THE UNIVERSITY OF CALGARY

Nitric Oxide and Form-Deprivation Myopia.

by

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ABSTRACT

Deprivation of form-vision induces myopia, characterized by a negative refractive error and ocular enlargement. Fujikado et al. (1997) proposed that nitric oxide, produced in retinal cells, causes form-deprivation myopia (FDM) in chicks. Work in our lab raises questions about the validity that study's results. Therefore, the effects of NO donors/inhibitors on FDM and normal ocular development were further tested.

Chick eyes were goggled or remained open, and injected with SNP (NO donor), L-NAME, L-NMMA or L-NIO (NOS inhibitors) and later refracted, measured and weighed. Histological and immunocytochemical techniques were used to assess retinal condition. Nitrate and nitrite levels were measured in retina and RPE of eyes treated with L-NIO to determine whether NO production was inhibited. Some eyes were treated with SNP and IBMX, later sectioned and labelled with antibodies raised to cGMP to resolve whether SNP treatment resulted in increased NO availability within the retina.

10 nmol SNP stimulated cGMP production in amacrine and bipolar cells with no effect on development. In goggled eyes, 100 nmol SNP reduced ocular size and myopic refraction while damaging photoreceptors (mainly rods), causing swelling, disorganization, shortening and thinning of rod outer segments accompanied by potential autophagy and DNA fragmentation in the photoreceptor layer. No other retinal cell type appeared to be damaged. 1000 nmol SNP retarded ocular growth and destroyed the retina, leaving a pigmented scar. While L-NAME prevented FDM inconsistently at 16-20 µmol, it had no effect at lower doses. Other inhibitors showed no effect, despite suppressing NO levels.

At sub-toxic doses, SNP and NOS-inhibitors did not affect normal ocular development or FDM. Therefore, NO does not appear to regulate normal ocular growth or myopiagenesis. High doses of NO are retinotoxic, and the associated effect on photoreceptors/RPE and eye growth is consistent with previous reports that normal or myopic growth requires intact photoreceptors.

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CHAPTER ONE

Introduction

Vision and Myopia in Brief

Vision is the predominant sensory modality that most vertebrates use to perceive the world around them. It is no wonder that much research is aimed at better understanding this fascinating sense, and how vision disorders occasionally occur. Some vision disorders are very common; one particular disorder, myopia, or near-sightedness, is estimated to affect 25% of the North American and Western European populations (Fledeluis *et al.* 1983, Sperduto *et al.* 1983).

The shape of the eye, in part, determines of the quality of vision (Figure 1.1). In an appropriately shaped eye, the refractive mechanisms in the anterior eye segment focus images of distant objects onto the retina. This is done while the accommodative mechanisms, which allow the eye to focus on objects at closer distances, are at rest. However, should the posterior eye segment grow excessively, the retina would come to lie beyond the focal plane of the projected image. This results in the condition known as myopia, with the predominant symptom being blurred vision of distant objects.

History of Myopia and Myopic Research

Myopia has been a subject of medical interest for many centuries. As early as 1632 it was known that this condition was caused by excessive elongation of the eye (reviewed by Dunphy 1970). Many theories addressing the cause of axial elongation have arisen, ranging from excessive pressure on the eye from extra- and intra-ocular muscles, and gravity pulling the front of the eye while peering downward at a book, to tension from short optic nerves pulling on the back of the eye. Even with these many imaginative theories, the exact cause of myopia still remains a mystery.

Modern research has focused primarily on two loci that may control eye shape, accommodative structures of the eye and the neural retina itself. The idea that accommodative structures cause myopia rose out of the observation that near-sightedness was common in individuals required to spend considerable time focusing on near objects, such as reading books. Support for this hypothesis came from studies showing that treatment of myopic school children with eye drops containing atropine, a muscarinic antagonist, reduced the progression of their myopia (Gimbel 1973, Bedrossian 1971,

Brenner 1985). In humans, muscarinic receptors are found in both the ciliary muscles responsible for accommodation as well as in the muscles that constrict the iris. Such drug treatment leads to paralysis of these muscles, suppression of accommodation and dilation of the iris. It seemed logical that if suppression of accommodation led to reduced myopia then accommodation might be able to produce a signal that causes excessive ocular growth and myopic refractive error. However, little attention was given to the possibility that muscarinic receptors exist in other ocular tissues and that these alternate sites of activity might be the source of the suppression of myopia.

In order to study better this phenomenon of excessive eye growth researchers rely upon a method for experimentally inducing myopia. This involves degrading the image reaching the retina, resulting in the reduction of high contrast, similar to viewing images greatly out of focus. This contrast reduction is accomplished either by suturing the eyelid shut or by fixing a translucent goggle over the eye. Both methods deprive the eye of form-vision and produce excessive ocular growth and myopic refractive error. This "Form-Deprivation" has become a standard method of producing myopia in research animals.

With the advent of form-deprivation myopia (FDM) both Hodos & Kuenzel (1984) and Wallman *et al.* (1987) showed that partial-field goggles induced myopic ocular growth only in regions where the retina was form-deprived. Wallman *et al.* (1987) suggested that this was strong evidence that the retina, and not accommodative mechanisms, regulated visually guided ocular growth. As accommodation is believed to focus images uniformly across the retina, it seems unlikely that such mechanisms could produce growth-controlling signals in this precise manner. This evidence suggests that the origin of the message responsible for myopic eye growth may be visual processing in the retina.

Other studies supported this, demonstrating that retinal input to the brain and feedback to the eye were not essential for the eye to grow excessively. Various investigations revealed that removal of pathways leading to the eye (ciliary ganglionectomy - Wildsoet *et al.* 1993, Schmid and Wildsoet 1996) as well as pathways leading from the retina (optic nerve section - Troilo *et al.* 1987, Wildsoet and Pettigrew, 1988; optic nerve transmission blockage – McBrien *et al.* 1993) did not significantly

affect the development of form-deprivation myopia in chickens. These lines of evidence strongly implicate the retina as the origin of the myopic growth signal.

Functional Anatomy of the Eye (Figures 1.1 and 1.2)

Light is refracted as it passes through the cornea. It then enters the crystalline lens, where it is further refracted to focus on a plane that matches that of the retina in a properly focused eye. For the eye to view sharp images of objects at various distances, a mechanism exists to modify its refractive strength. This mammalian accommodative mechanism exists as a network of suspensory ligaments and ciliary muscles, which suspend the lens from the wall of the eye. While the ciliary muscles are at rest, the suspensory ligaments pull the lens into a relatively flat shape with the least refractive power, allowing distant objects to be seen in focus. When the ciliary muscles contract, they slide forward towards their attachment near the base of the iris, causing the ligaments to shift forward into a smaller area and release their tension on the lens. This allows the lens to assume a spherical shape with greater refractive power, in order to view near objects. In the avian eye, this mechanism is augmented to include peripheral muscle fibers of the iris which contract and apply force to the lens through the ciliary processes and lens annular pad (Glasser *et al.* 1995). This constricts the equatorial diameter of the lens, increasing its curvature, thickness and refractive power. Birds can also flatten the curvature of the cornea (decreasing its refractive strength) through contraction of the anterior ciliary muscles (Glasser *et al.* 1994).

Retinal Morphology and Physiology (Figure 1.3)

In order to determine how the retina controls ocular growth through the processing of visual stimuli, one must have an appreciation of retinal physiology. The visual information is carried from the photoreceptors, through the bipolar cells to the ganglion cells, where it leaves the retina via the ganglion cell axons in the optic nerve. In mammals, this information arrives in the lateral geniculate nucleus (as well as other nuclei) where it is further processed and segregated for delivery to higher processing centres. In birds, the ganglion cell axons directly innervate the optic tectum and other structures. The information in this "through" pathway is shaped at the photoreceptor-bipolar synapse by horizontal cell input. The horizontal cells produce delayed inhibitory responses, which are associated with both the development of "surround" inhibition and

Figure 1.1: Illustration of an emmetropic (top) and myopic (bottom) Eye. Light from a distant object enters the eye and is refracted at the cornea and lens to form a projected image at a focal point (fp). In the emmetropic eye this image is coincident with the retina. In the myopic eye, this image lies in front of the retina.

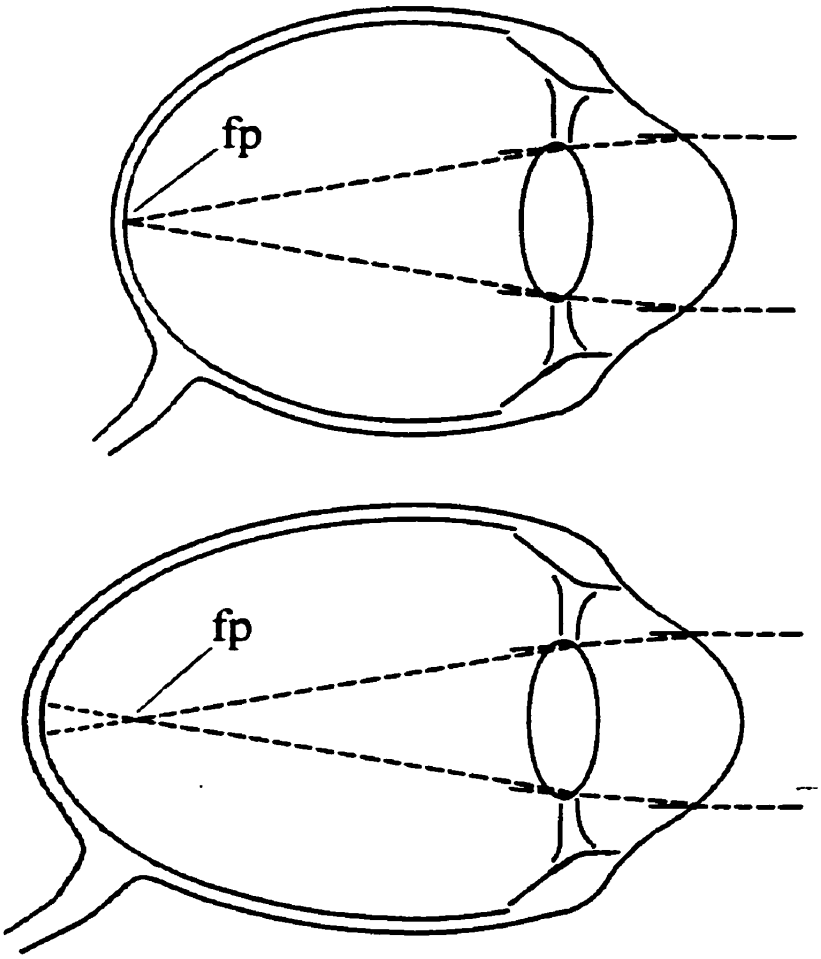
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Figure 1.2: General Eye Anatomy. Abbreviations: C: cornea, cho: choroid, CM: ciliary muscles, i:iris, L: crystalline lens, ON: optic Nerve, p:pupil, ret: retina, rpe: retinal pigment epithelium, scl: sclera, SL: suspensory ligaments, VH: vitreal humour.

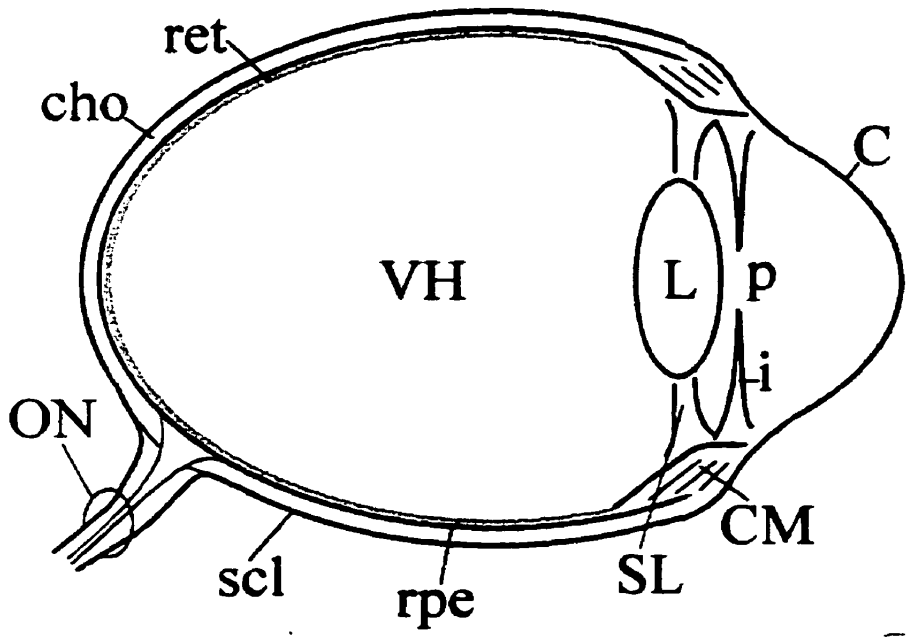
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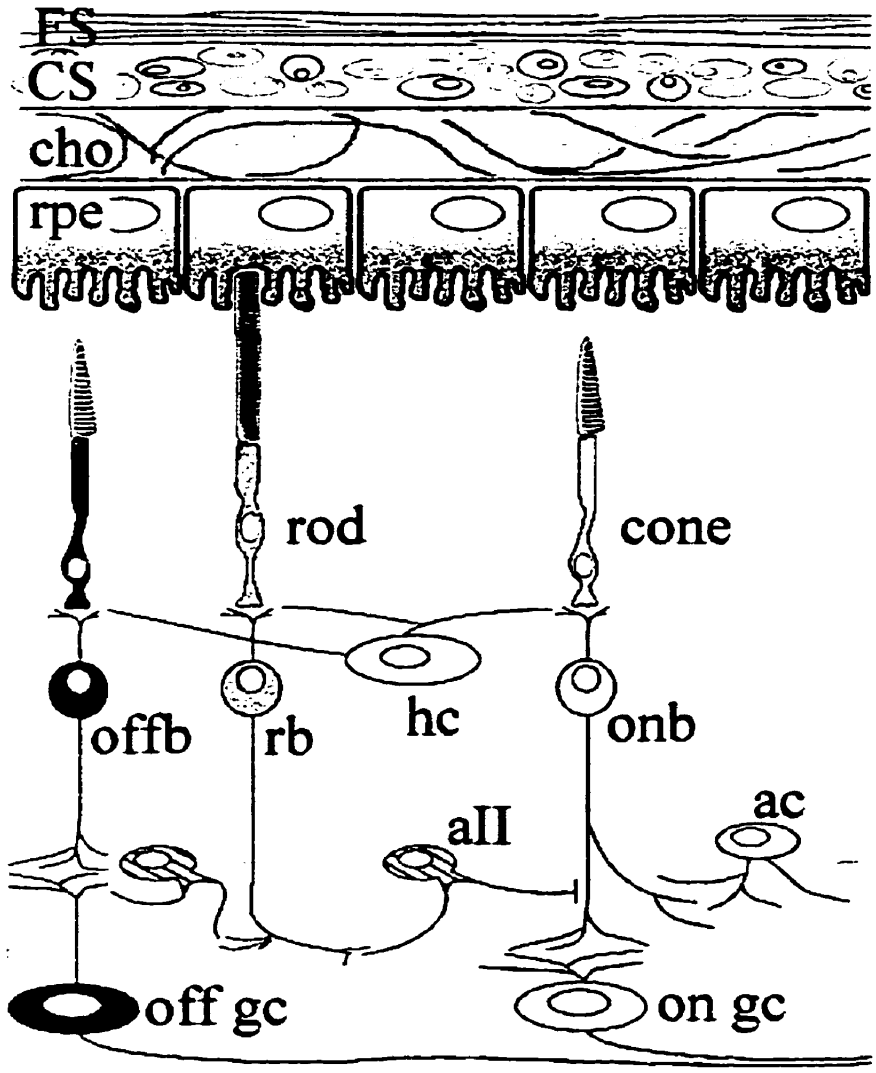
Figure 1.3: Retinal Morphology. Abbreviations: ac: generic amacrine cell, aII: AII amacrine cell, cho: choroid, cone: cone photoreceptor, CS: cartilaginous sclera, FS: fibrous sclera, hc: horizontal cell, off gc: off ganglion cell, offb: off bipolar cell, on gc: on ganglion cell, onb: on bipolar cell, rb: rod bipolar, rod: rod photoreceptor, rpe: retinal pigment epithelium.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sharpening of the temporal response of the bipolar cells. Amacrine cells provide synaptic input to shape ganglion cell responses to bipolar cells and are associated with both reducing the sustained bipolar cell response to transient ganglion cell response and detecting temporal changes in visual stimuli. However, the amacrine cells vary widely in morphology and likely physiology, and little is known about the myriad of roles that these cells likely play in visual processing.

Rod-Specific Physiology

Phototransduction marks the beginning of visual processing and signal delivery. Within the rod photoreceptors 11-cis retinal binds with scotopsin (protein moiety of rhodopsin, Shichida *et al.* 1990) to form rhodopsin, a light-sensitive photopigment. Photons of light excite rhodopsin, causing 11-cis retinal to change its conformation into all-trans retinal, initiating the degradation of the complex into its two components. As it degrades, it passes through a state known as metarhodopsin II. Metarhodopsin II activates transducin, which in turn activates phosphodiesterase, leading to the hydrolysis of cyclic-guanosine monophosphate (cGMP). Prior to hydrolysis, cGMP keeps sodium channels open, allowing ions to pass freely into the rod outer segment and keeping it in a depolarized state. By decreasing cGMP levels, light stimulus blocks this ion current. When coupled with the sodium efflux produced by ATPase activity in the rod inner segment, this results in a hyperpolarization of the photoreceptor. These electrical states influence glutamate release from the photoreceptor, with light-stimuli reducing glutamate release from the elevated rate that occurs in the depolarized "resting" state

Rod photoreceptors pass information to the ganglion cells via a number of pathways, mainly involving synaptic interactions with rod bipolar cells. Rod bipolar cells depolarize in response to illumination (which results in a decrease in rod glutamate release), through an deactivation of the metabotropic glutamate receptor, mGluR6. mGluR6 activates a phosphodiesterase to hydrolyze cGMP, resulting in blockage of a non-selective cationic conductance and hyperpolarization of the bipolar cell (a mechanism similar to that found in rod photoreceptors). Thus, a light-induced drop in glutamate release from rods results in decreased mGluR6 activity, heightened cGMP levels, influx of cations into and depolarization of rod bipolar cells.

In mammals, rod bipolar cells interact with AII amacrine cells to pass information to cone 'ON' bipolar cells and inhibit OFF-centre ganglion cells. AII amacrine cells have not yet been identified in the chicken retina, and thus the nature of the rod bipolar-AII amacrine-cone bipolar pathway can not yet be confirmed in this species. These cone bipolar cells are important considerations not only as part of the rod visual system, but also because they use cGMP in passing visual information, using a signal transduction cascade similar to rod bipolar cells..

Cone-Specific Physiology:

Despite the molecular analogy involved in cone- and rod-phototransduction, the kinetics of photoreception in these cell types differ (for review see Normann & Werblin 1974, Miller *et al.* 1994), indicating the use of distinct cell-type specific proteins. In particular, distinct cone types contain unique photopigments (photopsins or cone-opsins) instead of rhodopsin, with the differences of amino-acid sequence in these cone-opsins endowing differential sensitivity to light of specific wavelengths. This is the basis of colour vision.

Two major types of cone bipolar cells ('ON' and 'OFF' bipolar cells) are stimulated by cone photoreceptor activity. In a mechanism similar to rod bipolar cells, 'ON' bipolar cells respond to photoreceptor illumination by depolarizing. In contrast, 'OFF' bipolar cells respond by hyperpolarizing. Glutamate released by photoreceptors activates ionotropic receptors on 'OFF' bipolar cells, allowing for sodium ions to enter and the cell to depolarize. Light-induced reduction of glutamate release decreases the number of open ion channels and allows the 'OFF' bipolar cell to hyperpolarize.

Amacrine Cell Physiology

Of all the retinal cell types, amacrine cells appear to be the most diverse in morphology and varied in function, and to release the largest range of neuroactive agents. Amacrine cells receive their neural input from bipolar cells and other amacrine cells, and transmit to ganglion cells, other amacrine cells and even back to bipolar cells. In the pathway from photoreceptor to ganglion cell, the amacrine cell is the first neuron capable of firing action potentials instead of responding in a graded fashion (Marchiafava 1976). Many kinds of amacrines are known to release the neurotransmitters gamma-aminobutyric acid (GABA) and glycine (Wassle & Boycott 1991). Recent research

indicates that through the control of GABA and glycine release, amacrine cells can produce the transient responses typical of many ganglion cells directly by inhibiting ganglion cells, or indirectly through feedback to bipolar cells (Lagnado 1998, Nirenberg & Meister 1997). It seems that these cells are the processing powerhouses of the retina, likely responsible for many of the intricate capabilities of the retina.

Scleral Changes in Form Deprivation Myopia

FDM causes growth changes primarily within the sclera. As other ocular tissues are supported structurally by the sclera, their position is ultimately determined by scleral growth. Thus, as the sclera grows larger the other tissues recede from the anterior eye segment. The mammalian sclera consists of a single fibrous layer inhabited by fibroblasts and composed mainly of type I collagen with small amounts of type II collagen (Lee *et al.* 1981, Keeley *et al.* 1984). Changes associated with myopic eye growth in mammals include thinning of the sclera along with a decrease in collagen and proteoglycan content (Wallman and Adams 1987, Sivak *et al.* 1990). These findings are consistent with the theory that scleral expansion is due to a passive stretching of the tissue. However a number of subsequent studies suggest that scleral remodelling leads to expansion of mainly posterior regions (Norton and Rada 1995, Phillips and McBrien 1995, Guggenheim and McBrien 1996).

The chick sclera is composed of two distinct connective tissues, an external fibrous layer similar to that of mammals, and an inner, thicker cartilaginous layer. The cartilaginous sclera is composed of chondrocytes and an extracellular matrix predominantly made of the proteoglycan aggrecan along with collagen types II and IV (Gottlieb *et al.* 1990, Rada *et al.* 1991). Form-deprivation myopia affects these two scleral tissues in distinct fashions. The cartilaginous sclera thickens (Gottleb *et al.* 1990, Christensen and Wallman 1991) and experiences increases in chondrocyte DNA synthesis (Christensen and Wallman 1991), protein synthesis (McBrien *et al.* 1991, Christensen and Wallman 1991), and production of proteoglycans (Neville *et al.* 1991, Rada *et al.* 1991). Despite these increases, cell density decreases slightly in the myopic sclera (Gottlieb *et al.* 1990, Marzani and Wallman 1997). This is likely the result of an increase of proteoglycan deposition out-pacing the increase in mitotic division. Conversely, the fibrous sclera of the chicken responds to form deprivation by decreasing

synthesis of DNA and proteoglycans (Marzani and Wallman 1997), leading to thinning of the fibrous sclera. Kusakari *et al.* (1996) also demonstrated that FD-induced changes in the cartilaginous sclera varied over the width of the tissue. Regions of the cartilaginous sclera juxtaposed to the choroid had reduced cell density in response to FDM, while regions closer to the fibrous layer had much greater proliferating cell nuclear antigen (PCNA, indicative of cells undergoing division)-positive cell density than in control eyes. This indicated that regions lying farther from the fibrous sclera experienced heightened extracellular matrix production, while regions closer to the fibrous sclera underwent greater cell proliferation. This interaction between fibrous and cartilaginous scleral tissues was further supported by Marzani and Wallman (1997). They demonstrated that cartilaginous sclera could be persuaded to undergo appropriate synthesis changes by co-culturing with fibrous sclera tissue from form-deprived eyes.

Chemical Agents Implicated in the control of Myopic Ocular Growth

Once the retina was established as the likely origin of growth-controlling signals much attention was given to decipher what chemical agents might affect ocular growth.

Basic Fibroblast Growth Factor and Transforming Growth Factor Beta

Recognizing that chick FDM involved an increased production of scleral proteoglycans (Rada *et al.* 1991), Rohrer and Stell (1994) examined the effects of growth factors on myopic development in goggled chicks. Basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β) were selected for study because of the known "push-pull" interaction between these proteins when regulating extracellular matrix production. Their study revealed that exogenous bFGF led to a reduction of FDM, which could be partially countered by TGF-β co-treatment. Later, Seko *et al.* (1995) showed that ocular tissues from form-deprived eyes contained significantly less bFGF (sclera) and significantly more TGF-β (retina-RPE-choroid) than in control eyes. It was later demonstrated that most cell types within the chick retina possessed both bFGF and its receptors (Rohrer *et al.* 1997).

Honda *et al.* (1997) reported that the amount of TGF-β was reduced in chick FD eyes, and that exogenous TGF-β suppressed chondrocyte proliferation in scleral cultures. These findings contradict those of Rohrer and Stell (1994) and Seko *et al.* (1995).

Further, my own findings indicate that FDM did not affect endogenous levels of TGF-β within the chick ocular tissues (unpublished). These opposing views make it difficult to draw any conclusions on what role if any that TGF-β might play in the modulation of visually-guided ocular growth.

Dopamine Receptor Agonists/Antagonists

Research concerning dopaminergic mechanisms of myopic growth control constitutes the largest body of pharmacological evidence in FDM research and has led to a much greater understanding of the processes involved in the development of myopia. However, a clear answer pertaining to dopamine's role in myopia has not yet been finalized.

Stone *et al.* (1989) were first to demonstrate that levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were reduced in form-deprived eyes, a result repeatedly found by other studies (Rohrer *et al.* 1995, Guo *et al.* 1995, Weiss and Schaeffel 1993, Bartmann *et al.* 1994). Stone *et al.* (1989) also confirmed a dopaminergic effect on FDM by showing that the dopamine receptor agonist apomorphine suppressed axial elongation in FD chicks, and that this suppression was blocked by application of haloperidol, a dopamine receptor antagonist. Iuvone *et al.* (1991) showed that apomorphine also suppressed FDM in rhesus monkeys, to the point of hyperopia. Rohrer *et al.* (1993) showed that intravitreal injections of apomorphine were more efficacious than subconjunctival injections at suppressing FDM, suggesting a retinal or RPE site for this effect. That study also suggested that this outcome was mediated though the D2 receptor, as spiperone (a D2 receptor antagonist) abolished apomorphine's effect and SCH23390 (a D1 receptor antagonist) did not. Rohrer *et al.* (1995) further demonstrated that stroboscopic illumination (10 Hz), which had previously been reported to prevent FDM (Vingrys *et al.* 1991, Schwahn & Schaeffel 1997), could return the rate of dopamine synthesis to its unoccluded rate in goggled chick eyes.

Despite a wealth of support for the idea that reductions in dopamine occur with FD, some studies have shown that this relationship is not always certain. Continuous light (Bartmann *et al.* 1994), and the dopaminergic neurotoxin, 6-hydroxydopamine (Li *et al.* 1992, Schaeffel *et al.* 1994) have also been shown to suppress FDM in chicks, despite both treatments decreasing retinal dopamine levels (Bartmann *et al.* 1994, Dowling &

Ehinger 1978). Further, Schaeffel *et al.* (1995) showed that the dopamine antagonists sulpiride (D2 receptor-specific antagonist) and SCH23390 (D1 receptor-specific antagonist) also suppressed FDM. In that study reserpine and 5,7-dihydroxy-tryptamine (5,7-DHT) also served to suppress FD-induced myopic development at the same time as reducing retinal dopamine. With contradictory results showing both increases and decreases in dopamine associated with FDM, the role of dopamine in myopic development is left uncertain.

Vasoactive Intestinal Polypeptide

In 1988, Stone *et al.* demonstrated that juvenile macaques with fused eyelids expressed elevated vasoactive intestinal peptide (VIP) levels within a sub-set of amacrine cells and also developed myopia. Seltner and Stell (1995a) then demonstrated that intravitreal injections of VIP reduced, but did not eliminate FDM. Meanwhile, a VIP agonist (Hoffman LaRoche) failed to affect FDM, and two VIP antagonists (both modified VIP hybrids) completely abolished FDM (Seltner and Stell 1995).

Opiates/Enkephalins

Enkephalins were first linked to FDM when Ali *et al.* (1993) showed that Met^5-enkephalin was decreased in form-deprived chick eyes. Injection of the enkephalin-receptor non-specific antagonist naloxone blocked FDM in a dose-dependant manner, whereas the receptor non-specific agonist morphine had no effect (Seltner *et al.* 1997). In an attempt to determine which receptor sub-types were important in regulating myopia, Seltner *et al.* (1997) tested a number of opiate receptor subtype-specific agonists and antagonists, finding that both kappa receptor agonists and antagonists reduced FDM. Their study concluded that there was no definitive evidence that opiate receptors had a direct effect on FDM. This was further confirmed when Fischer *et al.* (1998b) showed that both the opiate receptor-active (L-) enantiomer and the opiate-inactive (D-) enantiomer of naloxone were equally effective in blocking FDM. This strongly suggests that opiate receptor agonists and antagonists influence FDM through non-opiate mechanisms. Other studies (Koyuncuoglu & Aricioglu 1991, Shukla & Lamaire 1991, Wong & Kemp 1991) have shown that certain opiate agonists and antagonists, including naloxone, can bind to NMDA receptors. This opens up the possibility that these agents might be preventing FDM through interactions with NMDA receptors.

N-Methyl-D-Aspartate and other Glutamate Agonists

As glutamate is a principal neurotransmitter of the retina, it is reasonable that it might be involved in the retinal pathways that regulate ocular growth. Also excitotoxicity caused by the over-stimulation of glutamate receptors has proven useful in studying visually guided ocular growth. NMDA-induced excitotoxicity was shown to cause excessive growth in non-deprived chick eyes and render eyes non-responsive to form-deprivation (Fischer *et al.* 1997). Fischer *et al.* (1998) further showed that quisqualic acid preserved the retina's ability to mediate ocular growth while destroying approximately 50% of all amacrine cells, including VIP-, met-enkephalin- and choline acetyltransferase-immunoreactive amacrine cells. Conversely, quisqualate spared tyrosine hydroxylase-containing cells, supporting the notion that dopamine is important in the modulation of FD-induced ocular growth. Treating eyes with quisqualic acid would become a method used to reduce retinal circuitry with the intent of abolishing any redundant ocular growth pathways and thus "simplifying" the retina.

Treatment of open eyes with kainic acid, another excitotoxic glutamate agonist, induces excessive ocular growth (Wildsoet and Pettigrew 1988b, Barrington *et al.* 1989, Ehrlich *et al.* 1990, Lauber and Oishi 1990). Ehrlich *et al.* (1990) further characterized the effects of kainic acid on ocular growth, by showing that while open eyes grew excessively, growth of occluded eyes was inhibited in relation to controls (occluded, saline-injected eyes). These growth changes were accompanied by severe retinal damage, including the destruction of photoreceptors, amacrine cells, bipolar cells and, to a lesser extent, ganglion cells (Ehrlich *et al.* 1990). Fujikado *et al.* (1996) explored the mechanisms leading to these growth changes and showed that at sub-toxic doses, kainic acid suppressed both 'ON' and 'OFF' bipolar cell responses to visual stimulus as recorded by electroretinogram, while inducing excessive ocular growth in open eyes. However, Golcich *et al.* (1990) had previously suggested that kainic acid selectively lesioned 'OFF' bipolar cells, leaving 'ON'-channels largely intact. Fujikado *et al.* (1996) and Crewther *et al.* (1996) also showed that the ON-channel blocker, L-amino-4-phosphonobutyrate (APB), induced hyperopia in open eyes. As kainic acid affects normal and FDM ocular growth in different ways, it is likely that they are the result of different regulatory pathways and may involve different loci of glutamate action.

Muscarinic Receptor Antagonists

This sub-field of myopic research originates from studies testing the efficacy of atropine for suppressing school-age myopia. In the chick paradigm, Stone *et al.* (1991) demonstrated that not only did atropine (a non-specific muscarinic receptor antagonist) suppress FDM in chicks, but so did pirenzepine, the defining M1 antagonist, while M2- and M3- specific antagonists showed no effect. This study also added support to the notion that muscarinic antagonist effects on FDM were via retinal interactions and not accommodative mechanisms, as accommodative musculature in the chick is mainly nicotinic. McBrien *et al.* (1993) used carbachol (general acetylcholine receptor agonist) and atropine on FD chicks in an attempt to better illustrate the retinal locus of FDM suppression. They showed that carbachol stimulated accommodation and pupillary constriction while atropine reduced FDM without reversing these carbachol-induced effects. Leech *et al.* (1995) further suggested the retinal locus of muscarinic growth control by showing that intravitreal injections of pirenzepine were significantly more effective in reducing excessive growth than were subconjunctival injections. Cottriall and McBrien (1996), through methods similar to those of McBrien *et al.* (1993), showed that M1-selective antagonists suppressed FDM in a mammalian species (tree shrew) via a non-accommodative mechanism. However, Fischer *et al.* (1998b) used ECMA- and quisqualic acid- retinotoxicity to demonstrate that atropine rescued eyes from FDM even after the destruction of cholinergic retinal pathways. This led them to conclude that muscarinic antagonists influenced myopia through either non-cholinergic or extra-retinal pathways. One study (Lind *et al.* 1998) showed that M1 antagonists reduced scleral cell proliferation and ECM production, concluding that such agents might affect FDM directly through the sclera. However, it must be considered that the doses required to elicit such scleral changes were only marginally different from those that were decided to be toxic. Also, no attempt was made to determine the site of this activity and no localizations of ChAT or mAChRs has been done on chick sclera. When all this is considered, it is hard to state convincingly that this may be a physiological and relevant mechanism.

Fos and other immediate early genes

Experiments conducted in our lab indicate that the immediate-early gene Fos might play a pivotal role in the modulation of myopic ocular growth. Rohrer *et al.* (1995) found that expression of c-Fos, a putative transcriptional regulator for tyrosine hydroxylase, was upregulated in dopaminergic amacrine cells in response to flickering light. Results indicate that an upregulation of Fos-like immunoreactivity is induced by goggle removal, an event that initiates ocular growth suppression allowing the eye to return to emmetropia (McGuire & Stell 1998). When injected into open eyes, antisense oligodeoxynucleotides (AODN) directed to identical sequences in the chick *c-fos* and *fra-2* genes induce mild increases in ocular growth (McGuire & Stell, 1999), yet do not affect growth in goggled eyes. Further, when injected into quisqualate-reduced retinas in open chick eyes, AODN induced excessive ocular growth, again not affecting goggled eyes. These findings suggest that genes of the Fos family in the retina may modulate vision-controlled ocular growth.

Recently it has been shown that the expression in amacrine cells (particularly those containing glucagon) of another immediate-early gene, ZENK is suppressed by conditions that produce excessive ocular growth and myopia (Fischer et al, Nature Neuroscience, in press), while being enhanced by conditions which inhibit ocular growth and produce hyperopia. These vision-dependent changes in ZENK expression are abolished by treatment with the NMDA-receptor antagonist MK-801. With these findings, Fischer *et al.* (1999) suggested that amacrine cells are able to detect the sign of visual defocus, and that this is influenced by NMDA-receptor activity. With the ability to detect the sign of defocus, amacrine cell activity could modulate ocular growth, inducing myopia or hyperopia in response to the visual environment.

Nitric Oxide/Nitric Oxide Synthase

A recent addition to the list of substances that may be involved in visually guided ocular growth control is nitric oxide, produced by nitric oxide synthase (NOS). NOS has been localized to the retina (Fischer & Stell 1999) and even claimed to modulate ocular growth (Fujikado *et al.* 1997). However, certain aspects of this modulation have raised serious questions concerning the validity of the results. This is the topic of this study and will be introduced in greater detail in sections to follow.

Photoreceptor Toxicity Eliminates Excessive Ocular Growth

Apart from selected pharmacological agents, research has also been conducted to determine the effects of toxic agents on FDM. Here I review the consequences of photoreceptor toxicity on the development of myopia.

Constant Light:

Constant light has been shown to suppress the excessive ocular growth associated with the development of FDM in chicks (Bartmann *et al.* 1994, Weiss & Schaeffel 1993). Previous studies had shown that constant light is sufficient to destroy both rod and cone photoreceptors in rats (La Vail 1976), though it was also shown that this treatment might affect rods to a greater extent (Cicerone 1976). However, what effect constant light might have on chick photoreceptors has not been studied and cannot be predicted. As chick ocular growth still responds to lens-induced defocus in constant light (Bartmann *et al.* 1994), it is likely that constant light spares many photoreceptors.

Tunicamycin:

Tunicamycin inhibits the process of linking oligosaccharides to glycoproteins (Takatsuki *et al.* 1975, Struck and Lennarz 1980). Rod and cone opsins are such glycoproteins and the treatment of eyes with this drug results in targeted degeneration of photoreceptors (Fliesler *et al.* 1984, Chambers *et al.* 1986). Ehrlich *et al.* (1990) later demonstrated that at doses sufficient to destroy photoreceptors, tunicamycin abolished excessive growth in goggled chicks, while leaving normal growth unaffected. The authors claimed these results indicated that photoreceptors played a critical role in modulating occlusion-induced ocular growth. However, the effective dose also severely damaged other retinal cell types. Despite the certainty that photoreceptor function is critical for visual regulation of ocular growth, it is my opinion that little can be drawn from that study to elucidate what role photoreceptors might have in modulating excessive ocular growth.

Formoguanamine:

Obara *et al.* (1985) showed that subcutaneous injection of formoguanamine (FG) into chicks resulted in degeneration of RPE and photoreceptors, leading to acute retinal detachment. Later, formoguanamine was used to inhibit the development of myopia in lid-sutured chicks (Oishi and Lauber 1988), resulting in ocular globe dimensions similar

to those of emmetropic eyes and the reduction of corneal diameter and anterior chamber depth compared to controls. Westbrook *et al.* (1995) later showed that intravitreal injections of FG suppressed FDM in occluded chicks without causing blindness, thus raising the question of whether myopic-rescue by FG is caused by photoreceptor degeneration.

These studies of photoreceptor toxicity have demonstrated that proper photoreceptor function is likely crucial for the typical development of myopic ocular growth. Whether this is derived by the photoreceptors' role in passing visual information to other retinal cell types, or whether it is intrinsic to photoreceptors, independent of other cell activities, has yet to be determined.

Human Retinal Disorders Coinciding with Myopia

A number of human visual disorders are associated with the development of negative refractive error. Of these, I will briefly discuss three; retinitis pigmentosa, complete congenital stationary night blindness (cCSNB) and Leber's congenital amaurosis (LCA).

Retinitis pigmentosa is the name given to a heterogeneous group of genetic diseases that leads to the degeneration of rods and cones (Milam *et al.* 1998). As the disease progresses it typically results first in night blindness and then complete blindness (Wong 1994). Progression of this disease is also associated with development of myopic refractive error in a high majority of cases (Sieving & Fishman 1978). Complete CSNB is also associated with moderate to severe myopic refractive error (Dry *et al.* 1993)(as opposed to incomplete CSNB which is associated with refractive errors encompassing emmetropia). Both types of CSNB are characterized by night blindness and equal decreases in visual acuity (Dry *et al.* 1993). However, while incomplete CSNB is associated with a greater decrease in cone function, complete CSNB has been shown to abolish rod photoreceptor function while only affecting cone function marginally (Miyake *et al.* 1986).

While the previous two conditions are associated with the development of myopia, LCA is largely accompanied by hyperopia (Wagner *et al.* 1985). This condition is characterized by loss of vision shortly after birth resulting in blindness or severely impaired vision. The electroretinograms of patients suffering from this condition show

greatly decreased retinal activity and patients exhibit either degeneration of, or deficits in development of, photoreceptors (Mizuno *et al.* 1977). In recent years, LCA has been mapped to mutations within the gene coding for retina-specific guanylate cyclase, linking this condition and its promotion of hyperopia to impaired cGMP production within photoreceptors (Perrault *et al.* 1996).

It would appear from these two conditions that rod function does play an important role in maintaining normal ocular growth.

Review of Nitric Oxide Biology

History:

The identification of nitric oxide as an endogenous biological agent originated in two separate scientific studies; the discovery of the vascular smooth muscle relaxing agent, endothelial-derived relaxing factor (EDRF - Furchgott & Zawadski, 1980), and the observation that nitrate content increased in the urine of endotoxin-treated rats (Wagner *et al.*, 1983). These two lines of research eventually identified nitric oxide as an endogenous signalling molecule produced in both endothelial cells (Ignarro *et al.* 1987, Palmer *et al.* 1987) and macrophages (Hibbs *et al.* 1987, Stuehr *et al.* 1989). The first evidence suggesting a neuromodulatory role for this molecule within the CNS was that cerebellar neuronal cultures released a factor with the physiological properties of nitric oxide (Garthwaite *et al.* 1988). Consequently, nitric oxide became recognised as mediating many biological processes, including immunoresponse, vascular tone and neuromodulation. The first indications that nitric oxide may be active in the retina came from descriptions of NADPH-diaphorase activity within the rabbit retina (Sagar 1986), a histological reaction later shown to co-localize quite reliably with nitric oxide synthase (NOS) (Matsumoto *et al.* 1993). The production of nitric oxide within the retina was firmly established when Dawson *et al.* (1991) localized NOS in amacrine and ganglion cells of the rat retina.

Activity/Physiology:

Nitric oxide (NO) is a highly labile molecule, able to diffuse through cellular membranes and having a lifespan of only seconds before being rendered biologically inactive (Snyder & Bredt, 1992). Readily diffusing across cellular membranes, it cannot be stored in vesicles and must be produced on demand. As no cell surface receptors have

been characterized to mediate NO cellular activity it is believed that NO elicits its biological influence directly within the target cells. By this thinking nitric oxide typically acts directly upon molecules which would normally be considered secondary messengers (Zhang & Snyder, 1995). The primary target of nitric oxide is soluble guanylyl cyclase (Arnold *et al.* 1977, Ignarro *et al.*, 1981), though others have been also been discovered (inhibition of various enzymes for glycolysis and iron metabolism as well as others: see Zhang and Snyder, 1995 for review). Binding strongly to the chelated iron of guanylyl cyclase, nitric oxide enhances its production of cyclic guanosine 3', 5'-monophosphate (cGMP) (Ignarro 1990, Bredt & Snyder 1992, Moncada & Higgs, 1993). Thus, many of nitric oxide's biological activities are the direct result of elevated cGMP levels.

Nitric oxide is quickly inactivated by spontaneous linkage to superoxides and haem proteins, such as haemoglobin (Murad *et al.* 1978, Martin *et al.* 1986, Ignarro 1989), or metabolized into nitrites and nitrates by various oxidases (Zhang and Snyder, 1995).

Nitric oxide mediates a number of different activities within the nervous system. In certain situations, the release of neurotransmitters from neurons is dependent on nitric oxide. Hirsch *et al.* (1993) demonstrated that blockade of nitric oxide production in brain synaptosomes inhibited NMDA-stimulated neurotransmitter release. Further, in nerve growth factor-treated PC-12 cells, acetylcholine and dopamine release was blocked by NOS inhibitors (Hirsch *et al.* 1993). Additionally, it has been suggested that nitric oxide may act as a retrograde messenger to promote long-term potentiation (Schuman & Madison 1991, O'Dell *et al.* 1991) and direct development of the maturing nervous system (Bredt & Snyder 1994). Nitric oxide has also been implicated in NMDA-neurotoxicity (Dawson *et al.* 1991, Dawson *et al.* 1993), apparently through a non-guanylyl cyclase mechanism (Lustig *et al.* 1992).

Nitric Oxide Synthase

Nitric oxide is produced by nitric oxide synthase (NOS), through the oxidization of L-arginine to yield L-citrulline and NO (Bredt & Snyder, 1994). Requiring five electrons to catalyze this reaction, NOS binds numerous electron donors: nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) (Bredt *et al.* 1992) and tetrahydrobiopterin (Kwon & Nathon 1989,

Mayer *et al.* 1991). NOS also has a binding site for calmodulin (Bredt & Snyder, 1990, Bredt *et al.* 1991).

NOS has three isoforms, each encoded by separate genes (Bredt *et al.* 1991, Xie *et al.* 1992, Sessa *et al.* 1992). The first isoform, neuronal NOS (nNOS, NOS I), is constitutively expressed within the cytosol of certain neurons of the CNS and PNS and its activity is dependent on calcium and calmodulin (Bredt & Snyder, 1990). Endothelial NOS (eNOS, NOS III) is also calcium-dependent and is expressed constitutively on the plasma membrane of vascular endothelial cells (Pollock *et al.*, 1991). The third isoform, inducible NOS (iNOS, NOS II) differs from the others in that it is expressed only when induced, particularly by cytokines and/or endotoxin, within phagocytes (Goureau *et al.* 1992). Induction by these factors is mediated through two recognition regions upstream of the gene's TATA box. Region one is an LPS-related response element and region two contains recognition sites for interferon gamma-related transcription factors (Lowenstein *et al.* 1993, Xie *et al.* 1993). Inducible NOS tightly binds a calcium-independent calmodulin-like subunit constitutively and is regulated transcriptionally (Cho *et al.* 1992), while the activities of nNOS and eNOS are modulated post-translationally by calcium levels, without apparent change in protein levels (Zhang & Snyder 1995). Because of these differences in regulation of the constitutive and inducible NOS isoforms, there are also marked differences in the dynamics of regulation. Neuronal and endothelial NOS activities are quickly modified whereas inducible NOS increases NO production only hours after an inducing stimulus, maintaining active for days (Nathan & Xie, 1994).

Of particular relevance to the field of FDM/NO research are the potential influences of glutamate, transforming growth factor beta (TGF-β) and fibroblast growth factors (FGF) on NOS. As reviewed above, glutamate and TGF-β/FGF may play roles in FDM (Fischer *et al.* 1997, Rohrer & Stell 1994, Seko *et al.* 1995). NMDA receptor activation triggers an influx of calcium through the ligand-gated channels. The calcium can bind to available calmodulin, which has the potential to activate either n- or e-NOS if it is present (Dawson *et al.* 1991). Drawing on results from Fischer *et al.* 1998c, which showed that NMDA-receptor activity promoted FDM, this link to NOS suggests that NO might also affect excessive ocular growth. Because of its calcium-independent activity, it is unlikely that inducible NOS can be influenced by NMDA activity in the same manner.

Instead, TGF-β has been shown to increase nitrite production (presumably through an induction of iNOS activity) in cultured lipopolysaccharides/interferon-γ-activated bovine RPE cells, while FGF is able to potently inhibit it (Goureau *et al.* 1993). This evidence, when combined with the work of Seko *et al.* (1995) and Rohrer & Stell (1994), advocates a role for NO in the control of myopic growth. If NMDA-receptors, TGF-β and FGF are relevant to myopic development, iNOS and nitric oxide would seem to be appropriate candidates to promote the ocular growth associated with myopia.

Nitric Oxide and the Chick Retina

Nitric oxide synthase has been detected in various cell types and tissues within the eyes of all species so far probed. Within the chicken retina, nNOS immunolabelling was present within a subset of ganglion cells, efferent fibres and their target cells, neural fibres of both plexiform layers, and four subtypes of amacrine cell (Fischer & Stell 1999), corresponding well with NADPH-diaphorase histochemistry. Meanwhile, NADPH-diaphorase alone was detected within Müller cells and photoreceptor ellipsoids. Further, nNOS was found in nerve fibres innervating the vascular smooth muscle of the choroid and diaphorase was detected in scleral chondrocytes, basal RPE and stromal tissues of the choroid (Fischer & Stell, 1999). As only NOS is thought to retain NADPH-diaphorase activity after paraformaldehyde fixation, cells that are labelled by both diaphorase and the antibody are believed to contain nNOS, while cells that are only labelled by diaphorase may contain a NOS isoform not recognized by the antibody. Fischer's & Stell's results agree with those done with NADPH-diaphorase in chick retina by Morgan & Miethke (1994), who labelled structures very similar to Fischer's & Stell's efferent fibres and target cells, and Paes del Carvalho (1996), who labelled photoreceptor inner segments as well as amacrine and ganglion cells. However, this NADPH-diaphorase reaction might be the result of an unknown, non-NOS enzyme that utilizes NADPH and is fixation-resistant. Still, the distribution of NOS in the chick eye is consistent with a role for nitric oxide in FDM.

Nitric Oxide and Myopia

Fujikado *et al.* (1997) reported that intravitreal injection of the NOS-inhibitor L-NAME suppressed the development of refractive error and axial lengthening in goggled chicken eyes, without affecting open eye development. Fujikado *et al.* (1997) suggested

that this was due to the ability of L-NAME to suppress NO-dependent ocular growth induced by form-deprivation. This result is consistent with observations implicating both glutamate and dopamine in the development of FDM (Fischer *et al.* 1998, Stone *et al.*, 1990, Rohrer *et al.* 1995). To clarify that nitric oxide was responsible for this effect, Fujikado *et al.* (1997) demonstrated that nitrite and nitrate levels (products of NO metabolism, collectively refered to as NO_x), a widely accepted measure of NO production, were significantly less in form-deprived chick retinas treated with L-NAME than in non-treated retinas. However, NO_x levels in form-deprived retinas were not significantly different from those of untreated retinas. Fujikado suggested that no difference in NO_x was detected because of the inadequacy of the test to resolve the products of the different NOS isoforms. Whereas L-NAME may inhibit all NOS isoforms and thus affect the isoform involved in FDM, measuring NO_x will detect all NOS activity and any changes due to form-deprivation may be masked by other NOS activities.

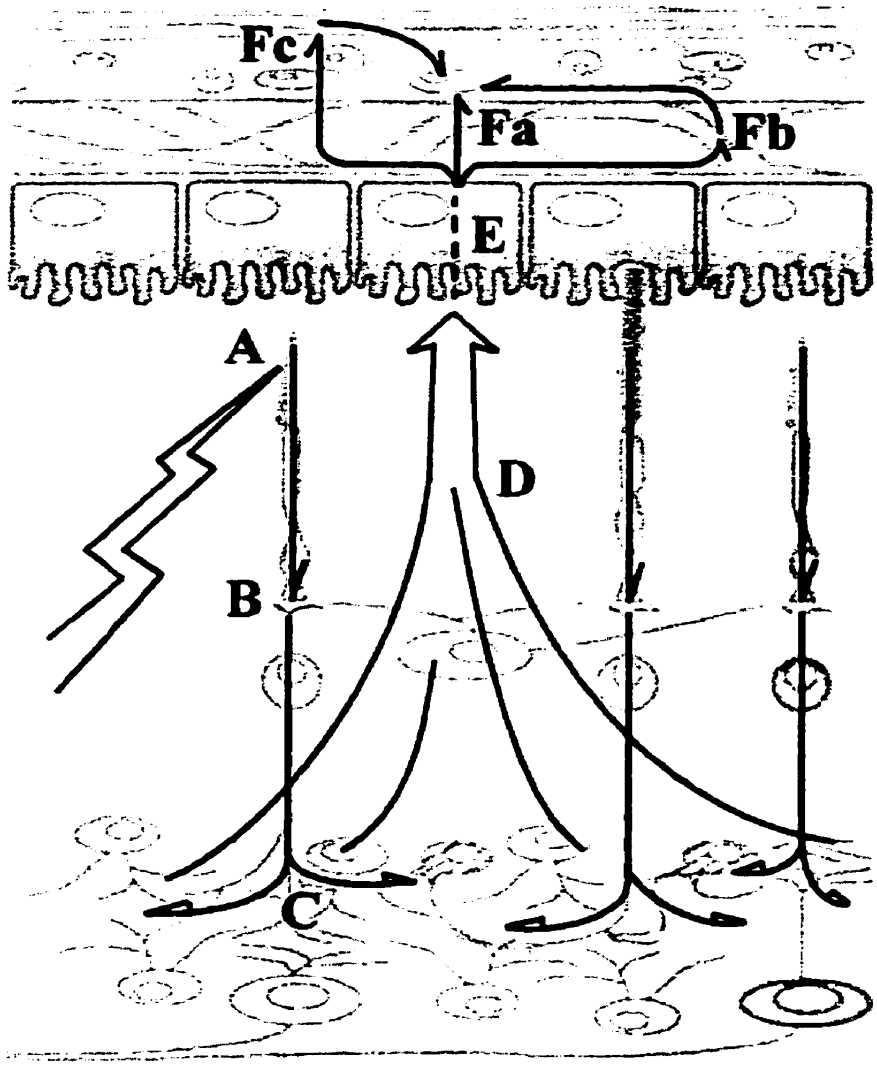
However, recent attempts in our lab to reproduce Fujikado's results have failed (Stell, unpublished). In these experiments, L-NAME and the NOS inhibitor L-NMMA, do not influence FDM except at extremely high doses, which could be toxic or elicit non-specific effects. Indeed, findings from Wellard *et al.* (1995) have indicated that L-NAME may not be a practical NOS inhibitor because of the large doses (IC_{50} = 19 mM) required to inhibit NOS activity in the intact chick retina, likely the result of an inability of L-NAME to gain entry into the retinal cells. This is compounded by the fact that even at doses lower than those used by Fujikado *et al.*, L-NAME has been shown to antagonize mammalian (canine) muscarinic acetylcholine receptors (Buxton *et al.* 1993). Since muscarinic receptors have been implicated in the mechanism of FDM in chicks (Stone *et al.* 1991), it is entirely plausible that L-NAME prevents FDM via muscarinic receptors. In addition to this, a recent report by Fujii *et al.* (1997) showed that the expression of iNOS and nNOS mRNAs decreased significantly in form-deprived chicks, a result contradictory to Fujikado's claims. These accounts seriously question the relevance of Fujikado's findings. It should be noted, however, that the pharmacology of chicken muscarinic receptors (Tietje & Nathanson 1991; Tietje *et al.* 1990) is quite different from that of the mammalian receptors studied by Buxton *et al.* (1993), and that

recent reports indicate that muscarinic mechanisms may not regulate normal or FD-induced ocular growth (Fischer *et al.*, 1998). Therefore, it remains uncertain whether toxicity or muscarinic receptor activity may play any role in the actions of L-NAME reported by Fujikado *et al.* (1997).

Model of Myopic Ocular Growth Figure 1.4

The retina is a complex sensory organ. Neural activities within the retina that modulate eye growth may be equally complex. It may prove helpful to have a conceptual model of how such growth control might be accomplished. Light is first received by the photoreceptors. The photoreceptors then communicate to the bipolar cells via glutamate receptors. Studies aimed at blocking information transmission through the bipolar cells have shown that both ON and OFF cone bipolar pathways (as well as rod bipolar paths) may be essential in distinct ways for proper ocular growth and development (Smith *et al.* 1985, Crewther *et al.* 1996, Fujikado *et al.* 1996). However, as bipolar cells do not appear capable of sampling the visual stimuli for defocus, I believe that the growth suppression induced by blockade of photoreceptor-bipolar communication is caused ultimately by depriving amacrine cells of essential inputs, leading to the signal changes that reduce growth. Amacrine cells have recently been shown to be capable of determining sign of defocus within the visual stimulus (Fischer *et al.*, 1999). Also, a number of different substances in amacrine cells are affected by form-deprivation. I believe that changes in amacrine cell activity come to influence RPE activity as the amacrine cell-released agents diffuse to the apical surface of the RPE. The RPE, upon reception of the amacrine signals alters its basal release of specific substances. These substances then travel into the choroid, likely interacting with, and altering the activities of, tissues and stimulating other signal changes. The summation of post-RPE signals that reach the sclera leads to the remodeling of the fibrous sclera resulting in lateral expansion of this tissue. In chickens, the chondrocytes of the sclera are induced to increase cell proliferation and production of extracellular matrix, in response to post-RPE signals and signals coming from the fibrous sclera. These scleral changes lead to an expansion of the sclera, axial elongation and the development of myopic refractive error.

Figure 1.4: Schematic Diagram of a Model for Visual Regulation of Ocular Growth. A: Photoreceptors receive visual input. B: Bipolar cells receive signals encoding visual stimuli from photoreceptors. C: Amacrine cell behaviour is influenced by bipolar cell activity. D: The substances released by amacrine cells diffuse to the apical surface of the RPE. E: The RPE alters certain activities in response to certain factors dispersed from the amacrine cells. F: The RPE releases altered levels of new factors. These factors may directly influence scleral growth (Fa), or may alter activities in the choroid (Fb) or the fibrous sclera (Fc) which ultimately affect scleral growth.


Fc
Fa
Fb
E
A
D
B
C

Hypothesis and Purpose

In this study, I have further explored the influence that nitric oxide has on the development of FDM in the chick model. The general hypotheses is:

"Nitric oxide does not play a physiologically relevant role in the development of form-deprivation myopia."

This hypothesis predicts that: "Neither the inhibition of NO production nor the augmentation of available NO will inhibit or promote the excessive ocular growth or development of refractive error associate with FDM. Such effects will only be observed when accompanied by non-specific activities or neurotoxicity." This statement provides the basis for the specific experimental hypotheses and stategies:

1. The treatment of chick eyes with NOS inhibitors will not influence the development of FDM through specific interactions with NOS, despite inducing a decline of retinal NO_x levels.

   Strategy: Both goggled and open eyes will be assessed for the effects of various doses of NOS inhibitors (L-NAME, L-NMMA, L-NIO) on ocular growth (normal or myopic). In the case of L-NIO, the retina and RPE of treated eyes will also be assayed for NO_x content to determine whether the drug effectively reduced nitric oxide availability within them.

2. The augmentation of available NO, via exogenous donors, will not influence the development of refractive error and excessive ocular growth associated with FDM through physiological mechanisms.

   Strategy: Eyes (goggled or open) will be treated with various concentrations of molecules known to liberate nitric oxide (sodium nitroprusside and L-Arginine). These eyes will then be measured (refractive error, axial length, wet weight) to determine the extent to which the agent and dose affected ocular growth (either normal or myopic). When necessary, the retinas of these eyes will also be examined microscopically using a number of immunolabels to determine the site and extent of damage due to the drug treatment. Further, eyes treated with SNP will be immunolabelled for cGMP to determine whether treatment with these compounds did result in increased retinal nitric oxide.

CHAPTER TWO

Methods and Materials

Animals

Male leghorn chickens (*Gallus gallus domesticus*) were obtained from the Lillydale Hatchery (Calgary, Alberta, Canada) on the day of hatching. Chicks were kept on a 12 hour light/ 12 hour dark cycle, at 25° Celsius, and were fed water and Purina chick starter pellets ad libitum. Seven days after hatching, chicks were segregated into groups of six, each group receiving a different treatment. Experiments were typically comprised of four or five different treatment groups.

Pharmaceutical Agents

This project utilized the drugs and chemical substances described in this section. Sodium nitroprusside (SNP, catalogue number S-151), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, cat.# A-161), N^G-monomethyl-L-arginine acetate (L-NMMA, cat.# M-125), L-N5-(1-iminoethyl) ornithine HCl, (L-NIO, cat.# I-134) and 3-isobutyl-1-methylxanthine (IBMX, cat.# A-007) were all obtained from Research Biochemicals International through Sigma . L-Arginine was obtained from Sigma (free base form, cat.# A 50006). SNP and L-Arginine are nitric oxide donors, while L-NAME, L-NMMA and L-NIO are inhibitors of NOS activity. IBMX is a phosphodiesterase inhibitor, used in this study to preserve cGMP synthesized within the retina.

General Procedures

Chicks were anaesthetized by inhalation of 1.5% halothane in equal parts N_2O and O_2, and the eyelids swabbed with 70% ethanol before injection. A 25-µl Hamilton syringe with a 26-gauge needle was used for injections. Injections were delivered through the eyelid into the dorsal region of the vitreal chamber. The right eye, acting as a control, was injected with 20 µl of sterile saline (0.9 % NaCl), while the left eye received the drug, dissolved in 20 µl of saline. Assuming a vitreous chamber volume of 180 µl, the maximum drug concentration in the vitreous is approximately one-tenth of that injected. The injections, as described above, were performed on days 7, 9, 11 and 13 (four-injection schedule), or days 7,9 and 11 (three-injection schedule) after hatching. In the case of L-NAME experiments, sub-experiments involved either multiple injections as above, or injections once on day seven (see L-NAME sub-section below for details).

To induce form-deprivation myopia, the left eye was fitted with a white translucent plastic goggle on the day of the first injection and the right eye was left unoccluded. The goggle remained over the left eye for the duration of the experiment.

On day 14, the ocular development of the chicks was examined. The chicks were refracted without anaesthesia or cycloplegia to determine refractive error, and then sacrificed by chloroform inhalation. Eyes were removed from the orbits and extraneous tissues were removed. The eyes were measured with digital calipers to determine axial length of the globe and then weighed.

Some pairs of treated and control eyes were further prepared for immunocytochemistry. These eyes were hemisected equatorially, and the vitreous humor removed. The resulting eye cups were fixed in a solution of 4% paraformaldehyde/ 3% sucrose in 0.1 M phosphate buffer, pH 7.4 at 20° C. for 30 minutes. At the end of the fixation, eye cups were washed for 10 minutes in 0.05M phosphate-buffered saline (PBS, 195 mM NaCl, 3 mM NaN3, pH 7.4). This wash was changed and repeated twice more. Eye cups were then cryoprotected by immersion in PBS plus 30% sucrose for 24 hours and then freeze-embedded in OCT (Tissue-Tek, Elkhart, IN) using liquid nitrogen. Sections approximately 13 μm thick were then cut through the centre of the fundus, placed on slides, air-dried and ringed with rubber cement.

Slides were washed three times in PBS to dissolve the OCT, and then incubated for 24 hours at 20° C in 200μl of PBS containing 5% normal goat serum, 0.3% Triton X-100, and diluted primary antiserum (See Table 2.1). They were then washed three times in PBS, and incubated for one hour in Cy3-conjugated goat-anti-mouse or goat-anti-rabbit immunoglobulin diluted in 200 μl PBS (Table 1). Slides labeled for LEP-100 were first treated in 4% formaldehyde/PBS for 30 minutes after dissolving the OCT and then washed three times in PBS. This extra exposure to formaldehyde was to ensure a strong fixation of the tissue required for proper labelling with the LEP-100 antibody.

After secondary labelling, slides were washed three times in PBS and then immersed in 4:1 glycerol : water, cover-slipped and sealed with nail-polish. Slides were then observed by epi-illumination fluorescence and photographed on Kodak Tmax film.NOS Inhibitors.

Table 2.1. List of antisera, their antigens, sources and appropriate ICC dilutions.

Table 2.1

Antiserum / Antibody	Antigen	Species/Type	Source	Working Dilution
C8666	Calbindin	mouse monoclonal	Sigma (Mississauga, Ontario)	1:200
pURXR.3	Calretinin	rabbit polyclonal	Dr. J.H. Rogers (U.of Cambridge)	1:1000
	cGMP	rabbit polyclonal	Dr. J. DeVente (U. of Netherlands)	1:400
1465	Choline acetyltransferase (ChAT)	Rabbit polyclonal	Dr.M.Epstein (U. of Wisconsin)	1:1000
R24	Gamma Aminobutyric Acid (GABA)	Rabbit polyclonal	Chemicon (Temecula, CA)	1:100
	Glutamic Acid Decarboxylase (GAD-65)	Rabbit polyclonal	Dr.C.Brandon (Chicago Medical School)	1:1000
LEP-100	Lysosomal membrane glycoprotein	mouse monoclonal	Hybridoma Bank (U. of Iowa)	1:50
M3	Unknown bipolar cell marker	mouse monoclonal	Dr.M.Chiquet (U. of Switzerland)	1:70
1473	Met-Enkephalin	Rabbit polyclonal	Dr.J.Walsh (UCLA)	1:1000
6761-6	neuronal NOS	Rabbit polyclonal	Abbott Labs	1:1000
Alpha-PA	Parvalbumin	Mouse monoclonal	Sigma (Mississauga, Ontario)	1:400
4D2	Rhodopsin	mouse monoclonal	Dr. B. Mulday (U. of B.C)	1:50
S-10	Somatostatin	Rat monoclonal	Dr. A. Buchan (U. of B.C.)	1:400
#16	Tyrosine Hydroxylase	Rabbit polyclonal	Dr.W.Tank (U. of Rochester)	1:1000
Anti-rabbit Cy3 (secondary Ab)	rabbit immunoglobulin	goat polyclonal	Boehringer Mannheim	1:1500
Anti-mouse Cy3 (secondary Ab)	rabbit immunoglobulin	goat polyclonal	Boehringer Mannheim	1:1500

L-NAME Experiments

Experiments were done on monocularly goggled chicks alone. The doses tested were 1.8 μmole, 3.0 μmole, 9.0 μmole, 16.2 μmole and 20 μmole of L-NAME per 20 μl of PBS vehicle injection, resulting in initial vitreal concentrations of approximately 9 mM, 15 mM, 45 mM, 81 and 100 mM L-NAME. Doses were given to chicks either only once on day seven after hatching (to replicate the injection protocol of Fujikado *et al.*, 1997) or every other day for a total of three or four injections (see Table 2.2). One week after the first injection, eyes were refracted, measured and weighed. Values for right open (control) eyes were then subtracted from the values of the left goggled (treated) eyes in each animal to compensate for injection effect and inter-individual variances.

L-NMMA Experiments

Experiments were done on monocularly goggled chicks alone. The doses tested were 1.6 nmol, 16 nmol, 160 nmol and 1.6 μmol L-NMMA per 20 μl of PBS vehicle injected. These doses, which give initial concentrations of approximately 8μM, 80μM, 800μM and 8000μM (respectively) in the vitreous, bracket the reported IC_{50} of 413 μM for suppression of NOS activity in intact chicken retina (Wellard *et al.* 1995). Injections were administered on three separate occasions, at two day intervals. Two days after the final injection the eyes were refracted, measured and weighed, and control eye values subtracted from treated eye values.

L-NIO Experiments

Experiments were conducted on monocularly goggled as well as ungoggled chicks. The doses were 0 nmol, 33 nmol, 100 nmol and 300 nmol L-NIO in sterile saline vehicle, giving initial vitreal concentrations of approximately 0, 167, 500 and 1500 μM. These doses therefore bracketed the reported IC_{50} of approximately 500 μmol for NOS activity in intact chick retina (Wellard *et al.* 1995). Injections were administered every other day for four injections. Two days after the last injections the eyes were refracted, measured and weighed, and the values for treated eyes were compared to those for control eyes as above.

In order to determine whether L-NIO acts as an effective inhibitor of NOS activity within the intact chicken eye, nitrate/nitrite levels were measured within the retina and RPE of L-NIO treated chickens. Nitrate/nitrite levels (NO_x) have been used before as an acceptable indicator of nitric oxide levels within the living creature. The

Table 2.2. List of L-NAME experiments, the doses tested, injection schedule and equivalence to doses used by Fujikado *et al.* (1997). Equivalents are reported as drug content within 30 µl of injection vehicle (as per Fujikado *et al.* 1997) required to produce approximate vitreal concentrations. **Bold** text indicates doses tested by Fujikado *et al.* (1997).

Table 2.2

Experiment Number	Dose (µmol/injection)	Equivalence to Fujikado	Injection Schedule
1	1.8	**60 mM**	Single Injection: Day 7
2	3.0	100.2 mM	4 Injections: Days 7, 9, 11, 13
3	9.0	300.6 mM	4 Injections: Days 7, 9, 11, 13
4	16.2	**540 mM**	Single Injection: Day 7
5	20.0	707.4 mM	Single Injection: Day 7
6	20.0		3 Injections: Days 7,9,11

eyes of all L-NIO-treated chicks were removed, hemisected and frozen in sterile saline. The eyes were then thawed and the retina and retinal pigmented epithelium (RPE) quickly removed into separate plastic test tubes containing 200 µl of sterile saline. Both retina and RPE were homogenised using a tissue grinder (Tissue Tearer, Biospec Products Inc, Oklahoma) and the sample tubes were sonicated for five minutes. Samples were then centrifuged at 5000 g for 15 minutes, and the liquid phase was transferred to new test tubes. Samples were then serially diluted, dot-blotted onto clean filter paper and allowed to dry. The paper was soaked in a Coomassie Blue solution (0.1% Coomassie Blue R-250, 40% ethanol, 10% acetic acid in distilled water) for five minutes and then destained (40% methanol, 10% acetic acid in distilled water) for one hour. The filter paper was then scanned into a computer and the central region (approximately 60% by diameter) of each sample dot was selected and its mean density recorded. These values were then substituted into an equation for a BSA standard curve (an appropriate logarithmic function) to determine the equivalent in milligrams of soluble protein contained in each sample.

100 µl of each sample was also added to 100 µl of Greiss Reagent (1% sulfanilamide, 0.1% naphthylethylene diamine, 2.5% H_3PO_4 in distilled water) and incubated at room temperature for ten minutes. Light absorbance at 550 nm was then read for each sample in an ELISA plate reader and NO_x content was quantified by comparison to a standard curve constructed by incubation of serial dilutions of $NaNO_2$ in Greiss Reagent. Resulting NO_x values were then divided by the amount of soluble protein in each sample to produce NO_x content values corrected for sample quantity. These values were used to represent the nitric oxide levels within the retina and RPE of the living chicken.

L-Arginine Experiments

L-arginine was tested for possible use as a competitive specificity control with the NOS-antagonists. Experiments were conducted only on FD chicks. The doses were 0 nmol (PBS alone), 340 nmol, 1.14 µmol, 3.43 µmol, 11.4 µmol and 34.3 µmol L-Arg in 20 µl of sterile PBS, yielding vitreal concentrations of 0, 1.7, 5.7, 17, 57 and 170 mM. Injections were administered every other day for three injections. Two days after the final injection, eyes were refracted, measured and weighed as above.

Sodium Nitroprusside Experiments

Experiments were conducted on ungoggled as well as monocularly occluded chicks. The doses tested were 0 nmol (saline alone), 10 nmol, 100 nmol and 1000 nmol per 20 µl injection, yielding vitreal concentrations of approximately 0 µM, 50 µM, 500 µM and 5 mM [respectively]. These injections were delivered at two day intervals for four injections. Two days after the final injection all eyes were refracted, measured and weighed. Eyes were selected at random from each dose group of the goggled chicks for sectioning and immunocytochemical labeling.

Slides were prepared for immunocytochemistry to detect DNA damage by nick end-labelling (TUNEL method) as follows. Once these slides were ringed with rubber cement, they were washed for ten minutes in PBS, then for ten minutes in PBS plus 0.3 % TX-100, and finally twice more in PBS. The slides were then incubated for one hour at 37°C in a solution containing: 0.25% dCTP-conjugated Cy3, 1.6% 3'terminal deoxynucleotidyl transferase, 20% 5X reaction buffer and 78.15% sterile H_2O (percent volume per final volume). All reagents were obtained from GibcoBRL. Slides were then washed three times in PBS and mounted in 4:1 glycerol : water solution, cover-slipped and sealed with nail-polish.

A separate experiment was also conducted to determine the effect of SNP injection on cGMP synthesis. The left eyes of the selected chicks were injected with either 0 nmol (saline alone), 4 nmol, 10 nmol, 40 nmol or 100 nmol of SNP. The injection vehicle also contained 170 nmol of IBMX, to inhibit phosphodiesterase activity and thereby enhance the accumulation of newly synthesized cGMP within the retina. Two hours later chicks were sacrificed and the eyes fixed as described above, but for 8 hours instead of 30 minutes, and then sectioned and labelled immunocytochemically with an antiserum to cGMP (Table 2.1).

Statistical Analysis and Experimental Replication

All experiments were conducted at least twice to ensure reproducibility. Micrographs presented in this study represent what was seen typically under the microscope. Data were analyzed using GraphPad's "Instat" 3.01 statistical software utilizing the Kruskal-Wallis method followed by Dunn's post-test. Statistical significance is identified on figures using asterisks.

Table 2.3. Master List of Dose Equivalents. This table shows the mole contents (in the injected medium) for all the drugs injected in this study, along with the equivalent molar concentrations (in the injected medium) and the approximate final concentration of drug in the vitreal chamber of the eye immediately after injection. For comparison, the drug doses used by Fujikado *et al.* (1997) are also re-calculated. For our studies, the volume of injected medium is 20 µl and the end vitreal volume is estimated to be 200 µl (180 + 20), while Fujikado's volume of injected medium was 30 µl and the end vitreal volume is estimated to be 210 µl (180 + 30).

Table 2.3

Description	Content of Injected Media	Concentration of Injected Media	Resulting in a vitreal concentration of:
Fujikado's L-NAME	1.8 µmol	**60 mM**	8.6 mM
	5.4 µmol	**180 mM**	25.6 mM
	10.8 µmol	**360 mM**	50.9 mM
	16.2 µmol	**540 mM**	76.1 mM
Our L-NAME	1.8 µmol	90 mM	9 mM
	3.0 µmol	150 mM	15 mM
	9.0 µmol	450 mM	45 mM
	16.2 µmol	810 mM	81 mM
	20.0 µmol	1 M	100 mM
L-NMMA	1.6 nmol	80 µM	8 µM
	16 nmol	800 µM	80 µM
	160 nmol	8.0 mM	800 µM
	1.6 µmol	80 mM	8000 µM
L-NIO	0 µmol	0 µM	0 µM
	33 nmol	1.67 mM	167 µM
	100 nmol	5 mM	500 µM
	300 nmol	15 mM	1500 µM
L-Arginine	0 nmol	0 mM	0 mM
	340 nmol	17 mM	1.7 mM
	1.14 µmol	57 mM	5.7 mM
	3.43 µmol	170 mM	17 mM
	11.4 µmol	570 mM	57 mM
	34.3 µmol	1.7 M	170 mM
SNP	0 nmol	0 µM	0 µM
	4 nmol	200 µM	20 µM
	10 nmol	500 µM	50 µM
	40 nmol	2 mM	200 µM
	100 nmol	5 mM	500 µM
	1000 nmol	50 mM	5 mM

Appendix to Techniques

Chemical Agents:

Sodium Nitroprusside: The exact mechanism leading to the liberation of nitric oxide from this compound is not fully understood. However, it is clear that the production of NO from SNP requires either light irradiation or chemical reduction, and does not seem to proceed spontaneously. Various biological reactions resulting in NO-release have been alluded to, including attack from thiolate anions (Feelisch 1998) and degradation by a membrane-bound enzyme requiring either NADH or NADPH as a cofactor (Bates *et al.* 1991, Kowaluk *et al.* 1992).

L-Arginine: This amino acid is the substrate of nitric oxide synthase. It was originally used in this study to act as a competitive blocker of NOS-inhibitor activities. However, it can also be used as a nitric oxide donor, by driving the equilibrium of the NOS reaction to the right. Exogenous L-arginine should have a potent effect since the endogenous synthesis of this amino acid is deficient in chickens (Wu *et al.*, 1995).

Inhibitors: L-NAME, L-NMMA and L-NIO all inhibit NOS activity by competing with L-arginine for access to the enzyme's catalytic site. For a further characterization of these inhibitors see Wellard *et al.* (1995). Wellard *et al.* show that both L-NMMA and L-NIO are more efficient NOS inhibitors than L-NAME in the intact chicken retina (as represented by much lower ID_{50} values), because L-NAME is transported poorly into cells.

Cryoprotection

Tissue samples are soaked in a 30% (w/v) sucrose solution for a period of twenty-four hours. During this period the sucrose solution infiltrates the tissue until all endogenous fluids contain sucrose. The sucrose alters the freezing properties of these fluids so that they vitrify instead of forming unyielding crystals which would tear through cell membranes and cause severe tissue damage.

Effects of Goggling

Placing a translucent goggle over the eye of a chick for a prolonged period of time, in a typical diurnal light cycle, results in FDM. Form-deprivation myopia in chicks is characterized by development of refractive error as well as enlarging of ocular parameters such as vitreal chamber length, axial length and wet weight of the eye. The

goggle is fixed to the animal by gluing the outer rim of the goggle directly to the feathers surrounding the chick's eye and held in place for at least 40 seconds to ensure adhesion. Goggles are placed over one eye only. Studies have shown that goggling both eyes of a chick leads to unanticipated results, such as developing hyperopia instead of myopia (Schaeffel & Howland 1991). Thus bilateral goggling would be a poor choice for this study and unilateral goggling is the method of choice.

Greiss Reaction to Quantify Nitrite/Nitrate Levels

This reaction is done at a pH of roughly 1.5. Under acidic conditions, any nitrite/nitrate ions present react with the sulphanilamide, producing N-(1-naphthyl)ethylenediamine dihydrochloride. This substance is yellow-green in colour and its concentration can be quantified by measuring the absorbance of the sample at 550 nm (Vogel 1996).

Immunocytochemical Theory

Antibodies used for immunocytochemistry are typically of the immunoglobulin G (IgG) class. These antibodies are composed of dimerized chains, which themselves contain constant and variable regions. The surface structure of the variable regions complements the surface structure of the antigen used to induce them. This matching of molecular shape and charge allows the antibodies to bind to their specific antigen. This first process of immunolabelling produces a situation where the location of the antigen of interest is labeled by antibodies, all with a common constant region. The tissue can then be labeled with a second antibody that recognizes this constant region. Typically conjugated to this second antibody is a fluorophor of some sort (e.g. Cy3), that emits light of a specific wavelength when illuminated with light of a different specific wavelength. This method of indirect fluorophor labelling allows for improved antigen identification as it presents a larger region for binding of fluorophor-conjugated antibodies (the common region of the primary antibody) and reduces the binding hindrance associated with such large molecules (the primary antibody is smaller than the fluorophor-conjugated secondary anibody and can gain closer access to the tissue).

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Certain points of this protocol must be noted to achieve a proper appreciation of this method.

1. Antibodies are large molecules and do not penetrate tissue samples well. For this reason, the tissue must be treated with a non-ionic detergent like Tx-100. This detergent removes lipids from the cell membranes and provides for better penetration by the antibodies.
2. The antibodies are not the only "sticky" molecules within this experimental protocol. The tissue sample itself has molecules that will readily bind to solvated proteins, including antibodies. To prevent non-specific "reverse" labeling of this sort, the tissue is pre-treated with an inert protein wash (typically bovine serum albumin: BSA) that blocks these endogenous (non-specific, low-affinity) binding sites.

Paraformaldehyde Tissue Fixation

Formaldehyde reacts with the unprotonated N-terminal amino groups as well as a number of other amino acid side groups including epsilon-amino groups of lysine residues, aromatic rings, guanidine groups and amides (Larsson 1988). This reaction typically involves the formation of methylene cross-links, resulting in the characteristic fixing and hardening of the tissue. Paraformaldehyde solutions produce a weaker fixation than formaldehyde via similar chemical reactions. This proves to have both advantages and disadvantages. Weaker fixation leads to better conservation of antigenicity within the fixed tissue, yet leads to poorer preservation of tissue structure. The fixation process is executed at near neutral pH, similar to that found in living tissue. This produces an environment where the majority of N-terminal amino groups are protonated, excluding them from fixation reactions. However, fixation at high pH hinders penetration of the fixative (Eldred *et al.* 1983) and raises important issues of protein denaturation at a pH far from that of the cytoplasmic/extracellular environment.

Refraction Technique

This is accomplished with the aid of a set of optometry test lenses, a lens-holder apparatus and a streak retinoscope. The chicken is held in the left hand and positioned so that the eye being examined is directly behind the test lens of choice being held by the lens holder. The chick is held at arm's length and the retinoscope is brought up to the examiner's eye so that the examiner can observe the reflex from the chick's eye while

peering through the viewport of the retinoscope. The focused beam of light radiated by the retinoscope is slowly streaked over the surface of the chick's eye, from right to left, and the light reflected back from the retina is noted. Light that passes in an opposing direction to the streak (left to right) indicates a negative refractive error within the eye, the rate by which the reflex streaks across the retina indicates the magnitude of the refractive error. Hyperopic refractive error is indicated by a reflex that moves in that same direction as the streak but at a faster rate. A reflex that passes with the streak at the same rate as the streak represents emmetropia. The cumulative dioptic power of the test lenses is altered until this state of emmetropia is mimicked. This cumulative dioptic power describes the refractive error of the eye.

The characteristics of the reflected streak are determined by the refractive strength of the cornea and lens and their distance from the RPE. Should the RPE lie at the combined focal length of the lens and cornea (emmetropia), then light reflected off the RPE leaves the eye along a parallel path. This light does not converge before entering the observers' eye and thus the image of it is not flipped. The result is a reflex that moves with the streak. However, should the RPE lie outside the lens/corneal focal length (myopia) then, upon exiting the eye, the light is refracted strongly enough that it converges before it enters the observer's eye. The image is inverted and the reflex appears to move in a direction that opposes the streak.

TUNEL

Terminal deoxynucleotidyl transferase (TdT)-mediated UTP nick-end labelling (TUNEL) was used to visualize cells that are presumed to be undergoing cell death. This method uses TdT in an altered buffer which allows it to transfer nucleotides to the 3'-end of DNA, be it single-stranded (uneven break) or double stranded. With the addition of an excess of deoxyuridine triphosphate, TdT forms poly-deoxyuridine tails onto the 3' end of the DNA. These poly-deoxyuridine tails anneal to poly-deoxycytidine, which is conjugated to a Cy3 fluorophore. This leads to the fluorescence of all free DNA terminals present in a labelled cell. This has two important outcomes. First, all cells will be labelled to some extent, resulting in some background fluorescence. Second, all cells undergoing DNA fragmentation, likely as a result of programmed cell death, will have a much higher number of DNA 3' terminals, resulting in a much greater fluorescence.

CHAPTER THREE

Experimental Results For Treating Form-Deprived And Normal Eyes With NOS Inhibitors

Introduction

Previous Findings Concerning The Effect Nitric Oxide Has On FDM

Fujikado *et al.* (1997) reported that L-NAME blocks form-deprivation myopia in goggled chicks. L-NAME elicited this effect at doses ranging from 27 mM (vitreal concentration) to the highest dose they tested, 81 mM. This suppression of FDM manifested itself as significant reductions in both refractive error and axial length of the L-NAME-treated goggled eyes compared to saline-injected goggled control group. In contrast, L-NAME injections into unoccluded eyes had no effect on refractive error. Peculiarly, both groups of unoccluded eyes had developed apparently significant hyperopic refractive error measured six days after single injection (twelve days old) though this is not mentioned. To verify that L-NAME was indeed acting to inhibit NOS activity, Fujikado and co-workers measured the content of nitric acid metabolites (nitrites and nitrates, collectively referred to as NO_x) within the retina. They found that the level of NO_x in deprived eyes treated with L-NAME was significantly lower than that in deprived eyes treated with saline. The authors concluded from these results that L-NAME suppressed NO-dependent processes that caused the excessive growth of FDM without affecting normal ocular growth pathways. However, it was also shown that NO_x levels were not different in deprived and unoccluded eyes when measured after six days of goggling. It stands to reason that if nitric oxide suppression leads to rescue from the effects of form-deprivation, then myopic ocular development should be accompanied by an increase in production of nitric oxide, and its metabolites. The authors suggested that this discrepancy in results may be due to the inability of the NOx assay to differentiate between the activities of different NOS isoforms in various cell types. The activity of one isoform in one cell type might prove essential to FDM. Blanket NOS suppression within the retina would hinder the ocular growth process that depends on this NOS activity, while changes in the activity of this NOS might be obscured or counteracted by the greater or opposite activities of other NOS isoforms or other NOS-containing cells. However, this explanation is difficult to accept as Fischer & Stell (1999) identified a

limited number of sites where NOS may be active in the retina and Fujii *et al.* (1998) showed protein content for the three NOS isoforms to be similar in magnitude within the retina. It is likely that changes in retinal NO levels significant enough to alter ocular growth should manifest themselves as noticeable net changes in retinal NOx content as well.

Why should these results be questioned?

An alternative explanation, however, could be that NO does not play a role in FDM. The dose of L-NAME claimed by Fujikado *et al.* (1997) to suppress FDM (generating a vitreal concentration about 27 mM shortly after injection) is consistent with the IC_{50} (= 19 mM) reported for inhibition of NOS activity in isolated chicken retina (Wellard *et al.* 1995). At such a high dose, however, L-NAME may not be specific for NOS. For example, Buxton *et al.* (1993) have shown that at doses of 100 μM L-NAME can also act as an antagonist at mammalian (canine) muscarinic receptors. Consideration of the real possibility of L-NAME acting at non-specific sites and the apparent lack of NOS upregulation in association with myopia warrants a re-examination of the role of nitric oxide in modulating FDM and excessive ocular growth.

Purpose/Hypothesis

This set of experiments is designed to test whether inhibition of nitric oxide synthase, and ultimately a decrease in nitric oxide availability, has any effect on the development of myopia or normal ocular growth. It is postulated that inhibition of NOS does not affect such forms of ocular growth. The introduction of NOS inhibitors into the vitreous and retina will not affect ocular growth through specific interaction with NOS.

Choice of Agents

L-NAME was used in these experiments in order to draw direct comparisons with those experiments conducted by Fujikado *et al.* (1997). L-NMMA is utilized for its greater efficacy as a NOS-inhibitor in intact chick retina as compared to L-NAME (Wellard *et al.* 1995). L-NIO is used both for its improved efficacy in intact retina

(Wellard *et al.* 1995) and for its apparent lack of affinity for muscarinic receptors (Buxton *et al.* 1992).

Methods

The methods used in this set of experiments are detailed in chapter two.

Acknowledgements

The experiments involving the use of L-NAME and L-NMMA were conducted by my supervisor, Dr.W.K.Stell. The results from these experiments are reported here to complete the account of how nitric oxide affects the development of FDM.

Results

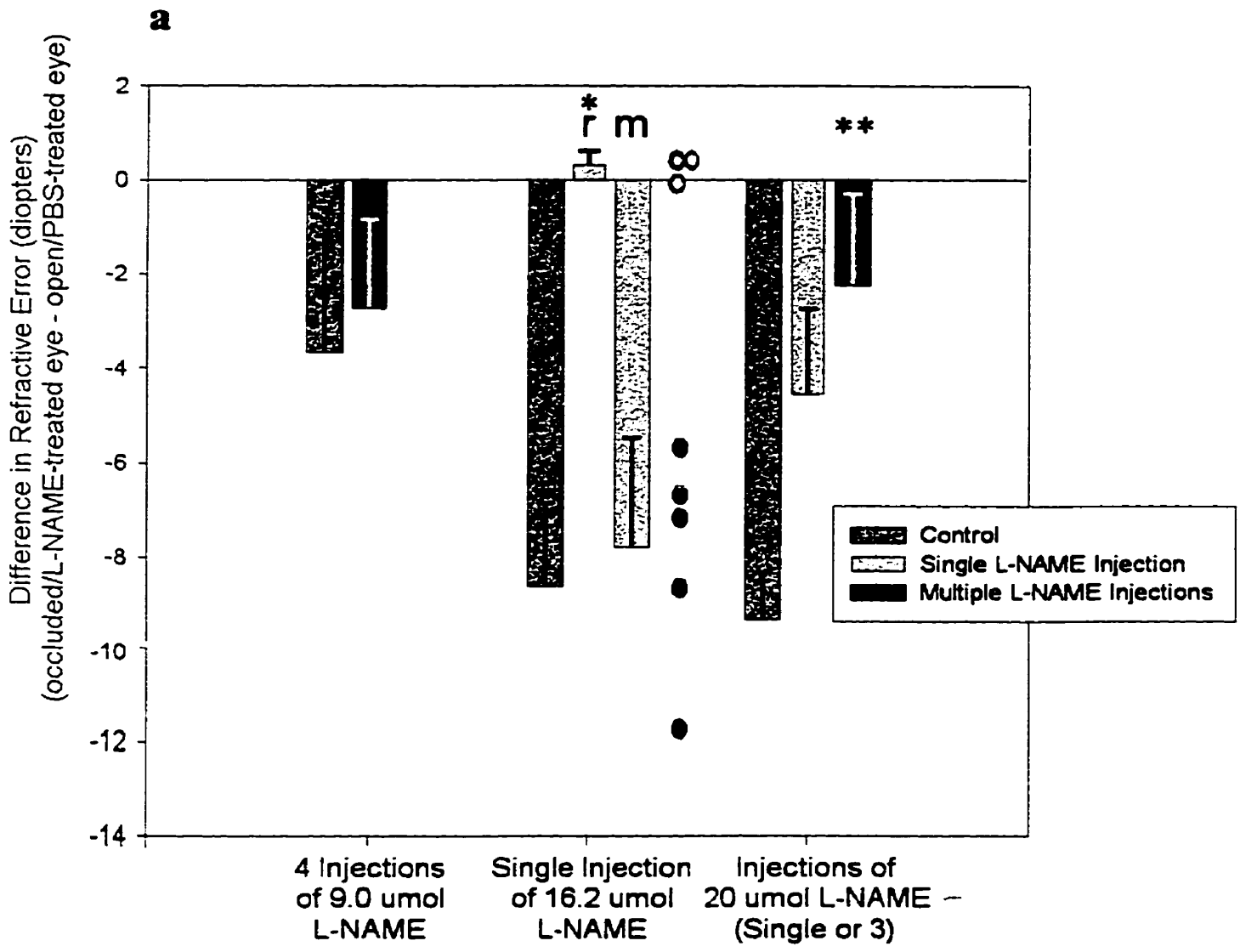
L-NAME treatment

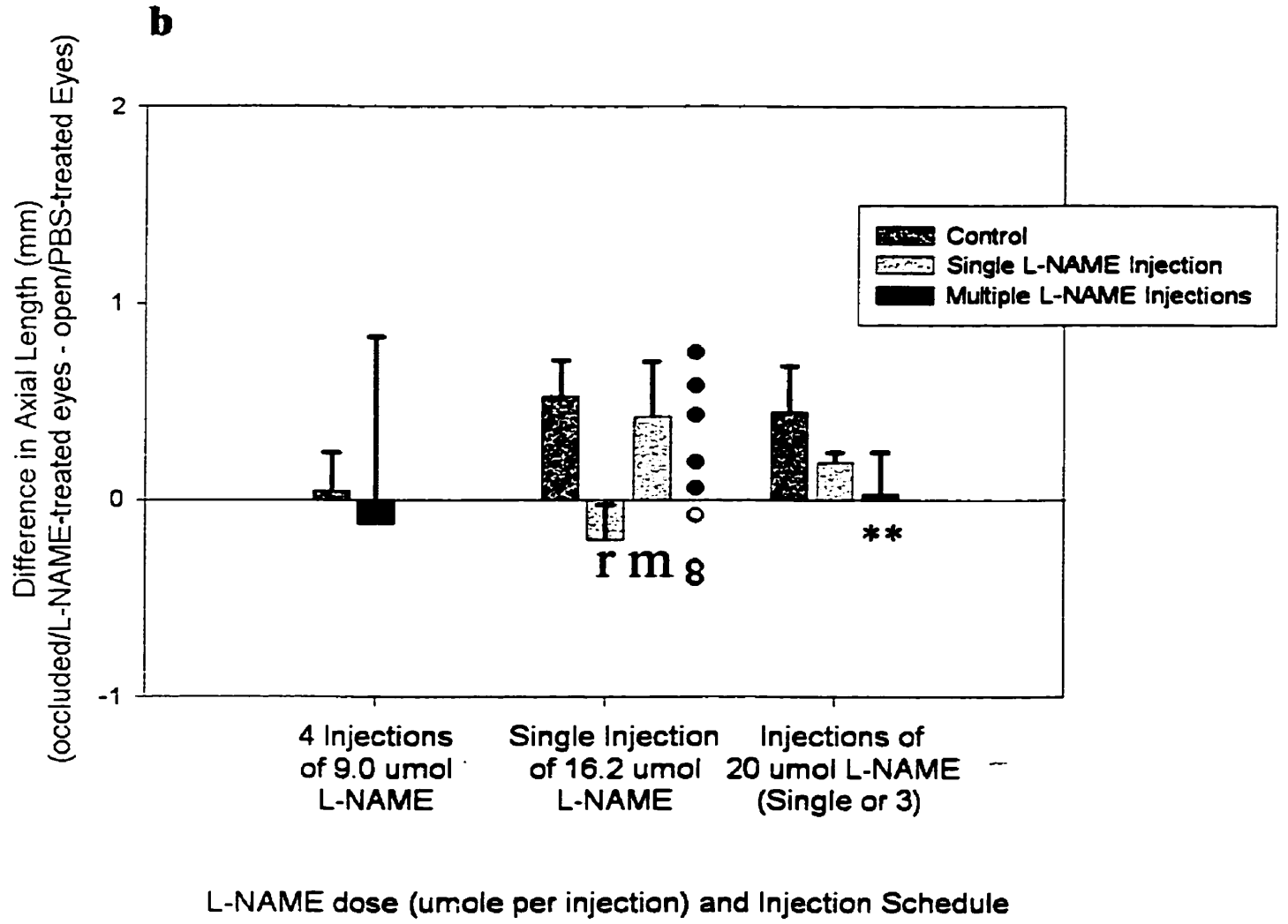
Our experiments using intravitreal injection of L-NAME did not replicate the effects reported by Fujikado *et al.* (1997) (Figure 3.1). Single injections of 1.8 (data not shown), 16.2 and 20 μmoles, and multiple injections of 3.0 μmol (data not shown) and 9.0 μmol L-NAME produced no consistently significant effect on goggle-induced myopic development as measured by refractive error, axial length or globe wet weight. Myopic development and excessive ocular growth were suppressed only when eyes were treated every other day for a total of three injections, each injection containing 20 μmol L-NAME per injection (producing approximately 100 millimolar vitreal concentration of L-NAME). These form-deprived eyes demonstrated considerable reductions in axial length and wet weight, to the extent that they proved to be not significantly different from their contralateral non-goggled, saline-injected control eyes. However, the mean difference in refractive error between goggled/L-NAME-treated eyes and their ungoggled/saline control eyes was -2.25 (±1.94 SD) diopters, significantly more myopic than the control eyes, yet still greatly reduced when compared to saline-injected goggled eyes. Dr. Andy Fischer (personal communication), used TUNEL labelling to show that no DNA fragmentation was present in chick eyes injected with any of these doses of L-NAME, suggesting that this reduction in myopia is not due to cellular damage or toxicity.

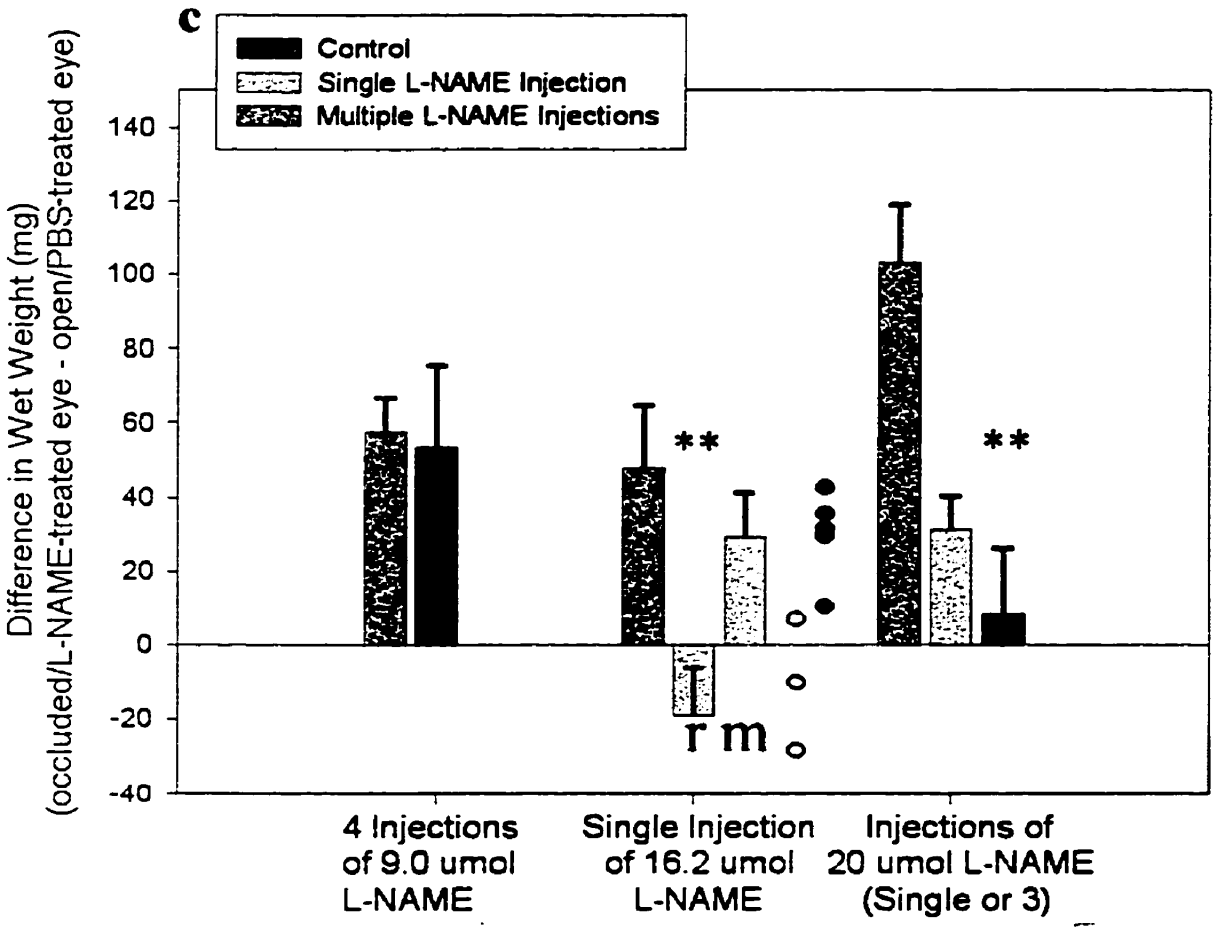
L-NMMA treatment

Assumed inhibition of NOS activity within the retina by the administration of L-NMMA also demonstrated no effect on the typical development of form-deprivation

Figure 3.1 Combined data showing the effects of intra-vitreal injections of L-NAME on myopic ocular development in goggled chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). Each experiment is grouped with its own control group. Treatment with 9.0 µmol L-NAME caused no change in the development of myopia. Eyes treated with 16.2 µmol L-NAME proved to be a bimodal population, with three chicks showing rescue from FDM (column labelled r), while the other five chicks were not affected and continued to develop myopia (column labelled m). In the case of doses of 20.0 µmol L-NAME, single injections proved unable to affect FDM, while multiple injections significantly reduced myopic development. The 16.2 µmol L-NAME dose used here is equivalent to the highest dose (540 mM X 30 µl) used by Fujikado *et al.* (1997), and the 9.0 µmol dose is equivalent to 167% drug content of the lowest effective dose (180 mM X 30 µl) reported by those authors. Depicted beside the graph bars for the 16.2 µmol treatment group are the individual data points (solid dots for the unaffected eyes, "m"; hollow dots for the rescued eyes, "r"). The two sub-populations within this group proved to be significantly different from the other in each of the three measured parameters (unpaired t-test: refractive error, $P<0.001$; axial length, $P<0.01$; wet weight, $P<0.01$). Doses represent absolute molar quantities injected per treatment. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. N = 6 (9.0 µmol L-NAME and 20.0 µmol L-NAME) or n = 8, 3, 5 (16.2 µmol L-NAME, control, rescued, myopic respectively), error bars represent standard deviation.


a
Difference in Refractive Error (diopters)
(occluded/L-NAME-treated eye - open/PBS-treated eye)
2
0
-2
-4
-6
-8
-10
-12
-14
*
**
Control
Single L-NAME Injection
Multiple L-NAME Injections
4 Injections of 9.0 umol L-NAME
Single Injection of 16.2 umol L-NAME
Injections of 20 umol L-NAME – (Single or 3)
L-NAME dose (umole per injection) and Injection Schedule


b
Difference in Axial Length (mm)
(occluded/L-NAME-treated eyes - open/PBS-treated Eyes)
2
1
0
-1
Control
Single L-NAME Injection
Multiple L-NAME Injections
r m 8
**
4 Injections of 9.0 umol L-NAME
Single Injection of 16.2 umol L-NAME
Injections of 20 umol L-NAME (Single or 3)
L-NAME dose (umole per injection) and Injection Schedule


c
Control
Single L-NAME Injection
Multiple L-NAME Injections
Difference in Wet Weight (mg)
(occluded/L-NAME-treated eye - open/PBS-treated eye)
140
120
100
80
60
40
20
0
-20
-40
**
**
r m
4 Injections of 9.0 umol L-NAME
Single Injection of 16.2 umol L-NAME
Injections of 20 umol L-NAME (Single or 3)
L-NAME dose (umole per injection) and Injection Schedule

myopia. At all doses tested (1.6 nmol, 16 nmol, 160 nmol and 1.6 μmol L-NMMA per 20 μl of PBS vehicle injected), resulting in initial vitreal concentrations of approximately 8μM, 80μM, 800μM and 8 mM, L-NMMA had no significant effect on the refractive error, axial length or wet weight (Figure 3.2) of goggled eyes as compared to the typical development of similarly goggled chicks. Although a zero-dose control was not included in this preliminary experiment, it should be noted that we have never failed to induce myopic enlargement and refractive error in goggled, saline-injected chicks.

L-NIO treatment

The influence of L-NIO on ocular development was tested on both ungoggled and monocularly goggled chicks. Both sets of experiments were replicated once to confirm the reproducibility of the results.

On both occasions tested, L-NIO had no effect on undeprived eyes (Figures 3.3.1 & 3.3.2). Even at the highest concentration (three times the reported IC_{50} for L-NIO suppression of NOS in intact retina) the axial length and wet weight of the treated eyes did not differ significantly from those of control eyes injected with saline alone. The refractive error of these eyes proved not to be different from that in emmetropia.

When form-deprived chicks were treated with L-NIO, the occluded eyes developed in typical myopic fashion while the control eyes remained emmetropic. This experiment was performed twice, each trial demonstrating similar results (Figures 3.4.1 & 3.4.2). Despite L-NIO treatment the deprived eyes developed considerable myopic refractive error and their axial length and wet weight increased. There was no indication of difference between the drug-treated eyes and saline-injected goggled eyes.

Determination Of NO_x Levels In Ocular Tissues

On both the occasions that this experiment was performed, NO_x concentrations in the retinas of the eyes treated with the highest dose of L-NIO were significantly less than in the control eyes of the same chicks (Figures 3.5.1 & 3.5.2). Table 3.1 shows that NO_x levels were reduced maximally by 69% in the retinas of form-deprived eyes treated with the highest does of L-NIO as compared to those of saline-treated form-deprived eyes (data from trial two). The RPE of L-NIO-treated goggled eyes showed an optimal 90%

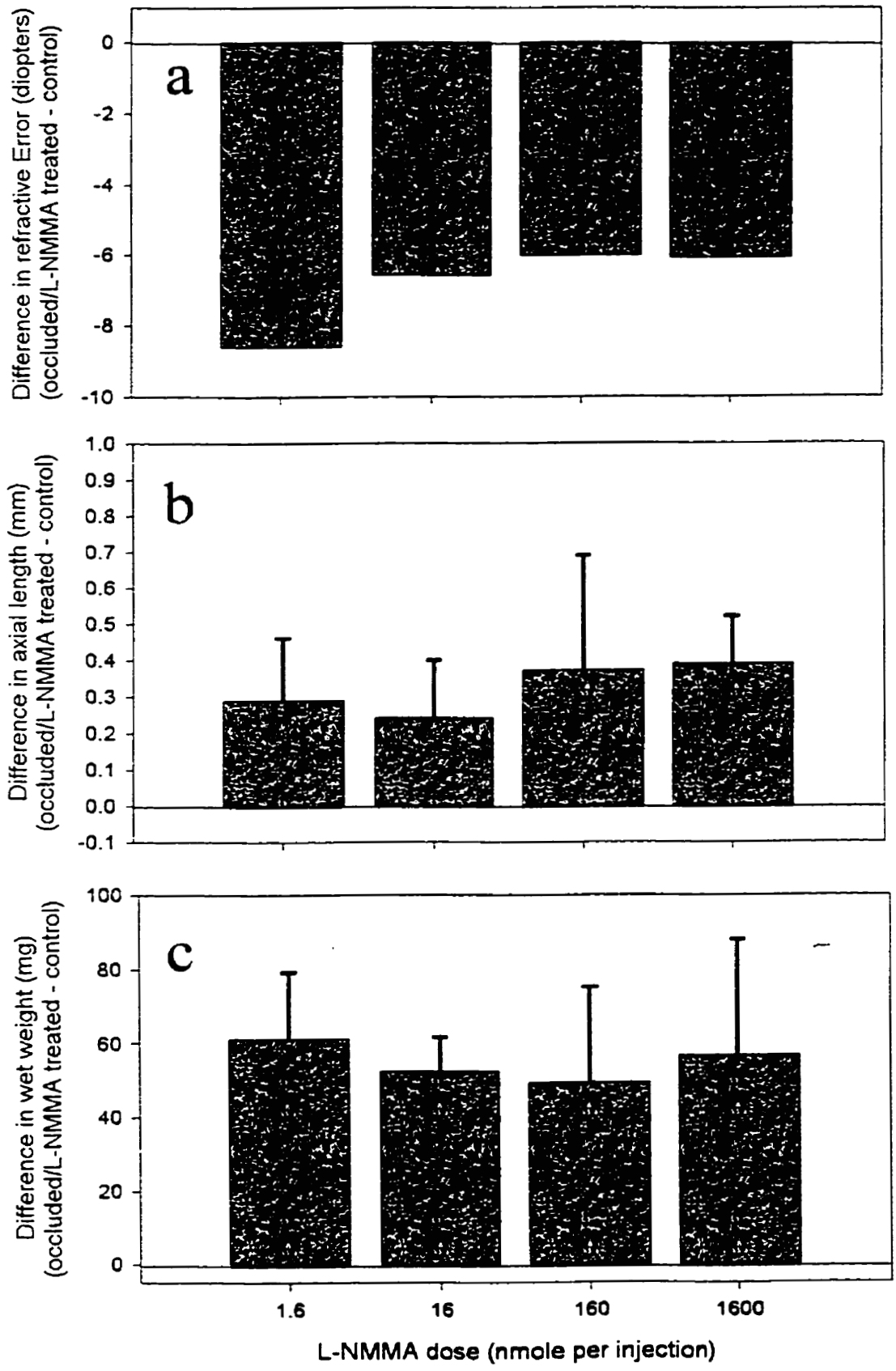

a
Difference in refractive Error (diopters)
(occluded/L-NMMA treated - control)
0
-2
-4
-6
-8
-10
b
Difference in axial length (mm)
(occluded/L-NMMA treated - control)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
c
Difference in wet weight (mg)
(occluded/L-NMMA treated - control)
100
80
60
40
20
0
1.6
16
160
1600
L-NMMA dose (nmole per injection)

Figure 3.3.1. Trial One showing the effect of intra-vitreal injections of L-NIO on growth of open chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). No significant effect on FDM was observed. Doses represent drug content within injected medium. n = 6, error bars represent standard deviation.

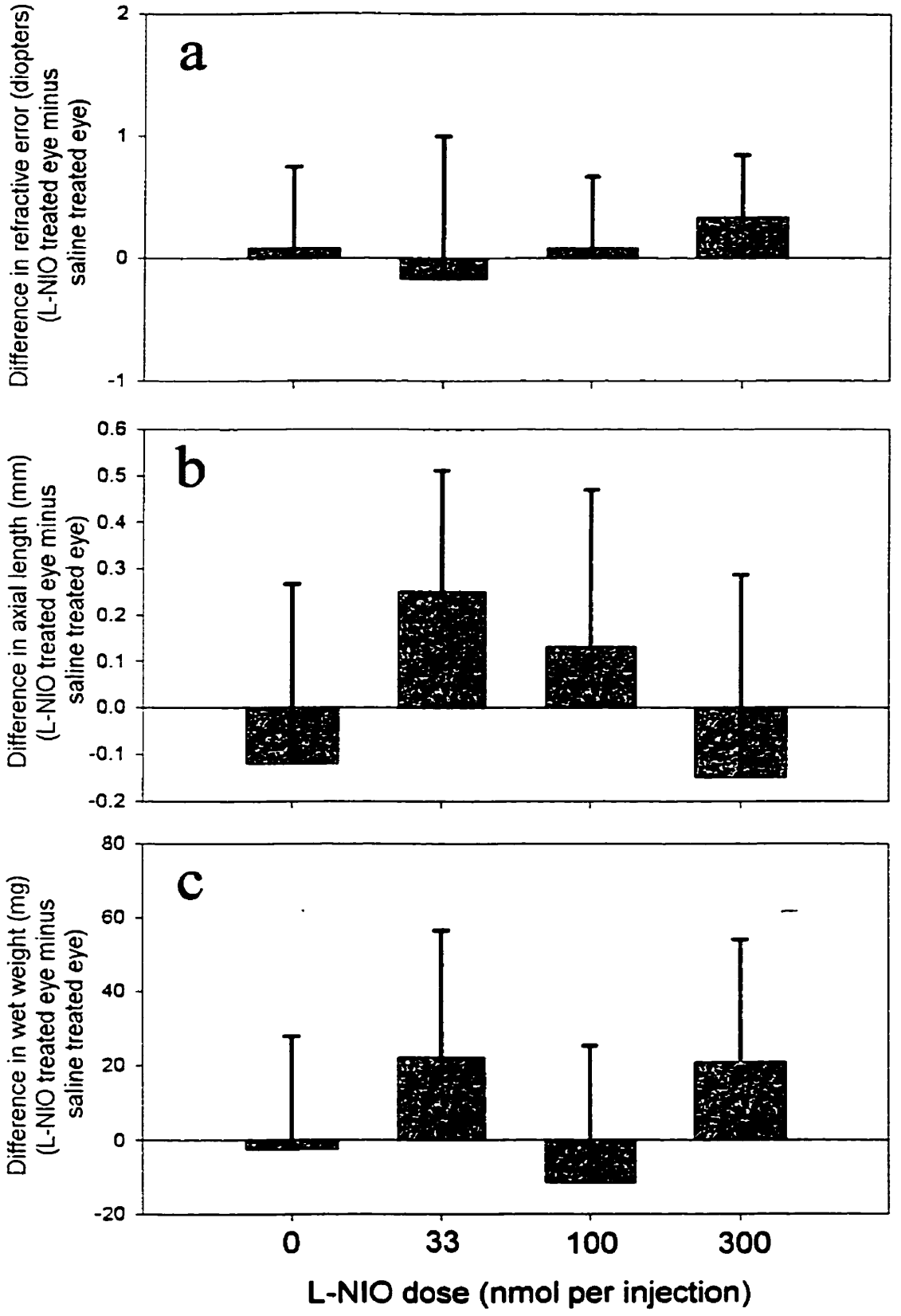

a
b
c
Difference in refractive error (diopters)
(L-NIO treated eye minus
saline treated eye)
Difference in axial length (mm)
(L-NIO treated eye minus
saline treated eye)
Difference in wet weight (mg)
(L-NIO treated eye minus
saline treated eye)
2
1
0
-1
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
80
60
40
20
0
-20
0
33
100
300
L-NIO dose (nmol per injection)

Figure 3.3.2. Trial Two showing the effect of intra-vitreal injections of L-NIO on growth of open chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). No significant effect on FDM was observed Doses represent drug content within injected medium. n = 6, error bars represent standard deviation.

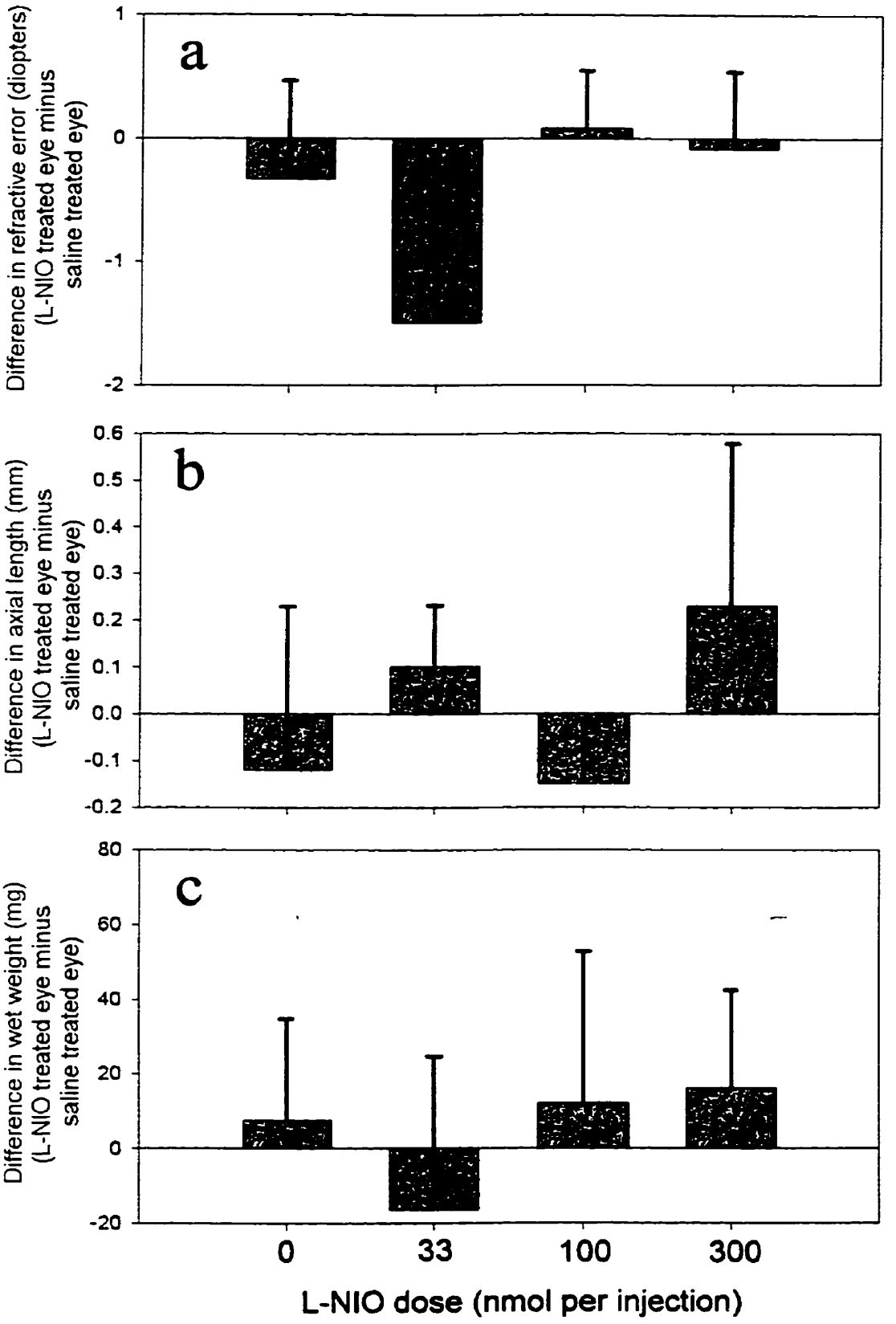

a
b
c
Difference in refractive error (diopters)
(L-NIO treated eye minus
saline treated eye)
Difference in axial length (mm)
(L-NIO treated eye minus
saline treated eye)
Difference in wet weight (mg)
(L-NIO treated eye minus
saline treated eye)
1
0
-1
-2
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
80
60
40
20
0
-20
0
33
100
300
L-NIO dose (nmol per injection)

Figure 3.4.1. Trial One showing the effect of intra-vitreal injections of L-NIO on the development of FDM in goggled chicks (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). No mean values were shown to deviate significantly from the typical development of FDM Doses represent drug content within injected medium. n = 6, error bars represent standard deviation.

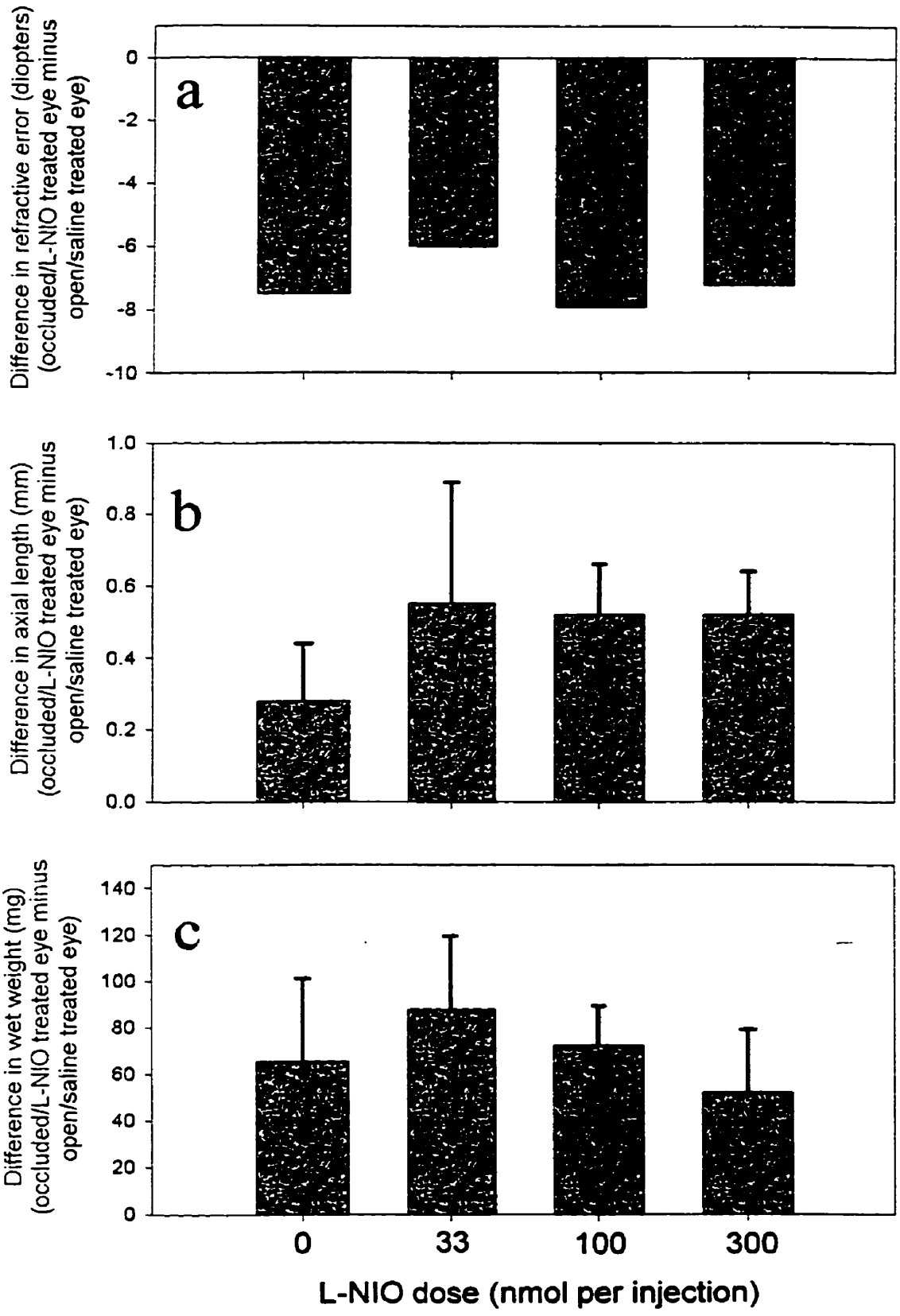

a
Difference in refractive error (diopters)
(occluded/L-NIO treated eye minus
open/saline treated eye)
0
-2
-4
-6
-8
-10
b
Difference in axial length (mm)
(occluded/L-NIO treated eye minus
open/saline treated eye)
1.0
0.8
0.6
0.4
0.2
0.0
c
Difference in wet weight (mg)
(occluded/L-NIO treated eye minus
open/saline treated eye)
140
120
100
80
60
40
20
0
0
33
100
300
L-NIO dose (nmol per injection)

Figure 3.4.2. Trial Two showing the effect of intra-vitreal injections of L-NIO on the development of FDM in goggled chicks (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). No mean values were shown to deviate significantly from the typical development of FDM. Doses represent drug content within injected medium. n = 6, error bars represent standard deviation.

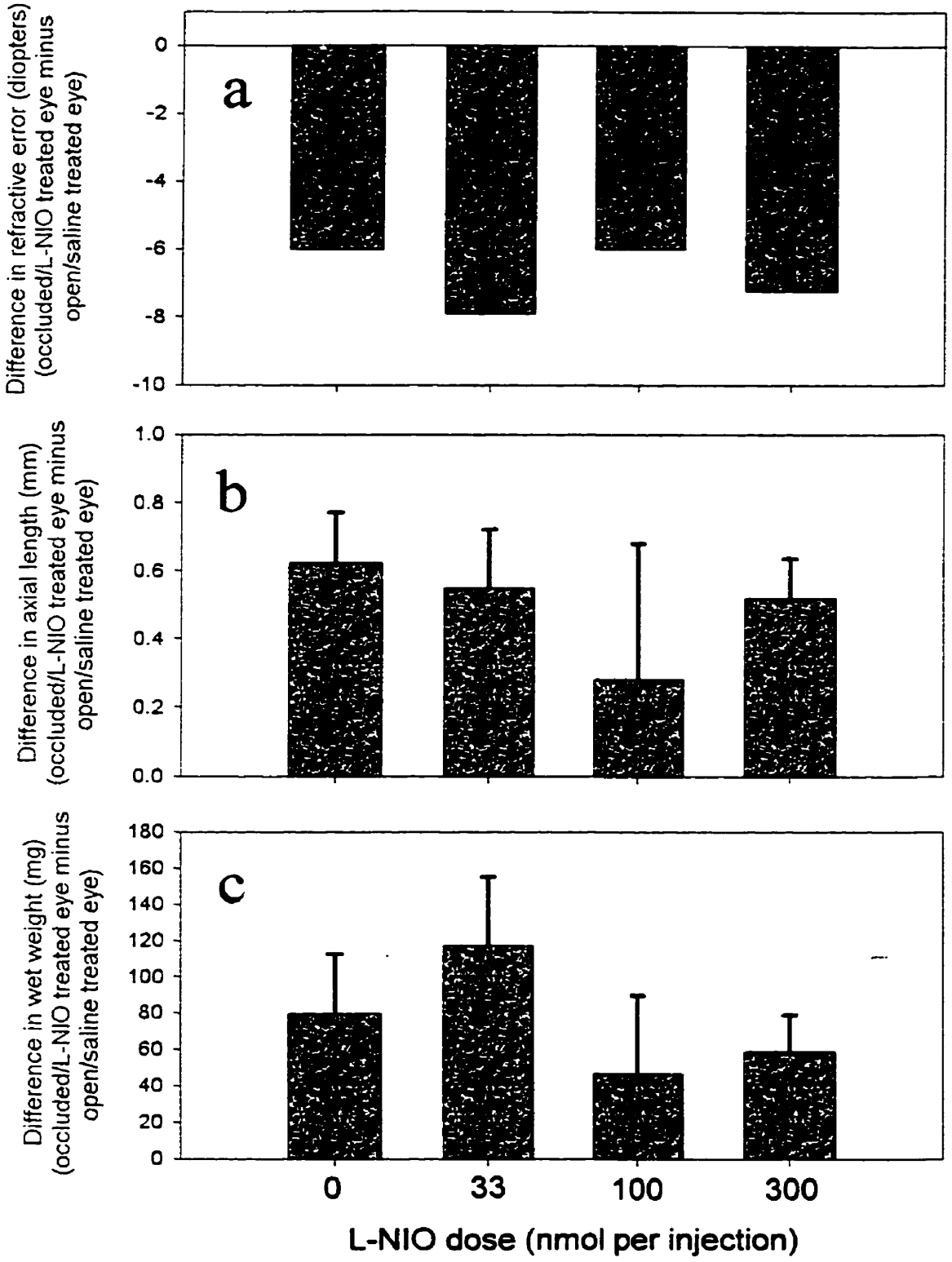

Difference in refractive error (diopters)
(occluded/L-NIO treated eye minus
open/saline treated eye)
a
0
-2
-4
-6
-8
-10
Difference in axial length (mm)
(occluded/L-NIO treated eye minus
open/saline treated eye)
b
1.0
0.8
0.6
0.4
0.2
0.0
Difference in wet weight (mg)
(occluded/L-NIO treated eye minus
open/saline treated eye)
c
180
160
140
120
100
80
60
40
20
0
0
33
100
300
L-NIO dose (nmol per injection)

Figure 3.5.1. Trial One showing the effect of intra-vitreal L-NIO injections on nitrite/nitrate levels in the retina and RPE of goggled chicks. The largest dose of L-NIO proved to reduce NOx levels significantly in both RPE and retina. Doses represent drug content within injected medium. ** $P<0.01$. $n = 6$, error bars represent standard deviation.

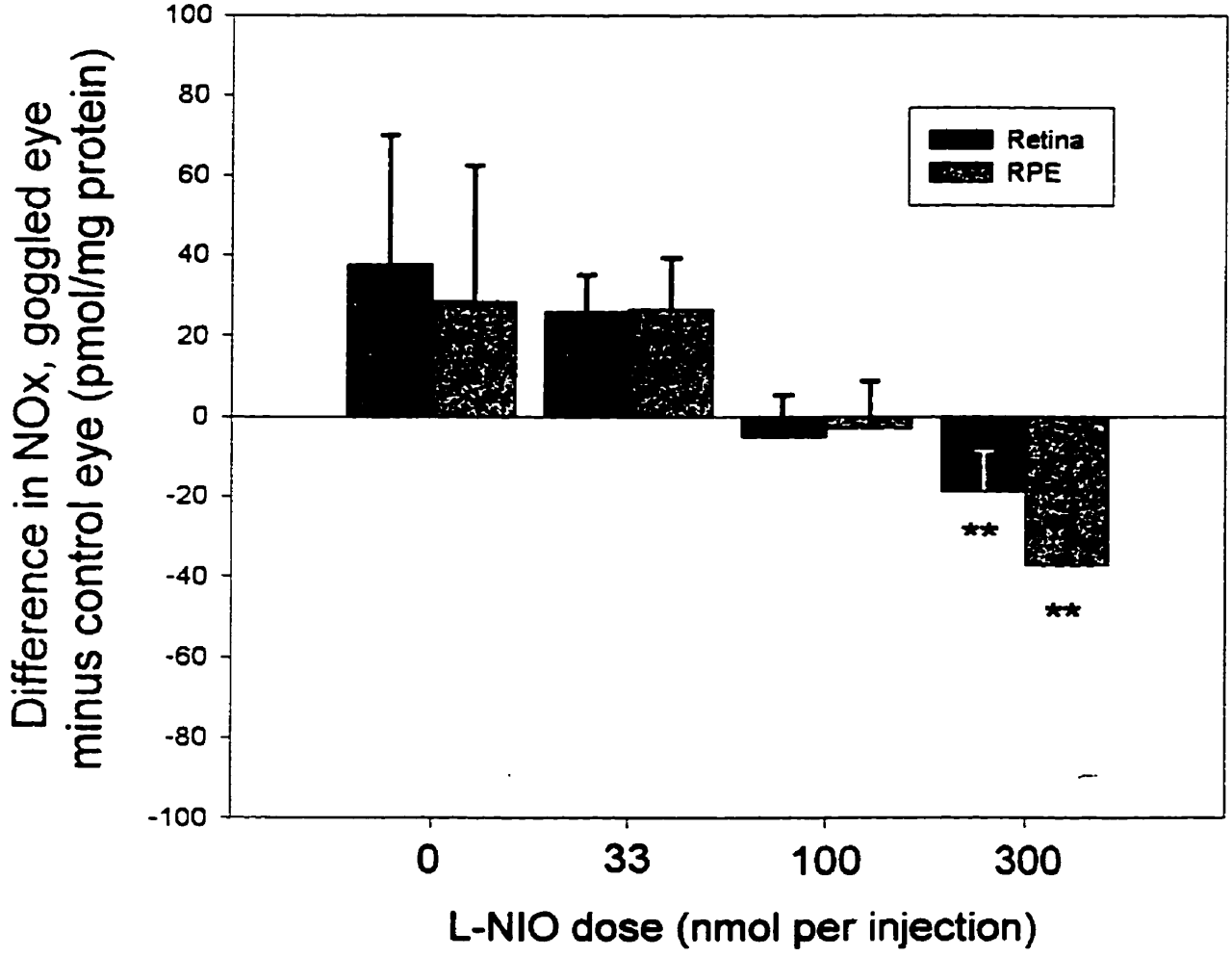

Difference in NOx, goggled eye minus control eye (pmol/mg protein)
100
80
60
40
20
0
-20
-40
-60
-80
-100
Retina
RPE
**
**
0
33
100
300
L-NIO dose (nmol per injection)

Figure 3.5.2. Trial Two showing the effect of intra-vitreal L-NIO injections on nitrite/nitrate levels in the retina and RPE of goggled chicks. The two largest dose of L-NIO proved to reduce NOx levels significantly in both RPE and retina. Doses represent drug content within injected medium. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. $n = 6$, error bars represent standard deviation.

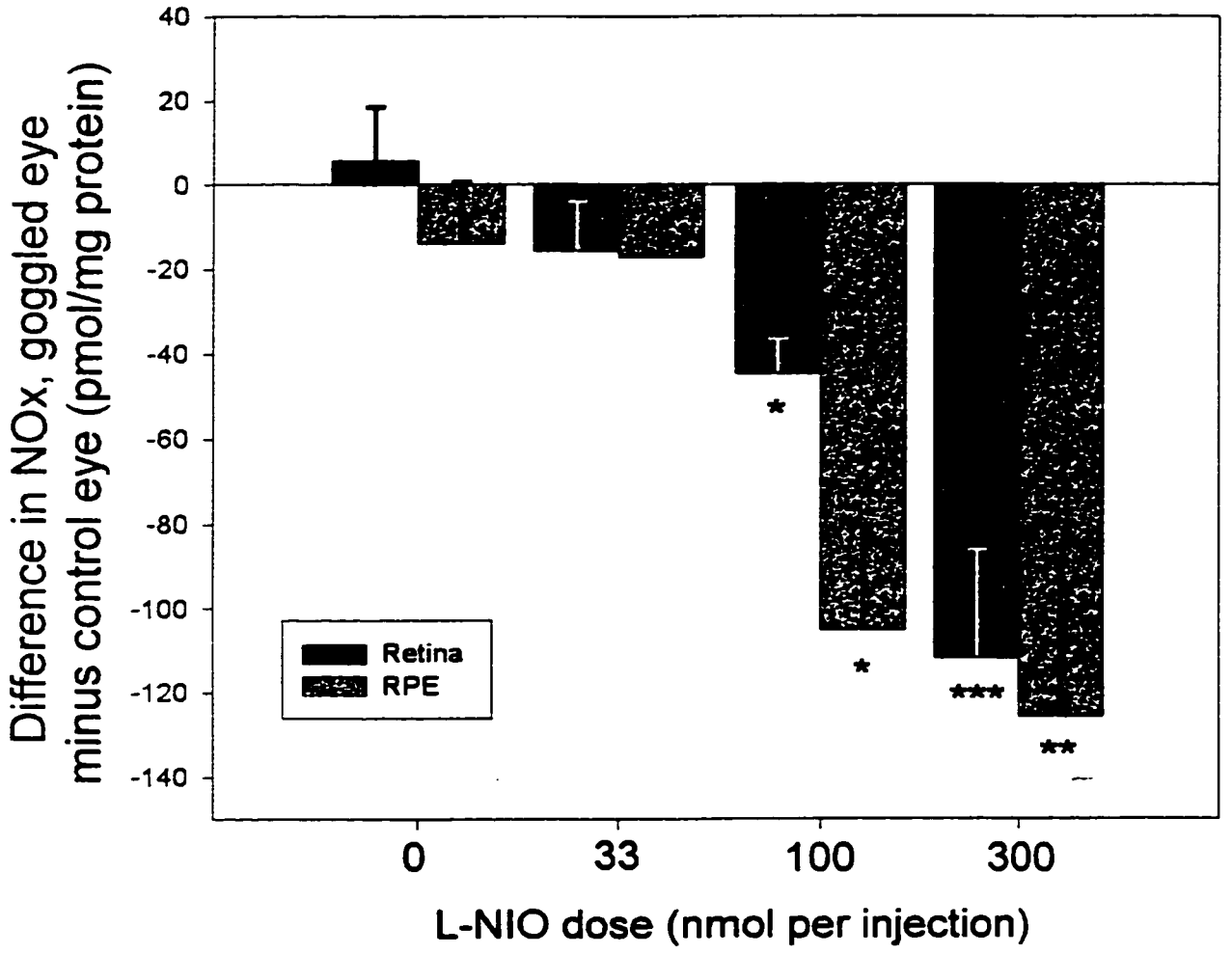

Difference in NOx, goggled eye minus control eye (pmol/mg protein)
40
20
0
-20
-40
-60
-80
-100
-120
-140
Retina
RPE
*
*
***
**
0
33
100
300
L-NIO dose (nmol per injection)

Table 3.1 Mean NOx levels in the retina and RPE of goggled chick eyes injected with L-NIO, from data obtained in trial two (Figure 3.5.2, n = 6). NOx levels in the contralateral open, saline-injected eyes were subtracted from the NOx level in the goggled eyes injected with various doses of L-NIO. Percent reduction was calculated for the averaged data [(control eyes – treated eyes)/control eyes)].

Table 3.1

Group	Treated Group (pmol/mg, mean)	Control Group (pmol/mg, mean)	% Reduction
Retina			
0 µM L-NIO	136.5	130.7	-4.5
167 µM L-NIO	128.1	143.5	10.7
500 µM L-NIO	122.5	167.2	26.7
1500 µM L-NIO	50.6	162.4	68.8
RPE			
0 µM L-NIO	124.1	137.7	9.9
167 µM L-NIO	115.8	132.9	12.8
500 µM L-NIO	27.7	132.6	79.1
1500 µM L-NIO	13.9	139.7	90.0

reduction in NO_x levels compared to those of control eyes (data from trial two). On the second trial of this experiment (Figure 3.5.2), eyes treated with 100 nmol L-NIO also demonstrated significantly reduced NO_x in both retina and RPE. This was not true for the first trial. Such significant declines in NO_x levels within the retina and RPE give fair indication of a suppression of NOS activity by L-NIO. Based on the results of trial two, L-NIO inhibited NOS activity with an ED_{50} of 650 μM in retina and 350 μM in RPE. These values are in reasonably close agreement with those calculated by Wellard *et al.* (1995) of 501 μM in chick retina.

The results of the first trial of this experiment (Figure 3.5.1) may be unreliable because the BSA protein had been previously prepared and frozen, then thawed for use. Some of the protein in these standards may have left solution and been incapable of being re-dissolved, leaving the standards with lower protein concentrations than reported. This would lead to greater NO_x values in samples that contained protein levels near those of the greatest affected standards. When the second trial was being prepared it was realized that the original protein standards might not be as accurate as necessary, due to protein precipitation. To avoid experiencing this problem again, protein standards were freshly prepared and used for sample calibrations in trial two. For this reason, trial two represents the superior set of data.

These results demonstrate that the ineffectiveness of L-NIO against FDM was not due to failure to suppress NOS activity. It is likely that most, if not all, NO-dependent signal pathways and mechanisms were greatly suppressed by L-NIO-induced decline in nitric oxide synthesis. The fact that myopic ocular development continued, unhindered by the drug-treatment, strongly suggests that nitric oxide and NOS do not play an essential role in development of FDM.

nitric oxide, allowing the molecule to reach the retina before releasing substantial amounts of NO *in situ* before being degraded to NO_x. Furthermore, SNP was selected for the constancy of its donation product, almost exclusively producing nitric oxide in its uncharged state. Immunocytochemical agents were chosen on the basis of previous studies or in order to evaluate the levels of damage incurred by the ocular tissues.

Methods

The methods used in this set of experiments are detailed in chapter two.

Acknowledgements

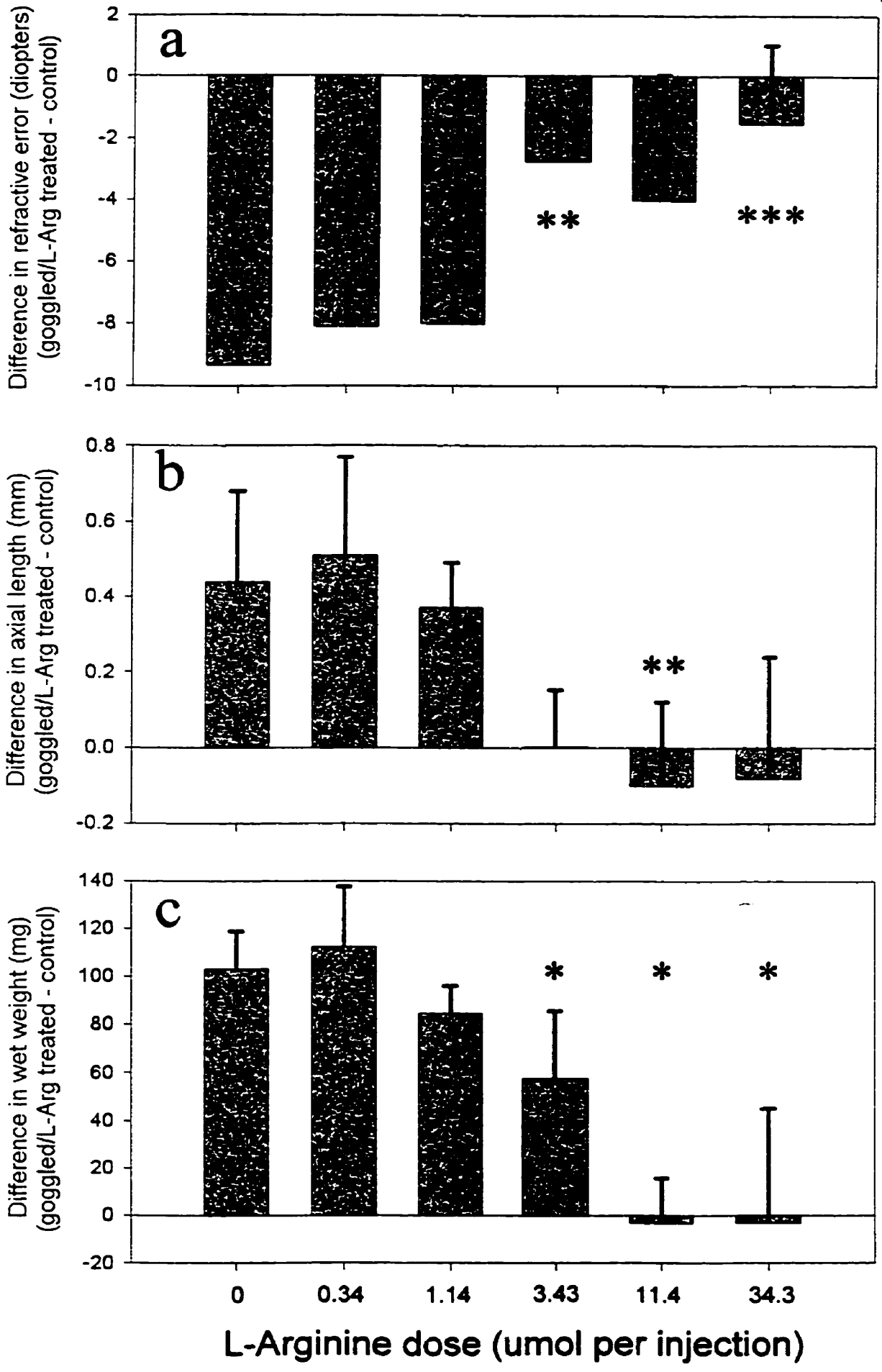
The L-Arginine experiments were done by Dr.W.K.Stell, as part of the preliminary study that gave rise to this thesis project. Dr. A.J.Fischer conducted the immunocytochemical labeling of cGMP in response to ocular injections of SNP. As in the previous section, the results from these experiments are reported here to complete the account of the effects of nitric oxide on FDM development.

Results

L-Arginine Results

L-arginine reduced myopic refraction and ocular enlargement of FDM in a dose-dependent fashion (Figure 4.1). For all parameters the ED_{50} was about 2.8 µmol per injection, corresponding to an initial concentration of 12 mM in the vitreous. However, suppression was not uniform across the three measured parameters. All eyes treated with $\geq$3.43 µmol L-arginine demonstrated refractive errors not significantly different from the emmetropic state. Decreases in axial length, compared to saline-injected goggled eyes, proved to be significant only in the 11.4 µmol L-arg treatment group (Figure 4.1b). Once again, the axial lengths of the three highest treatment groups were not significantly different from those of the contralateral undeprived eyes.

Figure 4.1. The effects of intra-vitreal injections of L-arginine on form-deprivation myopic development in goggled chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). Doses of 3.43 µmol L-arginine or greater generally displayed a suppressing effect on FDM. Doses represent drug content within injected medium. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. n = 6, error bars represent standard deviation.


a
Difference in refractive error (diopters)
(goggled/L-Arg treated - control)
2
0
-2
-4
-6
-8
-10
**
***
b
Difference in axial length (mm)
(goggled/L-Arg treated - control)
0.8
0.6
0.4
0.2
0.0
-0.2
**
c
Difference in wet weight (mg)
(goggled/L-Arg treated - control)
140
120
100
80
60
40
20
0
-20
*
*
*
0
0.34
1.14
3.43
11.4
34.3
L-Arginine dose (umol per injection)

SNP Results

Animal Behaviour

After the treatment period the goggles were removed from the SNP-treated eyes, and each eye was tested for response to a finger abruptly moved into the field of view. Sighted animals responded by flinching, moving the head away from the finger or demonstrating other escape behaviours. Blinded animals did not respond to the finger unless contact was accidentally made. When using eyes that were subjected to the highest SNP dose (1000 nmoles per injection) chicks were visually unresponsive, whereas when using control eyes and eyes treated at other doses they were visually responsive.

Unoccluded Eyes Treated With SNP

Each of the three times this experiment was conducted, only the highest dose of sodium nitroprusside had an effect on ocular development (Figures 4.2.1 & 4.2.2 & 4.2.3). 1000 nmol of SNP caused blindness and severely reduced both axial length (Figures 4.2.xb) and wet weight (Figures 4.2.xc) of the eyes treated with the drug. While equatorial circumference or diameter was not measured, the fact that 1000 nmol SNP treatment resulted in disc-shaped eyes suggests that axial growth was affected more than equatorial ocular growth (data not shown). SNP doses from 10 nmol to 100 nmol per injection spared the vision of the treated eye, which did not develop an ammetropia of any sort.

Form-Deprived Eyes Treated With SNP

On all three occasions that this experiment was conducted, FDM in goggled eyes was suppressed by either 100 nmol or 1000 nmol of SNP (Figures 4.3.1 & 4.3.2 & 4.3.3). In contrast, typical myopic ocular growth was unaffected in eyes injected with 10 nmol SNP or saline.

Treatment with 1000 nmol SNP blinded the treated eye, and caused severe reductions in axial length (Figures 4.3.xb) and wet weight (Figures 4.3.xc). These eyes were even smaller and weighed less than the contralateral saline-injected open eyes. Once again, eyes were disc-shaped, indicating a greater suppression of axial length than equatorial growth (data not shown). Despite blindness, these eyes appeared to be emmetropic (Figures 4.3.xa), but, the light reflex was weak, pinkish and diffuse.

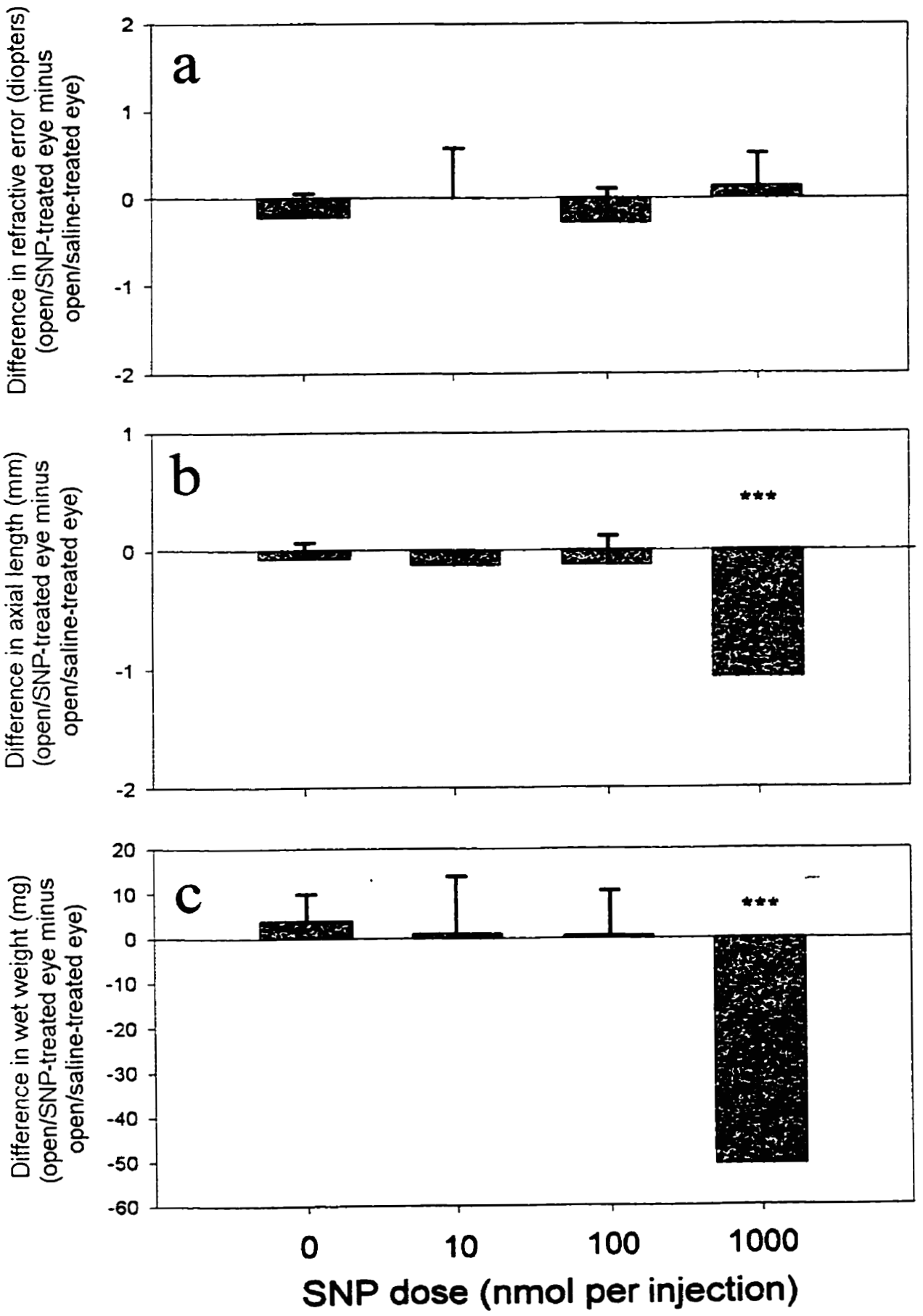

a
Difference in refractive error (diopters)
(open/SNP-treated eye minus
open/saline-treated eye)
2
1
0
-1
-2
b
Difference in axial length (mm)
(open/SNP-treated eye minus
open/saline-treated eye)
1
0
-1
-2
***
c
Difference in wet weight (mg)
(open/SNP-treated eye minus
open/saline-treated eye)
20
10
0
-10
-20
-30
-40
-50
-60
***
0
10
100
1000
SNP dose (nmol per injection)

Figure 4.2.2. Trial two showing the effect of intra-vitreal injections of SNP on ocular development in open chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). Eyes did not develop refractive errors at any dose. Both axial length and wet weight were significantly reduced in eyes treated with the highest dose. Doses represent drug content within injected medium. *** $P<0.001$. $n = 6$, error bars represent standard deviation.

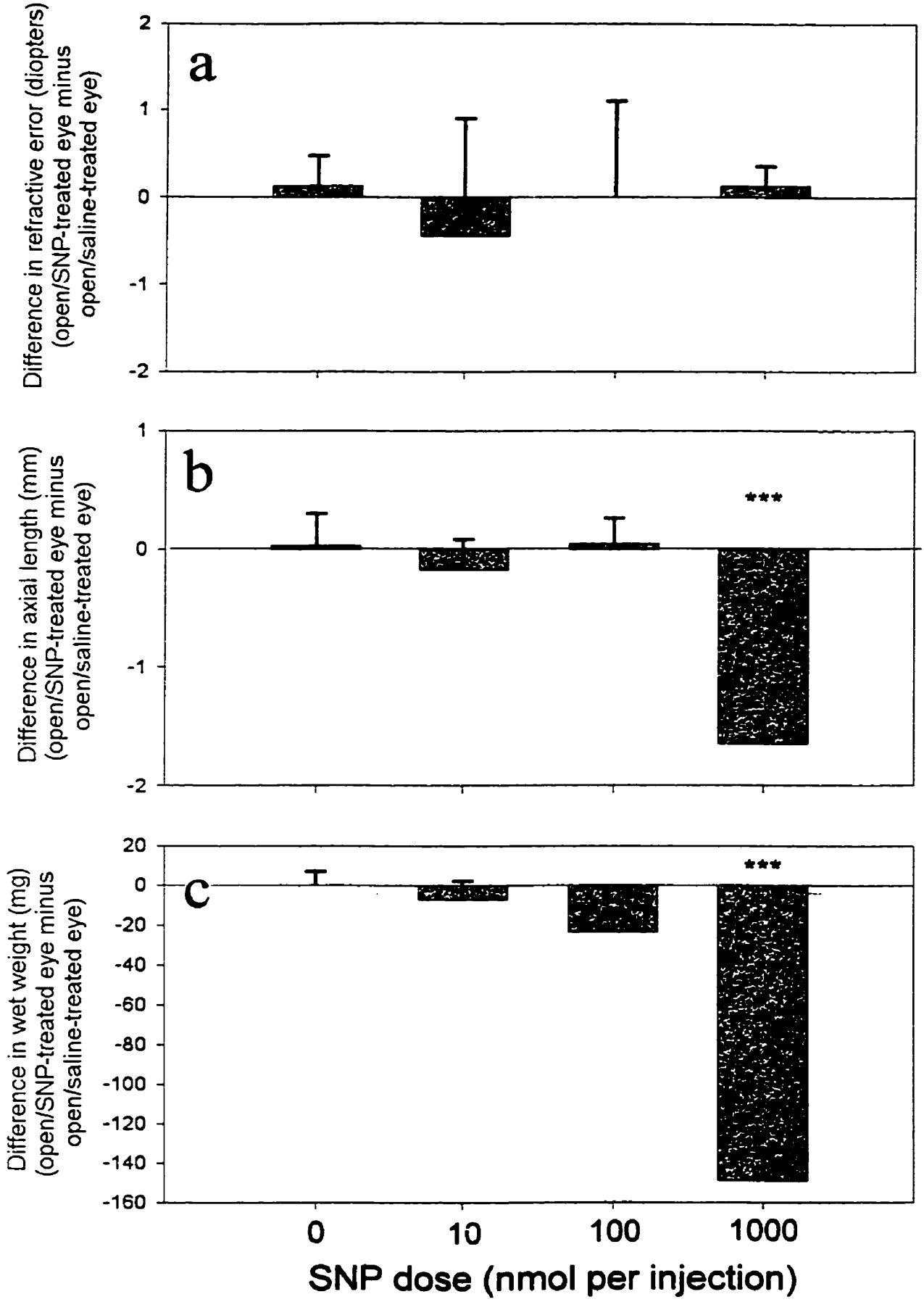

a
Difference in refractive error (diopters)
(open/SNP-treated eye minus
open/saline-treated eye)
2
1
0
-1
-2
b
Difference in axial length (mm)
(open/SNP-treated eye minus
open/saline-treated eye)
1
0
-1
-2
***
c
Difference in wet weight (mg)
(open/SNP-treated eye minus
open/saline-treated eye)
20
0
-20
-40
-60
-80
-100
-120
-140
-160
***
0
10
100
1000
SNP dose (nmol per injection)

Figure 4.2.3. Trial three showing the effect of intra-vitreal injections of SNP on ocular development in open chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). Eyes did not develop refractive errors at any dose. Both axial length and wet weight were significantly reduced in eyes treated with the highest dose. Doses represent drug content within injected medium. *** $P<0.001$. $n = 6$, error bars represent standard deviation.

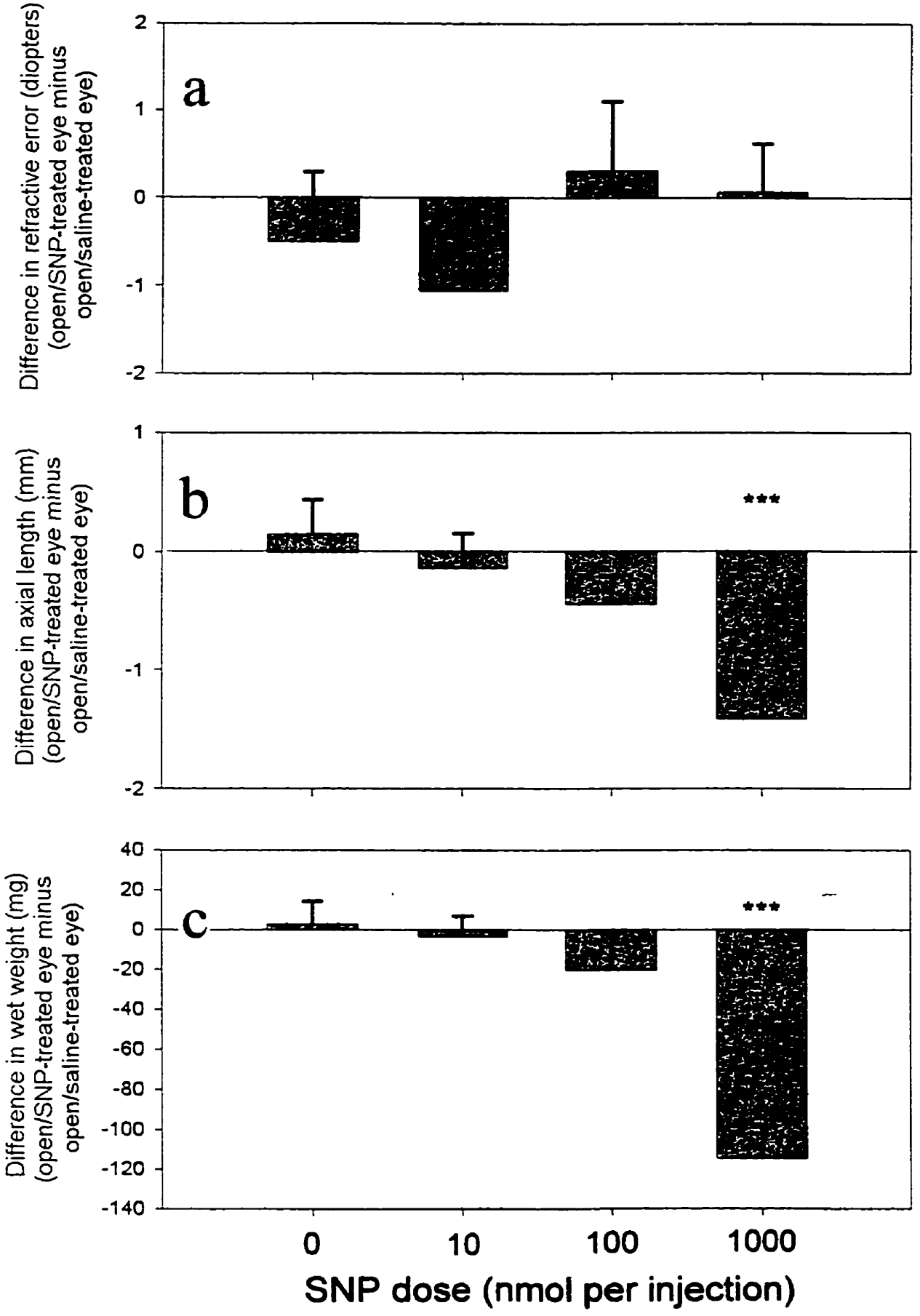

a
Difference in refractive error (diopters)
(open/SNP-treated eye minus
open/saline-treated eye)
2
1
0
-1
-2
b
Difference in axial length (mm)
(open/SNP-treated eye minus
open/saline-treated eye)
***
1
0
-1
-2
c
Difference in wet weight (mg)
(open/SNP-treated eye minus
open/saline-treated eye)
***
40
20
0
-20
-40
-60
-80
-100
-120
-140
0
10
100
1000
SNP dose (nmol per injection)

Figure 4.3.1. Trial one showing the effect intra-vitreal injections of SNP on development of FDM in goggled chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). Doses of 100 nmol SNP and 1000 nmol SNP significantly suppressed myopic development. Doses represent drug content within injected medium. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. $n = 8$, error bars represent standard deviation.

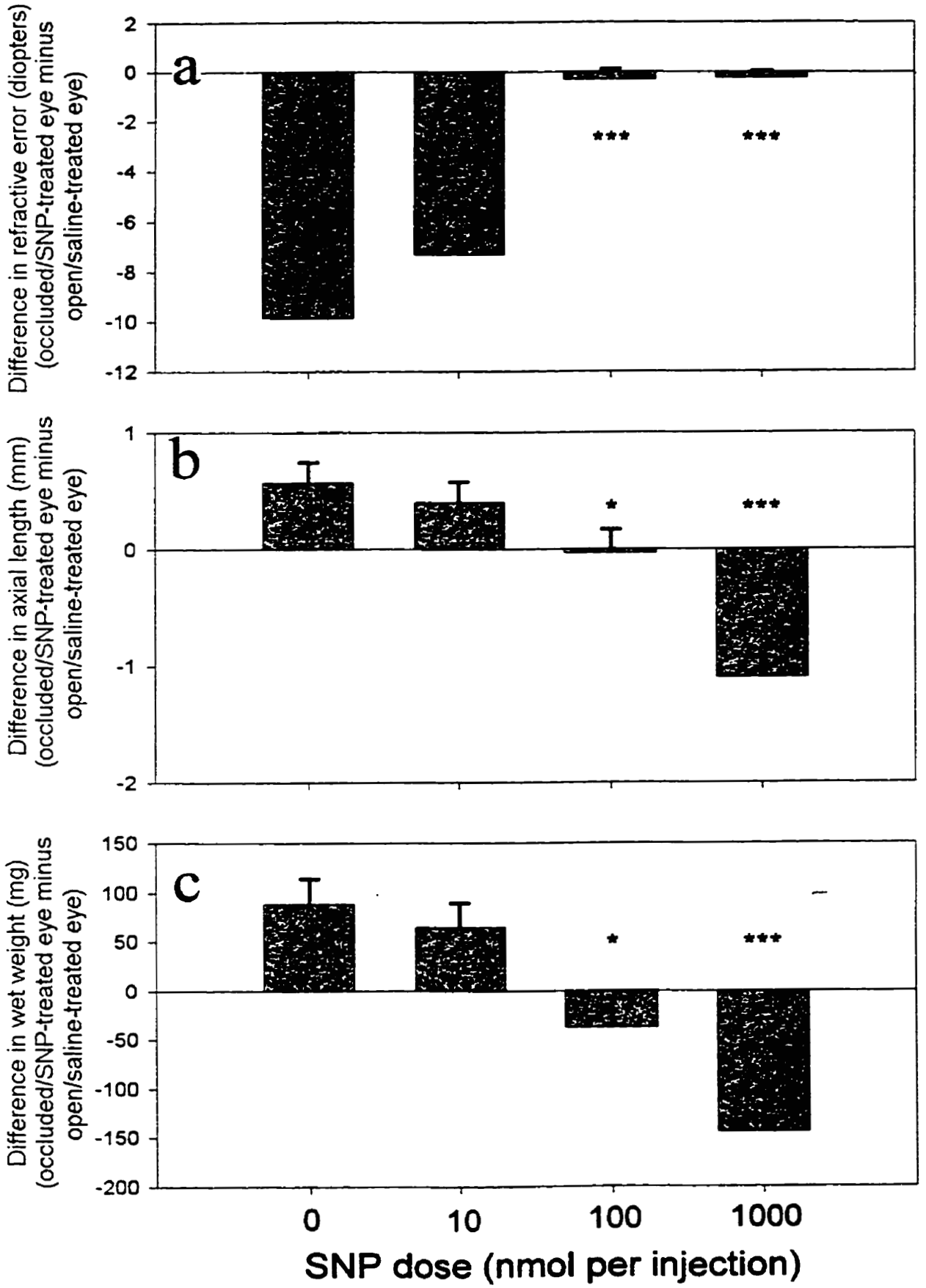

a
Difference in refractive error (diopters)
(occluded/SNP-treated eye minus
open/saline-treated eye)
2
0
-2
-4
-6
-8
-10
-12
***
***
b
Difference in axial length (mm)
(occluded/SNP-treated eye minus
open/saline-treated eye)
1
0
-1
-2
*
***
c
Difference in wet weight (mg)
(occluded/SNP-treated eye minus
open/saline-treated eye)
150
100
50
0
-50
-100
-150
-200
*
***
0
10
100
1000
SNP dose (nmol per injection)

Figure 4.3.2. Trial two showing the effect intra-vitreal injections of SNP on development of FDM in goggled chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). Doses of 100 nmol SNP and 1000 nmol SNP significantly suppressed myopic development. Doses represent drug content within injected medium. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. n = 8, error bars represent standard deviation.

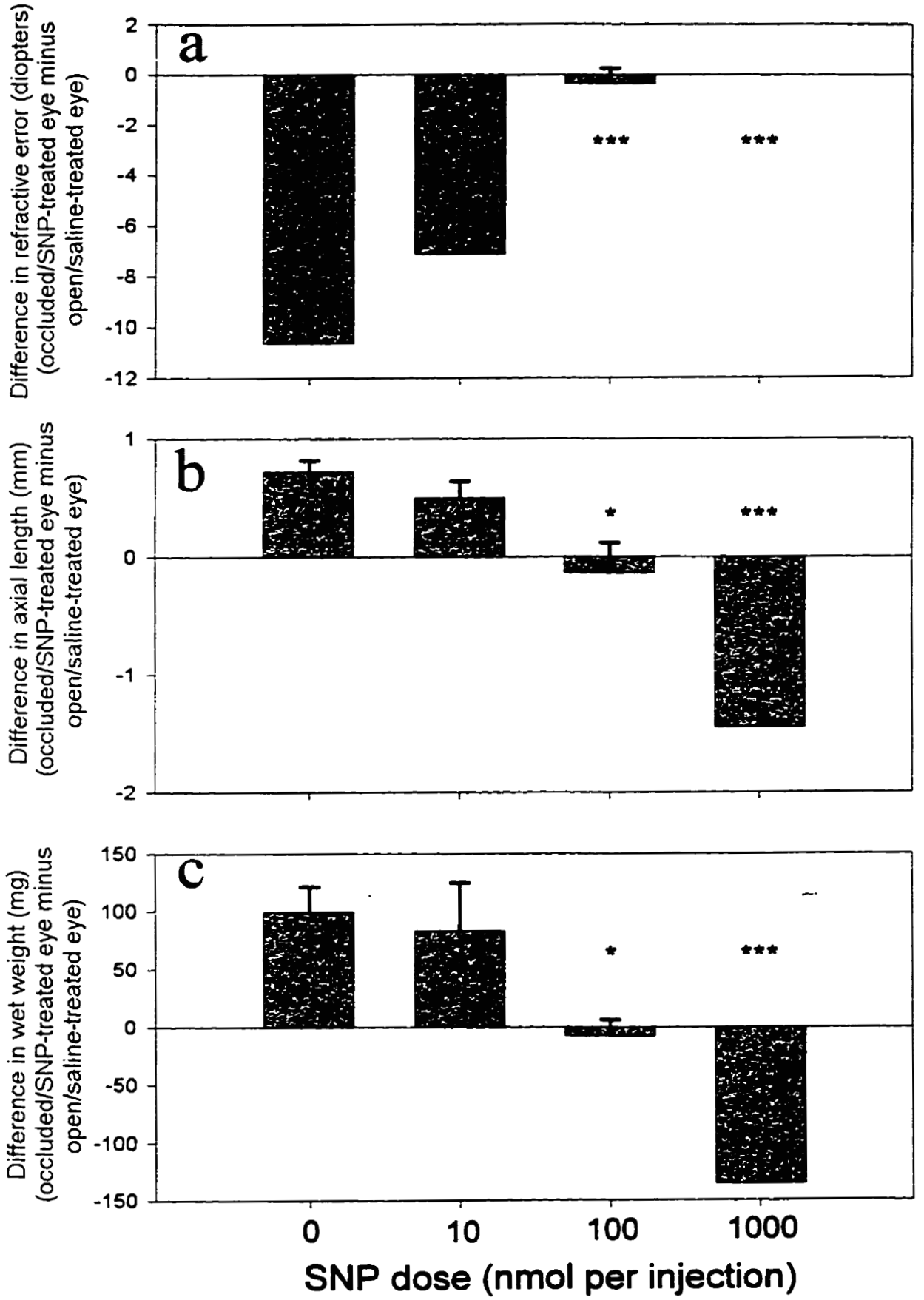
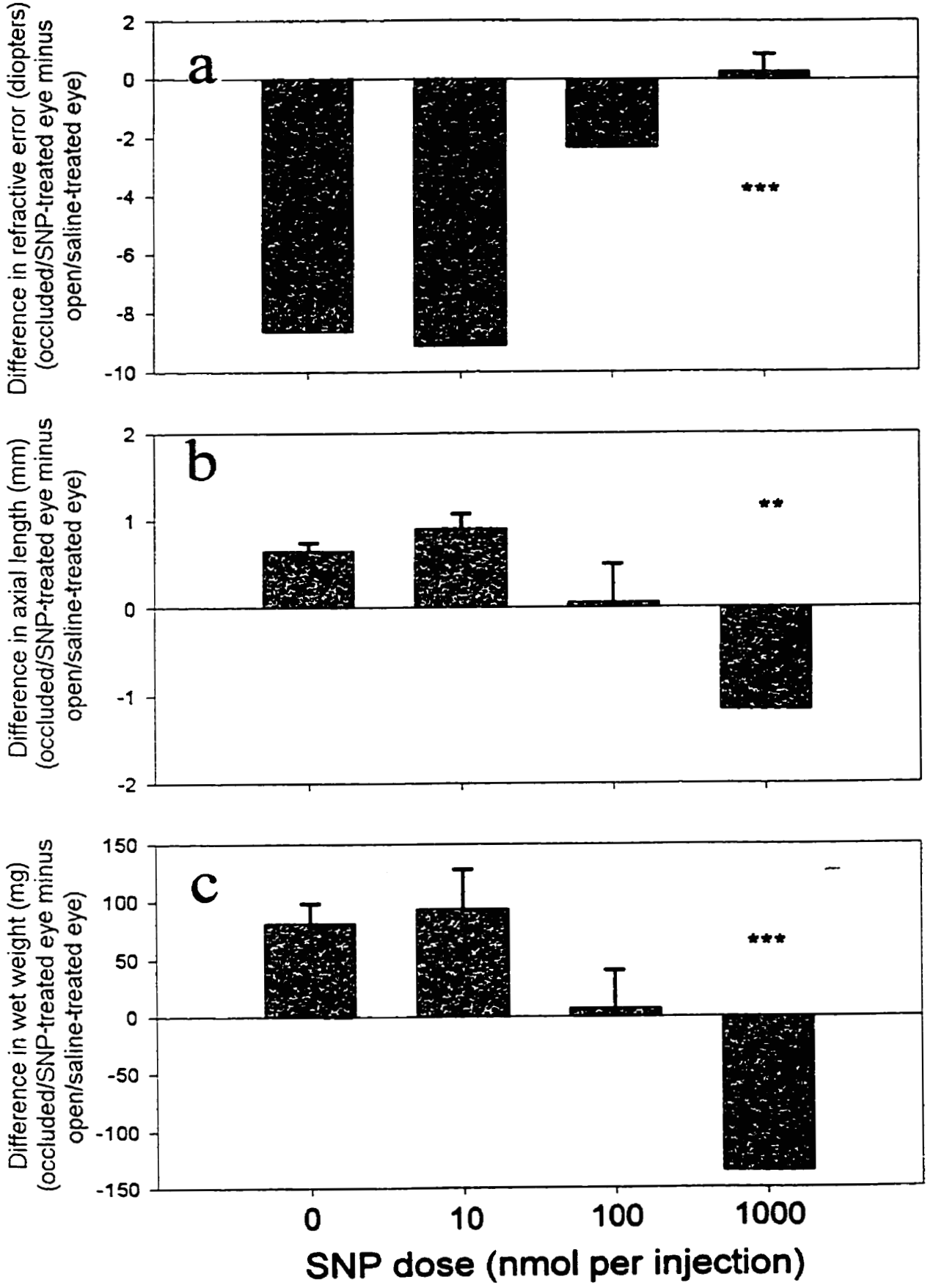

a
Difference in refractive error (diopters)
(occluded/SNP-treated eye minus
open/saline-treated eye)
2
0
-2
-4
-6
-8
-10
-12
***
***
b
Difference in axial length (mm)
(occluded/SNP-treated eye minus
open/saline-treated eye)
1
0
-1
-2
*
***
c
Difference in wet weight (mg)
(occluded/SNP-treated eye minus
open/saline-treated eye)
150
100
50
0
-50
-100
-150
*
***
0
10
100
1000
SNP dose (nmol per injection)

Figure 4.3.3. Trial three showing the effect intra-vitreal injections of SNP on development of FDM in goggled chick eyes (**a.** Refractive error **b.** Axial Length **c.** Wet Weight). Doses of 1000 nmol SNP significantly suppressed myopic development. Doses represent drug content within injected medium. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. n = 8, error bars represent standard deviation.


a
Difference in refractive error (diopters)
(occluded/SNP-treated eye minus
open/saline-treated eye)
2
0
-2
-4
-6
-8
-10
***
b
Difference in axial length (mm)
(occluded/SNP-treated eye minus
open/saline-treated eye)
2
1
0
-1
-2
**
c
Difference in wet weight (mg)
(occluded/SNP-treated eye minus
open/saline-treated eye)
150
100
50
0
-50
-100
-150
***
0
10
100
1000
SNP dose (nmol per injection)

These abnormalities in reflex are likely due to retinal damage and RPE perforation, leaving an uneven surface to reflect light and resulting in a diffuse light reflex. Lens damage may also attribute to the diffusion of the reflected light. When these eyes were hemisected for fixation and immunolabelling, the retinas were noted to be milky in colour and the underlying RPE was observed to be recticular in appearance, instead of sheet-like. This was clear indication of the damage to be seen under the microscope.

Goggled eyes treated with 100 nmol SNP also failed to develop FDM, while retaining vision and without grossly obvious ocular tissue damage (see the next section). These eyes showed significant reductions to both axial length (Figures 4.3.xb) and wet weight (Figures 4.3.xc) compared to the saline-injected goggled group, and closely resembled their contralateral undeprived eyes. Refractive errors in these eyes were significantly reduced compared to the saline-injected goggled eyes, and were not significantly different from emmetropia (Figures 4.3.xa).

Immunocytochemistry/Microscopy Results

After treatment with 1000 nmol SNP injection into either open or goggled eyes, the neural tissue of the retina was damaged. Microscopical evaluation showed that the retina was completely destroyed, replaced by a layer of scar tissue, and that the RPE had become dysplastic and infiltrated the retinal scar (Figure 4.4 right, right micrograph of Figures 4.5 – 4.9). Retinas treated with 100 nmol SNP showed mild damage and disorganisation, limited to the photoreceptor layer of the retina (best seen in Figure 4.8.1/2 & Figure 4.9, not clearly evident in Figure 4.4 centre). TUNEL labelling indicated that many photoreceptors in eyes treated with 100 nmol SNP contained considerable amounts of fragmented DNA, while saline-injected FD eyes did not (Figure 4.5 centre). This indicates that photoreceptor cells undergo apoptosis in the 100 nmol SNP eyes and not in controls. Also at this dose, immunolabelling with antiserum to LEP-100 (Figure 4.6 centre) showed increased amounts of this lysosomal membrane glycoprotein in the photoreceptor layer, suggesting a heightened level of cell digestion (autophagy) associated with increased cell death or damage. Antiserum to calbindin, used to label the cones of the photoreceptor layer, showed little or no effect on cone structure at 100 nmol SNP (Figure 4.7 centre). However, antibody to rhodopsin revealed a number of insults to the rod photoreceptor population (Figure 4.8.1 & 4.8.2 & 4.8.3). These include a reduction in density of rod photoreceptors in

Figure 4.4. Toluidine blue-stained, vertical sections of retinas taken from chick eyes after treatment with SNP. Micrographs represent retinas of goggled eyes inected with: left, saline (control); centre, 100 nmol SNP; right, 1000 nmol SNP. With this method there are no apparent differences between saline-treated and 100 nmol SNP-treated retinas at this magnification. However, retinas treated with 1000 nmol SNP appear to be greatly degenerated and infiltrated with pigmented cells. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar = 50 μm.


RPE
R
INL
IPL
GC

Figure 4.5. TUNEL labelling showing DNA fragmentation in vertical sections of retina, from goggled chick eyes after treatment with SNP. Doses: left, saline (control); centre, 100 nmol SNP; right, 1000 nmol SNP. The control retina shows no DNA fragmentation, while 100 nmol SNP induces DNA fragmentation limited to the photoreceptor and outer nuclear layer and 1000 nmol SNP induces wide-spread fragmentation throughout all nuclear layers (except the ganglion cell layer) of the retina. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar = 50 μm.

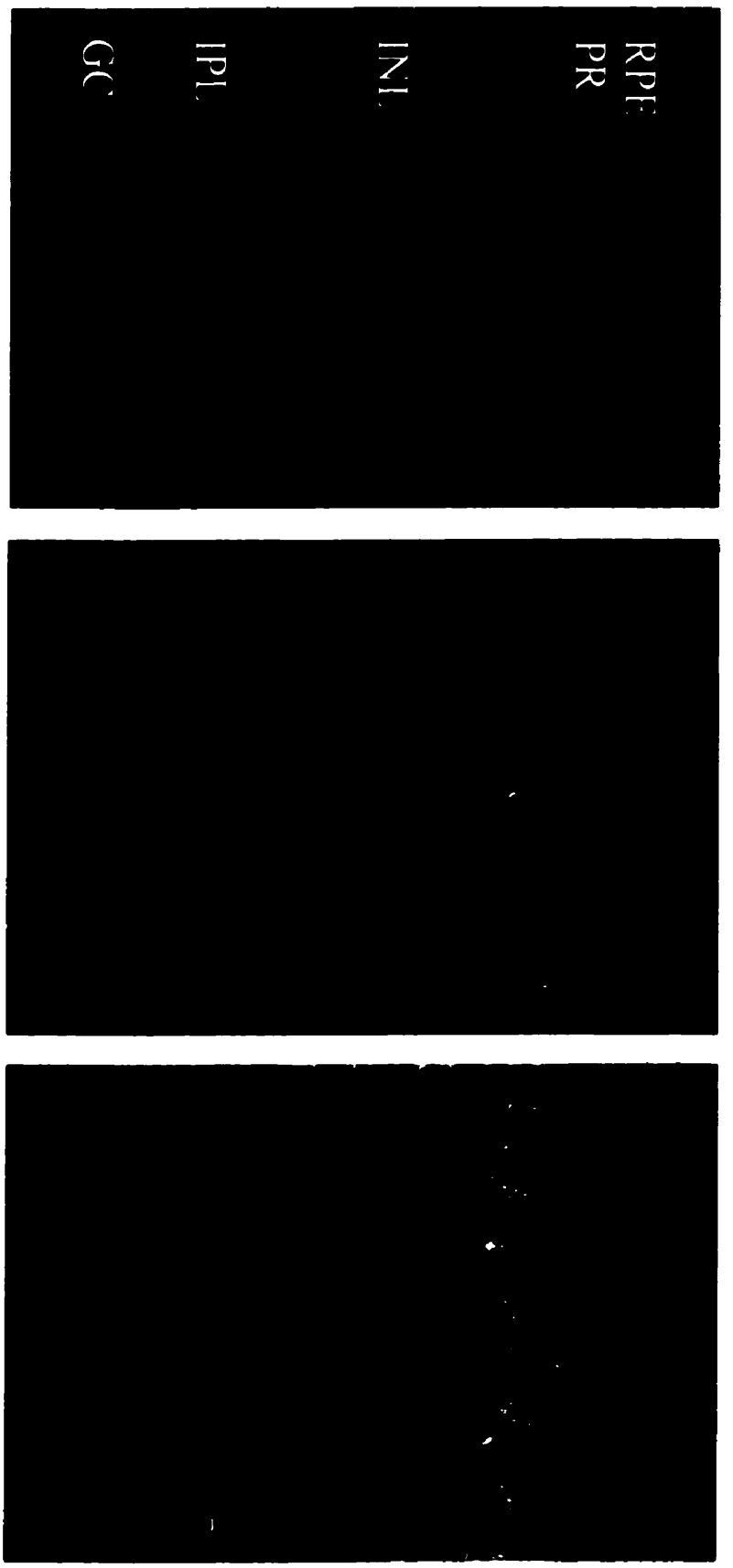

RPE
PR
INL
IPL
GC

Figure 4.6. Vertical sections from the retinas of goggled chick eyes treated with SNP, labelled for lysosomal membrane (LEP-100). Micrographs represent retina of goggled eyes injected with: left, saline injections (control); centre, 100 nmol SNP; right, 1000 nmol SNP. The control retina shows no labelling for lysosomal activity, while 100 nmol SNP causes labelling limited to the photoreceptor layer and 1000 nmol SNP treatment causes labelling throughout the degenerated retina. Abbreviations: RPE: retinal pigment epithelium, PR: photoreceptors, INL: inner nuclear layer, IPL: inner plexiform layer, GC: ganglion cell layer. Scale bar = 50 µm.

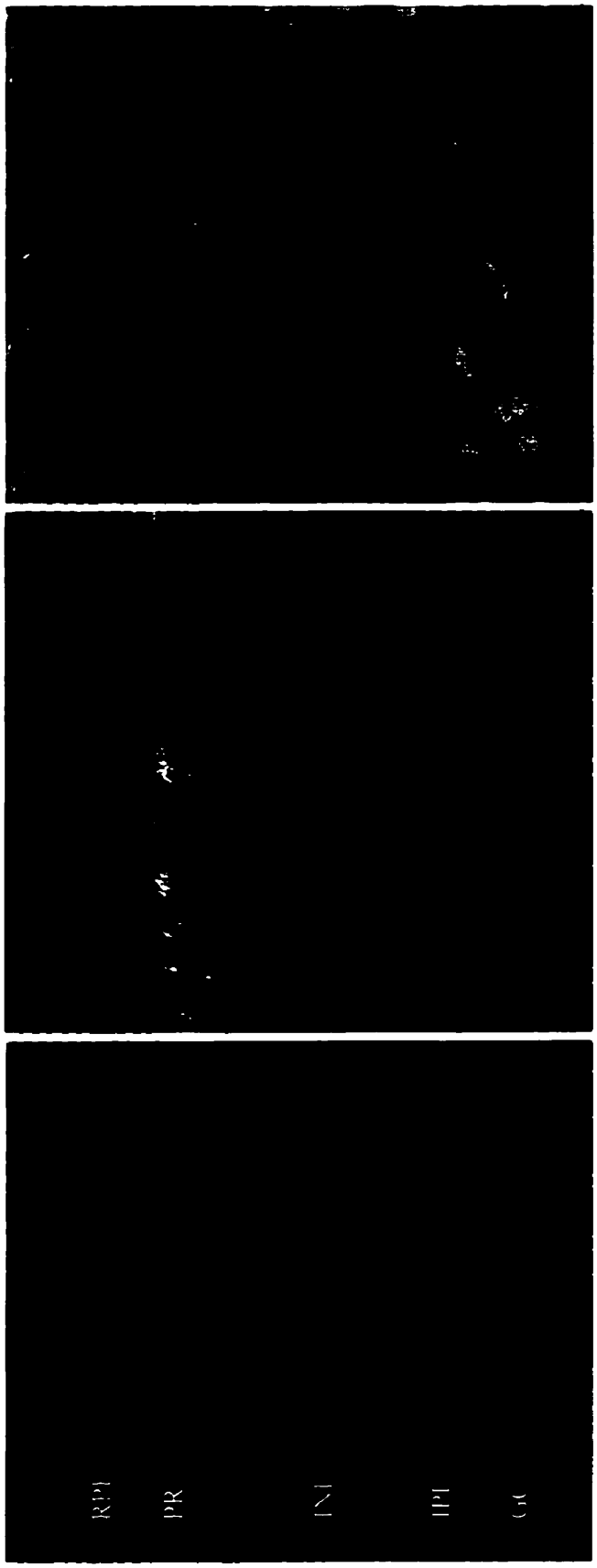

RPE
PR
INL
IPL
GC

Figure 4.7. Vertical sections taken from retinas of goggled eyes treated with SNP, and then labelled with antiserum directed against calbindin. Micrographs represent retina of goggled eyes injected with: left, saline (control); centre, 100 nmol SNP; right, 1000 nmol SNP. There was no difference in labelling between control and 100 nmol SNP-treated retinas, while eyes treated with 1000 nmol SNP showed faint, dispersed labelling thoughout the the outer region of the degenerating retina. Several pigment cells have invaded the photoreceptor layer in centre micrograph. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar = 50 μm.

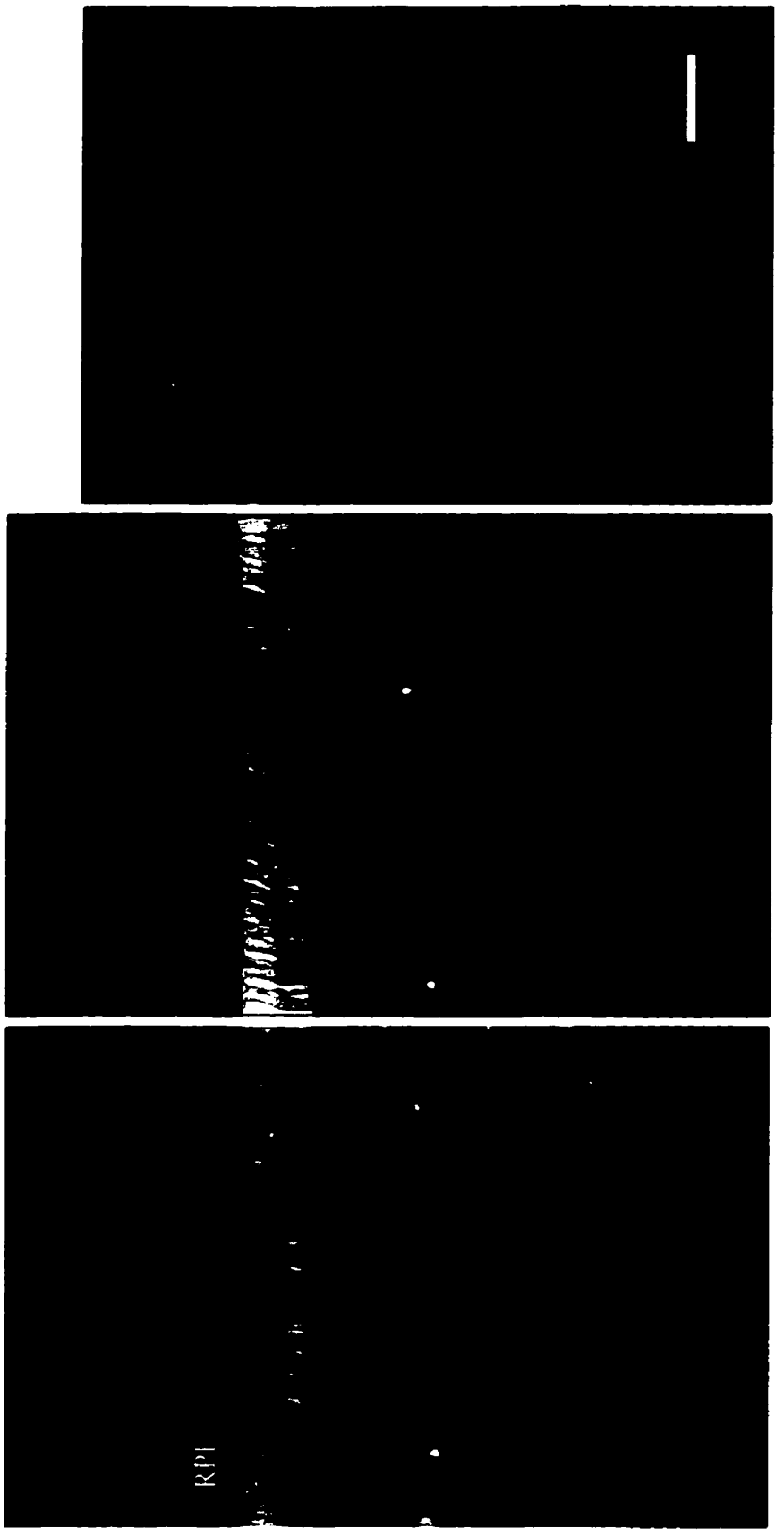
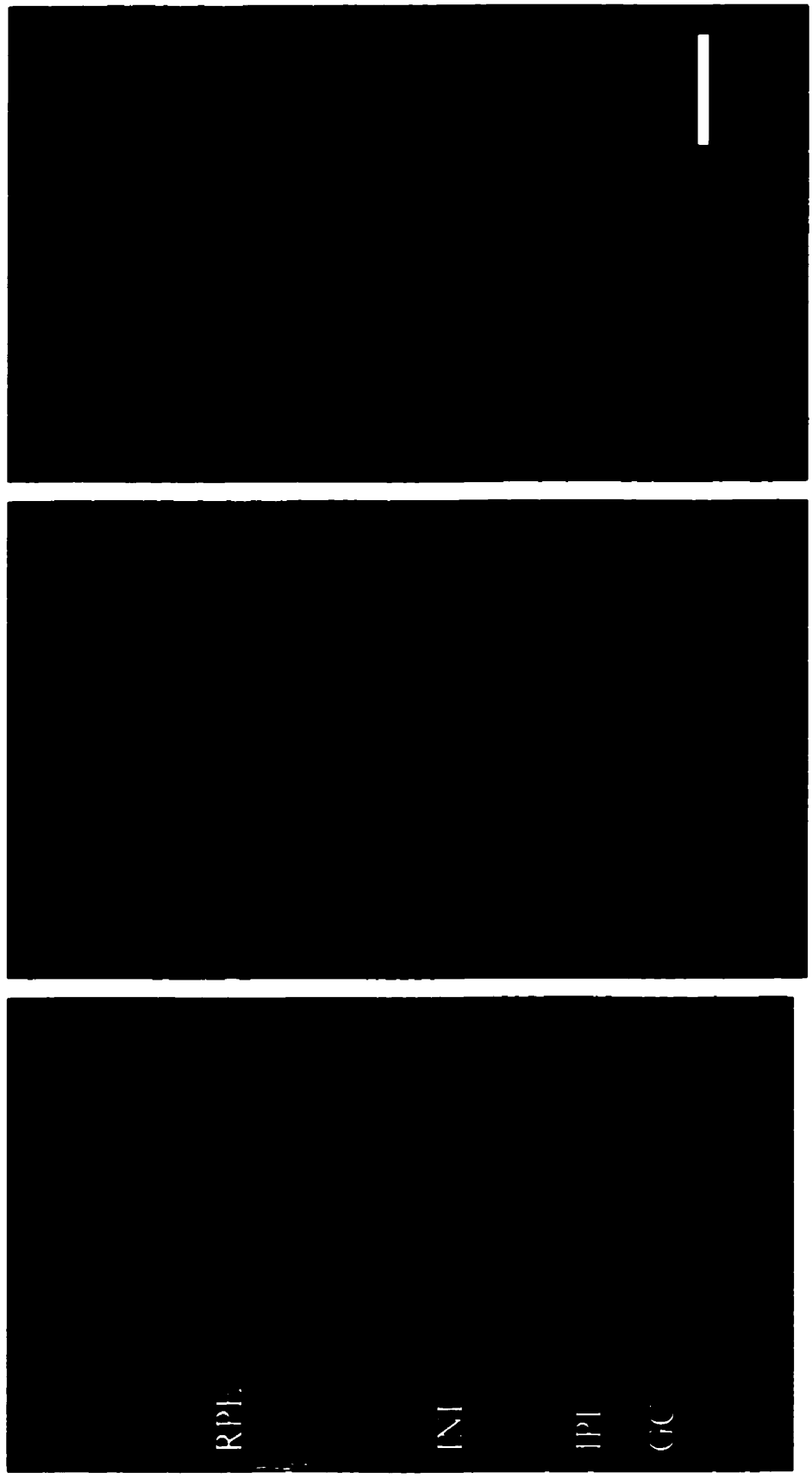

RPI

Figure 4.8.1 & 2. Vertical sections taken from retinas of goggled eyes treated with SNP, and then labelled with antisera directed against rhodopsin. Fig.4.8.1: Micrographs represent retina of goggled eyes treated with: left, saline (control); centre, 100 nmol SNP; right, 1000 nmol SNP. Fig.4.8.2: Micrographs represent retina of goggled eyes treated with: a, saline (control); b,c,d, further examples of 100 nmol SNP. Retinas treated with 100 nmol SNP showed thinned-out rod photoreceptors that appeared to have shortened, disorganized and possibly swollen outer segments. Retinas treated with 1000 nmol SNP were degenerate and showed faint labelling throughout the outer regions of the retina. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar = 50 μm.


RPL
INL
IPL
GC

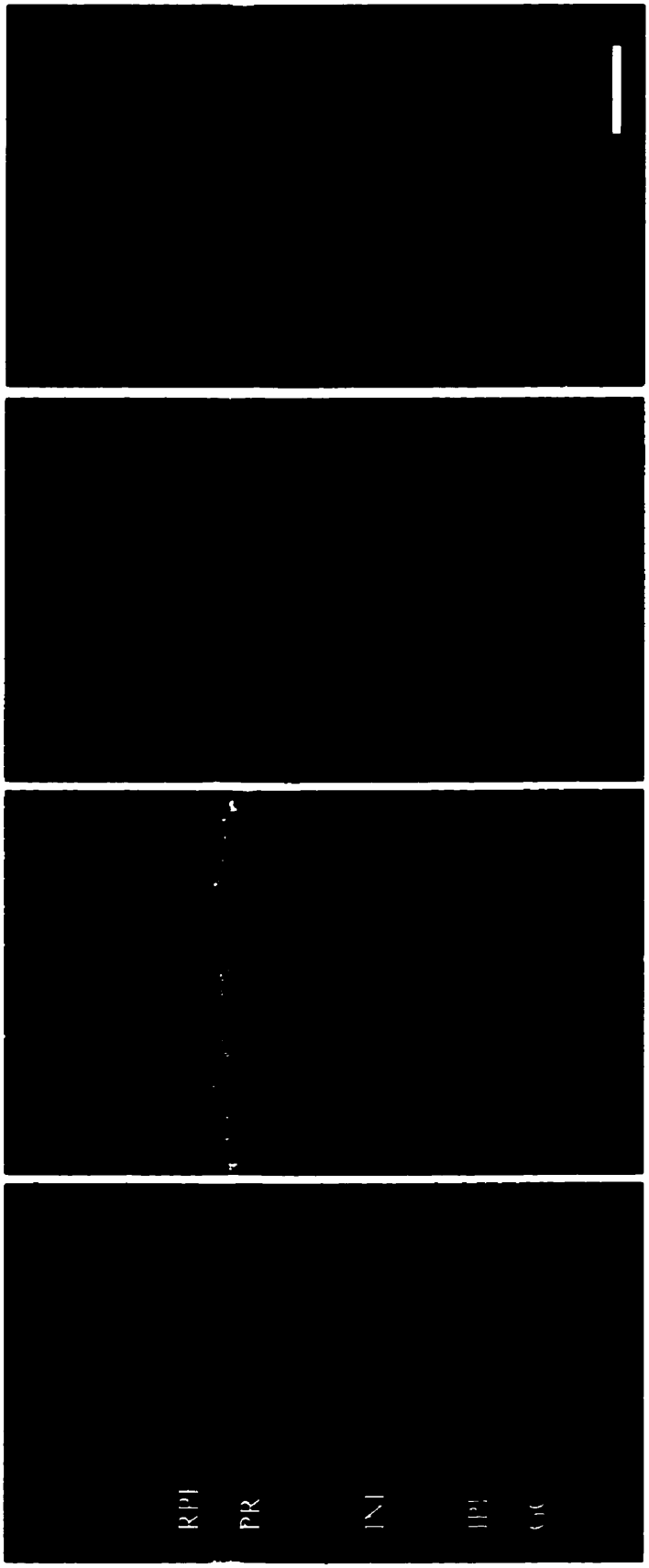

RPE
PR
INL
IPL
GCL

Figure 4.8.3. Vertical sections taken from retinas of goggled eyes treated with SNP, and then labelled with antisera directed against rhodopsin. These sections are viewed at a magnification of 400X. Micrographs represent retina of goggled eyes treated with: left, saline (control); right, 100 nmol SNP. The rod outer segments of retinas treated with 100 nmol SNP are shortened, swollen and disorganized compared to those in control retinas. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer. Scale bar = 50 μm.

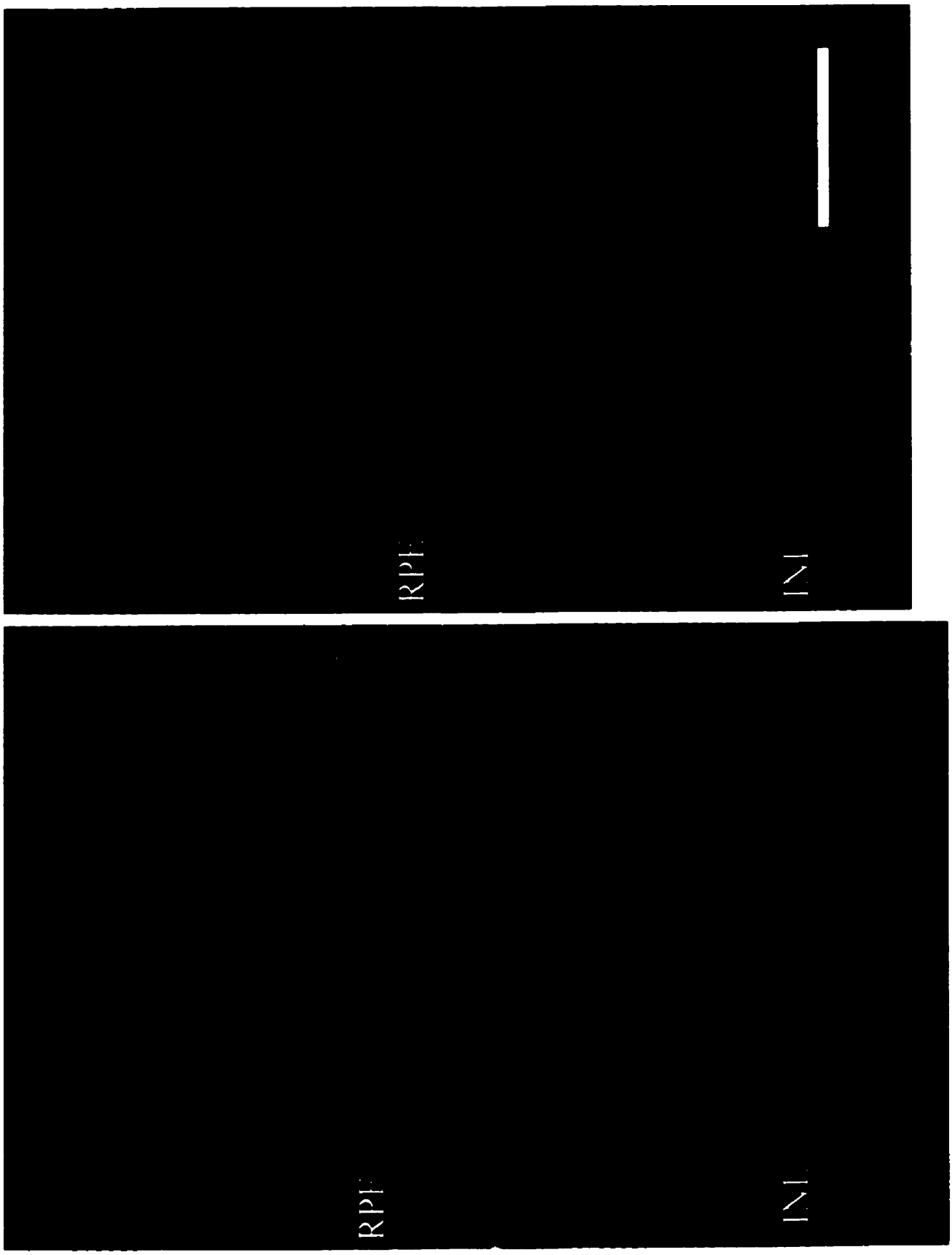
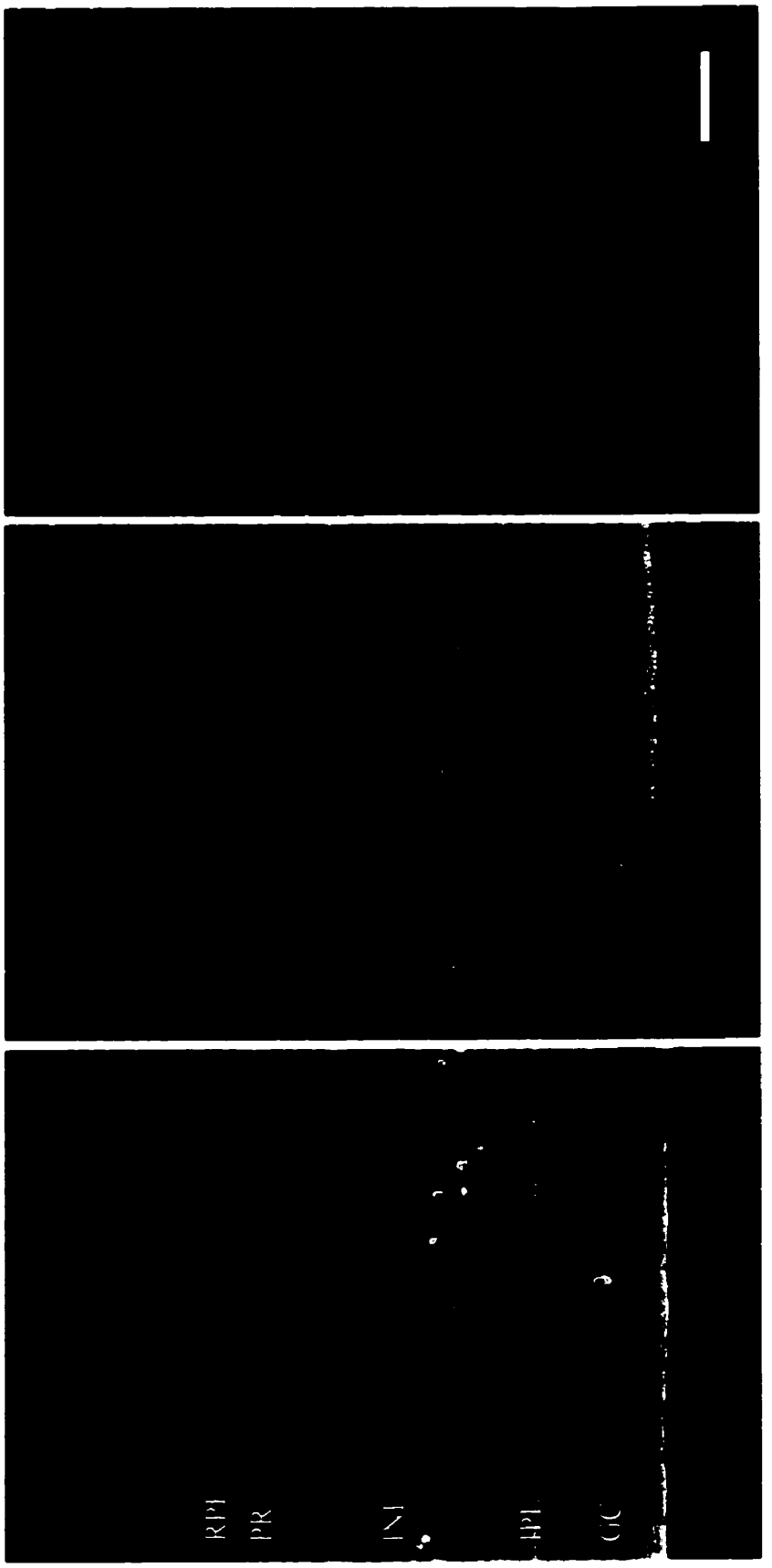

RPE
INL
RPE
INL

Figure 4.9. Vertical sections taken from retinas of goggled eyes treated with SNP, and then labelled with antiserum to calretinin. Micrographs represent retina of goggled eyes treated with: left, saline (control); centre, 100 nmol SNP; right, 1000 nmol SNP. There was no difference in labelling between control and 100 nmol SNP-treated retinas, while retinas treated with 1000 nmol SNP showed dispersed and disorganized labelling limited to regions expected to be labelled within intact retina. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar = 50 μm.


RPE
PR
INL
IPL
GC

some regions (Figure 4.8.1d & 4.8.2 centre), as well as shortening (Figure 4.8.1c&d) and possible swelling of the rod outer segments (ROS)(Figure 4.8.1b&c) and disruption of ROS organization (Figure 4.8.1b). Calretinin labelling, used as a widely expressed retinal label, showed no changes in horizontal, bipolar, amacrine or ganglion cells in response to 100 nmol SNP (Figure 4.9).

Other bipolar- or amacrine cell-specific markers likewise failed to reveal any changes in protein expression or cell structure. For example, antibodies to choline acetyltransferase (ChAT) revealed the four expected (Fischer *et al.* 1998d) amacrine cell types (types I and III localized to the INL and type II within the ganglion cell layer) along with the double lamination of the IPL at approximately 20% and 70% depths, all intact after ≤100nm SNP (Figure 4.10). Even at 1000nm SNP, ChAT labelling was still evident, though highly disrupted, in isolated regions where retinal degeneration was not yet complete (data not shown).

Antiserum to tyrosine hydroxylase labelled amacrine cells that were sparsely distributed, with cell bodies at the INL/IPL border and primary neurites projecting to 35% IPL depth, where they divided into a number of secondary neurites (Figure 4.11). These secondary neurites further extended to an IPL depth of about 75%. Arborizations, presumed to extend from the main neurites of these amacrine cells, were present at about 0%, 35% and 75% IPL depths, as previously reported in the avian retina (Su & Watt, 1987). This pattern persisted after 100 nm SNP treatment, and as for ChAT immunoreactivity, TH labelling could be seen in 1000nm SNP-treated retinas, though the pattern of labelling was highly disrupted (data not shown).

Parvalbumin labelling revealed the somata of a set of amacrine cells that were abundant throughout the proximal INL as well as three bands of neurites within the IPL. No difference could be discerned between the labelling patterns in control and 100 nmol SNP-treated eyes (Figure 4.12). Two of these bands (0% and 70% IPL depth) were strongly labelled, while the third (40% IPL depth) possessed considerably weaker labelling. This pattern of labelling was typical of that found in other studies on the chick retina (Fischer *et al.* 1998). It was disrupted at 1000nm SNP, yet distorted labelling remained even at this highest dose.

Figure 4.10. Vertical sections taken from retinas of goggled eyes treated with SNP, and then labelled with antiserum directed against choline acetyltransferase (ChAT). Micrographs represent retina of goggled eyes treated with: left, saline (control); right, 100 nmol SNP. There was no difference in labelling between control and 100 nmol SNP-treated retinas, both labelling all sub-types of ChAT-IR amacrine cell, projecting to two laminae within the IPL. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar = 50 μm.

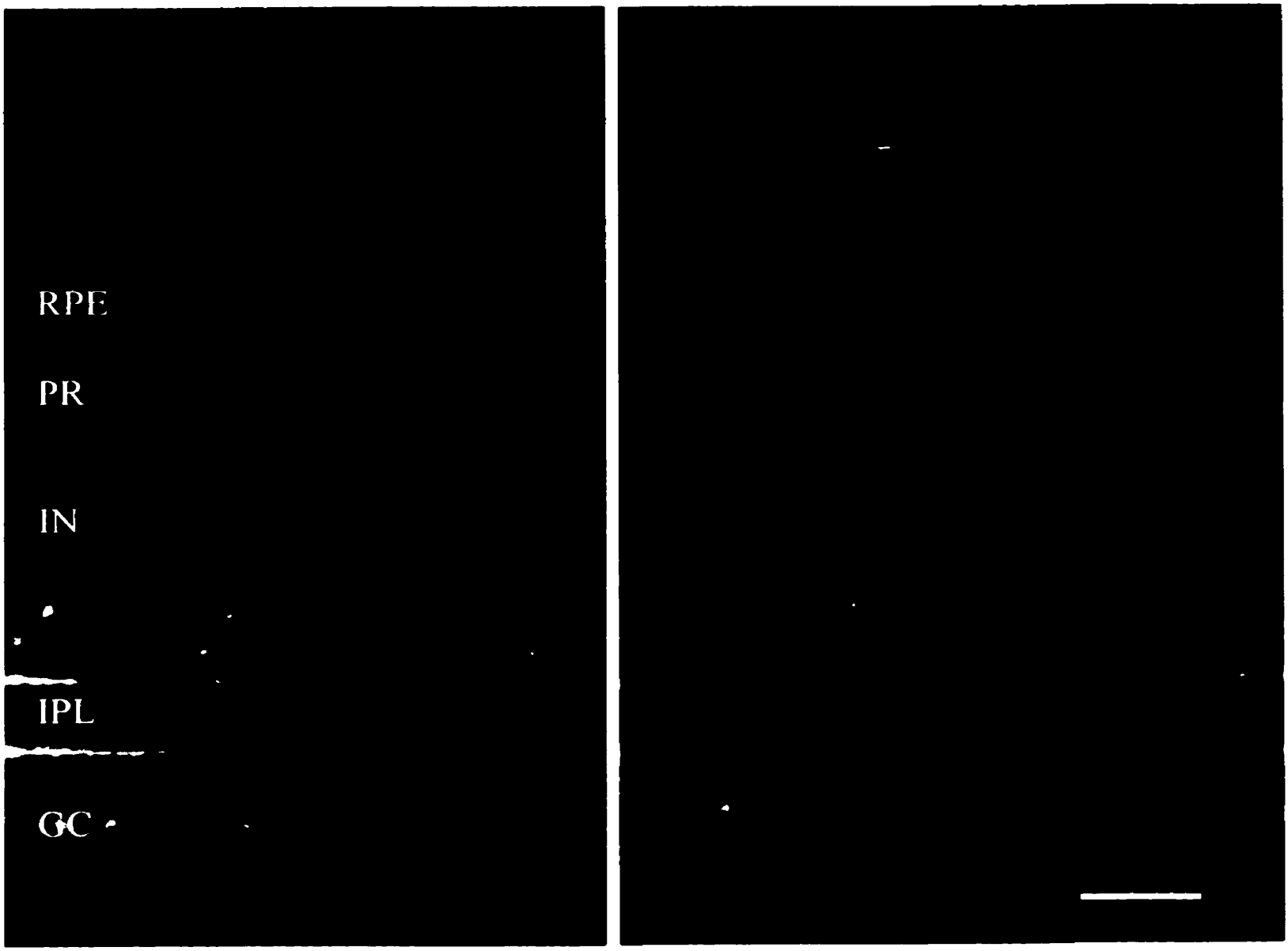

RPE
PR
IN
IPL
GC

Figure 4.11. Vertical sections taken from retinas of goggled eyes treated with SNP, and then labelled with antisera directed against tyrosine hydroxylase. Micrographs represent retinas of goggled eyes treated with: left, saline (control); right, 100 nmol SNP. Both control retinas and retinas treated with 100 nmol SNP demonstrated labelling within sparse amacrine cells and primary neurites which project to 3 laminae within the IPL; no difference could be discerned between the two conditions. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar = 50 μm.

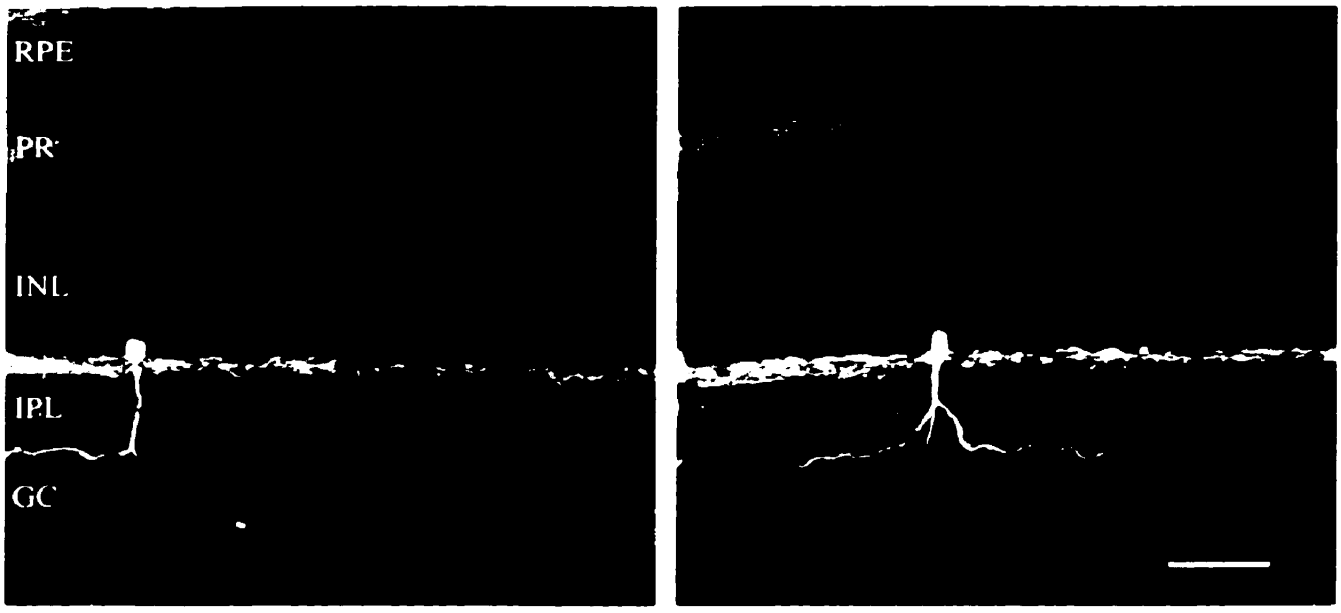
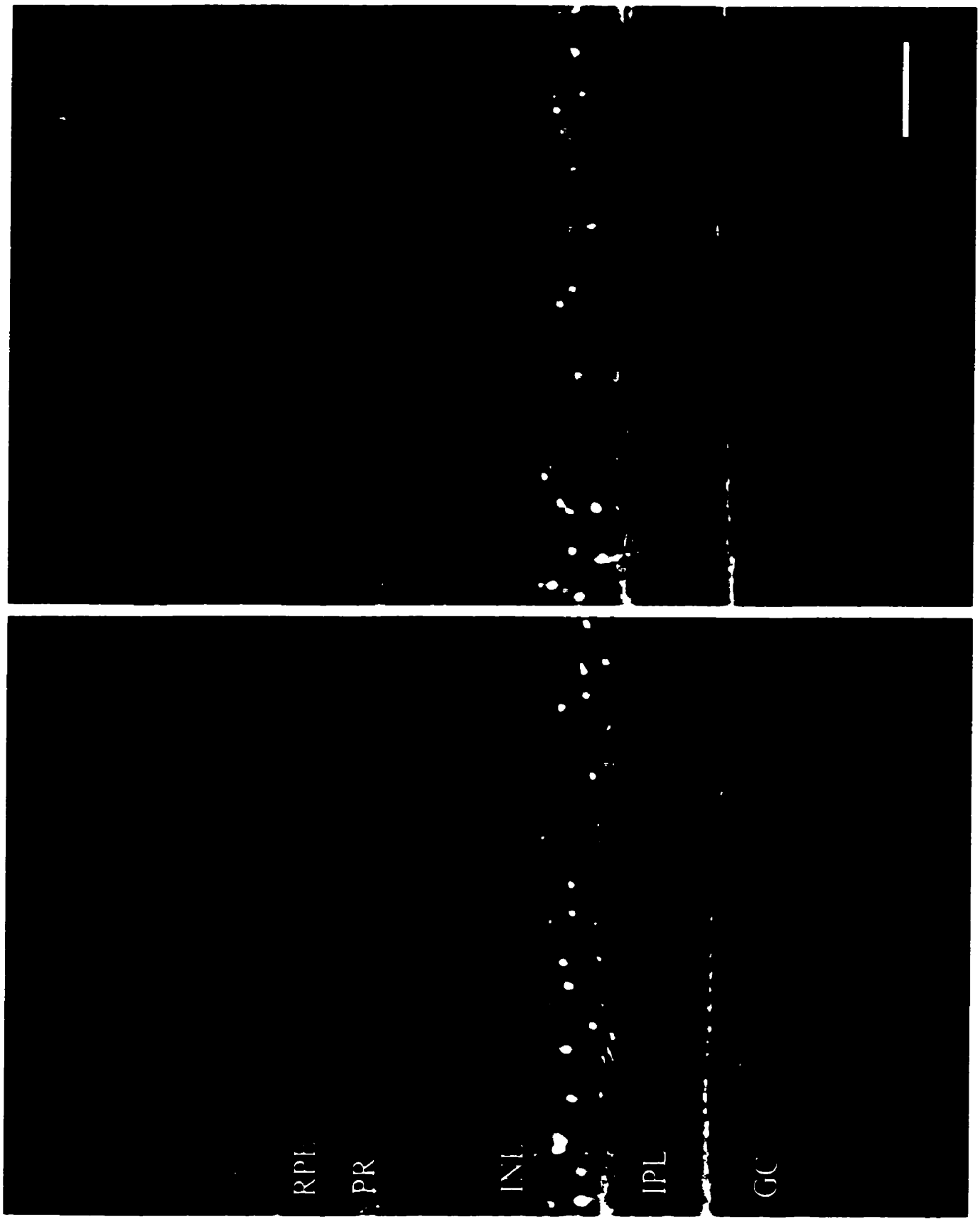

RPE
PR
INL
IPL
GC

Figure 4.12. Vertical sections taken from retinas of goggled eyes treated with SNP, and then labelled with antiserum to parvalbumin. Micrographs represent retinas of goggled eyes treated with: left, saline (control); right, 100 nmol SNP. Both control and 100-nmol SNP-treated retinas showed no difference in their labelling of amacrine cells and various laminae within the IPL. Abbreviations: RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar = 50 μm.


RPE
PR
INL
IPL
GC

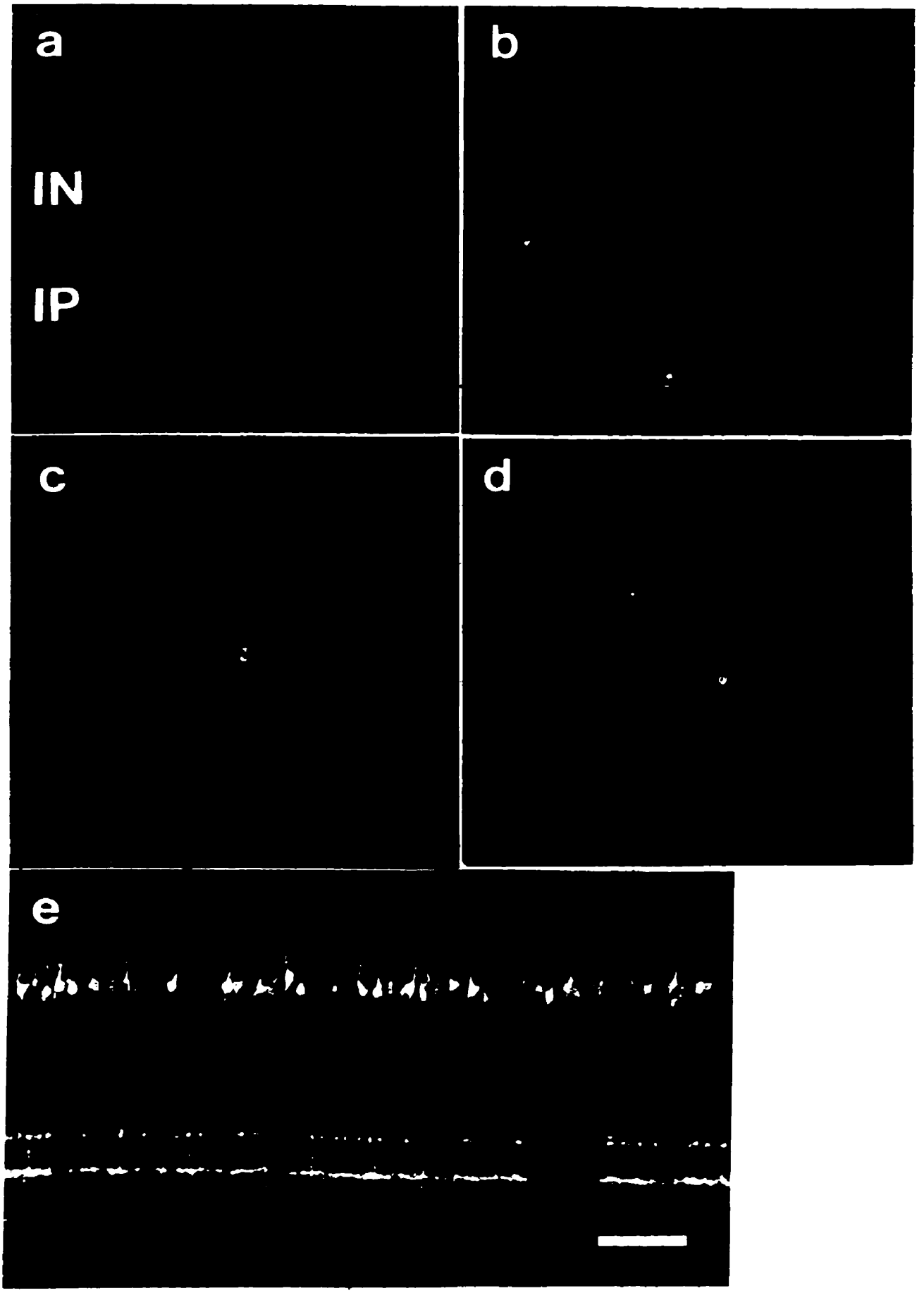
Both Met-enkephalin and somatostatin were localized to a set of relatively abundant amacrine cell somata near the middle of the INL (data not shown). Unfortunately, I was unable to label the neurites of these cells. The discernable characteristics of these cells did not appear to vary with drug treatment. These cells were damaged only by the highest dose of SNP, coinciding with the massive retinal damage that was also occurring. The description of these amacrine cells matches that of the ENSLI (enkephalin-neurotensin-somatostatin-like-immunoreactive) cells that have been previously characterized (Brecha et al. 1979, Watt *et al.* 1985, Morgan *et al.* 1994)

Immunolocalization of Cyclic GMP After SNP Treatment

Retinas treated with IBMX demonstrated an upregulation of cGMP production when co-treated with SNP. When eyes were injected with IBMX alone, cGMP-immunoreactivity was detected only weakly in a small number of nondescript amacrine cells (Figure 4.13a). However, when these eyes were also injected with four nmol SNP, cGMP-labelling was revealed in three distinct amacrine cell subtypes (Figure 4.13b&c). Eyes that received 10 nmol SNP along with IBMX exhibited cGMP-immunoreactivity in the above-mentioned amacrine cells as well as weak labelling in a subset of bipolar cells (Figure 4.13d). The cGMP-immunoreactivity present in the amacrine cells at lower levels of SNP was absent in retinas co-treated with 40 nmol (or 100 nmol) SNP and IBMX, while the labelling within the bipolar cells had become quite intense (Figure 4.13e).

The three sub-types of cGMP-immunoreactive amacrine cells labelled in four to ten nmol SNP-treated eyes were characterized by the locations of their somata, as well as their neurite patterns. The first type of amacrine cell was moderately abundant, with somata located in the proximal INL and one primary neurite that stratified at approximately a 30% IPL depth (Figure 4.13b). The second presumed amacrine cell sub-type had somata sparsely distributed throughout the GCL, forming one primary neurite that stratified at about 70% IPL depth (Figure 4.13b). These cells were identified as displaced amacrine cells rather than ganglion cells because their apparent lack of axons. The final subtype of amacrine cells had somata sparsely distributed throughout the central INL and formed one primary neurite. The neurites of these amacrine cells projected to

Figure 4.13. Micrographs localizing cGMP in the retinas of chick eyes treated with intravitreal co-injections of 170 nmol IBMX in sterile saline containing: **a**, 0 nmol SNP (control); **b**, 4 nmol SNP; **c**, 10 nmol SNP; **d**, 10 nmol SNP; **e**, 40 nmol SNP. Subfigures **b** and **c** demonstrate the laminae of the IPL to which the amacrine cells project and ramify, while subfigure **d** possibly shows the laminae to which the bipolar cells, amacrine cells or both project and ramify within. Abbreviations: IN, inner nuclear layer; IP, inner plexiform layer. Scale bar = 50 μm.


a
IN
IP
b
c
d
e

and usually bifurcated upon entrance to the IPL, and spread in a diffuse pattern from a 30% to a 80% IPL depth (Figure 4.13c).

The labelled bipolar cells had abundant somata in the distal INL (Figure 4.13d&e). They formed Londolt's clubs that extended to the outer limiting membrane and possessed dendrites within the OPL. Each bipolar cell extended an axon proximally to bistratify at about 55% and 75% IPL depths. Although these cGMP-immunoreactive bipolar cells shared morphological similarities with other histologically distinct subsets of bipolar cells, they did not label for other bipolar cell markers including M3, calbindin, or cellular retinoic acid binding protein (result not shown).

CHAPTER FIVE

Discussion of Experimental Results

In this study I set out to re-evaluate the claims of Fujikado *et al.* (1997) that nitric oxide is involved in the promotion of excessive ocular growth associated with FDM in chicks. Certain aspects of Fujikado's results made it necessary to determine whether the action of L-NAME was mediated through specific NOS inhibition or due to non-specific actions. I have shown here that neither pharmacologically effective doses of NOS inhibitors (doses reported here or in previous reports to inhibit NO production) nor sub-toxic doses of NO donors had an effect on ocular growth in either normal or form-deprived eyes. In fact, effects on FDM and ocular growth were observed only when the agent in question was administered at a very high dose, capable of causing non-specific actions or toxic damage to the retina.

NOS Inhibitor Effects On Ocular Growth

Clearly, inhibition of nitric oxide synthase is not sufficient to affect either the development of FDM or normal ocular growth. Even multiple injections of 9.0 µmol L-NAME (content of injected media), approximately 167% of Fujikado's minimal effective dose (concentration of injected media = 180 mM L-NAME), proved incapable of affecting FDM development. The next highest dose (16.2 µmol L-NAME) rescued eyes from FDM only inconsistently, suppressing refractive error and excessive growth in some individuals while leaving others completely unaffected. Only multiple 20 µmol injections, each resulting in a vitreal concentration five times the reported IC_{50} for this molecule (Wellard *et al.* 1995) had a uniform and significant effect on FDM.

Our experiments went on to show that two other NOS inhibitors, L-NMMA and L-NIO, were completely ineffective at influencing FDM or normal ocular development. These discrepancies between our results and those of Fujikado *et al.* (1997) and the disparity between our effective dose of L-NAME and its reported IC_{50} (Wellard *et al.* 1995) suggest that L-NAME influences FDM non-specifically. In fact, the lowest L-NAME dose reported by both studies to effectively suppress FDM (approximate vitreal concentrations: ours, 100 mM L-NAME; Fujikado's, 25.6 mM) far exceed those that have

been reported to antagonize muscarinic receptors in canine colonic smooth muscle (Buxton *et al.* 1993: 100μM). Buxton *et al* also showed that other NOS inhibitors that were not alkyl esters, such as L-NMMA and L-NIO, had no activity at mAChRs. At first, it would seem plausible that this ability to antagonize muscarinic receptors could account for L-NAME's capacity to suppress FDM, contrary to other NOS inhibitors. This notion is supported by the demonstration that mAChR antagonists prevented FDM in chicks (Stone *et al.* 1991). However, two other reports make such a mechanism action for L-NAME unlikely. First, the pharmacology of chicken muscarinic receptors (Tietje & Nathanson 1991; Tietje *et al.* 1990) is quite different from that of mammalian receptors, raising the question of whether L-NAME could still antagonize mAChRs in chicks as it does in canines (Buxton *et al.* 1993). Secondly, Fischer *et al.* (1998) showed that atropine could rescue eyes from FDM even after the destruction of cholinergic retinal pathways, suggesting that muscarinic antagonists influence myopia through either non-cholinergic or extra-retinal pathways. Consideration must also be given to the findings of Lind *et al.* (1998) that showed mAChR antagonists reduced chick scleral cell proliferation and ECM production *in vitro* despite the inability to localize mAChR or ChAT to that tissue (Fischer *et al.* 1998a), further suggesting non-specific activities for these substances. Therefore, it remains uncertain how L-NAME influences FDM development and whether or not this may involve mAChR antagonism.

The highest dose of L-NIO proved to be incapable of altering any ocular growth despite reducing the concentration of NO_x within the retina (by 69% in trial 2) and RPE (by 90% in trial 2). This reduction in NO_x levels suggests that L-NIO did inhibit NOS activity and that suppression of NO production had no physiologically meaningful effect on either normal ocular growth or FDM within the chick. However, it should be noted that while L-NIO treatment significantly reduced NO_x content in both tissues, some NO_x did remain in the samples. With this in mind, it is possible that FDM does require NO, and that L-NIO treatment is inadequate to reduce NO below the levels sufficient to affect FDM. This reasoning might explain the differences in results between this and Fujikado's study. Fujikado et al. (1997) reported that L-NAME treatment reduced retinal NO_x to levels much lower (8.45 pmol/mg) than L-NIO did in this study (63 pmol/mg). However, the method of measurement is considerably different between these two

studies. While Fujikado used retinal plugs (with RPE removed), standardizing them against the total protein content of the retinal plug, I extracted the whole retina and standardized the results against total soluble protein of the retina. Considering the unusually high content of intrinsic membrane protein (e.g. rhodopsin) in rods and cones, it is possible that the total soluble protein obtained from the retina is considerably less than the total protein content, leading to greater NO_x values per milligram in this study compared to the other. If this is the case, and both our control values are equivalent, then it is noteworthy that Fujikado's L-NAME only suppressed retinal NO_x by 21%, compared to the 69% for my study. This is consistent with the report of Wellard et al. (1995), that L-NIO is a more effective NOS inhibitor than L-NAME in chick retina.

The most plausible interpretation of Fujikado's results is that L-NAME reduces FDM through a nitric oxide-independent mechanism. L-NAME only suppressed FDM at doses that are proven sufficient to act non-specifically. Meanwhile, two other NOS inhibitors, including one that was shown to reduce NO_x levels at the doses used, had no effect on ocular development at all. It is questionable whether L-NAME's non-specific activity involves mAChR antagonism as recent studies have dismissed muscarinic involvement in FDM (Fischer *et al.* 1998b). These results indicate that decreases in NOS activity and NO availability are not sufficient to block FDM

As mentioned earlier, in this study I was able to detect a significant decrease in NO_x levels in the RPE as a result of L-NIO treatment. Fischer *et al.* (1999) demonstrated that chick RPE cells were NADPH-diaphorase positive but did not label for nNOS. RPE also did not label for eNOS (Fischer and Stell, personal communication). They suggested that either a NOS isoform not recognized by these antibodies existed or an uncharacterized fixation-insensitive non-NOS diaphorase existed within the RPE. My findings lend credence to the notion that a NOS isoform, possibly inducible NOS, may exist within the chick RPE. This is further supported by other studies showing iNOS expression with the RPE of other species (Goureau *et al.* 1993, 1995, 1997; Vodovotz *et al.* 1996; Kurenny *et al.* 1995). However, it is also possible that, under normal conditions, nitrates (as a result of NO degradation) may diffuse or be transported into the RPE where they accumulate to control levels. Studies have shown that the RPE does accumulate anions, namely chloride and carbonate (Gallemore & Steinberg, 1989a, b;

Hughes *et al.* 1984; Miller and Farber 1984), possibly as part of a mechanism to regulate ocular pressure. While the exact mechanism through which the RPE accumulates these ions is not known, it is plausible that nitrates could also be collected in a similar fashion. A significant drop in retinal NO levels would lead to decreased availability of nitrates for accumulation within the RPE, resulting in a reduction in nitrate levels within the RPE as well. Thus, with the results of this study, it cannot be determined with certainty whether L-NIO acted directly on the RPE to decrease NO_x levels or whether the effect observed in the RPE was the result of a decreased transport/accumulation of NO_x into these cells due to reduced availability from retinal sources.

NO Donor Effects On Ocular Growth

Exogenous sources of nitric oxide do not appear to affect FDM or normal ocular development in a physiologically significant manner. L-arginine and SNP only suppress such development at doses likely to induce non-specific or toxic responses.

L-Arginine was originally screened as a potential antagonist to NOS inhibitors, to test the specificity of L-NAME. Unexpectedly, it was found to suppress FDM on its own. Exogenous L-arginine should be able to act as a NO-donor (by shifting the NOS equilibrium towards NO production), raising the possibility that NO might prevent, rather than cause, FDM. This notion is supported by the results of Fujii *et al.* (1997), who showed that elevated expression of certain NOS isoforms (nNOS: mRNA; iNOS; protein and mRNA) was associated with FDM in chick retina. However, in our study, injections that resulted in vitreal concentrations of L-arginine less than 17.2 mM (content of injected media: 3.43 μmol) did not suppress FDM, leading to a calculated ED_{50} = 12 mM for L-arginine suppression of FDM. This greatly exceeds the ED_{50} for L-arginine at either its membrane transporter (ED_{50} = 140 μM for transport into murine macrophages, Bogle *et al.* 1992) or NOS (ED_{50} = 3.5 μM for eNOS in swine endothelial tissue cultures, Palmer *et al.* 1988). Although the mode of action of L-arginine against FDM remains unclear, it is noteworthy that neither the closely related amino acid L-lysine, nor the neutral agent sucrose, affected FDM reliably at an equivalent molar dose (data not shown). These other results would argue against L-arginine's imposing its influence on FDM through general amino acid, acidity or osmotic effects. L-arginine may prove to

affect FDM through an increase in NO levels, or through non-specific activities similar to those involved with L-NAME suppression of FDM.

The actions of L-arginine observed here might also be due to effects on NMDA-receptors. Previous studies have suggested that L-arginine, or its close derivatives, has the ability to interact with glutamate-gated cation channels (Marc 1997, Yang & Reis 1999). Marc reported that arginine can gain entrance to retinal neurons through these channels, while Yang & Reis suggest that 4-(aminobutyl) guanidine (also known as agmatine), a close derivative of L-arginine, blocks neuronal NMDA-receptor activity by interacting with the receptor via a moiety common to both agmatine and L-arginine. If L-arginine can act as an NMDA-receptor antagonist it is reasonable that it would suppress FDM in ways similar to other NMDA-R antagonists such as dextromethorphan, MK801, and AP5 (Fischer *et al.* 1998c).

SNP had no effect on FDM or normal ocular growth at doses less than 100 nmol. To verify that this was not due to a failure to deliver NO, we probed SNP-treated retinas with antiserum to cGMP, based on the assumption that by stimulating soluble guanylyl cyclase NO leads to an accumulation of cGMP. Injections of 4 nmol or 10 nmol SNP evoked cGMP synthesis in amacrine cells, whereas doses of 10 nmol, 40 nmol or 100 nmol SNP evoked cGMP synthesis in bipolar cells. These results indicate that all assayed doses of SNP were effective in donating nitric oxide to the retina and that SNP affected normal ocular development and FDM only at doses higher than those required to induce cGMP synthesis. This suggests that SNP and/or NO may influence ocular development via paths other than the induction of physiological levels of cGMP.

In open eyes, only the highest SNP dose was able to suppress growth, by means of drastic reductions in axial length and wet weight accompanied by blindness and abnormal light reflex. A detailed examination of these eyes showed that their retinas had suffered great damage. Possible mechanisms through which this damage occurred will be discussed later. It is clear, however, that a functioning retina and RPE are required for normal growth regulation (Raymond and Jackson 1995); a concept further supported by the fact that the destruction of these tissues by high doses of the cholinotoxin ECMA (AF64A) was also found to result in abnormally small eyes (Stell *et al.* 1997). Similarly, 1000 nmol SNP severely hindered growth of goggled eyes, leaving the retinas almost

completely destroyed and replaced by scar tissue that is heavily infiltrated with pigmented cells from the dysplastic RPE.

FDM was also suppressed by 100 nmol SNP; the deprivation-induced increases in axial length and wet weight were abolished, refraction was emmetropic, the light reflex was normal and the animals reacted to visual stimuli presented to the treated eye. Microscopic examination of these eyes showed the retinas to be much less damaged than those treated with 1000 nmoles. TUNEL and LEP-100 labelling revealed damage mainly in the photoreceptor layer. Antisera to rhodopsin and calbindin revealed that rod, and not cone, photoreceptor populations had become partially depleted and displayed shortened, disorganized and possibly swollen outer segments. Immunolabelling failed to reveal damage to other cell types within the retina. It is likely that the suppression of FDM caused by 100 nmol SNP is due to NO-induced photoreceptor damage and not some other action of NO, such as the disruption of regulatory retinal circuitry caused by other toxins (Lauber *et al.* 1990, Erlich *et al.* 1990, Westbrook *et al.* 1995, Schaeffel *et al.* 1995, Fischer *et al.* 1997).

According to a few reports, certain antibodies raised against rhodopsin also label type II single cones (Szel *et al.* 1985, Araki *et al.* 1984) in the chicken retina. Examinations of the amino acid sequences of the chicken photopigments indicate that the rhodopsin antibody (Molday & MacKenzie 1983) used in this study may cross-react with the chicken green cone opsin. The epitope to which this antibody binds is not clearly defined, and the only information available is that it binds to a region within the N-terminus between amino acids 2 and 39 of bovine rhodopsin. This region shows 82% homology to chick rhodopsin and 68% homology with chick green opsin, though, in the case of the green opsin, perfect homology seldom extended beyond 5 amino acids in a row. As the photoreceptors that were labelled by this antibody appear to be quite uniform in their morphology when examined with epi-fluorescence microscopy, it is difficult to determine with the available data whether cones were labelled as well as rods. Despite possible misidentifications of cones as rods, it still appears that the above description of rod destruction is valid although such rod damage may have been underestimated. The majority of cone photoreceptors still appear to be unaffected by the 100 nmol SNP treatment.

At this point, I am satisfied to conclude that at the highest dose administered, SNP suppresses ocular growth through a complete disruption of retinal (and RPE) function and subsequent elimination of visually guided ocular growth cues. 100 nmol SNP suppressed myopiagenesis, possibly as the result of degeneration and elimination of rod function. The mechanisms of retinal degeneration and how they might affect ocular growth are explored in following sections.

Note on Previous Studies Using SNP-Treatments

In our experiments, we utilized a wide range of SNP doses, resulting in vitreal concentrations ranging from 20 μM to 5 mM (4 nmol to 1000 nmol, respectively). At doses ranging from 20 μM to 200 μM (40 nmol), we were able to demonstrate activation of guanylyl cyclase resulting in elevation of cGMP levels. At doses of 500 μM (100 nmol) and higher, SNP was neurotoxic. This is consistent with the results from other studies that have also used SNP as a nitric oxide donor. In rat brain tissues, 10 μM SNP increased cGMP levels (Berkelmans *et al.* 1989). Similarly 100 μM SNP was shown to affect soluble guanylyl cyclase maximally in bovine rod outer segments (Margulis *et al.* 1992). SNP has been shown to inhibit depolarization-induced dopamine release in rabbit retina, an effect that saturates at 300 μM (Djamgoz *et al.* 1995). SNP continues to affect systems in reversible ways beyond these referenced concentrations, however neurotoxicity begins to also play a role. At 1 mM SNP caused remarkable neurotoxicity in cultured fetal rat retina after only ten minutes of exposure (Kikuchi *et al.* 1997). The present work represents the most comprehensive long-term study to date of SNP dose effects on the functions and structure of neural retina.

SNP Treatment and Causes of Retinal Damage

As mentioned in the previous section, a sufficiently high dose of SNP causes significant damage to the retina. This retinal toxicity presents itself in two forms; either as photoreceptor damage at 500 μM (100 nmol) SNP, or complete retinal degeneration at 5 mM (1000 nmol) SNP. It is possible that this neurotoxicity is an action of either nitric oxide (for review see Zhang and Snyder 1995) or SNP itself. Upon review of the literature, I am inclined to believe that independent mechanisms cause these two forms of

toxicity with massive retinal damage masking an underlying photoreceptor toxicity at the highest SNP dose. For this reason, I will first discuss the possible causes and mechanisms through which the massive retinal damage may have occurred, followed by an exploration of causes leading to the apparently selective destruction of rod photoreceptors.

Massive Retinal Degeneration

The retinal deterioration that occurs after exposure to 5 mM (1000 nmol) SNP is likely due to multiple mechanisms of toxicity resulting from the metabolism of SNP. As SNP consists of a ferrous ion bound to five cyanide moieties and a nitrosyl group, its metabolism can lead to the liberation of multiple molecules of cyanide (Feelisch 1998) along with the expected nitric oxide.

Cyanide Toxicity:

At modest doses, the cyanide released from the SNP molecule can be absorbed by various circulatory and cellular mechanisms. These include absorption by methemoglobin in erythrocytes (Friederich and Butterworth 1995), transsulphuration by rhodanase in mitochondria (Friederich and Butterworth 1995), and reaction with hydroxocobalamin (vitamin B12b) to form cyanocobalamin (vitamin B12) (Kulig 1991). Each of these mechanisms renders the cyanide molecule either non-toxic or nearly so. However, once these reservoirs are exhausted, cyanide toxicity can set in.

Through a reversible process, cyanide binds to the ferric iron of cytochrome oxidase, inhibiting its ability to transfer electrons from cytochrome c to molecular oxygen (Brittain & Greenwood, 1976). The result is a block of aerobic metabolism leading to metabolic acidosis, and functional anoxia. Because of the extreme sensitivity of retinal neural tissues to metabolic insult, it is expected that this should lead to severe toxicity. In fact, a study examining the effects of metabolic inhibition in retinal explants showed significant elevations of extracellular GABA, aspartate and glutamate accompanied by tissue swelling and minor deterioration after only 30 minutes exposure to 5 mM potassium cyanide (Zeevalk and Nicklas 1991). As the injections of SNP that led to

massive retinal damage in my study have the potential to deliver 25 mM cyanide, it is quite plausible that this toxicity is the direct result of cyanide poisoning.

Nitric Oxide Toxicity:

Considering the calculated rates of the known reactions involving nitric oxide *in vivo,* three main reactions can be assumed to exhaust the majority of available NO (Beckman and Koppenol 1996); 1) the activation of guanylyl cyclase, 2) sequestration of NO by oxyhemoglobin and 3) reaction with superoxide to form peroxynitrite. In the non-vascular retina of the chick it is uncertain how important the second reaction is. As SNP-induced guanylyl cyclase synthesis in the retina is limited to a small subpopulation of amacrine cells, bipolar cells and photoreceptors it is unlikely that GC could produce the widespread destruction that occurs with the highest dose of SNP. However, as a small portion of superoxide is unavoidably released during the reduction of molecular oxygen to water (in the aerobic metabolic process), it should be available in all retinal cells. This radical is highly reactive and could be quite destructive to tissues, if it weren't readily converted to molecular oxygen and hydrogen peroxide by superoxide dismutase (SOD), an enzyme that is nearly ubiquitous. However, at high concentrations, nitric oxide can outcompete SOD for the available superoxide radicals, yielding peroxynitrite (Cudd and Fridovich, 1982). This molecule is quite reactive and is capable of oxidizing lipids and nucleic acids, damaging cell membranes and modifying protein tyrosine residues (Demiryurek *et al.* 1998). While no studies have demonstrated the destructive capacity of peroxynitrite in the retina, one study has correlated its presence with retinal degeneration associated with autoimmune uveitis (Wu *et al.* 1997). Should the highest SNP dose produce large quantities of nitric oxide, it could outcompete SOD across all regions of the retina, resulting in abundant peroxynitrite, causing a massive wave of cellular oxidation and total destruction of retina.

Studies conducted within the last decade (for review see Zhang & Snyder 1995) have also uncovered many other potential targets of nitric oxide activity. Though they are too abundant to name here, many of these reactions share a common consequence of cellular metabolic inhibition. In this respect, the outcome of many of these reactions would resemble that for cyanide toxicity discussed above, and thus would largely complement it.

The presence of either peroxynitrite or cyanide in sufficient amounts is probably adequate to produce widespread retinal destruction. However, as both agents are likely present as a result of high SNP treatment, this mass retinal destruction probably can be attributed to the simultaneous actions of both these agents.

Rod Photoreceptor Degeneration

Several possible mechanisms might lead to the specific degeneration of rod photoreceptors induced by 500 μM SNP. As previously mentioned, two of the principal nitric oxide reactions are the activation of guanylyl cyclase, resulting in elevated production of cyclic GMP, and the formation of peroxynitrite by reaction with superoxides. Exogenous nitric oxide can also suppress the phagocytosis of shed rod outer segments by the RPE (Becquet *et al.* 1994), which is associated with photoreceptor degeneration (Edwards & Szamier 1977). However, the physical evidence (consistent accumulation of cellular debris between RPE and photoreceptors) that is required for this to be a feasible explanation is not present. All of these activities have the potential to cause rod photoreceptor-specific toxicity and degeneration. However, despite the specificity of the damage at this dose, cyanide poisoning cannot be completely ruled out. Below is a discussion of how these activities might lead to photoreceptor degeneration.

Cyanide Toxicity:

The photoreceptors are arguably the most metabolically active cell type in the retina. This is mostly due to the constant transport of ions out of the inner segment (Ames *et al.* 1986, 1992), and the rapid turnover of cGMP (Ames *et al.* 1992). Thus, it is conceivable that photoreceptors may be more susceptible than other retinal cells to toxicity resulting from cyanide-induced metabolic inhibition. However, the damage in this study seems to affect rod photoreceptors in preference to cones. In order for cyanide toxicity to preferentially affect rods, these cells would have to maintain a higher metabolic rate in a photopic environment, when the injections were performed. However, in these conditions rods are less responsive than cones to visual stimuli and are in a continuous state of hyperpolarization as a result of a closing of the cGMP-gated ion channels in the outer segments. It would make sense that in this state photoreceptors have lower metabolic demands, as has been suggested (Wang *et al.* 1997). The fact that

cones are still responsive to visual stimuli and thus maintain some level of dynamic cytosol ion modulation leads me to believe that cones would have a higher metabolism in light and would be more vulnerable to cyanide poisoning. As this is not what is observed, cyanide poisoning is not likely to be the causative agent in the photoreceptor toxicity associated with 500 μM SNP treatment.

Peroxynitrite-Induced Photoreceptor Degeneration:

At first consideration, the reaction product of nitric oxide and superoxide, peroxynitrite, does not seem a likely cause of the rod toxicity. The susceptibility of photoreceptors to this form of cell damage again lies in their high metabolic activity, making these cells likely to have the highest superoxide production within the retina. Thus, when exposed to abundant nitric oxide, it is within these cells that the greatest reallocation of superoxide to the production of peroxynitrite is liable to occur. Yet, as discussed above, this reasoning would suggest that cones would be more vulnerable than rods.

However, the localization of the superoxide detoxifying enzymes supports the idea that rods may have greater susceptibility than cones to peroxynitrites. While the majority of the retina is protected from oxidative damage by the activity of multiple enzymes (superoxide dismutase, peroxidases and catalase), rod outer segments appear to possess only SOD (Armstrong *et al.* 1981). This lack of diversity in protection mechanisms may allow nitric oxide to recruit more superoxide within the rods for the production of peroxynitrite, resulting in oxidative injury limited principally to the rods.

A similar mechanism has been proposed to explain the rod-specific cell damage associated with eyes of rats exposed to ultraviolet light. Oguni *et al.* (1996) demonstrated that this destruction of rod outer segments and dysplasia of the RPE was colocalized with a lack of Mn^{++} superoxide dismutase immunoreactivity and presumably an abundance of superoxides within these cells. As reaction with nitric oxide to produce peroxynitrite appears to be a major component of superoxide-associated cell toxicity (Munzel *et al.* 1997), an abundance of superoxide within the rods could scavenge any available nitric oxide, resulting in selective toxicity in those cells.

If rod photoreceptors are truly less capable than cones of disposing of superoxides, an increased availability of nitric oxide may be all that is required to result in excessive peroxynitrite production and rod-specific photoreceptor degeneration. In this light, peroxynitrite seems to be a credible causative agent for the observed retinal damage.

Cyclic GMP-Induced Photoreceptor Degeneration:

Elevated cellular levels of cGMP have been implicated in a number of retinal conditions involving the degradation of photoreceptor cells (Lolley and Farber 1976, Farber and Lolley 1977, Aguire *et al.* 1978, Sandberg *et al.* 1990, Wong 1994, Kommonen *et al.* 1996). In most cases, the pathology has appeared to be largely limited to the rod photoreceptor cells and the outer segments of these cells. Of particular relevance to SNP-induced photoreceptor toxicity is the condition incurred by retinal degenerative (rd, C3H) mice, in which the photoreceptors begin to degenerate around the eighth day after birth to a point where, around day 21, only cones remain (Carter-Dawson *et al.* 1978). Lolley and Farber (1976) demonstrated elevated cGMP within these photoreceptors at least one day prior to the initiation of cell degeneration. This increased cGMP content was later shown to result from a disabling mutation in the beta subunit of rod-specific cGMP phosphodiesterase (PDE) (Bowes *et al.* 1990, 1993, Pittler and Baehr 1991). Ulshafer *et al.* (1980) showed that treating human retinas with a cGMP analogue was sufficient to induce rod photoreceptor degeneration while sparing cones. This demonstrated that the toxicity leading to rod destruction is likely to be caused indirectly by excessive cGMP and not an immediate result of the PDE mutation itself. That study (Ulshafer *et al.* 1980) also demonstrated that rods were more susceptible than cones to excessive cGMP levels, as well as being the photoreceptors in which the dysfunctional PDE was expressed in rd mutants.

Owing to nitric oxide's ability to upregulate guanylyl cyclase activity, treating retinas with a nitric oxide donor is certain to result in greater levels of cGMP. As rod photoreceptors appear to be especially vulnerable to large increases in cGMP, a sufficiently high dose of SNP should lead to rod photoreceptor degeneration. Such reasoning does adequately explain the observed results in the most parsimonous fashion.

Cyclic GMP has been accredited with both cytotoxic and cytoprotective characteristics on a tissue-specific basis. Augmentation of cGMP has been associated with the prevention of motor neuron degeneration (Weill and Greene 1984), and protecting cells against excitatory amino acid-induced damage in cerebellar slices (Garthwaite and Garthwaite, 1998). Cyclic GMP also appears to play a role in the protective properties of nitric oxide against trophic factor-deprived PC12 cells and sympathetic neurons (Farinelli *et al.* 1996). In contrast, the accumulation of cGMP is associated with excitatory amino acid-induced cytotoxicity in cortical neurons (Frandsen *et al.* 1992, Lustig *et al.* 1992), nerve cell death in response to glutathione depletion (Li *et al.* 1997), and nitric oxide-induced endothelial apoptosis (Suenobu *et al.* 1999). Of course, heightened levels of cGMP are also linked to photoreceptor degeneration in rd mice (Lolley *et al.* 1977, Bowes *et al.* 1990).

Despite this lengthy history demonstrating cGMP's involvement in both inducing and inhibiting cellular damage, little is known about the exact mechanism by which this might occur. In the case of photoreceptors, cGMP is involved in opening cell surface cationic channels. Prolonged periods of heightened cGMP levels may lead to a pathological state of depolarization, which might result in severe energy depletion of the photoreceptors. In turn, this might induce an apoptotic fate for the energy-depleted cells (Richter *et al.* 1996). Another, more appropriate mechanism involves an excessive influx of calcium ions through the cGMP-gated channels. Various studies have indicated that large excesses of calcium can induce cell degeneration and death through numerous mechanisms involving apoptosis and other methods of cell death (for reviews see Benjamin & Berezesky 1995, McConkey & Orrenius 1997). With this in mind, the excessive influx of calcium caused by prolonged cGMP activity may be the key to the observed rod photoreceptor damage. However, this does not explain how SNP induces toxicity in rods while sparing cone photoreceptors and 'ON' bipolar cells. To complicate matters, Farber and Lolley (1976) showed that calcium levels did not increase significantly in rod photoreceptors prior to degeneration in rd mice.

An increase of cGMP seems to be the best explanation for the rod degeneration in response to nitric oxide donation. The rod-specific susceptibility to nitric oxide agrees with a cGMP-mediated mechanism, since past studies suggest vulnerability to elevated

cGMP levels is cell-type specific. One problem with this explanation is the lack of upregulation of cGMP immunolabelling in photoreceptors of eyes treated with both SNP and IBMX. It is also possible that the IBMX treatment was not sufficient to block PDE activity within the photoreceptors. It is improbable that nitric oxide could affect GC in bipolar cells as well as amacrine cells without also affecting the enzyme in photoreceptors (Koch *et al.* 1994).

All this considered, it must not be forgotten that peroxynitrite is also a favourable candidate as the causative agent of photoreceptor damage. As is the case with the massive retinal degeneration associated with 5 mM (1000 nmol) SNP treatment, rod-specific photoreceptor damage is likely to be the result of many of the above described mechanisms, likely involving both cGMP- and peroxynitrite-dependent retinotoxicities.

Retinal Damage and Form-Deprivation Myopia

How Does Massive Retinal Degeneration Suppress Myopic Ocular Growth?

When treated with 1000 nmol SNP, the retina was severely disrupted, and the RPE had become seriously dysplastic, ceasing to exist as an epithelium and infiltrating the scar that was forming in place of the retina. Not surprisingly, these eyes suffered severe ocular growth retardation, particularly in the anterio-posterior axis, while equatorial dimensions were affected to a much lesser extent. Previous studies have shown similar outcomes for ocular growth in response to massive retinal damage. Ehrlich *et al.* (1990) showed that treating eyes with tunicamycin blocked myopic ocular growth significantly only at doses sufficient to cause massive retinal toxicity. Similarly, the destruction of retinal tissue with the cholinotoxin ECMA (AF64A) produced abnormally small, hyperopic eyes (Stell *et al.* 1997). As 5mM SNP toxicity affected the growth of both occluded and undeprived eyes, it better resembles the ECMA results which also affected both ocular treatments, while tunicamycin only affected growth in form-deprived eyes.

Alternatively, the observed suppression of growth may be the result of substantial damage to the RPE. This tissue acts as a barrier between the choroid and the retina and mediates all chemical communication between them, including nutritive support for the retina and photoreceptors, and presumably the relaying any growth signals coming from

the retina to the sclera. It is likely that upon reception of growth signals from the retina the RPE releases its own signals that influence scleral growth. Should the RPE be sufficiently disrupted, it is conceivable that these RPE signals may cease to function properly, extinguishing any influence either the retina or the RPE has on ocular growth, including potential "grow" signals.

It appears that axial and equatorial ocular growth are controlled by separate mechanisms, since 1000 nmol SNP treatment had minimal effect on equatorial growth while greatly retarding axial elongation. Other studies have similarly differentiated between axial and equatorial growth behaviours (Stone *et al.* 1995, 1990, 1989, Norton *et al.* 1995, McBrien *et al.* 1993, Wildsoet and Pettigrew 1988). This suggests that axial length is regulated by retinal signals, while equatorial growth might be largely controlled by retinal-independent mechanisms. In fact, some of these retinal signals may constitute a 'go' growth signal, promoting fundal scleral growth as a part of normal retinal functioning. Thus, severe retinal destruction would abolish any retinal signals affecting axial growth and might bring this growth largely to a halt, while minimally affecting equatorial growth.

Of course, it cannot be ruled out that 5mM SNP treatment may affect tissues as peripherally located as the choroid or sclera, and may be disrupting their normal functions in such a way as to inhibit typical axial growth. However, it is hard to see how such a method could produce differential affects on axial and equatorial growth. The damage to the retina caused by this dose of SNP is sufficient to disrupt retinal function, and probably eliminates any growth-regulating signals originating there, leading to a decline in ocular growth rate.

How Does (Rod) Photoreceptor Toxicity Inhibit Myopiagenesis?

At 100 nmol SNP, FDM was suppressed in goggled eyes while photopic vision remained functional. This treatment specifically damaged rod photoreceptors, causing DNA fragmentation in photoreceptors and disrupting rod outer segments, while not affecting other retinal cell types in an obvious manner.

It makes sense that vision-guided ocular growth would be compromised in the absence of all visual stimuli. This would be the case if all photoreceptors were damaged

beyond the ability to function (Oishi and Lauber 1988, Bartmann *et al.* 1994, Weiss & Schaeffel 1993), and might be argued to be so with 100 nmol SNP if it had not been verified that treated eyes still possessed sight. With the severity of rod damage that occurs, as seen in immunocytochemical observations, it is possible that these chicks are without rod function. Yet, because of the retention of vision at photopic light levels, cones within the retinas of these chicks still appear to function.

It remains unclear how the specific elimination of rods might lead to the abolition of myopic development. They are not likely capable of relaying the high spatial or temporal frequency or defocus information that seem to be vital for normal ocular growth. Estimates and predictions have placed the maximal visual acuity of the chick to be 1.5 cycles per degree (Over & Moore 1981), four to six cyc/deg (Demello *et al.* 1992), or 12.9 cyc/deg (Ehrlich 1981). Meanwhile, the spatial frequency that best suppressed the effects of form-deprivation is 0.86 cycles per second (Schmid & Wildsoet 1997). No studies have been conducted to determine the visual acuity of the chick in scotopic conditions, however it is likely to be considerably less than that in photopic light. Regardless of rod acuity, rods have been shown to be insignificant contributors to visual information in photopic lighting conditions in which cones operate (Normann & Werblin 1974, Schaeffel *et al.* 1991). By this reasoning, loss of the rod contribution to the visual stimulus at photopic levels due to rod damage is not likely the cause of suppression of myopic ocular development. However, a number of conditions appear to affect rods almost exclusively and correlate with the development of negative refractive error. As discussed in the introductory chapter, human cCSNB involves the complete loss of rod photoreceptor function, while only marginally affecting cones (Miyake *et al.* 1986). This condition is typically accompanied by the development of myopic refractive error (Dry *et al.* 1993). Another human condition, retinitis pigmentosa, first progresses as a near-specific degeneration of rod photoreceptors (Milam *et al.* 1998), resulting in night blindness (Wong 1994) as well as myopia in a majority of cases (Sieving & Fishman 1978). It may be the case that deletion of rods, or their glutamate output, might lead to the suppression of myopia through rendering the 'ON' pathway inactive. Meanwhile, conditions such as retinitis pigmentosa, cCSNB, and retinal degeneration in rc mice

might result in myopia through making rods pathologically active, increasing glutamate release and driving the 'ON' system.

It is possible that despite retention of visual capabilities, cone function has been compromised enough to eliminate the transmission of visually regulated growth signals. However, it is hard to imagine how such a degradation without complete loss of cone-mediated vision could result in suppression, rather than potentiation, of abnormal ocular growth. In fact, studies have shown a significant positive correlation between cone dystrophies and myopic development (Sadowski & Zrenner 1997, Mantyjarvi & Tuppurainen 1989), making this postulate unlikely. Heightened cGMP within other cell types of the retina should also be considered as possible causes of myopic growth suppression. However, cGMP in amacrine cells appears to be upregulated at doses as low as 20 μM (4 nmol) SNP and in bipolar cells at 50 μM (10 nmol) SNP, without affecting FDM. These changes in cGMP levels also did not appear, at any dose below 5 mM (1000 nmol) SNP, to cause DNA fragmentation in these cells. This makes it unlikely that cGMP-induced activities or toxicities in bipolar or amacrine cells are necessary for the suppression of FDM being explored here.

Another possible mechanism leading to suppression of myopia is either the authentic involvement of nitric oxide in promoting normal ocular growth or the ability of exogenous nitric oxide to stimulate cellular machinery that itself is involved in promoting normal ocular growth. This notion is supported by the findings of Fujii *et al.* (1998) and Neal *et al.* (1998). Fujii and co-workers (1997) showed that iNOS (mRNA and protein) and bNOS/nNOS (mRNA) decreased in the retina-RPE-choroid of form-deprived eyes relative to control eyes, indicating a possible correlation between development of FDM and a decrease in NOS expression. Neal and associates (1998) found that flickering lights caused an upregulation of nitric oxide production within the rabbit retina. Vingrys *et al.* (1991) and Schwahn & Schaeffel (1997) have shown that exposing goggled chicks to flickering light greatly reduces the development of myopic refractive error and excessive axial growth, although in the case of Schwahn's & Schaeffel's study suppression of myopia was largely attributed to corneal flattening. It is possible that this increase of nitric oxide in response to flickering visual stimulus triggers some pathway that regulates and promotes normal ocular growth, the elimination of which might open

the door for myopic ocular development. However, this proposal is directly in conflict with evidence presented in other studies, as examined below.

Neal and associates (1998) used L-amino-4-phosphonobutyrate (APB), a glutamate agonist at mGluR6 receptors, to block the responses of 'ON' bipolar cells to light in rabbit, leading to an inhibition of flicker-dependent increases in retinal nitric oxide levels. When combined with the inability of either glycine or cis-2,3-piperidinedicaarboxylic acid (PDA) to block this increase in nitric oxide, the authors concluded that flicker-induced increases of nitric oxide were the result of 'ON' bipolar activity. It would follow that if the upregulation of nitric oxide due to flicker was associated with an emmetropiziing signal, then blockade of that signal should at least result in the potentiation of myopiagenesis. This is not supported by the literature. In fact, Fujikado *et al.* (1995) showed that open chick eyes treated with APB developed hyperopic refractive errors and shortened axial length, indicating a suppression of normal ocular growth opposing changes that would be associated with myopia. APB was also shown to induce hyperopic refractive error and decreases in axial growth when injected into kitten eyes (Smith *et al.* 1991). This is opposite what would be expected if flicker-induced nitric oxide release maintained normal development and its absence induced myopia. However, another study (Crewther *et al.* 1996) suggests that APB may have differential effects on normal ocular growth and FDM, as APB had no effect on the growth rate of occluded eyes, while decreasing the growth of open eyes in chicks.

Finally, the facts that FDM is not suppressed until after the maximal effects of SNP treatment on cGMP are achieved, and that this suppression is invariably accompanied by photoreceptor injury, strongly support a photoreceptor-damage-based mechanism. This mechanism is NO-specific in that nitric oxide donation has the apparent ability to damage rod photoreceptors selectively. If other, NO-independent methods led also to a discriminating effect on rods, they too should result in a blockade of myopiagenesis.

What does this knowledge accomplish?

This study is the first account of an artificially induced specific elimination of rod photoreceptors, leading to a cessation of myopic ocular development while leaving

normal ocular growth intact. This finding suggests that rods are essential in detecting the visual stimuli which induce myopiagenesis, offering a concrete link between retinal degenerative diseases which selectively affect rod photoreceptors and the myopia they commonly cause. While rods appear to play a fundamental role in myopiagenesis, they do not influence normal ocular growth in any significant way. Meanwhile, nitric oxide has been shown not to affect form-deprivation myopia in any manner short of toxic destruction of retina tissues, contrary to previous reports. Through the proper use of SNP, a new method has been established for inducing rod photoreceptor degeneration without the loss of cone function, sight, or visual regulation of ocular growth. Through this method, both mechanisms of photoreceptor degeneration and possibly subsequent regeneration can be studied and modelled.

Future Directions

L-NAME does not appear to elicit a NOS-specific effect on FDM. However, at higher doses there still remains a suppression of excessive ocular growth. The mechanism of this suppression has yet to be adequately explained. It has been suggested that this may involve antagonism of muscarinic receptors. However, recent studies have shown that muscarinic receptors may themselves play no role in visually guided ocular growth (Fischer *et al.* 1998). To determine whether L-NAME exerts its influence on FDM through muscarinic receptors, one could test L-NAME-induced FDM suppression in occluded eyes treated with quisqualic acid. Fischer *et al.* (1998) have shown this method to be an acceptable means to remove nearly all muscarinic receptors from the chick retina (this is the method that was used to disqualify retinal muscarinic receptors from playing a role in visually guided ocular growth). If the eyes maintain the ability to develop FDM that can be suppressed by L-NAME, then it is safe to say that L-NAME does not influence myopia through muscarinic receptors. With the current knowledge derived from this study, it could be concluded that L-NAME possesses the ability to suppress FDM through interactions at yet another NOS-non-specific site.

SNP has been shown to rescue ocular growth from FDM through the rod-specific degeneration of photoreceptors, without inducing the complete loss of vision. Yet, the exact means by which SNP induces this rod toxicity is not completely understood. It

should prove possible to evaluate the relative roles of cGMP and peroxynitrite in this rod degeneration. Co-treating chick eyes with 100 nmol SNP and the guanylyl cyclase inhibitor, LY83583, will demonstrate the ability of nitric oxide to induce photoreceptor degeneration in the absence of GC activity. Should rod photoreceptors continue to degenerate, it would appear that the activation of GC and subsequent increases in rod cGMP levels are not important in nitric oxide-induced photoreceptor destruction. This test can be complemented with an assay that treats the chick eyes with dibutyryl cyclic guanosine monophosphate (dbcGMP), a cGMP agonist (as used by Ulshafer *et al.* 1980), which can show whether increases in cGMP-like substances can induce the observed rod-degenerative effect. Evaluating peroxynitrite's involvement in rod degeneration could be carried out using co-treatment with SNP and various superoxide scavengers including aminoguanidine. By removing superoxides from the system, the potential for peroxynitrite production is reduced. Rescue from photoreceptor degeneration would suggest that nitric oxide induced photoreceptor degeneration through the toxic effects of peroxynitrite.

Since animals respond to visual stimuli presented to rod-degenerated eyes, it is evident that vision does still exist after 100nmol SNP treatment. However, the extent of cone receptor damage cannot be judged objectively by the methods used in this study. This would have to be evaluated in order for SNP to be used as an acceptable means of inducing rod-specific photoreceptor degeneration.

Conclusions

1. Neither augmentation nor suppression of nitric oxide availability is essential for either normal ocular growth or the development of FDM.
2. Only at doses sufficient to cause (apparently selective) rod photoreceptor damage does nitric oxide suppress FDM.
3. Our results suggest that intact rod photoreceptors may be required for the typical development of FDM, while seeming to be inconsequential to normal ocular growth.
4. At very high doses nitric oxide severely retards both normal ocular growth and myopiagenesis and completely destroys the retina, leaving only a scar.
5. Nitric oxide-induced photoreceptor damage may be due to increases in levels of reactive molecules, cyanide or peroxynitrite, or dependent on heightened levels of cGMP in rod photoreceptors.
6. SNP-induced photoreceptor damage could be a useful model for elucidating both mechanisms of damage and repair in photoreceptors, and the roles of rod pathways in ocular growth regulation.

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