus in either the nasal or temporal visual field resulted in myopia development restricted only to these regions with retinal blur (Diether & Schaeffel, 1997). That partial form deprivation or defocus resulted in localized myopia but not in the unrestricted retinal regions also suggests that postnatal regulation of eye growth may be dependent on visual experiences (Diether & Schaeffel, 1997; Smith III et al., 2009; Wallman et al., 1981).

### *1.3. Visual Regulation of Eye Growth*

Several studies have supported the hypothesis that a visual feedback mechanism exists that guides postnatal eye growth. As previously mentioned above, form deprivation and lens wear in animal studies result in myopia. Similar results were found in humans where disruptions in visual experiences also resulted in myopia (Nathan, Kiely, Crewther, & Crewther, 1985; Rabin, Van Sluyters, & Malach, 1981), suggesting the importance of visual input in the regulation of eye growth. Perhaps the strongest evidence for the visual regulation of eye growth comes from studies showing the linear relationship between the magnitude and direction of spectacle lens power and the refractive and axial responses in the chick model. Studies have shown that spectacle lens induced defocus resulted in the

development of refractive errors in chicks that are consistently associated with changes in the rate of ocular growth (Irving, Sivak, & Callender, 1992). The use of negative lenses, for example, which create hyperopic defocus, resulted in myopia development as axial length increased. On the other hand, the use of positive lenses, which create myopic defocus, resulted in the development of hyperopia, as axial length decreased (Irving et al., 1992). It has been further suggested that the eye has the ability to distinguish between different types of defocus (Diether & Schaeffel, 1997) and to respond accordingly by using luminance and chromatic contrast changes to distinguish between myopic and hyperopic defocus (Rucker & Wallman, 2012). This suggests that the quality of the visual experiences may be important as well. The eye's compensatory response to defocus supports a role for visual stimulus in regulating ocular growth (Miles & Wallman, 1990; Rucker & Wallman, 2009, 2012). Moreover, recovery of form deprivation myopia with unrestricted normal vision has been reported in chicks (Wallman & Adams, 1987), tree shrews (Siegwart Jr & Norton, 1998), and monkeys (Smith III, Hung, Kee, & Qiao, 2002), providing additional evidence of visually guided mechanisms in the regulation of eye growth.

### *1.4. Ocular Growth and the Sclera*

Evidence from animal studies has shown that changes in the biosynthesis rates in the sclera underlie changes in eye growth. As eye growth decreases, a decrease is seen in scleral glycosaminoglycan (GAG) synthesis. Conversely, as eye growth increases, scleral GAG synthesis also increases. Results have also shown an increase in axial elongation and thinning of the sclera with myopia development, suggesting a role for scleral involvement in

regulating ocular growth (reviews: McBrien & Gentle, 2003; Wallman, 1990). Active scleral remodeling and changes in scleral GAG synthesis within the posterior sclera have been reported in a number of species subjected to form deprivation (chicks: Christensen & Wallman, 1991; Rada & Matthews, 1994; tree shrews: McBrien, Lawlor, & Gentle, 2000; monkeys: Funata & Tokoro, 1990; Rada, Nickla, & Troilo, 2000). Similar results were reported in response to spectacle lens wear in chicks (Wildsoet & Wallman, 1995; Nickla, Wildsoet, & Wallman, 1997) and tree shrews (Frost & Norton, 2007), indicating that the sclera is in fact involved in eye growth. Additionally, it has been shown that different layers of the sclera have different responses. The chick sclera, for example, is composed of an outer fibrous layer and an inner cartilaginous layer. During form deprivation, an increase in GAG synthesis occurs in the cartilaginous layer resulting in the thickening of this layer. The opposite occurs in the fibrous layer, where there is a decrease in scleral GAG synthesis and thinning of the fibrous layer of the sclera (Gottlieb, Joshi, & Nickla, 1990). These changes in the fibrous layer of the chick sclera in myopic eyes are similar and analogous to what is seen in the mammalian fibrous sclera despite the differences observed in the cartilaginous layer. To further support the sclera's role in the regulation of ocular growth, research in chicks showed that inhibition of scleral proteoglycan synthesis inhibited axial elongation in deprived chick eyes (Rada, Johnson, Achen, & Rada, 2002). Moreover, gene knock out studies in zebrafish causing downregulation of the gene responsible for an important proteoglycan in the sclera resulted in scleral thinning and ocular changes characteristic of axial myopia (Yeh, Liu, Kao, Huang, Hu, Chien, & Wang, 2010). McBrien et al. (2000) specifically showed that scleral GAG synthesis in formed deprived tree shrews was

decreased but upon removal of the occulders, increased levels of scleral GAG synthesis were detected as early as 24 hours after unrestricted normal vision, suggesting a role of the sclera in visually guided mechanisms of eye growth. Furthermore, research shows that there is a circadian rhythm in scleral proteoglycan synthesis, with higher levels of synthesis during the day than at night (Nickla, Rada, & Wallman, 1999). This rhythm appears to be associated with rhythms in ocular growth as eye growth increases during the day and decreases at night, thus, providing evidence that the sclera may determine growth.

### *1.5. The Role of the Choroid*

The choroid, a vascular layer, is now known to have multiple functions that may include regulation of eye growth. In humans, the choroid provides the main blood supply to the outer retina, while in birds it is responsible for supplying nourishment to the entire retina. The choroid also functions in thermoregulation and secretion of growth factors and other molecules. Additionally, the choroid has been shown to be a secretory tissue, and has been hypothesized to secrete molecules involved in regulating the growth of the sclera and in turn ocular growth (reviews: Nickla & Wallman, 2010; Summers, 2013).

Research has shown a correlation between choroidal thickness and refractive error states. Work by Wallman, Wildsoet, Xu, Gottlieb, Nickla, Marran, Krebs, and Christensen (1995) in chicks showed that in response to myopic defocus with plus lenses, the choroid thickens to bring the retina to the plane of the image. The opposite occurs in hyperopic defocus with minus lenses, where the choroid thins. Similar results were also reported in primates where thicker choroids were associated with hyperopic eyes and thinner choroids

associated with myopic eyes, although to a lesser degree than in chicks (Troilo, Nickla DL, & Wildsoet, 2000). Recently, the choroid in myopic children has also been reported to be significantly thinner than their non-myopic counterparts using optical coherence tomography (OCT; Read, Collins, Vincent, & Alonso-Caneiro, 2013).

Choroidal responses to defocus have also been shown to be rapid and transient. In fact, research showed that only 10 minutes of lens wear resulted in significant choroidal thickness changes measured 2 hours later (Zhu, Park, Winawer, & Wallman, 2005). However, the underlying mechanism regulating these changes is unknown. It was hypothesized that nitric oxide may play a role in modulating choroidal thickness. Work by Nickla and Wildsoet (2004) using the non-specific nitric oxide inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), showed that it prevented the defocus-induced thickening. Results also showed that the effects were rapid, transient, and dose dependent, suggesting a role for nitric oxide in modulating the transient choroidal thickness changes. Additional studies showed that transient choroidal thickening was consistently associated with brief periods of unrestricted vision that inhibited ocular growth (Nickla, 2007). Moreover, a recent study reported choroidal thickening in response to bright lights in chick eyes, (Lan, Feldkaemper, & Schaeffel, 2013), implicating the choroid in ocular growth regulation.

It has been further suggested that choroidal thickness changes form the rapid part of the compensatory response to defocus in chicks, preceding ocular growth rate changes (Wallman et al., 1995; Zhu et al., 2005). In myopic defocus, ocular growth inhibition results in a decrease of eye growth, whereas in hyperopic defocus, eye growth increases. Along with the observed ocular growth rate changes is an observed change in scleral GAG

synthesis. It has been hypothesized that the choroid regulates scleral GAG (Rada & Palmer, 2007), possibly through the secretion of regulatory molecules as co-culturing the sclera with the choroid of recovery chick eyes inhibited scleral GAG synthesis (Marzani & Wallman, 1997). Research has shown that changes in choroidal thickness have been associated with changes in GAG synthesis (Nickla et al., 1997).

### *1.6. Potential Candidate Signaling Molecules*

Although research has shown evidence supporting the role of both the choroid and sclera in the regulation of ocular growth, the molecules involved and the signaling pathways mediating these responses are unclear. The specific molecules involved in the signaling cascade regulating ocular growth remains unknown but several studies have implicated a number of potential messenger molecules. These include vasoactive intestinal peptide (VIP; Raviola & Wiesel, 1988), retinoic acid (Mertz & Wallman, 2000; McFadden, Howlett, & Mertz, 2004), glucagon (Ashby, Kozulin, Megaw, & Morgan, 2010; Feldkaemper & Schaeffel, 2002), opiates (Seltner, Rohrer, Grant, & Stell, 1997), serotonin (George, Schmid, & Pow, 2005), and melatonin (Rada & Wiechmann, 2006), to name a few.

One of the more widely studied molecules has been acetylcholine and its role in myopia development. Interest in this molecule resulted from the hypothesis that excessive accommodation may cause myopia. As previously mentioned, an association between near work and myopia onset has been suggested. However, research has shown that myopia can be induced in species without functional accommodative systems (McBrien, Moghaddam, New, & Williams, 1993). The use of muscarinic agonists has been shown to cause choroidal

thinning associated with ocular growth (Nickla, Zhu, & Wallman, 2013), support a role for acetylcholine in the choroidal response. On the other hand, atropine, a non-selective muscarinic antagonist, has been shown to reduce myopia progression in chicks (Stone, Lin, & Laties, 1991). In fact, atropine caused growth inhibition in chicks, which have nicotinic acetylcholine receptors. Similar results have been reported in clinical trials with atropine. Administration of atropine drops has been shown to slow the progression of low to moderate myopia in children (Chua, Balakrishnan, Chan, Tong, Ling, Quah, & Tan, 2006). However, atropine drops have unwanted visual side effects such as pupil dilation, loss of accommodation, and near vision blur. In addition, its inhibitory effects were not sustainable as cessation of atropine use resulted in a return of myopia (Chia, Chua, Wen, Fong, Goon, & Tan, 2014). Thus, alternative treatment options are needed that have lower side effects and more long term efficacy in myopia inhibition.

Interestingly, it has been hypothesized that an interaction between the muscarinic and dopaminergic pathways exists in regulating ocular growth. It was reported that atropine increased the release of dopamine from cellular stores in chick eyes (Schwahn, Kaymak, & Schaeffel, 2000). However, the combination of a dopamine agonist and atropine to study the interaction between the two pathways showed that utilizing both pathways did not result in a greater inhibition of myopia development (Schmid & Wildsoet, 2004). Results specifically showed that the combined effects of apomorphine, a non-specific dopamine agonist, and atropine were not additive or as effective as the administration of atropine alone. This suggests that perhaps both molecules are involved in the same pathway with effects at different sites. It has also been suggested that dopamine may in fact act upstream of the

muscarinic pathway, suggesting a role for dopamine in the regulation of ocular growth (review: Feldkaemper & Schaeffel, 2013).

### *1.7. Dopamine (DA)*

Dopamine (DA) has also generated great interest and perhaps is the most promising of all the putative signaling molecules. As a retinal neuromodulator, dopamine has multiple functions in the brain with regard to affect, reward, and movement. It has been suggested that DA functions in regulating circadian rhythm and cell survival (review: Witkovsky, 2004). There is also strong evidence from previous animal studies implicating a role of dopamine in regulating ocular growth.

In the retina, dopamine has been found in "amacrine-like" cells in the inner nuclear and inner plexiform layers (Kramer, 1971) and with multiple targets. A recent review on dopamine by Feldkaemper and Schaeffel (2013) summarized the role of DA in controlling receptive field sizes at the molecular level to aid in spatial tuning of the retina. Specifically, it modulates the conductance of the gap junctions between rods and cones, regulates horizontal cell coupling, controls conductance of amacrine II-amacrine II junctions, and mediates ganglion cell coupling as well. This suggests dopamine has a number of targets at the level of the retina. In fact, staining for dopamine receptor subtypes showed the presence of D2 receptors in the inner and outer plexiform layers, as well as in the photoreceptor inner and outer segments of a number of species (Wagner, Luo, Ariano, Sibley, & Stell, 1993). Dopamine receptor subtypes were also present in the ganglion cell layer and at the proximal margin of the inner nuclear layer of mammals and chick (Wagner et al., 1993). In addition,

Ohngemach, Hagel, and Schaeffel (1997) showed in their study that the vitreous contained only one-tenth, the choroid about one-third, and the sclera about one-twentieth of the retinal dopamine content, suggesting that the retina may be the major source of DA release. The retinal pigment epithelium (RPE) has also been suggested as another potential target (review: Rymer & Wildsoet, 2005). It is likely that the RPE may be a target of DA due to the presence of immunoreactive dopamine receptor subtypes and their mRNA in the basal region of the RPE (Rohrer & Stell, 1995).

Dopamine has been hypothesized to play an inhibitory role in regulating ocular growth in animal models. Work by Stone et al. (1989) showed a reduction in levels of retinal dopamine in form deprived chick eyes. Similar results were also found in primate models (Ivone, Tigges, Fernandes, & Tigges, 1989). Reduction of DA levels has also been reported with negative lens wear in chicks (Guo, Sivak, Callender, & Diehl-Jones, 1995). These changes in retinal DA levels were localized to the areas of induced myopia, suggesting a role for dopamine in ocular growth regulation (Ohngemach et al., 1997; Stone, Pendrak, Sugimoto, Lin, Gill, Capehart, & Liu, 2006). Subsequent work by Iuvone, Tigges, Stone, Lambert, and Laties (1991) looking at the effects of dopamine agonists on eye growth showed that injections of apomorphine and quinpirole prevented form deprivation myopia (FDM) in both chick and monkey models. In addition, DA agonists were also found to inhibit the development of myopia induced by negative lenses (Schmid & Wildsoet, 2004). Results indicating that dopamine agonists were capable of inhibiting myopia development in both paradigms of myopia provided additional evidence to support that DA is indeed involved in ocular growth regulation. Intravitreal injections of dopamine itself in rabbit eyes

were also able to inhibit FDM (Gao, Liu, Ma, Zhong, Wu, & Ge, 2006). Collectively, these studies suggest a role of dopamine in inhibiting ocular growth.

As previously mentioned, spending time outdoors is protective against myopia development in children (Jones et al., 2007; Rose et al., 2008). It is believed that this effect is mediated by dopamine. In animal studies, bright lights were protective against FDM in chicks and monkeys (Megaw, Morgan, & Boelen, 1997; Ashby, Ohlendorf, & Schaeffel, 2009; Cohen, Peleg, Belkin, Polat, & Solomon, 2012). Additionally, retinal dopamine levels fluctuate on a diurnal rhythm—increasing during the day and decreasing at night (Megaw, Boelen, Morgan, & Boelen, 2006). This suggests a correlation between light intensity and DA levels. In fact, dopamine levels were reduced only during the daytime in FDM (Stone et al., 1989). Conversely, levels of DA were shown to increase in recovery studies. The removal of translucent goggles in formed deprived chick eyes to allow normal vision, resulted in an increase in DA levels within 2 days of goggle removal (Pendrak, Nguyen, Lin, Capehart, Zhu, & Stone, 1997). However, if eyes subjected only to bright lights were injected daily with the dopamine antagonist, spiperone, the protective effect of light was abolished (Ashby & Schaeffel, 2010). Furthermore, injecting spiperone prior to daily periods of unrestricted vision in form deprived chick eyes, prevented the ameliorative effects of vision on the development of myopia (McCarthy, Megaw, Devadas, & Morgan, 2007), supporting the role of dopamine in mediating the protective effects of bright lights.

It is thought that dopamine exerts its effects through specific receptor subtype mediated mechanisms. Specifically, it was suggested that the ocular growth inhibition effects of DA were mediated through a D2 pathway. The D2 receptors are responsible for

inhibiting adenylate cyclase, which results in a decrease of cAMP (review: Witkovsky, 2004). Studies with quinpirole, the D2 specific dopamine agonist, have resulted in an inhibition of both FDM and lens-induced myopia (LIM; McCarthy et al, 2007; Nickla, Totonelly, & Dhillon, 2010). Further supporting the hypothesis that D2 receptor mediated mechanisms regulate ocular growth, intravitreal injections of the D2 dopamine antagonist, spiperone, prevented the growth inhibition of the DA agonist, apomorphine, in form-deprived eyes (Rohrer, Spira, & Stell, 1993). However, studies in this lab showed that spiperone is not as effective in LIM as it was in FDM, suggesting that the D1 receptor family may also be involved in regulating ocular growth (Nickla et al., 2010; Nickla & Totnelley, 2011). Despite these results, the prevailing theory remains that the inhibitory effects of dopamine is mediated through a D2 specific pathway.

Furthermore, work in this lab has shown an association between dopamine and a choroidal response. Treatment with the D2 specific agonist, quinpirole, resulted in a transient but highly significant thickening of the choroid within 3 hours of treatment (Nickla et al., 2010). This suggests that dopamine may be working at or above the level of the choroid. Immunohistochemistry studies indicated the expression of dopamine receptor proteins in the choroid plexus of rats (Mignini, Bronzetti, Felici, Ricci, Sabbatini, Tayebati, & Amenta, 2000). It has been suggested that the choroid plexus is under dopaminergic control (Townsend, Ziedonis, Bryan, Brennan, & Page, 1984), with D2-like receptors found in the sympathetic nerve fibers supplying the choroid plexus epithelium and vasculature (Mignini et al., 2000). Moreover, injections of DA in form-deprived rabbits resulted in scleral thickening (Lin, Chen, Ge, Cui, Wu, Tang, Tan, Zhong, & Gao, 2008). These results

are consistent with other studies implicating dopamine in ocular growth inhibition and suggest that dopamine affects both choroidal and scleral thicknesses.

### *1.8. Nitric Oxide (NO)*

Animal studies have also implicated the involvement of the inhibitory molecule nitric oxide (NO) in regulating ocular growth. Nitric oxide is a ubiquitous, gaseous neurotransmitter that is a potent vasodilator and smooth muscle relaxant. In the eye, NO is produced by many different cell types including retinal amacrine and ganglion cells, Müller cells, retinal pigmented epithelium, and nerve fibers in the outer and inner plexiform layers (Fischer & Stell, 1999; Yamamoto, Bredt, Snyder, & Stone, 1993). It has also been reported in cells of the choroid, sclera, and possibly the ciliary ganglion (review: Goldstein, Ostwald, & Roth, 1996). The presence of NO among multiple cell types suggests that nitric oxide may have multiple functions within the retina and the eye. Research indicates that NO modulates voltage-gated ion channels in rod photoreceptors and retinal blood flow regulation (review: Goldstein et al., 1996). Nitric oxide also appears to play an important role in the vasodilation of various tissues such as the iris, ciliary body, and choroid (Nilsson, 1996). Moreover, stimulation with steady or flicker light has been reported to increase NO production in carp retina (Sekaran, Cunningham, Neal, Hartell, & Djamgoz, 2005). This suggest that NO may be involved in ocular growth regulation as animal studies mentioned above have shown bright lights to be protective against FDM. In form-deprived chick eyes, all three nitric oxide synthase (NOS) isoforms were expressed in the retina, RPE, and choroid ((Fujii, Honda, Sekiya, Yamasaki, Yamamoto, & Saijoh, 1998). However, only inducible NOS (iNOS) was

significantly reduced in form-deprived eyes, suggesting the involvement of specific isoforms in ocular growth regulation (Fujii et al., 1998).

As briefly mentioned above in subsection 1.5, nitric oxide is hypothesized to play a role in modulating choroidal thickness and thus ocular growth. The use of L-NAME, a non-specific NOS inhibitor, prevented choroidal thickening in response to myopic defocus with plus lenses and recovery in previously form-deprived chick eyes (Nickla & Wildsoet, 2004). Additional studies showed that injections of L-NAME prior to the daily removal of diffusers for 2 hours inhibited the protective effects of brief vision with eyes becoming significantly more myopic (Nickla, Wilken, Lytle, Yom, & Mertz, 2006). These studies also showed that L-NAME inhibited NO synthesis and dis-inhibited scleral glycosaminoglycan synthesis in positive lens wearing eyes (Nickla et al., 2006). Subsequent studies trying to elucidate the potential source of NO showed that injections of N-omega propyl-L-arginine (NPLA), the specific neuronal NOS (nNOS) inhibitor, had the same effects as L-NAME (Nickla, Damyanova, & Lytle, 2009). Both NPLA and L-NAME inhibited the transient choroidal response and showed dis-inhibition of growth responses. Specifically, injections of NPLA inhibited the shift in hyperopic refractive error with plus lenses and showed a dis-inhibition in axial growth as axial length increased in these treated eyes (Nickla et al., 2009). NPLA also showed effects at the level of the choroid by preventing the transient choroidal thickening to myopic defocus within just 7 hours of treatment. These studies suggest that NO maybe involved in ocular growth regulation.

*1.9. Dopamine and Nitric Oxide*

It appears that both dopamine and nitric oxide have inhibitory effects in ocular growth and exert effects on the choroid in response to defocus. However, it is unknown if these two inhibitory molecules act within the same pathway and if so whether dopamine acts upstream of nitric oxide or vice versa. The retina is hypothesized to be the main source of DA and research showing that choroidal and scleral dopamine levels were not affected by deprivation suggests that other messenger molecules must relay the information to the sclera (Ohngemach et al., 1997). This other messenger may possibly be nitric oxide. Both DA release and NO production are believed to be controlled by light (Feldkaemper & Schaeffel, 2013), supporting a common link between the two molecules. Additionally, animal studies provide evidence to support the hypothesis that dopamine may regulate nitric oxide release. The use of dopamine agonists, for example, has been shown to increase nitric oxide production in rat brains (Melis, Succu, & Argiolas, 1996). Histological staining further supports this theory that dopamine regulates NOS activity (Morris, Simpson, Mundell, Maceachern, Johnston, & Nolan, 1997) with dopaminergic terminals potentially providing direct input to neurons responsible for NO release (Fujiyama & Masuko, 1996). Moreover, whereas the application of exogenous DA to dark-adapted retinas significantly enhanced the production of NO, the inhibition of DA activity suppressed the light-evoked release of nitric oxide (Sekaran et al., 2005). These studies indicate that DA may act upstream of nitric oxide in the signaling cascade regulating ocular growth.

### *1.10. Purpose*

The purpose of this study is to elucidate the signaling cascade regulating ocular growth inhibition. This study aims to determine where in the signaling cascade dopamine and nitric oxide exert their effects and whether these two molecules act in the same pathway. If they are indeed involved together, it seeks to understand the relationship between the two molecules. Specifically, it will determine which molecule acts upstream of the other. We further hypothesize that choroidal thickening is part of the signaling cascade mediating ocular growth inhibition. Another aim of this study is ascertain whether the regulatory mechanisms differ between the two paradigms of myopia—form deprivation and negative lens-induced.

## 2. Method/Materials

### *2.1. Animals*

White Leghorn (*Gallus gallus domesticus*) chicks acquired as eggs were hatched and raised under 12h light/12h dark cycles with continuous supplies of food and water. The light intensity during the light cycle was approximately 300 lux. For all experiments the right eye served as the experimental eye and the fellow left eye as the untreated control. Care and use of the chicks abided to the Association for Research in Vision and Ophthalmology (ARVO) Resolution for the Care and Use of Animals in Research.

### *2.2. Experimental Design*

12-day-old chicks reared for 5 days were fitted with either translucent diffusers or -10D lenses on the right eye to induce form deprivation- or lens induced- myopia, respectively. The left eye served as the experimental control. Diffuser or lenses used were designed with Velcro rings and attached to the matching ring glued to the feathers around the orbit of the right eye. This method allowed for the ease of removing and replacing diffusers/lenses during subsequent measurements and injections.

On day 0, prior to any application of diffusers or lenses, baseline ocular measurements of axial length, choroidal thickness, and anterior chamber depth were made using a high frequency A-scan ultrasonography. Chicks were then given a daily intra-vitreal injection around mid-day for a total duration of 4 days. A total volume of 20µl of either (a) quinpirole (n=20, diffusers; n=12, lenses), (b) $N^{\omega}$-propyl-L-arginine (NPLA) (n=4, diffusers; n=6, lenses), (c) a combination of NPLA and quinpirole (10µl ea) (n=19, diffusers; n=17,

lenses), (d) combination of L-$N^5$-(1-Iminoethyl)-ornithine (L-NIO) and quinpirole (10µl ea) (n=12, diffusers; n=12, lenses), or (d) saline (n=26, diffusers; n=23, lenses) was used accordingly. Prior to injections on day 4, ocular dimensions were collected and then again 3h post-injections to measure potential choroidal changes. On the final day, in addition to ocular measurements with the A-scan ultrasonography, refractive error measurements were made with a Hartinger's refractometer. All measurements and injections were completed with the chicks placed under isoflurane inhalation anesthesia.

*2.3. Injections*

All injections were done over the superior temporal sclera of the experimental right eye at approximately the same time each day for 4 days total. To ensure all injections were made at the same site, a small notch was created in the matching Velcro ring. The ring was then glued to the feathers surrounding the right eye with the notch oriented over the superior temporal direction. Prior to injections, the chicks were anesthetized using an inhalation mixture of isoflurane/0.8% oxygen anesthesia. The diffuser/lenses were removed and the injection site was cleaned with an alcohol pad. A 30-gauge needle was then inserted at about a 45-degree angle through the skin of the superior temporal aspect of the eye. A total volume of 20µl was injected at a steady rate into the vitreous and the needle was held in place for about approximately 30 seconds. The skin surrounding the injection site was then held tightly with forceps as the needle was slowly withdrawn from the injection site. Immediately after completion of each injection, the diffuser/lenses were re-attached to the matching rings. The chicks were watched for an additional 15 minutes post-injection to

monitor for any adverse events from the injection or anesthesia used.

The drugs used in the experiments were quinpirole, a dopamine D2 specific agonist, NPLA, a neuronal nitric oxide synthase (NOS) inhibitor, and L-NIO, a specific endothelial NOS inhibitor. All drugs were obtained from TOCRIS Biosciences and prepared by dissolving into 0.75% saline. The same saline used for dissolving the drugs was used for the saline control injections. The concentrations of the drugs used were 10nmol quinpirole and 400nmol NPLA, and 300nmol L-NIO. For the combination drug injections, double the concentration of each drug was used.

*2.4. Ocular Measurements with A-scan Ultrasonography*

Ocular measurements were obtained using a high frequency A-scan ultrasonography system equipped with a 30MHz transducer. Briefly, chicks were placed on a movable stage and anesthetized using isoflurane inhalation anesthesia. The A-scan probe was positioned to align with the optical axis of chick eye to maximize the frequency peaks of the sound waves of the cornea, lens, and retina. A total of 8 traces per eye were taken, analyzed, and averaged to calculate the axial length, vitreous chamber depth and choroidal thickness. Axial length is defined as the distance from the front of the cornea to the front of the sclera. Vitreous chamber depth is defined as the distance from the back of the lens to the front of the retina. Changes in axial length and vitreous chamber depth were both reported as the rate of change in micrometers per 4 days. Choroidal thickness is defined as the distance from the back of retina to the front of the sclera. Changes in choroidal thickness were reported as the rate of change in micrometers per 3 hours.

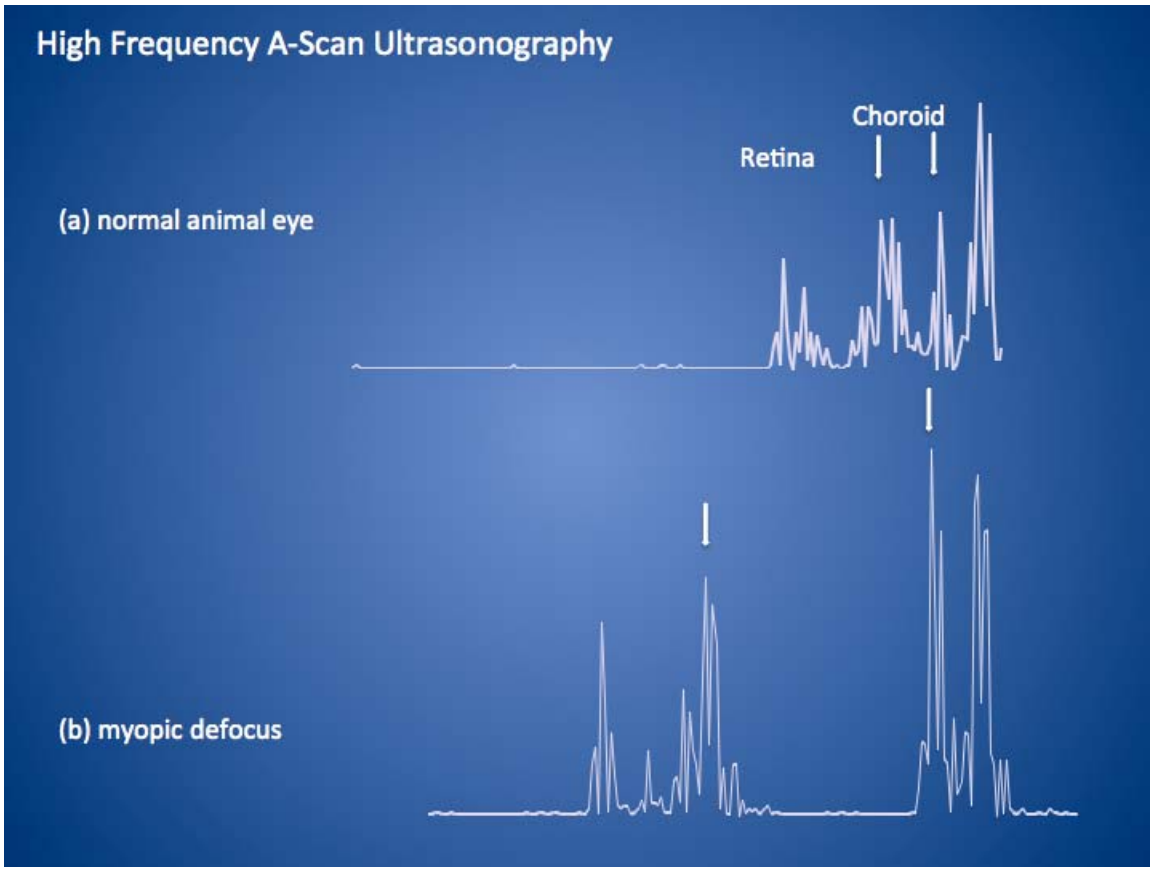



Fig 1: Example of the observed change in choroidal thickness from high frequency A-scan ultrasonography sound wave trace. White arrows indicate the location of the choroid.

*2.5. Refractive Error Measurements with Hartinger's Refractometer*

Refractive error measurements of both eyes were measured on the final day of experiments. Chicks were placed on a movable stage and anesthetized. Horizontal and vertical mires were maximized for each measurement using the Hartinger's refractometer. A total of four measurements per eye were taken and averaged for the final refractive error. Results were reported in diopters (D).

*2.6. Eye Cup Preparation & Nitric Oxide Measurement*

To determine the effects of quinpirole on nitric oxide (NO) levels in the eyes, a pilot experiment was completed to prepare eye cups for measurement of NO levels in quinpirole treated form-deprived eyes. Care was taken to euthanized chicks at the end of experiments via intraperitoneal injection of kenolog. Eyes were then enucleated post-mortem. The anterior portion of the eye, vitreous, and pecten were carefully removed to avoid damaging the retina. Eye cups were rinsed gently rinsed in media to remove any debris, placed into 6-well plates, and incubated overnight in RPMI media. The media was collected after 24 hours and aliquots were immediately frozen. Measurements of NO were done using an in-NO amperometric system. A total of 12 pairs of eye cups were obtained. Results were reported in pAmps.

*2.7. Statistical Analysis*

One-way ANOVA was used to determine statistical significance between experimental and saline control groups for refractive error, axial length/vitreous chamber depth changes, and transient choroid thickness measurements. Additional post-hoc Bonferroni tests were completed for comparison between treatment groups and saline groups if ANOVA tests showed significant differences across groups. Two-tailed student t-tests were used to test statistical significance between untreated fellow eyes and experimental groups.

## 3. Results

**Table 1**: Sample size, means, standard errors, and p-values for both myopia paradigms

| | Quin, n=20 | Quin+NPLA, n=19 | NPLA, n=4 | Quin+L-NIO, n=12 | Saline, n=26 | Fellow | ANOVA p-value |
|---|---|---|---|---|---|---|---|
| RE | -3.4 (0.4) | -5.9 (0.6) | -4.2 (1.0) | -5.8 (0.7) | -6.2 (0.4) | 0.2 (0.1) | **0.001** |
| AL | 330 (34) | 492 (38) | 422 (18) | 530 (47) | 444 (24) | 399 (10) | **0.0064** |
| VCD | 275 (29) | 401 (31) | 312 (31) | 440 (49) | 384 (26) | 211 (11) | **0.0042** |
| CT | 21 (4.6) | 19 (6.0) | 32 (18) | -5.0 (11) | 3.0 (7.0) | -10 (4.0) | 0.0795 |

Abbreviations: Quin, quinpirole; RE, refractive error; AL, axial length; VCD, vitreous chamber depth; CT, choroidal thickness

Table 1a. Sample size, mean, standard errors (in parenthesis), and p-values for the experimental and control groups subjected to form deprivation (FD). The "fellow" group included the combined data from all untreated contralateral eyes of each group. P-values of statistical significance are bolded.

| | Quin, n=12 | Quin+NPLA, n=17 | NPLA, n=6 | Quin+L-NIO, n=12 | Saline, n=23 | Fellow | ANOVA p-value |
|---|---|---|---|---|---|---|---|
| RE | -0.4 (0.9) | -3.5 (0.6) | -5.1 (0.8) | -2.4 (0.8) | -4.8 (0.5) | 0.2 (0.2) | **0.0007** |
| AL | 315 (26) | 457 (57) | 604 (47) | 451 (41) | 469 (43) | 317 (14) | 0.0861 |
| VCD | 253 (37) | 407 (32) | 530 (40) | 403 (33) | 378 (29) | 224 (13) | **0.0044** |
| CT | 31 (5.0) | 0.0 (9.0) | 19 (9.0) | 17 (8.0) | -9.0 (9.0) | -1.5 (5.0) | **0.0118** |

Table 1b. Sample size, mean, standard errors (in parenthesis), and p-values for the experimental and control groups subjected to negative lens wear. The "fellow" group included the combined data from all untreated contralateral eyes of each group. Abbreviations are the same as above. P-values of statistical significance are bolded.

### *3.1. Refractive Error*

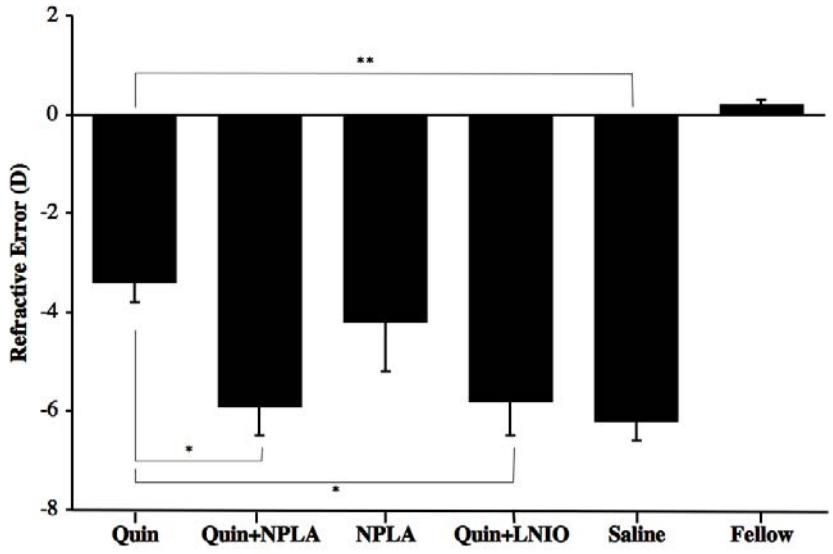
As expected, results showed quinpirole prevented the development of myopia in both

form-deprived and lens-wearing eyes (Fig. 2). Eyes reared with negative lenses and treated with quinpirole (n=12) were less myopic than saline treated eyes (n=23) (-0.4D vs. -4.8D; ANOVA p-value= 0.0007, post-hoc p-value= 0.0004). The same held true for eyes subjected to form deprivation and injected with quinpirole (n=20) (-3.4D vs. -6.2D; ANOVA p-value= 0.001, post-hoc p-value= 0.0008). However, in formed-deprived eyes, this inhibitory effect of quinpirole was prevented when it was combined with either NPLA (n=19) or L-NIO (n=12) (-3.4D vs. -5.9D and -5.8D; p= 0.0095 and 0.0481, respectively). Eyes treated with the combination of quinpirole and NOS inhibitor (NPLA or L-NIO) became as myopic as the saline treated eyes (n=26) (Fig 2a). No differences were seen in the refractive errors of these eyes compared to the saline controls (-5.9D and -5.8D vs. -6.2D; p=1.0).

Interestingly, in the lens-wearing condition, only the combination of NPLA with quinpirole significantly diminished the inhibitory effect of quinpirole alone (Fig. 2b). The combination of NPLA with quinpirole (n=17) resulted in the development of myopia in comparison to quinpirole only treated eyes (-3.5D vs. -0.4D; p=0.0198). The effects of NPLA alone (n=6) were similar to the saline control (-5.1D vs. -4.8D; p=1.0). Although co-injection of L-NIO and quinpirole (n=12) also resulted in myopia development, the effect was not statistically significant compared to the quinpirole only treatment (-2.4D vs. -0.4D; p=0.6973).

**Figure 2: Refractive error changes**

**A.**




**B.**

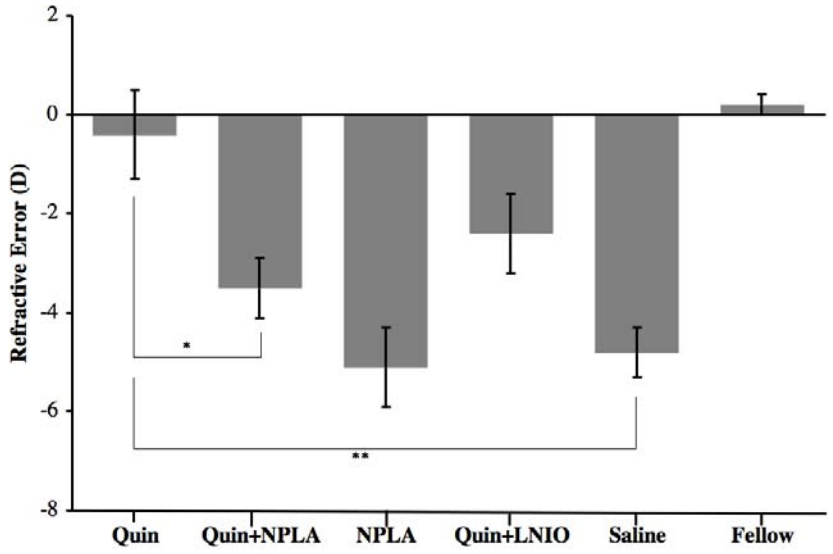



Fig 2. Change in refractive error in diopters (D) at the end of 4 days of form deprivation (A) or negative lens-wear (B) in drug/saline injected eyes and untreated fellow eyes. Bars represent standard error. Fig 2a FD ANOVA p-value= 0.001, post-hoc p-values: *p<0.05, **p<0.001; Fig 2b lens ANOVA p-value= 0.0007, post-hoc p-values: *p<0.05, **p<0.001.

*3.2. Ocular Growth: Vitreous Chamber Depth and Axial Length*

The inhibition of myopia development by quinpirole was consistent with results indicating inhibition of ocular growth (Figures 3 and 4). Figure 3b showed quinpirole inhibited the elongation of the vitreous chamber depth (VCD) compared to saline controls in the lens paradigm (ANOVA p=0.0044). Quinpirole treatment in lens-wearing eyes resulted in shorter vitreous chamber depths than saline controls (253 μm/4d vs. 378 μm/4d; p=0.0584). Differences in VCD changes also reached statistical significance in form-deprived eyes (Fig. 3a; ANOVA p=0.0042). However, there was no difference between quinpirole-injected eyes and saline controls in formed-deprived eyes (275 μm/4d vs. 384 μm/4d; p=0.0847). Additionally, there was no difference in VCD elongation in quinpirole treated eyes from that of untreated fellow eyes in both paradigms (FD: 275 μm/4d vs. 211 μm/4d; lens: 253 μm/4d vs. 224 μm/4d; p>0.05).

The quinpirole induced myopia inhibition observed in both paradigms was prevented with co-injections of quinpirole with either NOS inhibitor. This dis-inhibition of VCD elongation resulted in eyes growing significantly longer compared to eyes treated with quinpirole alone. In form deprivation conditions (Fig. 3a), quinpirole alone resulted in a shorter vitreous chamber depth compared to the combination of quinpirole and NPLA (275 μm/4d vs. 401 μm/4d; p=0.0341). Similar results were found with co-injections of quinpirole along with the other NOS inhibitor, L-NIO (275 μm/4d vs. 440 μm/4d; p=0.0071). Injections of NPLA alone were no different from saline controls (312 μm/4d vs. 384 μm/4d; p=1.0). Results from eyes subjected to lens-wear also showed dis-inhibition of ocular growth with co-injections of either NOS inhibitor (Fig. 3b). Co-injections of either NPLA or L-NIO

and quinpirole in lens-wearing eyes resulted in significant elongation compared to quinpirole only injections. The combination of quinpirole with NPLA resulted in longer VCD of 407 μm/4d compared to 253 μm/4d found in eyes treated with only quinpirole ($p=0.0056$). Significant elongation was also found with co-injections with L-NIO (403 μm/4d vs. 253 μm/4d; $p=0.0162$).

Axial length changes as shown in figure 4 had similar trends in ocular growth inhibition as those found in vitreous chamber depth changes for both paradigms. Differences in axial length changes were statistically significant in form-deprived eyes but not for lens-wearing eyes (FD ANOVA $p=0.0064$; lens ANOVA $p=0.0861$). However, in both conditions measurements of axial lengths from quinpirole injected eyes did not differ from that of saline controls. Despite failing to reach statistical significance, quinpirole treatment did result in shorter axial lengths in comparison to saline injection (FD: 330 μm/4d vs. 444 μm/4d, $p=0.2855$; lens: 315 μm/4d vs. 469 μm/4d, $p=0.126$). The increased variability in the axial length measurements maybe due to an injection effect that usually results in a decrease in anterior chamber growth and ultimately affects axial length (measured from the front of the cornea to the front of the sclera).

Effects on axial length changes with the addition of NOS inhibitors resulted in similar trends mirroring those found with changes in vitreous chamber depth. In Figure 4a, results from form-deprived eyes co-injected with either NOS inhibitor and quinpirole showed eyes significantly grew longer than eyes treated with quinpirole alone (quin+NPLA vs. quin: 492 μm/4d vs. 330 μm/4d, $p=0.0232$; quin+LNIO vs. quin: 530 μm/4d vs. 330 μm/4d, $p=0.0111$). This same trend was observed in the lens paradigm but did not reach statistical significance

(Fig. 4b). Negative lens-wearing eyes co-injected with quinpirole and NPLA resulted in axial length elongation compared to quinpirole alone (457 μm/4d vs. 315 μm/4d; p=0.1824). The combination with LNIO also resulted in the dis-inhibition of ocular growth with eyes growing faster than with quinpirole only (451 μm/4d vs. 315 μm/4d; p=0.2798).

**Figure 3A: Vitreous chamber depth changes**

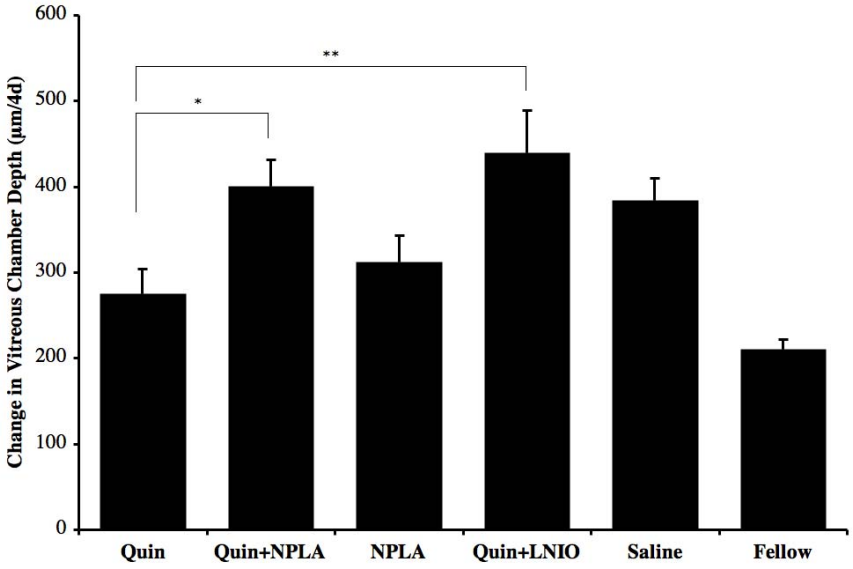



Fig 3a. Vitreous chamber depth changes in micrometers over 4 days (μm/4d) in form-deprived eyes for experimental and control groups. Bars represent standard error. FD ANOVA p-value= 0.0042, post-hoc p-values: *p<0.05, **p<0.01.

**Figure 3B: Vitreous chamber depth changes**

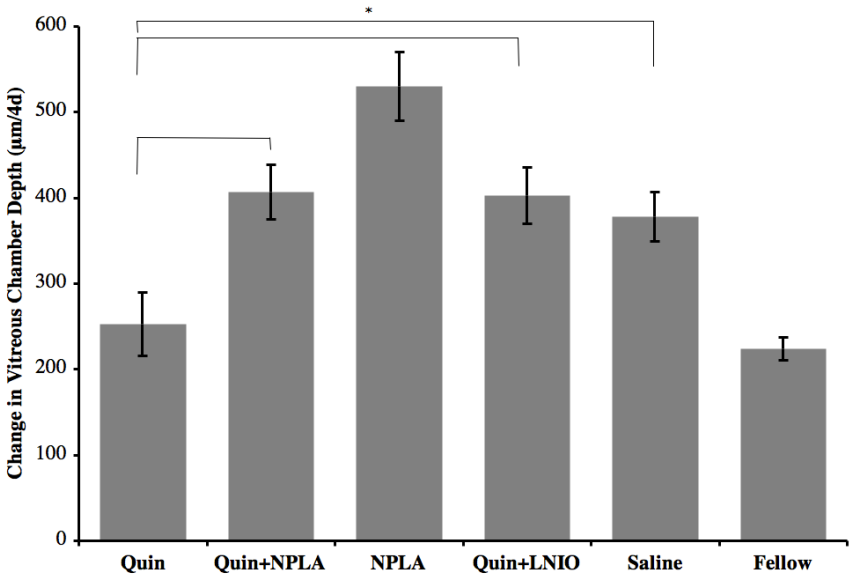
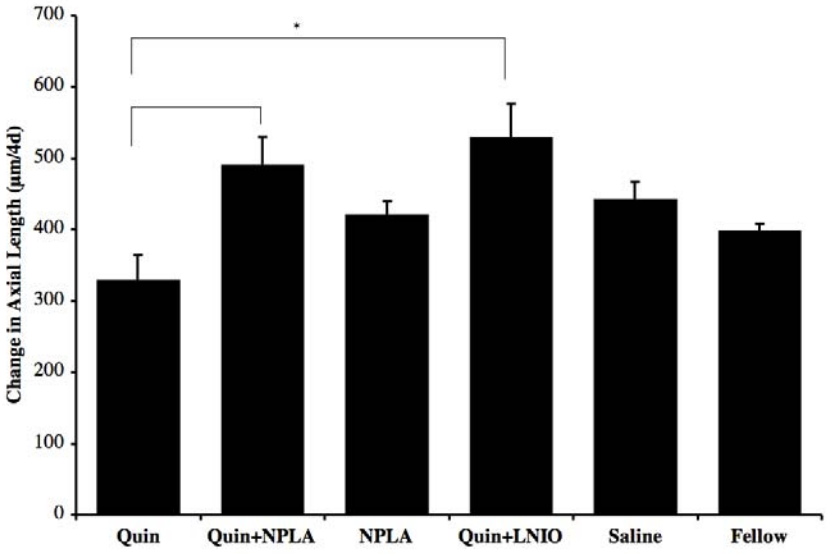



Fig 3b. Vitreous chamber depth changes in micrometers over 4 days (µm/4d) in lens-wearing eyes for experimental and control groups. Bars represent standard error. Lens ANOVA p-value= 0.0007, post-hoc p-values: *p≤0.0584.

**Figure 4: Axial length changes**

**A.**




**B.**

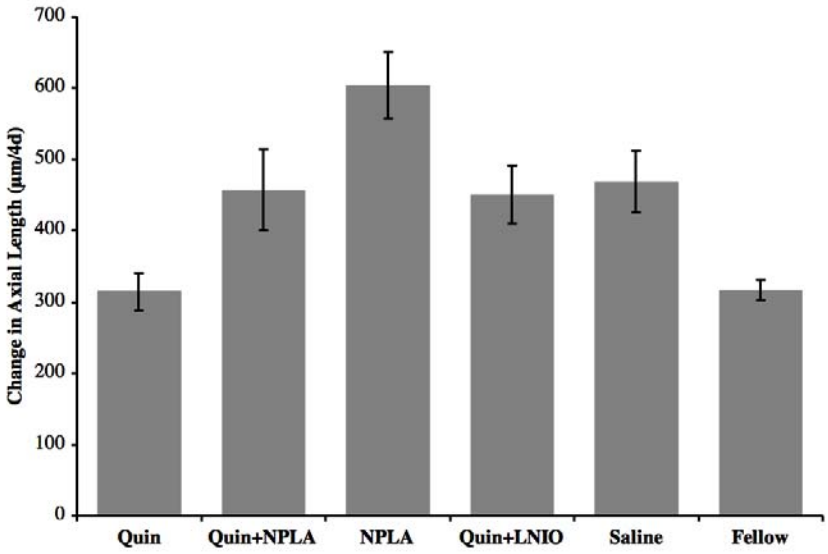


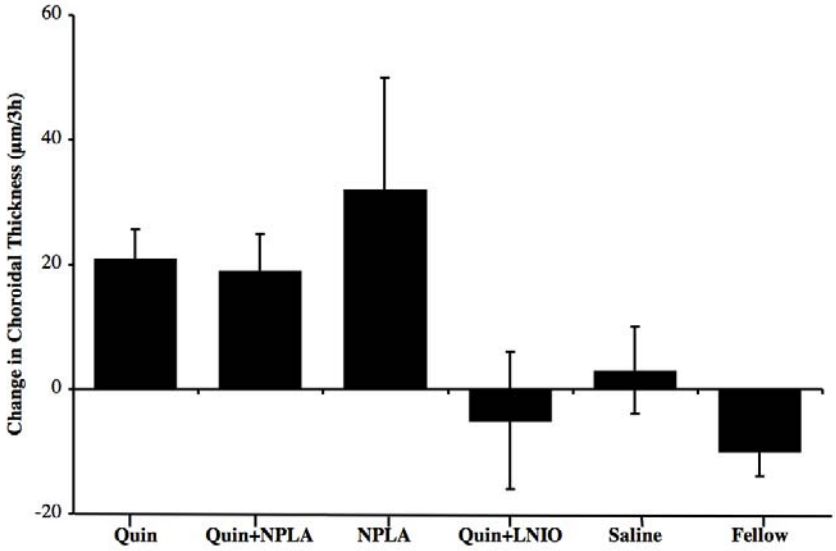

Fig 4. Changes in axial length in micrometers over 4 days (µm/4d) in form-deprived (A) or lens-wearing eyes (B) for experimental and control groups. Bars represent standard error. Fig 4a FD ANOVA p-value= 0.0064, post-hoc p-values: *p<0.05; Fig 4b lens ANOVA p-value= 0.0861.

*3.3. Transient Choroidal Thickening*

Surprisingly, results on transient choroidal thickening showed a difference between the two paradigms (Fig. 5). Differences in transient choroidal response were only statistically significant in the lens paradigm (FD ANOVA, p=0.0795; lens ANOVA, p= 0.0118). As previously shown in this lab, quinpirole injection resulted in a transient increase in choroidal thickness. Results here were consistent with that finding, as negative lens-wearing eyes had a significant increase in choroidal thickness 3 hours post-injection compared to saline controls (31 μm/3h vs. -9.0 μm/3h; p=0.0147). However, what has not yet been shown is the effect of NPLA in combination with quinpirole on the transient choroidal response to defocus. Results in Figure 5b showed that co-injections with NPLA inhibited the transient choroidal thickening found in eyes treated with quinpirole alone (quin+NPLA vs. quin: 0.0 μm/3h vs. -9.0 μm/3h; p=0.0798). Co-injections with the other NOS inhibitor, L-NIO, had no effects in lens-wearing eyes (p=1.0). In form-deprived eyes, neither combinations of NPLA nor L-NIO with quinpirole inhibited the quinpirole induced choroidal thickening response (quin+NPLA vs. quin: p=1.0; quin+L-NIO vs. quin: p=0.5463).

**Figure 5: Transient choroidal thickness changes**

**A.**




**B.**

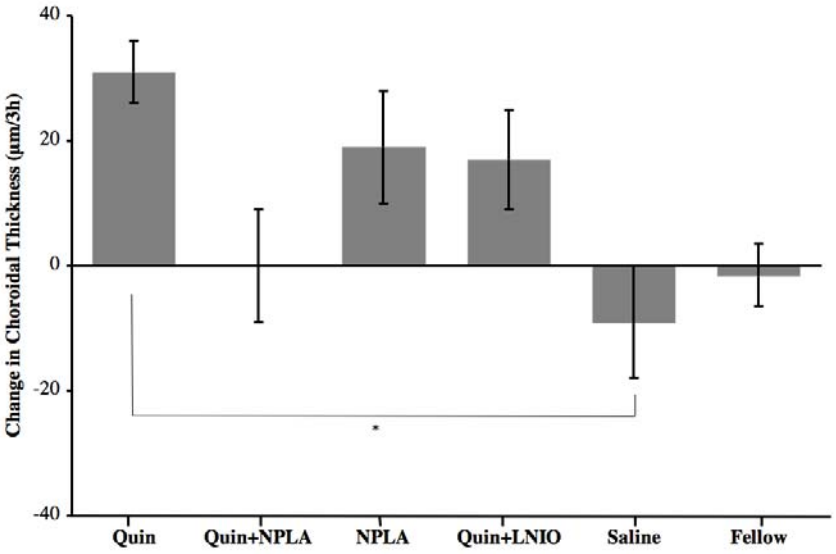



Fig 5. The rate of transient choroidal thickness change 3h post-injection in micrometers over 3 hours (µm/3h) for formed-deprived (A) and lens-wearing eyes (B). Bars represent standard error. Fig 5a FD ANOVA p-value= 0.0795; Fig 5b lens ANOVA p-value= 0.0118, post-hoc p-values: *$p<0.05$.

*3.4. Nitric Oxide Levels*

Results in figure 6 from a pilot study on the effects of drug injections on nitric oxide levels in the eye showed that quinpirole significantly increased the level of nitric oxide (NO) in form-deprived eyes. Quinpirole treatment resulted in higher measureable levels of NO compared to untreated fellow eyes (606 pAmps vs. 387 pAmps; p=0.016). The standard error for this data set was large but maybe due to the relatively small sample size. Effects of quinpirole in lens-wearing eyes were not tested.

**Figure 6: Nitric Oxide Levels**

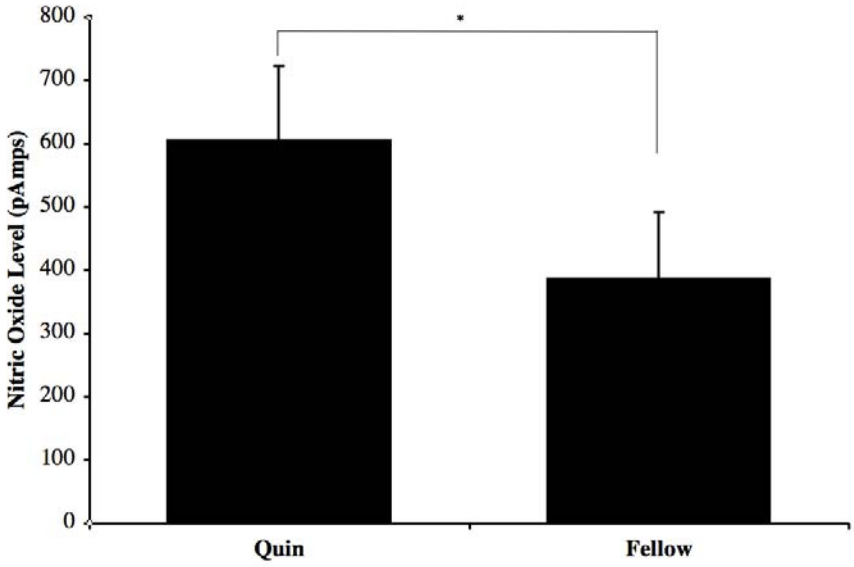



Fig 6. Nitric oxide levels in eye cups measured in pAmps from formed-deprived eyes treated with quinpirole and untreated fellow control eyes. Bars represent standard error. *p=0.016.

**4. Discussion**

The results from this study support the hypothesis that dopamine acts upstream of nitric oxide in the signaling cascade mediating ocular growth inhibition, with nitric oxide release being essential in mediating these responses as the combination of nitric oxide synthase (NOS) inhibitors and quinpirole prevented the induced ocular growth inhibition found with quinpirole alone. This dis-inhibition of ocular growth was consistent for both form deprivation- and lens induced-myopia, suggesting that the mechanism mediating ocular growth is similar in both paradigms despite some existing evidence indicating otherwise (review: Feldkaemper & Schaeffel, 2013; Nickla & Totonelly, 2011). Interestingly, results also showed that the specific neuronal NOS (nNOS) inhibitor, N-ω-propyl-L-arginine (NPLA) prevented the transient choroidal thickening induced by quinpirole but only in the negative lens wear condition. These results are consistent with previous studies in this lab that showed similar effects of NPLA on inhibiting choroidal thickness response but in eyes subjected to myopic defocus with positive lenses (Nickla et al, 2009). If these two inhibitory molecules are involved in the same signaling cascade mediating ocular growth inhibition, the question remains which ocular tissues secrete these signaling molecules? Moreover, what are their designated targets?

### *4.1. Dopamine Release and DA Signaling*

It is generally believed that the dopaminergic amacrine cells are responsible for the release of dopamine in the signaling cascade regulating ocular growth (review: Feldkaemper & Schaeffel, 2013). As previously discussed, substantial evidence from animal studies support the hypothesis that DA is involved in ocular growth inhibition with the retina

potentially being the major source of DA release. In comparison to the retina, DA levels of the vitreous were only one-tenth, the choroid only one-third, and the sclera only one-twentieth of retinal levels (Ohngemach et al., 1997). In addition, animal studies have shown a reduction of vitreal levels of dihydroxyphenylacetic acid (DOPAC), the dopamine metabolite, in both form deprivation (Megaw et al, 1997) and negative lens wear (Ohngemach et al., 1997; Guo et al., 1995). In fact, DOPAC is considered to be the best indicator of dopamine release. Levels of DOPAC in the retina were also decreased in eyes subjected to negative lens wear. Conversely, in eyes subjected to myopic defocus with positive lenses (Gou et al, 1995) or recovering from form deprivation myopia (Pendrak et al, 1997), DOPAC levels were increased. However, DOPAC levels were not affected in the choroid or sclera (Ohngemach et al., 1997). This suggests that the retina is the source of DA release. In this current study, results showing inhibition of the quinpirole induced choroidal thickening in lens wearing chick eyes with co-injections of NPLA were consistent with the hypothesis that DA is released upstream of the choroid. If dopamine is indeed released by the amacrine cells within the retina, it is possible that it may act on the RPE, which contains DA receptor subtypes (Rymer & Wildsoet, 2005). Thus, the retina or RPE are potential sources of dopamine release responsible for mediating ocular growth regulation.

Previous studies also suggest dopamine regulates nitric oxide release (Fujiyama & Masuko, 1996; Melis et al., 1996; Morris et al., 1997). In this study, NO levels were significantly increased in quinpirole treated form-deprived eyes compared to fellow untreated control eyes providing additional evidence that DA modulates NO release. To note, the standard error for the eye cup data set was large but this is possibly due to the relatively small

sample size. Moreover, the effects of quinpirole on nitric oxide levels in lens wearing eyes were not tested. Despite these limitations, increased NO levels measured in the eye cups were statistically significant and in line with the hypothesis that DA is upstream of NO. Additionally, previous studies using the nNOS inhibitior, NPLA, has been shown to transiently dis-inhibit ocular growth and transient choroidal thickening in positive lens wearing eyes, supporting a link between nitric oxide, choroidal thickening, and ocular growth inhibition (Nickla et al., 2009). Moreover, dopamine agonists apomorphine and quinpirole both induce transient choroidal thickening, also linking DA in growth inhibition and supporting the hypothesis that dopamine acts upstream of the choroid (Nickla et al., 2010). Furthermore, current findings in this study showed that both NOS inhibitors, NPLA and L-NIO, prevented the quinpirole induced ocular growth inhibition providing additional evidence to support this hypothesis. However, further research is needed to determine if there is a direct link between DA and NO or if there are other secondary messengers involved.

### *4.2. Nitric Oxide Release and NO Signaling*

Evidence from this study and previous studies suggests that nitric oxide acts downstream of DA and works at or above the level of the choroid in mediating ocular growth. As previously discussed, NO is a ubiquitous, gaseous neurotransmitter that is present in the retina, RPE, choroid, and ciliary ganglion (Fischer & Stell, 1999; review: Goldstein et al., 1996; Yamamoto et al., 1993). Thus, any of these could be the source of nitric oxide. If it is assumed that NO acts directly on the choroid to mediate thickening as

previous studies have suggested (Nickla & Wildsoet, 2004; Nickla et al., 2009), it is unlikely that the source of NO is in the retina. Any release of nitric oxide from the retina would require NO to traverse a significant distance to exert its effects. To reach the choroid, NO would need to pass through the RPE, Bruch's membrane, and the high volume blood flow of the choriocapillaris. Therefore, dopamine may cause increased NO release from sources within the choroid. Possible sources include the intrinsic choroidal neurons (ICNs) or the axon terminals of parasympathetic inputs to the choroid. The ICNs can control the non-vascular smooth muscles within the choroid and modulate choroidal blood flow (Nickla & Wallman, 2010). Furthermore, it was hypothesized that the non-vascular smooth muscles respond to NO with relaxation causes thickening, thus mediating changes in choroidal thickness in response to defocus (Wallman et al., 1995). It is also possible that the RPE may be the source of NO. If so, then nitric oxide would still need to diffuse through Bruch's and the choriocapillaris. Additional research is needed to answer this question.

Previous work in this lab showed that of the three NOS inhibitors tested, only NPLA had similar effects as the non-specific inhibitor, L-NAME, in eyes subjected to myopic defocus (Nickla et al., 2009), suggesting that the neuronal isoform was potentially involved in the signaling cascade regulating ocular growth inhibition. However, a number of isoforms of nitric oxide synthase (NOS) are present within the eye including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). In this current study, NPLA, an nNOS specific inhibitor, and L-NIO, a relatively non-selective NOS inhibitor, both prevented the growth inhibition effects of quinpirole. This dis-inhibition of ocular growth was consistent for both form deprivation- and lens induced-myopia. If it is assumed that each NOS inhibitor

exerts effects on separate isoforms, with NPLA on nNOS, and L-NIO on either iNOS or eNOS, then (1) if quinpirole was effective on all isoforms of NOS, neither inhibitor alone should have prevented the quinpirole induced growth inhibition; (2) if quinpirole's effects are mediated through the nNOS isoform, then only NPLA would be effective at preventing quinpirole's effect; (3) if quinpirole acted only on eNOS or iNOS, then L-NIO should inhibit quinpirole's effects, but NPLA should be ineffective. The current finding in this study was that both inhibitors were effective at preventing quinpirole's effects, suggesting that perhaps L-NIO is exerting cross-over effects on nNOS. This is perhaps due to the multiple injections protocol in this current study with daily injections over a 4 day period in comparison to the single injection protocol for 1 day in the previous study. Although unlikely, another explanation for the difference in the effectiveness of L-NIO in preventing the growth inhibition effects of quinpirole in this present study but its ineffectiveness in preventing the positive-lens induced ocular growth inhibition in the previous study is that dopamine (quinpirole) does not mediate the effects of myopic defocus. However, this explanation is not supported by existing research. In fact, current data shows that quinpirole mediates the effects of form vision or myopic defocus as discussed in subsection 1.7. Thus, evidence supports the hypothesis that nitric oxide acts downstream of DA and works at or above the level of the choroid in mediating ocular growth.

### *4.3. The Choroid and Ocular Growth*

As discussed earlier, the choroid has also been implicated in regulating ocular growth. Previous work in this lab has shown that quinpirole induced ocular growth inhibition in

negative lens wearing eyes was associated with transient choroidal thickening (Nickla et al., 2010), suggesting that perhaps the two are in the same pathway. On the other hand, ocular growth stimulation has been associated with choroidal thinning as seen in both form deprivation- and lens induced-myopia (Nickla & Wallman, 2010), indicating that perhaps these are also in the same pathway. In this study, both NOS inhibitors prevented the ocular growth inhibition effects of quinpirole. Results also showed that co-injections of NPLA with quinpirole did inhibit the transient choroidal thickening response, as would be expected if a thinner choroid was part of the growth-stimulating pathway. However, co-injections with L-NIO were ineffective at preventing the transient choroidal thickening. This suggests that choroidal thinning is not an integral part of the growth-stimulating pathway as both inhibitors prevented the quinpirole induced growth inhibition but only one prevented the choroidal thickening. This suggestion is supported by findings from several other studies. In one study, double parasympathectomy inhibited eye growth in form-deprived eyes but did not prevent the choroidal thinning response (Nickla & Schroedl, 2012). In another, muscarinic agonists caused choroidal thinning in normal eyes but this had no effect on eye growth (Nickla et al., 2013). Surprisingly, results also showed that NPLA prevented the transient choroidal thickening induced by quinpirole but only in the negative lens wear condition, suggesting that the mechanisms regulating ocular growth may differ between the two paradigms.

*4.4. Mechanism Differences Between Myopia Paradigms?*

It has been suggested that the regulatory mechanism of ocular growth inhibition in lens induced myopia (LIM) and form deprivation myopia (FDM) is in fact the same. A number of studies have report similar findings in these two paradigms of myopia, providing substantial evidence to support this claim. First, FDM and LIM both result in myopia development due to excessive axial elongation. Second, as previously mentioned above, reduction in dopamine levels have been shown in form deprived (Stone et al., 1989) and lens wearing eyes (Guo et al., 1995). Third, the use of dopamine agonists has prevented myopia development in both paradigms (Schmid & Wildsoet, 2004), further implicating a role of dopamine in FDM and LIM. Finally, both FDM and LIM showed thinning of the choroid associated with ocular growth. In this study, the combination of NOS inhibitors and quinpirole prevented myopia inhibition and ocular growth inhibition in both form deprivation- and lens induced-myopia, suggesting a similar mechanism mediating ocular growth in both paradigms.

Despite strong evidence supporting the role of dopamine in both paradigms, more recent research has shown that the mechanism for myopia development may differ between lens induced- and form deprivation-myopia due to differences in response to light, effects of dopamine and dopamine antagonists, and parasympathetic influences. First, it was shown that constant light inhibited FDM but not LIM (Bartmann, Schaeffel, Hagel, & Zrenner, 1994). Specifically, form deprived chicks reared under constant light for 8 days showed a significant reduction in form deprivation myopia. In contrast, lens wearing chicks were unaffected by constant light. In addition, effects of dopamine agonists in the two paradigms

have been reported to be different in guinea pigs (Dong, Zhi, Pan, Xie, Qin, Lu, Mao, Chen, Willcox, Qu, & Zhou, 2011). Although dopamine agonists were shown to inhibit both FDM and LIM in chicks and monkeys, studies with the guinea pig showed only prevention of form deprivation myopia with the dopamine agonist, apomorphine. Moreover, spiperone, the D2 dopamine antagonist, prevented the inhibitory effects of brief periods of normal vision in FDM but was ineffective with LIM (Nickla & Totnelley, 2011). Furthermore, different rates of ocular response to lens and diffusers have been reported (Kee, Marzani, & Wallman, 2001). Further adding to the debate is a recent study suggesting differences in parasympathetic influences in FDM and LIM (Nickla & Schroedl, 2012). Sectioning of the autonomic part of cranial nerve VII which provides input to the pterygopalatine ganglia plus ciliary ganglionectomy resulted in inhibition of myopia development in form deprived chick eyes but not in negative lens wear. Results from this current study further support the hypothesis that the underlying mechanisms may differ, as NPLA prevented the transient choroidal thickening induced by quinpirole in the lens paradigm but not in form deprivation. In fact, neither NOS inhibitors, NPLA nor L-NIO, were effective at preventing the quinpirole induced transient choroidal thickening in form-deprived eyes. This suggests that there maybe different mechanisms acting in the two paradigms at the level of the choroid. The difference may be attributed to reported physiological differences in the choroids of form-deprived eyes in comparison to lens wearing eyes. Form-deprived eyes in one study showed pathological changes in the RPE due to hypo-osmolarity of the choroids (Junghans, Crewther, Liang, & Crewther, 1999). It also implies that the choroidal response is mediated by a non-nitrergic mechanism, further suggesting that perhaps another molecule may

modulate the choroidal response in FDM. Certainly there is evidence to support a similar underlying mechanism in both form deprivation- and lens induced-myopia, but conflicting evidence also exists supporting the converse view that the mechanisms are in fact different. Thus, further research is needed to determine this.

### *4.5. Conclusion*

In conclusion, results suggest that dopamine does indeed act upstream of nitric oxide and the choroidal response in the signaling cascade mediating ocular growth inhibition in both myopia paradigms. The dis-inhibition of the quinpirole induced choroidal thickening by the neuronal specific NOS inhibitor, NPLA, in only lens wear suggests that choroidal thinning is not required for ocular growth stimulation. Furthermore, the lack of inhibition of the choroidal thickening with either NPLA or L-NIO in the form deprivation paradigm suggests that perhaps the mechanism regulating the choroidal response may in fact differ between the two.

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