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Visual induction of Fos in amacrine cells regulates ocular growth and refraction in chick

by

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Abstract

The eye depends on visually-guided mechanisms in order to develop emmetropically, or without net refractive error. In the absence of appropriate visual stimuli (form-rich) aberrant ocular growth occurs and results in an enlarged and myopic eye. Pathways that respond to growth-modulating stimuli and regulate eye growth are located primarily in the retina. The focus of this thesis was to identify retinal neurons that specifically respond to the type of visual stimuli required for emmetropization and then to modulate the activity of these cells in order to evaluate their role in ocular growth-control.

The expression of Fos, an immediate early gene product, was used as an indicator of cell activation in response to the onset of emmetropizing stimuli. A population of amacrine cells responded to a switch from diffuse to form-rich visual stimulation by upregulating Fos expression. Using antisera to specific Fos isoforms, it was found that both c-Fos and Fra-2, but not Fra-1 or FosB, were upregulated in response to form-rich visual stimulation. The onset of form vision resulted in an immediate but brief increase in c-Fos-IR and a delayed and prolonged increase in Fra-2-IR.

Antisense oligodeoxynucleotides (AODN) were used to block the expression of Fos (all Fos isoforms), Fra-2, or c-Fos in the retina in order to assess the role of these proteins in growth-control. AODN were applied to eyes exposed to diffuse or form-rich visual stimulation to test whether the development of myopia or emmetropia respectively would be affected. In order to simplify the retina, by eliminating specific populations of amacrine cells, some eyes were treated with

quisqualate (QA) prior to AODN treatment. Eyes exposed to form-rich stimuli, and treated with AODN to block Fos or Fra-2, developed mild myopia. QA-treated eyes exposed to form-rich stimuli and treated with AODN to block Fos, Fra-2, or c-Fos developed extreme myopia. The results indicate that c-Fos and Fra-2 contribute, in varying degrees, to ocular growth-restraint. In addition there is at least one QA-sensitive, but Fos-AODN-insensitive, pathway that also contributes to a growth-restraining signal.

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Abbreviations and Symbols

AMPA	Aminohydroxy methylisoxazole propionic acid
AODN	Antisense oligodeoxynucleotide(s)
AODN-Fos	Antisense oligodeoxynucleotide directed to a transcript sequence common to all fos genes
AODN-Fra-2	Antisense oligodeoxynucleotide directed to a transcript sequence specific to the chicken fra-2 gene
AODN-c-Fos	Antisense oligodeoxynucleotide directed to a transcript sequence specific to the chicken c-fos gene
AODN-Fos-SCRAM	Scrambled control for AODN-Fos
AODN-Fra-2-SCRAM	Scrambled control for AODN-Fra-2
AODN-c-Fos-SCRAM	Scrambled control for AODN-c-Fos
AP-1	Activator protein 1
AP-1-RE	Activator protein 1 response element
APB	Amino-4-phosphonobutyrate
Ca^{2+} /CRE	Calcium / cAMP response element
CaM	Calcium- calmodulin
CREB	cAMP-Response element binding protein
c-Fos	Cellular homologue of Fos protein
c-fos	Cellular homologue of Fos gene or transcript
c-Fos-LIR	c-Fos-Like Immunoreactivity
cGMP	cyclic guanidine monophosphate
Cy3	Cyanin fluorophore
D	Diopters

ENSLI	Enkephalin, neurotensin, somatostatin – like immunoreactive
FDM	Form-deprivation myopia
FITC	Fluoroscein isothiocyanate
Fos	Fos protein
fos	Fos gene or transcript
Fos-LIR	Fos-like Immunoreactivity
Fra-2	Fos-related antigen"2" protein
fra-2	Fos-related antigen"2" gene or transcript
Fra-2-LIR	Fra-2-like Immunoreactivity
GCAP	Guanylate cyclase associated protein
GCL	Ganglion cell layer
GF	Growth factor
5-HT	5-hydroxytryptamine
ICC	Immunocytochemistry
IgG	Immunoglobulin G
INL	Inner nuclear layer
IR	Immunoreactivity
MAP	Mitogen-activated protein kinase
MC	Monoclonal
mGluR-6	Metabotropic glutamate receptor subtype 6
n	number of chicks per treatment
NADPH	Nicotinamide adenine dinucleotide phosphate-diaphorase

NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
Nmol	Nanomole
nNOS	Neuronal nitric oxide synthase
OPL	Outer plexiform layer
P	Post-natal day
PB	Phosphate buffer
PBS	Phosphate buffered saline
PC	Polyclonal
PDGF	Platelet-derived growth factor
PKA	Protein kinase A
PKC	Protein kinase C
QA	Quisqualic acid
RPE	Retinal pigmented epithelium
SIE	Sis-Inducible element
SRE	Serum response element
SRF	Serum Response Factor
TH	Tyrosine hydroxylase
TX-100	Triton X-100
μl	Microliter
μm	Micrometer
VGCC	Voltage-gated calcium channels

Chapter 1

INTRODUCTION

Myopia: An important medical disorder

Myopia, or nearsightedness, is a common disorder of the visual system that afflicts greater than one quarter of the world's population (Fledeluis et al.,1983; Sperduto et al., 1983). In myopia, abnormal elongation of the eye's vitreous chamber causes the image of distant objects to be focused in front of rather than on the retina (fig. 1.1). Accommodative mechanisms of the eye are often insufficient to correct for the short-falling image and myopes require corrective lenses or refractive surgery in order to compensate for the blurred vision of distant objects. In addition, myopia is associated with increased susceptibility to sight-threatening conditions such as retinal detachment.

Myopia frequently begins in childhood and progresses through adolescence and adulthood. Twin studies have shown that there is a genetic and heritable component to myopia (Li et al., 1987; Hammond et al., 1999). In addition, conditions associated with myopia have been mapped to specific loci (i.e. Young et al., 1998a,b have mapped familial high myopia to loci 12q and 18p). Developmental myopia, however, is generally acquired as a result of prolonged visual experiences associated with reading and close work (Curtin, 1985).

Form-deprivation model of myopia:

Emmetropia is the condition of the eye in which the image of distant objects is focused on the retina with the accommodative mechanisms of the eye at rest. In order to achieve emmetropia the eye depends on visually-guided growth cues to

match the axial length of the vitreous chamber to the refractive characteristics of the lens and cornea (Wallman, 1993). Depriving the eye, and thus the retina, of necessary contrast and spatial frequencies (form) results in the development of form-deprivation myopia (FDM). Myopia can be induced in experimental animals such as the chick (Wallman, 1978), the tree shrew (Sherman et al., 1977), and monkeys (Weisel & Raviola, 1977) by goggling with a translucent occluder or by eyelid suturing.

In the chick, induced myopia results in increases in axial length, equatorial width, weight of the eye, and negative refractive error due to the backward expansion of the cartilaginous sclera. A comparison of axial length changes and scleral glycosaminoglycan synthesis with respect to light phase transitions suggests that there is a delay of approximately 6 h between the end of the light phase and the effect on scleral growth (Devadas & Morgan, 1996). Robust changes in the ocular size of a form-deprived eye develop quickly and are independent of changes in the contralateral eye, creating a valuable model in which to study developmental myopia.

In most cases a form-deprived eye can return to emmetropia if it is restored to a form-rich environment (Napper et al., 1995; Wildsoet et al., 1997)(may not be true for FDM induced in post-pubertal adolescent marmosets and monkeys (Troilo, et al., 1999; Smith et al., 1999)). This re-emmetropization is due, at least in part, to a forward expansion of the choroid (Wildsoet, 1997). In such a case the eye would have been form-deprived for several days and developed a significant amount of myopia. Thus the process of re-emmetropization due to restored form-

vision would be in response to retinal processing of positive defocus cues, similar to the development of hyperopia in response to high-power plus lens wear. It has been argued that normal emmetropization is the eye's response to and correction of small defocus errors (Wildsoet & Wallman, 1995). The application of plus lenses or minus lenses causes the eye to grow hyperopic or myopic respectively, and thus also serves as a good model for investigating ocular growth control (Schaeffel et al., 1988; Wildsoet & Wallman, 1995).

Daily interruption of form-deprivation for as little as 15 minutes is sufficient to inhibit FDM (Napper et al.,1995, 1997). In such a case the eye would have been form-deprived for no more than one day so that restored form-vision would result in clear form-vision rather than exposure to positive defocus. The restorative action of form-vision likely works through those pathways that provide contrast and spatial frequency cues to guide normal emmetropization (Grayson & McFadden, 1997; Schmid & Wildsoet 1997, Schwahn & Schaeffel, 1997).

There is much evidence to support the idea that lens-induced myopia is produced through different pathways from FDM. For example, FDM is largely unaffected by optic nerve section whereas the response to negative lens wear is greatly attenuated by optic nerve section (Troilo et al., 1987; Wildsoet & Pettigrew, 1988b; Wildsoet & Wallman, 1995). In addition –15D lens wear results in more myopia than complete form-deprivation by diffusers for the same short period of time (Wildsoet & Wallman, 1995). Even more compelling is that different subsets of cells are activated in response to restored form-vision (after short-term deprivation) than in response to defocus cues (Fischer, et al., 1999a).

Possible mechanisms of growth control: Myopia versus emmetropia:

Accommodation:

Myopia was first thought to result from mechanisms related to accommodation. Accommodation is the process by which the eye adjusts the shape of the lens (more or less curvature) in order to compensate for the distance of the viewed object (near or far respectively). It was hypothesized that accommodation or convergence, due to increased near work, caused myopia by a force created on the sclera and a resultant increase in intraocular pressure (Van Alphen, 1961; Young, 1975). This hypothesis was supported by studies showing that the progression of myopia could be slowed by the application of atropine (Gimbel, 1973; Bedrossian, 1979). Atropine is a muscarinic acetylcholine receptor antagonist, which acts at receptors in the ciliary muscles and the muscles responsible for iris constriction to block accommodation and pupil constriction. Muscarinic acetylcholine receptors also exist in the retina, RPE, choroid and ciliary body (Fischer et al., 1998a).

However, studies showed that accommodation was unnecessary for myopia to develop as myopia could be induced in chicks after blockade of the accommodative response by ciliary nerve section (Wildsoet et al., 1993; Schmid and Wildsoet, 1996). McBrien et al. (1993) showed that atropine prevented FDM in the presence of carbachol-induced accommodation or light-induced pupil constriction. In addition, Wallman et al. (1987) showed that partial occluders could result in an elongation of only the portion of the eye where form-vision was

deprived, a phenomenon neither accommodation nor increased intraocular pressure could account for.

Retinal Pathways:

In light of the evidence against accommodation-dependent mechanisms of myopia researchers began to look for other pathways through which atropine or other pharmacological agents could be acting to control growth. Much evidence was collected that supported the idea that the retina was the source of the growth-modulating signal. Myopia could be induced in eyes in which afferent and efferent retinal pathways were severed by optic nerve section (Troilo et al., 1987; Wildsoet and Pettigrew, 1988b), and in eyes in which retinal ganglion cell action potentials had been blocked by tetrodotoxin (McBrien et al., 1995).

Many pharmacological manipulations that target particular retinal pathways have been shown to influence the degree to which FDM develops. For example, dopamine agonists (Stone et al., 1989; Rohrer et al. 1993), opioid compounds (Seltner et al., 1997), basic fibroblast growth factor (Rohrer and Stell, 1994), muscarinic acetylcholine antagonists (Stone et al., 1991), antagonists to vasoactive intestinal peptide (VIP) (Seltner & Stell,1995), and antagonists to N-methyl-D-aspartate (NMDA) (Seltner et al., 1996), have been reported to reduce or eliminate FDM induction when applied intraocularly. Unfortunately, pharmacological studies are limited in specificity, as many pharmacological agents act at multiple receptor types and cell types. For example, Seltner et al. (1997) showed that naloxone, a non-specific opiate receptor blocker, prevented FDM. However, specific antagonists to opiate receptor subtypes were ineffective,

and both active and inactive forms of naloxone prevented myopia. It is likely that naloxone acts at other receptors such as glutamate-NMDA receptors or affects the release of enkephalin from ENSLI cells (Seltner et al., 1997).

Various neurotoxins, when applied to the retina, ablate subtypes of retinal neurons and alter or spare ocular growth control. For example, one week after treatment with the excitotoxin quisqualate (QA) approximately 40% of amacrine cells were ablated but ocular growth control remained intact (Fischer et al., 1998b).

Toxic doses of NMDA, kainate, and colchicine caused excessive ocular growth and a loss of response to form-deprivation (Wildsoet & Pettigrew, 1988a; Fischer et al., 1997; Fischer et al., 1999b). Treatment of the retina with tunicamycin, formoguanamine, or high doses of sodium nitroprusside caused severe photoreceptor damage and inhibited the development of form-deprivation myopia (Ehrlich et al., 1990; Oishi & Lauber, 1988; Gudgeon et al., in preparation). These results have been helpful for eliminating groups of cells likely not involved in growth control and creating a simplified retinal model in which to study potential regulators of growth control.

Visual Stimulus Requirements For Emmetropization:

Images degraded by defocus are reduced in contrast in proportion to the magnitude of defocus. The more contrast is degraded, the more one's ability to resolve spatial frequencies diminishes, beginning with the highest frequencies (Campbell & Green, 1965). It has been reported that sufficient contrast at particular ranges of spatial frequencies is required for emmetropization to occur.

Schmidt and Wildsoet (1997) restricted spatial frequencies experienced by chicks by placing them in a slowly rotating wallpapered cylinder. Wallpaper patterns were gratings of high (4.3 cycles/deg), medium (0.86 cycles/deg), or low (0.086 cycles/deg) spatial frequency. The results showed that spatial frequencies in the medium range were required for emmetropization. Schmid & Wildsoet (1997) also showed that daily interruption with restricted contrast stimuli (78%, 38%, and 9% of maximum) was effective in reducing the form-deprivation response, however, eyes stimulated with the 9% contrast were slightly more myopic (1.5D) at the end of the 10 day deprivation period. It was concluded that contrast does not specifically provide cues about defocus but must exceed a critical threshold for spatial frequency information to be detected. Similar results from Grayson and McFadden (1997) suggest that a spatial frequency of 1-2 cycles per degree is required for emmetropization (McFadden personal communication).

Given the type of visual stimulation that is required for emmetropization it is likely that the retinal neurons participating in growth control are amacrine cells. Amacrine cells are two stages downstream from the photoreceptors and are tuned to spatial and temporal features of complex visual stimuli (Werblin, 1972; Werblin & Copenhagen, 1974; Maguire et al., 1989; Sakai & Naka, 1989). Amacrine cells also express a great number and variety of transmitters and growth factors that are good candidates for growth-control regulators (Karten & Brecha, 1993). The following is a brief review of vertebrate ocular development and physiology from which an understanding of amacrine cell function can be drawn.

The Vertebrate Retina

Development:

In brief, vertebrate optic development begins at gastrulation where the involuting endoderm and mesoderm interact with the ectoderm that is destined to form the head. Head ectoderm is provided with signals that influence it to develop into lens tissue when it is in the correct position relative to the retina (Saha et al., 1989). Optic vesicles evaginate from the lateral walls of the diencephalon and induce the overlying ectoderm to differentiate into lens placodes. The optic vesicle is then reciprocally induced to invaginate and form the optic cup. The inner and outer layers of the optic cup begin to differentiate into neural retina and pigmented epithelium respectively. The neurons and glia of the neural retina differentiate into functional laminae beginning with the ganglion cells and concluding with the photoreceptors. Pleuripotent retinal progenitors are guided by progressive fate restrictions due to the changing external environment. Many neurotrophins, growth factors, hormones, morphogenetic factors, and transcription factors act to determine or bias retinal cell fate (review see Harris, 1997). The optic stalk, which connects the optic vesicles to the diencephalon, becomes the optic nerve as ganglion cell axons travel along it to make connections with the brain. For review see Gilbert (1994).

Phototransduction:

In a fully developed emmetropic eye light is refracted as it passes through the lens and cornea to fall on the retina. Photoreceptors, the first cells of the visual

pathway, have the cellular mechanisms necessary to convert the light signal to a neuronal signal via the phototransduction cascade.

Phototransduction occurs in the outer segment of the photoreceptor, a specialized region that lies adjacent to the retinal pigmented epithelium. Outer segments contain hundreds of flattened membraneous disks on which the initial steps of phototransduction take place. In the dark, photoreceptors are depolarized via the influx of Na^+ and Ca^{++} through cGMP-gated channels on the plasma membrane. These cGMP-gated channels are maintained in their open state by a high concentration of cGMP inside the cell. A dark current loop is maintained by the flow of K^+ out of voltage-gated K^+ channels in the inner segment of the photoreceptor. Under these conditions glutamate is constantly released from the synaptic terminal of the photoreceptor.

The following is a description of phototransduction in rods. Cones are very similar but differ mainly by having various cone opsins of different wavelength sensitivities to interact with 11-cis retinal. In addition, the membranous disks, on which phototransduction begins, are continuous with the plasma membrane in cones rather than pinched off as in rods. Phototransduction, in rods, begins with light initiating the isomerization of 11-cis retinal to all-trans retinal resulting in the activated Meta II state of rhodopsin. Meta II rhodopsin causes the activation of transducin via the exchange of GDP for GTP on transducin's alpha subunit. The alpha subunit dissociates from the $\beta\gamma$-subunits and interacts with, and activates phosphodiesterase. Phosphodiesterase then catalyzes the hydrolysis of cGMP to 5'GMP. Decreased cGMP levels cause the cGMP channels to close and the

photoreceptor to become hyperpolarized. Glutamate release from the synaptic terminal is thus inhibited.

As important as the phototransduction cascade is in the response to a light stimulus, equally important is the inactivation of the cascade and its return to dark-state conditions. Rhodopsin is inactivated by C-terminal phosphorylation and by the binding of arrestin. Transducin and phosphodiesterase are inactivated by the hydrolysis of GTP to GDP on transducin's alpha subunit. In addition to the hyperpolarization of the cell in response to the closed cGMP-gated channels, intracellular Ca^{++} levels drop. This decrease in Ca^{++} plays several important roles in phototransduction inactivation including activation of guanylate cyclase (mediated by GCAP proteins) to increase cGMP levels, regulation of the binding of recoverin to rhodopsin, and modulation of channel sensitivity for cGMP by calmodulin. (Reviews of photransduction include Molday, 1998; Baylor, 1997; Koutalos & Yau, 1993).

Retinal Physiology:

As early as 1892, Ramon y Cajal described in detail the retinal morphology of a variety of vertebrate species. He extrapolated much of the physiology that is being investigated today from the connections and stratification patterns of the cells he observed. The following is a generalized summary of the structure and function of the vertebrate retina. (For a review see Cajal, 1972; Kolb,1994).

The vertebrate retina is arranged in functional laminae with three nuclear layers and two neuropil layers. Beginning distally, the outer nuclear layer is comprised of the photoreceptor cell bodies and lies proximal to the retinal

pigmented epithelium. The inner nuclear layer contains somata of horizontal, bipolar, amacrine, and displaced ganglion cells. The ganglion cell layer is the most proximal nuclear layer and houses somata of ganglion cells and displaced amacrine cells. The outer and inner plexiform layers, distal and proximal to the inner nuclear layer respectively, contain the majority of synaptic connections arising from cells in their bordering nuclear layers.

Visual information traverses the retina along a basic "through" pathway comprised of photoreceptors, bipolar cells and ganglion cells and is shaped by lateral interactions with horizontal and amacrine cells. Each step shapes the output from retina to brain to reflect adequately the specific characteristics of the visual signal (i.e. temporal frequency, spatial frequency, luminance, contrast).

Cones and On/Off Pathways:

Cone photoreceptors begin the visual pathways responsible for bright light and color vision. Cones hyperpolarize in response to light stimulation and decrease levels of glutamate release via the phototransduction cascade. It is in the inner stratum of the OPL that the On and Off pathways are delineated. Hyperpolarizing cone bipolars connect by basal junctions to cone photoreceptors to begin the Off pathway. These Off-bipolars depolarize in response to glutamate stimulation via ionotropic AMPA and kainate receptor types. Depolarizing cone bipolars connect by invaginating synapses to cone photoreceptors to begin the On pathway. These On-bipolars hyperpolarize in response to glutamate stimulation at AP4-sensitive metabotropic receptors (mGluR-6) (Ueda, et al., 1997). Off-bipolars make contacts with Off retinal ganglion cell arbours in the outer portion of the inner

plexiform layer. On-bipolars make contact with On-ganglion cells in the inner portion of the inner plexiform layer. Thus, On and Off channels optimize information transfer from the retina to the brain by providing excitatory signals for both increases and decreases in light energy (Schiller, 1992).

Rods and On/Off pathways

Rod photoreceptors begin the pathway responsible for vision in dim light conditions. Like cones, rods hyperpolarize in response to light stimulation via the phototransduction cascade and decrease levels of glutamate release.

Rod photoreceptors synapse with one type of bipolar, the rod bipolar cell. The rod photoreceptor forms sign-inverting synapses with the rod bipolar primarily through the activation of a particular subset of metabotropic glutamate receptors, the mGluR-6 receptors (Ueda, et al 1997). Similar to the ON cone bipolar, the rod bipolar depolarizes in response to light. Many rod photoreceptors contact each rod bipolar, and thus much pooling of the signal occurs. In mammals it has been shown that the rod bipolar cells contact AII amacrine cells. The AII amacrines make gap junctions with ON cone-bipolar cells and make glycinergic connections with OFF ganglion cells. Thus the rod ON and OFF systems are delineated in the inner retina but still result in excitatory input to ON ganglion cells in response to light increment and to OFF ganglion cells in response to light decrement (Schiller, 1992). Ganglion cells receive input from bipolar cells and amacrine cells, often in a centre-surround organization, and transmit this information via action potentials to the brain.

Horizontal Cells

Horizontal cells reside in the distal portion of the inner nuclear layer and form ribbon-triad synapses with bipolars at invaginating cone-bipolar interactions (Stell, 1982). Horizontal cells are extensively coupled electrically and provide inhibitory feedback by releasing γ-aminobutyric acid (GABA), to act at GABA receptors A and B. This slow-sustained inhibitory (hyperpolarizing) response of the horizontal cells works to temporally sharpen the input from photoreceptor to bipolar via surround inhibition (Werblin & Dowling, 1969; Dowling & Werblin, 1969).

Amacrine Cells

Most amacrine cell bodies reside in the INL. Amacrine cells vary greatly in shape, size, stratification and function. While Cajal described over 15 types of mammalian amacrine cells, more recent estimates suggest that there are anywhere from 26 (MacNeil & Masland, 1998) to 40-50 types (Vaney, 1990). Electron microscopy and electrophysiological studies showed that amacrine cells make both pre- and post-synaptic contacts with bipolar cells, presynaptic contacts with ganglion cells, and pre- and post-synaptic contacts with other amacrine cells (Dowling & Werblin, 1969; Werblin & Dowling, 1969). Further studies showed that unlike other retinal neurons, amacrine cells are specifically responsive to complex visual stimuli (Werblin, 1972; Werblin & Copenhagen, 1974; Maguire et al., 1989; Sakai & Naka, 1989). It has been inferred from studies that use ganglion cell output as an indicator that amacrine cells can also modulate contrast-sensitivity (Smirnakis, 1997; Grzywacz et al., 1998). It has also been shown that through electrical coupling or action potentials amacrine cells can mediate antagonistic ganglion cell receptive fields (Bloomfield, 1992; Vaney, 1993; Cook &

McReynolds, 1998). Other studies report that amacrine cells can discriminate specific stimulus parameters such as wave-length (Ammermüller & Kolb, 1995a; Ammermüller et al., 1995b).

Glia

The major type of glial cell in the retina is the Müller cell. Müller cells extend radially from their cell bodies in the inner nuclear layer to the external limiting membrane and to the internal limiting membrane. They are known to serve several functions including the regulation of extracellular K^+ levels by spatial buffering currents, regulation of extracellular GABA and glutamate concentration by uptake via high affinity carriers, and the control of pH by the action of a Na^+-HCO_3- co-transport and carbonic anhydrase. Müller cells also express a number of voltage-gated and ligand-gated receptors that recognize neuroactive substances to trigger depolarization and intracellular calcium waves. (For review see Newman & Reichenbach, 1996). Microglia are present in the chick retina, however, astrocytes and oligodendrocytes are found primarily in the optic nerve fiber layer.

Strategy For Identifying Activated Amacrine Cells By Localizing Fos

A comparison of cell function with stimuli that modulate growth suggests that amacrine cells are the most likely regulators of ocular growth. It was necessary, however, to devise a way of identifying the particular amacrine cells that are responsive to stimuli that initiate increases or decreases in growth. An effective way to identify such cells is by localizing immediate-early gene products that are transcribed rapidly and briefly in activated and responsive cells.

Immediate-early genes are those genes activated first by an external stimulus, whose products may act as transcription factors to initiate the transcription of late-response genes. The activation of immediate early genes does not require *de novo* synthesis of some other protein. The induction of such genes is rapid and transient and thus useful for indicating the onset as well as duration of change in cell activity. In addition, the high transcriptional turnover rate of such genes makes them good targets for blockade with antisense oligodeoxynucleotides. An example of such a gene is *c-fos*, the cellular homologue of the viral oncogene *v-fos*, which was isolated from an osteosarcoma in mouse (Curran et al., 1984). Curran and Morgan (1985) were the first to use induction of *c-fos* as an activity indicator in PC12 cells treated with nerve growth factor (NGF). Since that time numerous investigators have used induction of *c-fos* alone or in combination with other immediate-early genes to identify cell activation (Anokhin, et al., 1991; Gudehithlu, et al., 1993; Hoffman et al., 1993; Sharp et al.,1995; Chaudhuri, 1997; Kaczmarek & Chaudhuri, 1997; for review see Herrera & Robertson,1996).

Fos Family of Immediate-early Genes

The *fos* family includes genes coding for c-Fos, FosB, Fra-1, and Fra-2 (fig. 2), and a splice variant called delta-FosB, collectively referred to as Fos (Nishina et al., 1990). The members of the *fos* family can all be upregulated by similar stimuli although separation of their temporal and spatial expression has been demonstrated (Chen et al., 1997; Sharp et al., 1990; Hoffman et al., 1993). The response characteristics of *fos-B* and *c-fos* induction are similarly rapid and transient (Muller et al., 1984; Zerial et al., 1989) whereas those of *fra-1*, *fra-2*,

and *delta-fosB* are more delayed and prolonged (Cohen & Curran, 1988, Matsui et al., 1990, Nishina et al., 1990). Of the *fos* family, only the *c-fos* and *fra-2* genes have been cloned in chicken (Fujiwara et al., 1987; Nishina et al., 1990). The regions of homology between the gene products of chick *fra-2*, chick *c-fos*, rat *fra-1* and mouse *fos B* are illustrated in figure 2 adapted from Nishina et al., (1990).

Fos proteins form dimers with members of the Jun family of immediate-early gene products. The dimers bind to the AP-1 site to either promote or inhibit the transcription of late-response genes, depending upon which of the Fos and Jun isoforms combine. Genes encoding Jun proteins that act as transcription factors include *c-jun*, *jun-B*, and *jun-D* (Hughes & Dragunow, 1995). Jun expression is characteristically upregulated for a longer period of time than c-Fos and FosB expression, similar to Fra-2 and Fra-1 (Hughes & Dragunow, 1995).

Regulation of Fos

Fos proteins are expressed in response to numerous types of cell stimulation. Stimuli that induce *fos* that are specifically relevant to this project include activation by glutamate via NMDA-glutamate receptors (Szekely et al., 1987; Das, 1997), nitric oxide (Ohki et al., 1995), dopamine D1 receptor (Young et al., 1991; Das, 1997) and growth factors (Curran & Morgan, 1985; Greenberg et al., 1985). Several intracellular signal transduction cascades involving PKA, PKC, CAM kinase and MAP kinase act to transfer the signal to the regulatory region of the *fos* gene where *cis*-acting elements, cAMP response element (Ca^{++}/CRE), serum response element (SRE) sis-inducible element (SIE), and activator protein 1

binding site (AP-1) act to control *fos* transcription. For example, stimulation that results in calcium influx via transmitter-gated or voltage-dependent ion channels targets Ca^{++}/CRE. The increase in calcium concentration causes the activation of CAM-kinase IV or cAMP and then protein kinase A. These factors phosphorylate CREB (cyclic AMP response element binding protein). Phosphorylated CREB no longer binds to the CRE upstream regulatory region of the *fos* gene and thus transcription of *fos* is initiated. See Figure 1 for a review (Curran & Morgan, 1987; Ginty, 1997; Kovacs, 1998).

Fos as an indicator of retinal activity

The first studies to use Fos expression as an indicator of active cells in retina were completed by Sagar & Sharp (1990), in which flickering light (3Hz) induced Fos expression in amacrine and ganglion cells of the rabbit. These results prompted Rohrer et al. (1995) to look for Fos expression in response to emmetropizing stimuli such as stroboscopic illumination or goggle removal in chicks. A frequency-dependent increase in Fos-like-immunoreactivity (Fos-LIR) was detected when chicks were exposed to stroboscopic light. Many of the active cells, indicated by Fos labeling, were found to be immunopositive for tyrosine hydroxylase. Rohrer et al. (1995) did not detect an increase in Fos-LIR when chicks were taken from form-deprivation to normal visual stimulation. Rohrer's results differ from those reported here. This discrepancy will be dealt with in the Discussion section. Yoshida et al. (1995) showed that flashing light induced c-fos and somatostatin mRNA in the inner nuclear layer and ganglion cell layer of rat retina. Subsequent studies in mice showed that induction of *c-fos* in retinal bipolar

cells by steady light or flashing light was absent in mice lacking mGluR-6 (Yoshida, et al. 1998). Recently, Fos expression was used to identify neurons in the chick retina that respond to optokinetic rather than stationary stimuli, with the finding that cholinergic and GABAergic circuits are responsive to movement (Araki & Hamassaki-Britto, 1998).

Many studies have shown that *fos* is activated in response to light onset and is under circadian regulation. Yoshida et al. (1993) showed that under a 12hr light /12hr dark cycle *c-fos* mRNA levels increased transiently for 30 min at light onset in the inner nuclear layer of the rat. A thirty min pulse of light in the dark period, or of dark in the light period, caused a similar increase suggesting that this increase in *c-fos* is in response to change in ambient illumination. Activation of *c-fos* in response to light onset in the rat retina can be suppressed by the nicotinic antagonist, mecamylamine, and the muscarinic antagonist, atropine, and attenuated by the NMDA receptor inhibitor, MK-801 (Gudehithlu et al., 1993). Studies in the rabbit retina showed that many nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH)-positive cells (nitric oxide producing cells) synthesize Fos proteins in response to light stimulation (Koistinaho et al., 1993b). Further studies showed that *fos* was induced in the majority of dopaminergic amacrines and one third of cholinergic displaced amacrines in response to light onset (Koistinaho & Sagar, 1995). In this case, however, MK-801 and mecamylamine did not block the light-induced Fos expression.

Various pharmacological manipulations of retinal circuits affect the expression of Fos. Injection of kainate into the rabbit eye induces Fos expression and protein

kinase C (alpha) activation in a subset of bipolar cells and induces Fos expression in a subset of amacrine and ganglion cells with a more delayed time course (Osborne & Barnett, 1992). Tetrodotoxin, applied to block action potentials in the retina, resulted in an increase in Fos induction by flashing light (Koistinaho, et al., 1993a). Fos expression was shown to be induced in Müller cells in response to excitatory amino acids (Pearlman et al., 1993) and in response to basic fibroblast growth factor (Cao et al., 1998). In turtle retina, Fos and Jun expression in amacrine and ganglion cells increased in response to excitatory amino acids but not in response to GABA (Yaqub, et al., 1995).

Fos expression has also been used to investigate cells activated during development (He, et al., 1998, Ohki, et al., 1996; Yu, et al., 1994) and during phospholipid synthesis (Bussalino, et al., 1998; Guido, et al., 1996). In addition, much attention has been given to *fos* induction in response to optic nerve crush (Robinson, 1994; Herdegen, et al., 1993), ischemic or focal injury (Otori, et al., 1997; Yoshida, et al., 1995,), photoreceptor degeneration due to constant light (Mallo, et al., 1995; Harada, et al., 1996) or in *rd* mice (Hafezi, 1998; Masana, et al., 1996; Huerta, et al., 1997; Rich, et al., 1997).

Antisense Oligodeoxynucleotides

One way of altering the expression of immediate early gene products such as Fos is to use antisense oligodeoxynucleotides (AODN). AODN are short chains of nucleic acids whose sequences are chosen to be complementary to and thus bind to a particular mRNA sequence. Translation of the targeted mRNA transcript is prevented by either physical blockade of the ribosome movement along the

mRNA or by the targeting of RNaseH to the DNA/RNA complex (Neckers, 1998; Branch, 1998). AODN 12-15 bases long readily gain access to cells without the need for vector-mediated gene transfer techniques or transgenics. The estimated specificity of a 12-mer AODN probe is greater than 95% (Falker et al.,1994). Increasing the length of the oligonucleotide does not necessarily enhance binding specificity, as more mRNA sequences may be complementary to some portion of the longer AODN strand. In addition longer chains are characteristically more toxic, and less easily taken up by the cell (Neckers, 1998; Branch, 1998). AODN 15 or 18 bases in length are commonly used as they maximize specificity and minimize toxicity and uptake problems. AODN probes are commonly designed to target the start codon but it has been reported that targeting anywhere in the sequence may be equally effective (Falker et al., 1994).

Linkage modifications (phosphorothiate, or methyl phosphonate), sugar modifications, and base modifications have been commonly used to change the properties of the antisense probe (Gerwitz et al., 1998). These modifications have proven useful for increasing the stability of the antisense probes by providing some resistance from RNases. Some of the modifications, such as the phosphorothioate linkage, more efficiently elicit the activity of RNase H (Agrawal, et al., 1990). The advantages of using modified probes are often outweighed by the prevalence of non-specific effects, increased toxicity, and activated immune responses associated with their use (Woolf, et. al, 1992; Branch, 1998; Gerwitz, et al., 1998).

Several controls are required when using AODN to block gene expression. Firstly, a scrambled probe having the same base composition as the antisense probe but random in sequence must be used to test for non-specific or toxic effects of the AODN. Such effects have been reported in many cases (Neckers, 1998; Branch, 1998). It is preferable to use a scrambled probe rather than a reverse order probe for a control as there are reports that reverse order probes can produce similar results to those of the antisense itself (Mileusnic et al., 1996). In addition, it is also important to do sequence similarity searches for a selected AODN as a match of only 13 of 15 base pairs has been reported to be 50 % effective for blockade of gene expression (Moulds, et al., 1995). Another way of testing for specificity of gene product blockade is to separately target two parts of the same gene to test whether similar results are produced (Nicot & Pfaff, 1997).

AODN have been used to block Fos expression and in some cases provide functional clues about Fos expression. In particular, a 50 ug dose of an 18-mer AODN was used to block c-Fos expression in the chick retina and provided functional clues about the contribution of c-Fos expression to the regulation of light / dark-controlled phospholipid synthesis (Guido, et al., 1996).

Objective:

The purpose of the study was to identify retinal neurons that are activated in response to stimuli required for normal ocular growth, and to test their role in growth control.

Hypothesis 1a: Circuits active during form-deprivation myopia and emmetropization can be mapped by localizing activity-dependent markers such

as immediate-early gene products, transcription factors or phosphorylated intracellular messengers.

Strategy:

Candidates for activity indicators include immediate early genes, transcription factors, and phosphorylated intracellular messengers because levels of these factors change quickly in response to stimulus changes. The candidate markers were screened immunocytochemically by localization in retinas from form-deprived eyes versus eyes switched from diffuse visual stimulation to form-rich visual stimulation. Promising candidates showed a differential expression between the diffuse versus form-rich stimulation.

Hypothesis 1b: Cells active in FDM and emmetropization will survive quisqualate treatment and still be labeled with the activity indicator (Fos).

Strategy:

QA-treated eyes were switched from diffuse to form-rich visual stimulation. The retinas were labeled immunocytochemically for Fos expression.

Hypothesis 1c: Fos isoforms (c-Fos, Fra-1, Fra-2, FosB) will be differentially upregulated in response to the restoration of form-vision.

Strategy:

Retinas of eyes switched from diffuse to form-rich visual stimulation were labeled with isoform-specific antisera and with antisera that recognize all Fos isoforms.

Hypothesis 1d: The cells identified by Fos expression in response to form-rich visual stimulation will be a subset of amacrine cells.

Strategy:

I looked for cell-type specific markers that are expressed by the Fos-labeled cells. Candidate markers include those for amacrines, displaced amacrines, bipolar, and ganglion cell subtypes. Identifying a cell-type specific marker that colocalizes with Fos may provide information about synaptic contacts (if the cell-type specific marker labels cytoplasm) or a specific neuroactive substance contained within the identified cells.

Hypothesis 2a: Intraocular injections of antisense oligodeoxynucleotides will competently block the Fos expression elicited by restoring form-vision.

Strategy:

Antisense oligodeoxynucleotides directed to a transcript sequence common to all *fos*-related genes (AODN-Fos) were injected into form-deprived eyes. Form-rich visual stimulation was restored and the resultant Fos expression was evaluated by immunocytochemical localization.

Hypothesis 2b: Chronically blocking retinal Fos expression will impair normal growth control.

Strategy:

AODN-Fos was injected daily for one week into both saline or QA-treated eyes for groups of goggled and open-eyed chicks. Ocular size, refractive error, and Fos expression were assessed at the conclusion of the experiment.

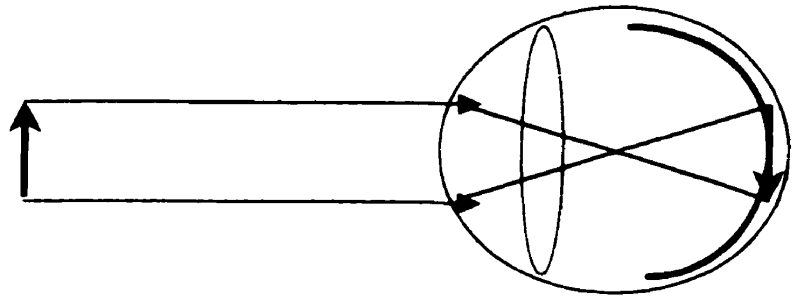
Hypothesis 2c: Antisense oligodeoxynucleotides directed to a transcript sequence specific to Fra-2, but not c-Fos, will mimic the actions of AODN-Fos.

Strategy: AODN-Fra-2 or AODN-c-Fos was injected daily for one week into saline or QA-treated open eyes. Ocular size, refractive error, and Fra-2 or c-Fos expression were assessed at the conclusion of the experiment.

Figure 1.1 Ocular characteristics of emmetropia, myopia, hyperopia. A) Emmetropia is the condition in which the eye is of appropriate length so that distant objects are focused on the retina when accommodation is at rest. The eye requires form-rich visual stimulation in order to develop emmetropically. B) Myopia is the condition of the eye in which abnormal elongation causes distant objects to be focused in front to the retina when accommodative mechanisms are at rest. Myopia can be experimentally induced by eliminating form-vision with a goggle or by applying a minus lens. C) Hyperopia is the condition in which the eye is shortened so that the image of distant objects is focused behind the retina when the accommodative mechanism is at rest. Hyperopia can be experimentally induced by applying a plus lens to the eye.

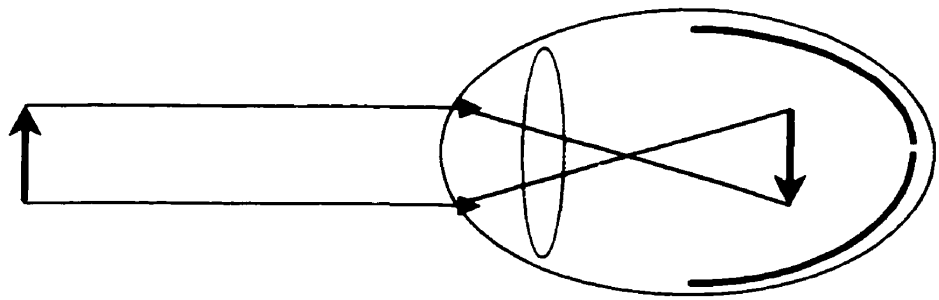
A

Emmetropia



B

Myopia



C

Hyperopia

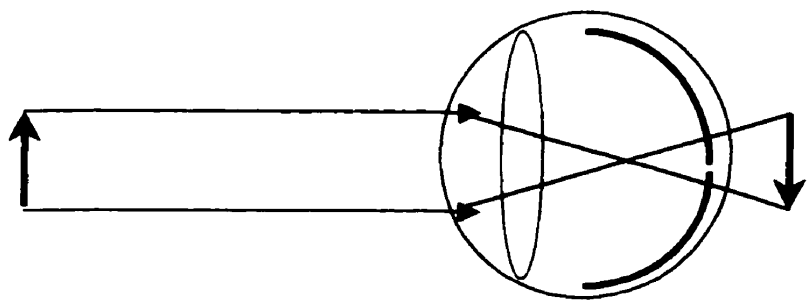


Figure 1.2: Regulation of *fos*. Intracellular signal transduction pathways and their target regulatory elements on the 5' flanking regulatory region of the *fos* gene. The regulatory elements include calcium-cAMP response element (Ca^{++}/CRE), activator protein-1 response element (AP-1-RE), serum response element (SRE), and sis-inducible element (SIE). These regulatory elements are targets of signal transduction cascades including the following factors: cAMP, protein kinase A (PKA), cAMP-response element binding protein (CREB); calcium-calmodulin-dependent kinases (CaM kinase); mitogen-activated kinase (MAP-kinase), transactivating serum response factor (SRF), ternary complex factor –ELK-1 (TCF/Elk-1); protein kinase C (PKC). These intracellular factors can be activated by cell stimulation in the form of increased intracellular calcium from voltage-gated calcium channels (VGCC) or NMDA-receptor (NMDA-R) activation, serum, growth factors (GF), platelet-derived growth factor (PDGF) (Kovacs, 1998; Ginty, 1997).

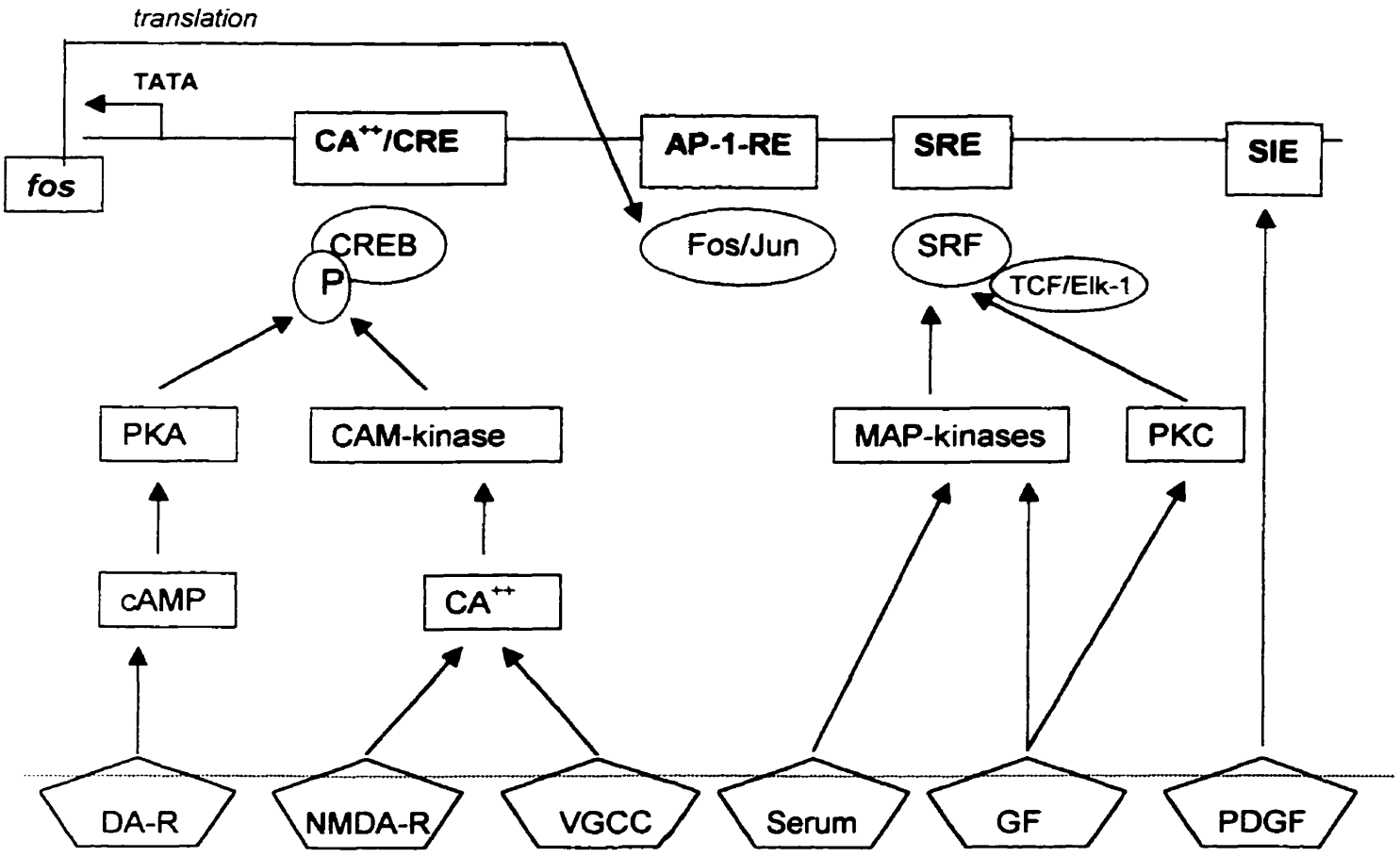
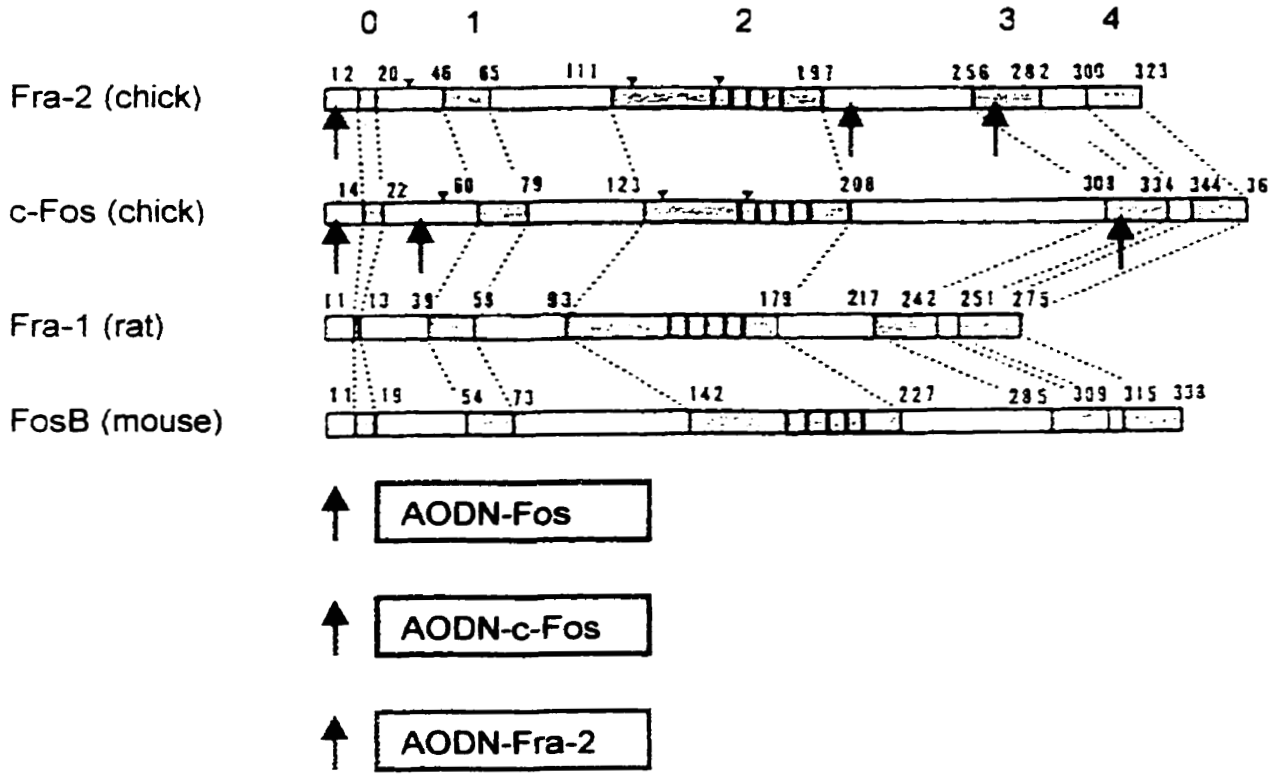

translation
TATA
fos
CA++/CRE
AP-1-RE
SRE
SIE
CREB
P
Fos/Jun
SRF
TCF/Elk-1
PKA
CAM-kinase
MAP-kinases
PKC
cAMP
CA++
DA-R
NMDA-R
VGCC
Serum
GF
PDGF

Figure 1.3: Comparison of the *fra-2, c-fos, fra-1*, and *fosB* gene products adapted from Nishina et al., 1990. Shaded regions (0 - 4) indicate regions of high homology between the gene products. Arrows indicate amino acid residues corresponding to transcript sequences targeted by AODN.


0
1
2
3
4
Fra-2 (chick)
c-Fos (chick)
Fra-1 (rat)
FosB (mouse)
AODN-Fos
AODN-c-Fos
AODN-Fra-2

Chapter 2

Materials & Methods:

Animals:

Newly hatched leghorns from Lilydale Hatcheries (Calgary, AB) were kept in a 12 hr light / 12 hr dark cycle (lights on at 07:00). Chicks were kept at approximately 25°C and given water and Purina chick starter *ad lib*. All experimental procedures were carried out in the lab at a temperature of approximately 20°C.

Switch from diffuse to form-rich visual stimuli:

P7 chicks were monocularly occluded with a contrast-degrading goggle to provide a diffuse visual stimulus. 24 hr later the contrast-degrading goggle was switched to a clear goggle of equal transmittance in order to restore form vision. Transmittance through the clear tinted goggles was 90.4% and through the contrast-degrading goggles was 88.1% of luminance observed through a goggle with the centre cut out . These measurements were made with the goggle sitting on a light source of 44000 lux and with a 1° spot luminosity meter positioned directly above the goggle. Eyes were harvested 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 24 hr, and 48 hr after goggle exchange.

Tissue fixation & sectioning:

Chicks were sacrificed by chloroform inhalation. Eyes were removed and hemisectioned equatorially, the eye cup separated from the gel vitreous, and placed into fixative (4% paraformaldehyde, 3% sucrose in 0.1M phosphate buffer pH 7.4) for 30 minutes. Eye cups were washed three times (10 min per wash) in

phosphate-buffered saline (PBS) (0.05M phosphate buffer, 195mM NaCl, 3mM NaN_3, pH 7.4), cryoprotected overnight in PBS + 30% sucrose, embedded in O.C.T. Compound (Tissue Tek, Miles Inc.), frozen in liquid nitrogen and mounted onto sectioning blocks. Sections 10 to 15 μm thick were cut, thaw-mounted onto subbed slides, air dried, and stored at -20°C.

Immunocytochemistry:

Slides were washed 3 times in PBS, then incubated overnight in the primary antiserum diluted to appropriate concentrations in antibody diluent (0.3% TX-100,+/- 0.1% NaN_3) plus 5% normal goat serum (NGS). Incubation in the primary solution was followed by three washes in PBS and incubation for 1 hr in secondary antibody diluted to an appropriate concentration in antibody diluent. The slides were washed three times in PBS, mounted in 4:1 (v/v) glycerol to water, and coverslipped. Immunoreactivity was observed with an epifluorescence microscope. Control slides were treated identically, except that the primary antiserum, or the secondary antiserum was excluded. Representative fields of view were photographed and negatives were scanned for blind assessment of cell counts. To obtain objective cell counts, all negatives were scanned in at 50% brightness, 50% contrast; contrast of the scanned image was then increased to 80% to eliminate large numbers of dimly labeled cells (Adobe Photoshop software). Only images of preparations that underwent ICC and photographic development at the same time were compared in this manner. Counting cells that fluoresced beyond an 80% contrast level made cell counts simpler and more objective. The results showed the same trends as those from counting

subjectively under the microscope. In some cases the data presented here (fig. 4.1, 4.2) represent subjective cell counts. For subjective counts only the brightest nuclei were counted and averages were taken from 30 fields of view per chick with n = 6 chicks per treatment.

Double Labeling

Simultaneous: When two antisera of different species were used to label the same retinal sections they were applied together at their appropriate dilutions for the primary incubation step. A Cy3-conjugated secondary antibody raised to one species and a FITC–conjugated secondary antibody raised to the other species were applied together at their appropriate dilutions for the second incubation step.

Sequential: When two antisera of the same species were used to label the same retinal sections the steps were as follows: primary incubation with "antiserum 1"; secondary incubation with Cy3-conjugated secondary antibody; primary incubation with "antiserum 2"; secondary incubation with FITC-conjugated secondary antibody. The process was repeated on separate sections but with the order of the primary antisera reversed. With this method it is possible to test whether "antiserum 2" labels cells in addition to those labeled by "antiserum 1", and vice versa.

Antisera:

Table 1 lists the primary antisera, including their antigens, species, type, working dilution, and source. Primary antisera to Fos proteins included those directed to a region of high homology between all Fos proteins (Riabowol et al., 1988); to mammalian Fra-1 (Riabowol et al., 1988); to mammalian Fra-2 (known

to cross-react with chicken, Santa Cruz Biotech); to a unique C-terminal portion of chicken c-Fos (Freeman & Rose, 1995); and to amino acids 79-131 of the N-terminus of mammalian Fos B (Chen et al., 1997). The specificity of the Fra-2 antiserum was tested by preadsorption with the immunizing peptide for two hours at 20°C. Fos-LIR always refers to immunoreactivity observed in tissue processed with the TF-6 antiserum that recognizes all Fos isoforms. Fra-2-LIR always refers to immunoreactivity observed in tissue processed with the Fra-2 antiserum that recognizes Fra-2 specifically. c-Fos-LIR always refers to immunoreactivity observed in tissue processed with the Eq-Fos antiserum that recognizes c-Fos specifically.

Primary antisera to markers of specific retinal cell types included those to somatostatin, vasoactive intestinal peptide, tyrosine hydroxylase, choline acetyltransferase, protein kinase C α subunit, glucagon, and parvalbumin. All of these antisera are routinely used in our lab to label distinct subsets of chick retinal neurons. The typical labeling patterns for these markers are illustrated in Fischer et al., 1998b.

Secondary antisera raised in goat to rabbit or mouse IgG and conjugated to Cy3 or FITC (Sigma) were used at working dilutions of 1:1500 and 1:150 respectively.

Antisense Oligodeoxynucleotides:

Table 2 lists the antisense oligodeoxynucleotide probes, including their sequences, the targeted sense sequences, the targeted residues, and their corresponding scrambled control probe. The antisense oligodeoxynucleotide

sequence to Fos (AODN-Fos) was chosen to target identical sequences in the chick *c-fos* and *fra-2* genes, contained within the third region of high homology of chick c-Fos, chick Fra-2, mammalian Fra-1 and mammalian FosB corresponding to residues #263 -267 of chick Fra-2 (Nishina et al.) and residues #315-319 of chick c-Fos. The antisense oligodeoxynucleotide sequences to Fra-2 (AODN-Fra-2^A and AODN-Fra-2^B) were chosen to target unique sequences in the chick *fra-2* gene corresponding to residues #205-209 and residues #8-12 of chick Fra-2. The antisense oligodeoxynucleotide sequences to c-Fos (AODN-c-Fos^A and AODN-c-Fos^B) were chosen to target unique sequences in the chick *c-fos* gene corresponding to residues #35-39 and residues #1-5 of chick c-Fos.

Sequences described as unique consist of a sequence of nucleotides that correspond to a region on the protein not highly homologous to other Fos proteins (as per Nishina et al., 1990) and do not show a high probability of targeting non-Fos mRNAs (as per Genbank sequence similarity searches). The criteria for accepting a target sequence were a) there were no sequences with similarity greater than 13/15 nucleotides, b) there were no similar sequences present corresponding to a protein known to be involved in retinal functioning (i.e. one sequence was eliminated for having a similarity to a sequence found in rhodopsin). Final sequences were selected with preference given to those that have a high G-C content but without more than 4 G-C pairs in a row (described as favorable for stability by Sczakiel, 1997; Gerwitz et al., 1998).

Scrambled probes consisted of a mixed arrangement of the antisense probe nucleotides with no more than 4 G-C pairs in a row. Sequence similarity searches

were also performed for scrambled probes with the same criteria for acceptance as antisense probes.

AODN probes were injected intraocularly at various doses (dissolved in 20 μL sterile saline) and at various times prior to goggle exchange to determine the optimum conditions for injection. Chicks were anesthetized with 1.5% halothane in 50% N_2O and 50% O_2 prior to injection. Twenty-five μL Hamilton syringes with 26 gauge needles were used to inject through the upper eyelid and dorsal coats of the eye into the vitreous chamber.

Ocular growth control experiments:

On the seventh day after hatching (P7) chicks were monocularly injected with 0 or 200 nmol of quisqualate (QA) in 20 μL of saline. At P14 half of the saline-treated group and half of the QA-treated group were subjected to six daily injections of AODN-Fos. The other half of each group was subjected to six daily injections of saline. This experiment was completed twice each for AODN-Fos in open eyes, AODN-Fos in goggled eyes, ODN-Fos scrambled probes in open eyes, AODN-Fra-2 in open eyes, ODN-Fra-2 scrambled probes in open eyes, AODN-c-Fos in open eyes, and ODN-c-Fos scrambled probes in open eyes. Eyes were assessed for refractive error with a streak retinoscope, ocular dimensions with digital calipers, and wet weight with a digital scale. At the conclusion of each growth control experiment at least two treated eyes from each of the following groups were fixed, sectioned, and processed for immunocytochemistry by the antisera specific to the Fos isoform(s) targeted by the probe used: QA/saline, QA/AODN, QA/SCRAM. The central regions of 4 sections of each eye were

examined for immunoreactivity. In each case all sections from the QA/saline and QA/SCRAM groups showed low levels of immunoreactivity qualitatively consistent with open eye levels for the targeted isoform. In each case all sections from the QA/AODN group showed no immunoreactivity for the targeted isoform. Unfortunately, only two animals per group were observed and immunoreactivity for isoforms other than the targeted isoform was not assessed.

Table 2.1: List of antibodies and antisera including their antigens, species, type, working dilution, and source. PC = polyclonal; MC = monoclonal

Antigen	ID	Species/Type	Working Dilution	Source
Fos	TF-6	Rabbit/PC	1:5000	Dr. K. Riabowol
Fra-1	Fra-1	Rabbit/PC	1:5000	Dr. K. Riabowol
Fra-2	SC-52	Rabbit/PC	1:1000	Sigma
FosB	N79-131	Rabbit/PC	1:400	Dr. Y. Nakabeppu
c-Fos	Eq-Fos	Rabbit/PC	1:10000	Dr. P. Sharp
Somatostatin	S-10	Rat/MC	1:300	Dr. A. Buchan
Vasoactive intestinal peptide	VP31	Rat/MC	1:80	Dr. A. Buchan
Tyrosine hydroxylase	#16	Mouse/MC	1:50	Hybridoma Bank
Choline acetyl-transferase	1465	Rabbit/PC	1:800	Dr. M. Epstein
PKC α-subunit	RPN536	Mouse/MC	1:50	Amersham
Parvalbumin	αPA	Mouse/MC	1:1600	Sigma
Glucagon	8305034	Mouse/MC	1:400	Dr. M. Gregor

Table 2.2: List of antisense oligodeoxynucleotide probes, including their sequences, the targeted sense sequences, the targeted residues, and their corresponding scrambled control probe. Refer to fig. 1.2 where arrows indicate amino acid residues corresponding to transcript sequences targeted by AODN.

AODN Probe	Sense 5' - 3'	Targeted	Scrambled Sequence
	AODN 5' - 3'	Residues	5' – 3'
AODN-Fos	GTGGTGACCTCGACA	#263-267 c-Fos	ACTCAGCAGCGTCTG
	TGTCGAGGTCACCAC	#315-319 Fra-2	
AODN-Fra-2[A]	AGCCTCCAGAGCGTT	#205-209 Fra-2	CGAGTCGACTCGTGA
	AACGCTCTGGAGGCT		
AODN-Fra-2[B]	AGCTTCGACACCTCC	#8-12 Fra-2	GTGAGTGAGTGGACC
	GGAGGTGTCGAAGCT		
AODN-c-Fos[A]	GACTCCTTCTCCAGC	#35-39 c-Fos	TCAGAGAGAGCGTGG
	GCTGGAGAAGGAGTC		
AODN-c-Fos[B]	GCCCTGGTACATCAT	#1-5 c-Fos	GACAGGTCAGTCGAT
	ATGATGTACCAGGGC		

Chapter 3

Results

Part 1: The Localization Of Cells Responsive To Form Vision

An emmetropizing stimulus induced Fos expression

Antisera to Fos and other candidate activity indicators were tested on retinas exposed to various changes in stimuli (deprived of form-vision, restoration of form vision, light adapted, dark adapted). Several of the candidates, including cGMP, zif268, and pERK, showed differential expression between a new stimulus and the control condition, but the most distinct and observable difference was in Fos protein expression. Fos expression refers to the sum of all Fos-isoform immunoreactivities.

Seven-day old chicks were treated with various stimuli for short durations in order to compare Fos expression in response to restored form-vision with expression after other types of visual stimulation. In an open eye, after 5 hrs of light exposure, low levels of Fos expression were observed with few cells labeled in the amacrine cell layer and the ganglion cell layer (fig. 3.1a). Goggle application itself resulted in a small increase in retinal Fos-LIR but the increase in expression was not evident 24 hr after goggle application. The number of Fos-LIR cells labeled at the end of a 24 hr goggling period was less than that of normal open eye levels (fig. 3.1b). The contrast-degrading goggle was applied for 24 hrs prior to goggle switching. The onset of form-rich visual stimulation, via isoluminant goggle switching, resulted in an increase in both the number of Fos-LIR cells and the labeling intensity. The increased Fos-LIR was observed in

nuclei in the proximal half of the inner nuclear layer (INL) and the ganglion cell layer (GCL) (fig. 3.1c). According to their location the former were probably amacrine cells and the latter displaced amacrine cells or ganglion cells. A separate group of chicks was treated with 200 nmol of quisqualate (QA) seven days prior to goggle application. An increase in Fos-LIR was again localized to the proximal INL and the GCL (fig. 3.1d). Although there were fewer Fos-LIR cells in the QA-treated retina than in the untreated goggle-switched retina, labeling intensity was comparable. In all cases goggle exchange occurred 4 to 6 hours after light onset so that the effects of circadian rhythm or environmental light/dark cues would be standardized. In addition, all chicks (open eye, goggled, goggle exchange) were harvested within one hour of one another, 6 to 8 hours after light onset.

Both the Fra-2 and c-Fos isoforms were upregulated in response to restoration of form-vision.

The antiserum used for labeling cells activated in response to the onset of form-rich stimuli (fig. 3.1) was raised to a region highly conserved between Fos isoforms (Fra-1, Fra-2, FosB, c-Fos) (Riabowol et al., 1988). Using antisera specific to each Fos isoform we screened for immunoreactivity in chick forebrain or gut, chick retina (newly hatched, or at P7 with no treatment), and chick retina in response to goggle-switching. All of the antisera, except that to Fra-1, produced nuclear labeling in at least one of these chick tissues. Only the antisera to Fos, Fra-2 and c-Fos produced retinal labeling in response to the goggle exchange (fig. 3.2). In all cases goggle exchange occurred 4 to 6 hours after light

onset so that the effects of circadian rhythm or environmental light/dark cues would be standardized.

Sequential labeling with antisera to Fos and then Fra-2 showed that all of the Fra-2 expressing cells are positive for Fos (fig. 3.3a). Sequential labeling with antisera to Fra-2 and then Fos showed that there is a small group of cells that are Fos-positive but not Fra-2-positive, and are thus most likely c-Fos expressing cells (fig. 3.3b).

Fra-2 and c-Fos are differentially upregulated in response to restored form-vision.

In order to determine the time course for upregulation of the Fos proteins, the chicks were binocularly goggled for 24 hr and then the right contrast-degrading goggle was exchanged for a clear goggle for various lengths of time. For this experiment goggle exchange was performed at light onset so that the period of restored form vision was completely within the light phase for all groups except the chicks exposed to 24hr of restored form-vision. The cell counts presented show the difference in immunoreactivity between treated and control chicks, quantified objectively from digitized images, in order to control for light-cycle variations in Fos expression. Fos-LIR, c-Fos-LIR, and Fra-2-LIR were localized by specific antisera on separate sections of the same chicks (n=6 per treatment).

After goggle exchange the amount of increase in Fos-LIR was detectable at 15min, reached a maximum at 4hr, and returned to control levels by 12hr (fig. 3.4 red). After goggle switching the amount of increase in Fra-2-LIR was detectable after 1hr , reached a maximum at 5hr, and returned to control levels by 12hr (fig. 3.4 blue). The increase in c-Fos-LIR in response to goggle exchange was

detectable after 15 min, reached a maximum at 30 min, and returned to control levels at 2 hr (fig. 3.4 green). Thus, in response to form-rich visual stimulation c-Fos is expressed rapidly and briefly whereas Fra-2 expression is delayed and prolonged. The sum of the c-Fos-IR and Fra-2-IR cells well approximates the number of Fos-IR cells. The antiserum to Fos recognizes both c-Fos and Fra-2 and thus the upregulation of total Fos-LIR reflects both the immediate, brief response of c-Fos and the delayed, prolonged response of Fra-2.

The cells that show increased Fos expression in response to form-vision are not immunoreactive for tyrosine hydroxylase (TH) or a number of other cell-type specific markers.

Previous studies have shown that Fos is upregulated in TH-IR cells in response to an emmetropizing stimulus (stroboscopic illumination) (Rohrer, et al., 1995). The cells activated in response to restored form-vision are not positive for TH (fig. 3.5). In addition, several other cell-type specific markers were tested to see whether they would colocalize with Fos. Antisera to somatostatin, vasoactive intestinal peptide, parvalbumin, choline acetyltransferase, and PKC α-isoform, did not label the subset of Fos-positive amacrine cells that are activated in response to goggle removal. Although we have not found a cell-type specific marker that identifies these activated cells, many remain to be tested.

Form-vision induced Fos expression is mediated through NMDA receptors

The increase in Fos expression in response to form-vision is sensitive to NMDA receptor blockade by MK-801 (fig. 3.6). This indicates that the cells of interest possess NMDA receptors through which activation of Fos is initiated.

Figure 3.1: An emmetropizing stimulus induces Fos expression. a) Control: Fos-LIR in the retina of a P8 chick left with an open eye. b) Control: Fos-LIR in the retina of a P8 chick after 24 hr of contrast-degrading goggle wear. c) Fos-LIR in the retina of a P8 chick after 24 hr of contrast-degrading goggle wear followed by 2 hr of clear goggle wear. Switching from diffuse visual stimuli to form-rich visual stimuli resulted in increased levels of Fos protein in cells in the amacrine cell layer. d) Fos-LIR in the retina of a P15 chick after treatment with 200nmol QA at P7, 24 hr of contrast-degrading goggle wear, followed by 2 hr of clear goggle wear. Scale bar = 50 μm. From top to bottom the arrows indicate the outer border of the inner nuclear layer, inner border of the inner nuclear layer, and the outer border of the ganglion cell layer.

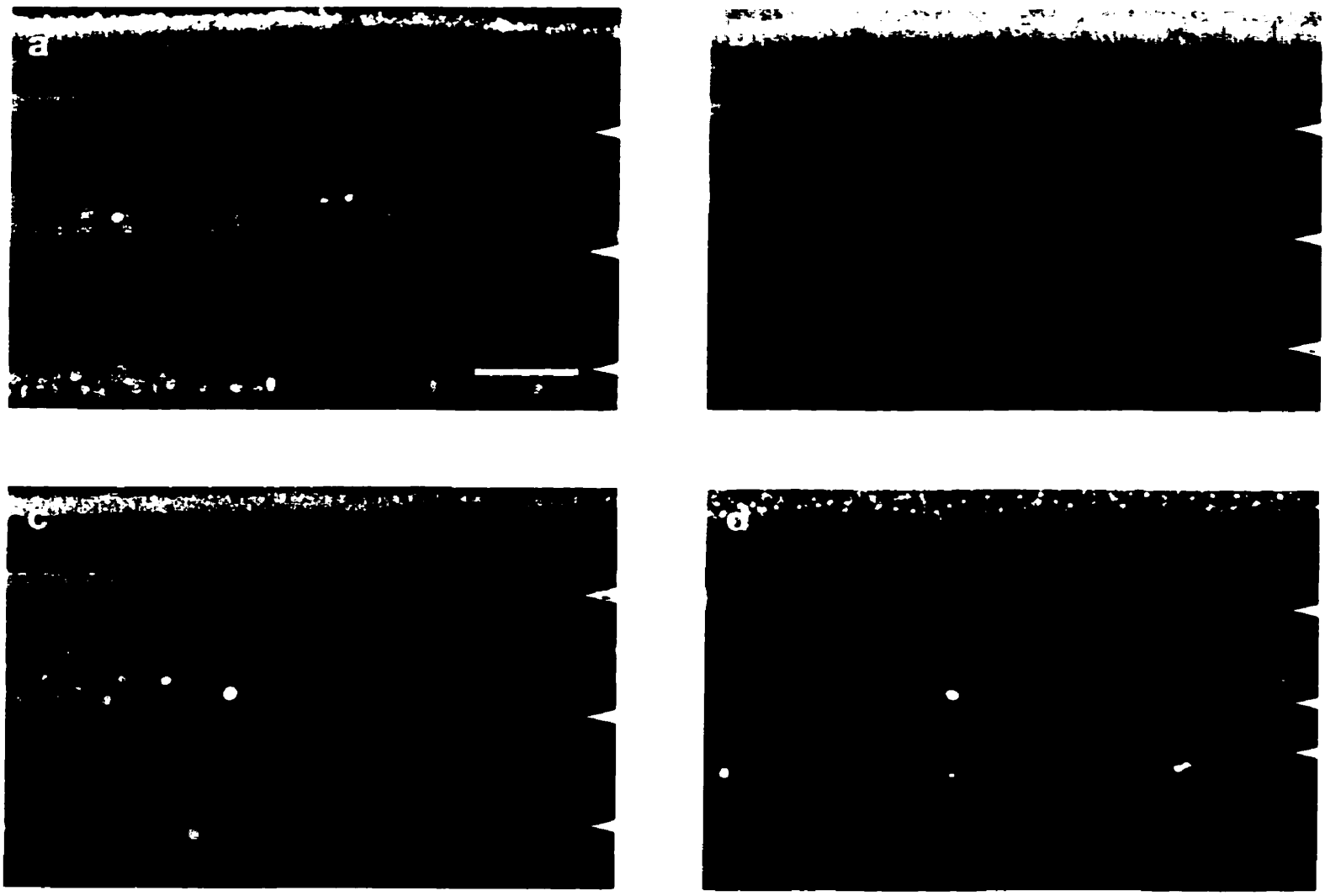

a
b
c
d

Figure 3.2: Both the Fra-2 and c-Fos isoforms are upregulated in response to restored form-vision. Immunolocalization of c-Fos, Fra-1, Fra-2, and FosB in a) chick forebrain or gut, b) chick retina after 24 hr of contrast-degrading goggle wear followed by 2 hr of clear goggle wear. Only the antisera to chick Fra-2 and c-Fos produced increased labelling in response to the onset of emmetropizing stimuli. Scale bar = 50 μm.

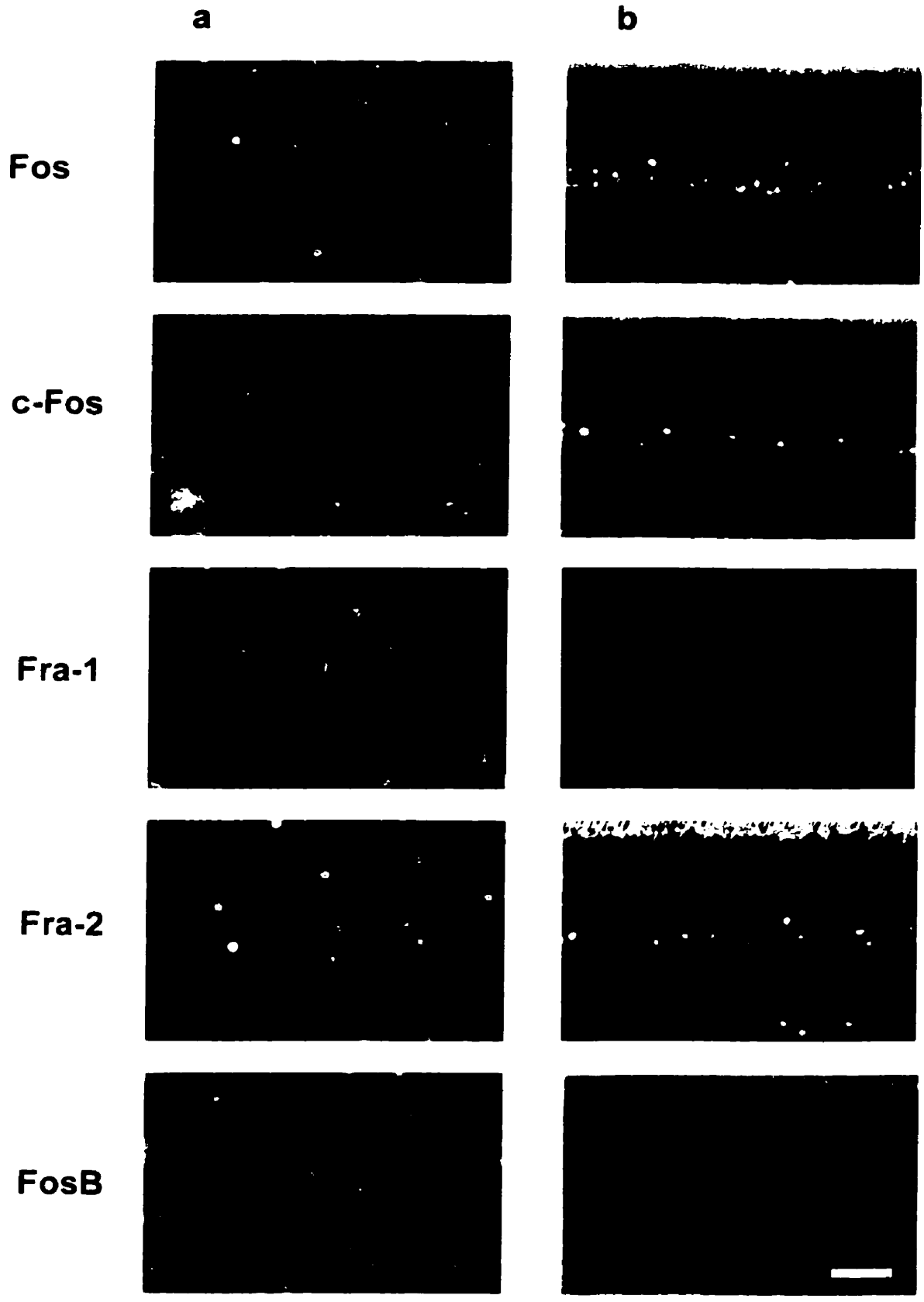

a
b
Fos
c-Fos
Fra-1
Fra-2
FosB

Figure 3.3: Colocalization of Fos-LIR nuclei and Fra-2-LIR nuclei illustrated by means of sequential labeling. a) Fra-2-LIR (red) (αFra-2 applied first) and Fos-LIR (green) in the retina of a P8 chick after 24 hr of contrast-degrading goggle wear followed by 2 hr of clear goggle wear. Cells labelled green in b) Fos-LIR (red) (αFos applied first) and Fra-2-LIR (green) in the retina of a P8 chick after 24 hr of contrast-degrading goggle wear followed by 2 hr of clear goggle wear. Cells that are labelled yellow or orange are immunoreactive for both the first and the second marker. Cells labelled green are immunoreactive for the second marker only. Scale bar = 50μm. . From top to bottom the arrows indicate the outer border of the inner nuclear layer, inner border of the inner nuclear layer, and the outer border of the ganglion cell layer.


a
b

Figure 3.4: Fra-2 and c-Fos are differentially upregulated in response to restored form-vision. Number of Fos-LIR (red), Fra-2-LIR (blue), c-Fos-LIR (green) nuclei per field in a treated eye minus the number in a control eye view (mean ± SD) at various lengths of time after goggle switch. Treated eyes were form-deprived for 24 hr and then restored to form-vision for various lengths of time. Control eyes were form-deprived for 24 hrs plus the duration of the treated eye's restored form-vision. Fos-LIR, c-Fos-LIR, and Fra-2-LIR were localized by specific antisera on separate sections. n=6 per interval of restored form-vision. Cell counts were obtained by objectively counting digitized images.

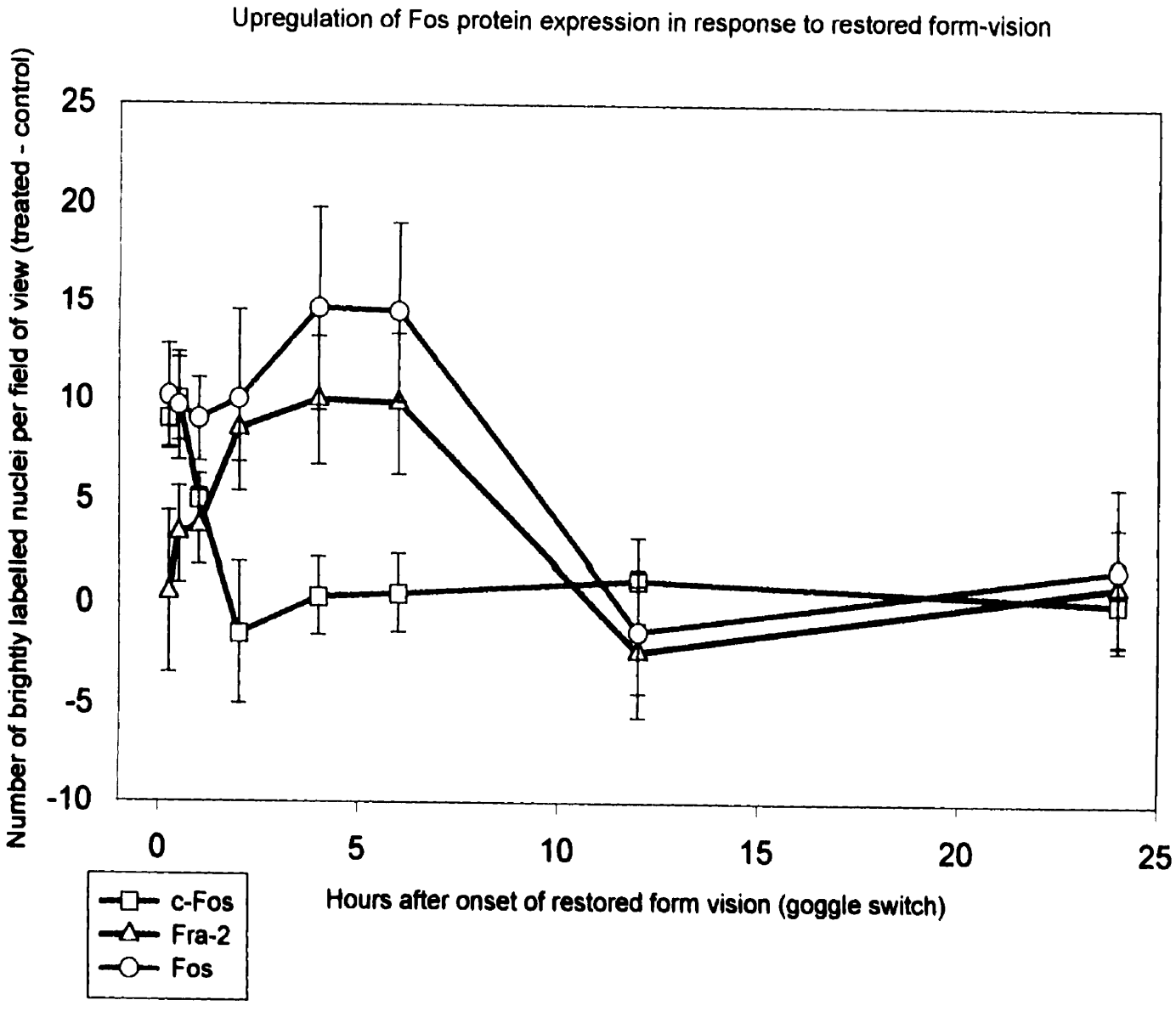

Upregulation of Fos protein expression in response to restored form-vision
Number of brightly labelled nuclei per field of view (treated - control)
25
20
15
10
5
0
-5
-10
0
5
10
15
20
25
Hours after onset of restored form vision (goggle switch)
c-Fos
Fra-2
Fos

Figure 3.5: The cells that show increased Fos expression in response to form-vision are not immunoreactive for tyrosine hydroxylase (TH). Fos-LIR (green) and TH-IR (red) in the retina of a P8 chick after 24 hr of contrast-degrading goggle wear followed by 2 hr of clear goggle wear. Goggle exchange was performed 5 hrs after light onset. Scale bar = 50 μm. From top to bottom the arrows indicate the outer border of the inner nuclear layer, inner border of the inner nuclear layer, and the outer border of the ganglion cell layer.

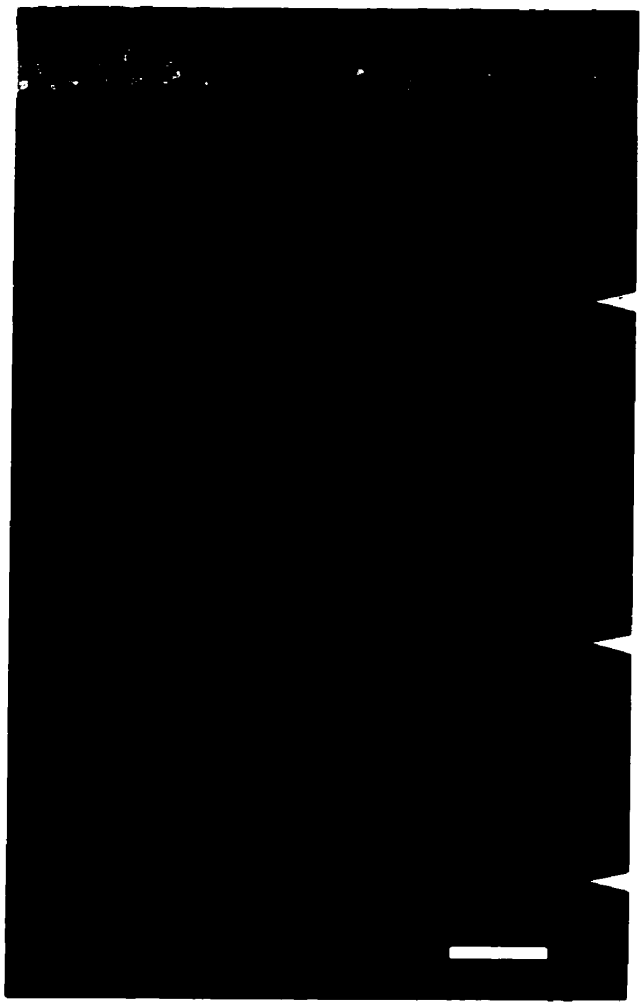
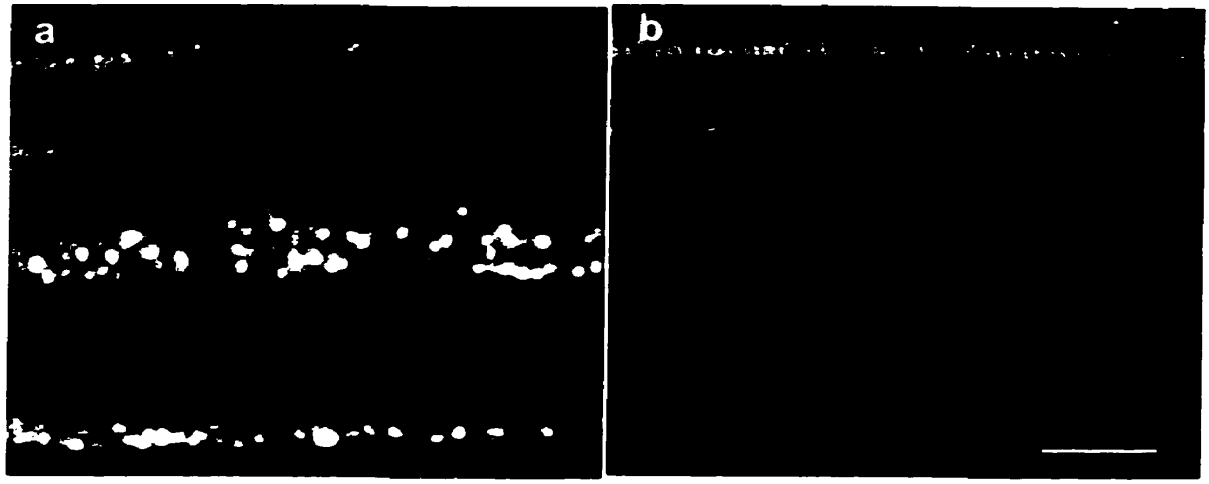


Figure 3.6: Form-vision induced Fos expression is mediated through NMDA receptors. a) Fos-LIR in the retina of a P8 chick after 24 hr of contrast-degrading goggle wear followed by 2 hr of clear goggle wear. b) Fos-LIR in the retina of a P8 chick injected with 40nmol MK-801 2 hr prior to goggle exchange. Goggle exchange was performed 5 hrs after light onset. Scale bar = 50 μm. From top to bottom the arrows indicate the outer border of the inner nuclear layer, inner border of the inner nuclear layer, and the outer border of the ganglion cell layer.


a
b

Chapter 4

Results

Part 2: Testing Whether Fos-LIR Cells Are Necessary For Ocular Growth Control

Intraocular injections of AODN-Fos block Fos protein expression.

Increases in Fos-LIR in response to the onset of emmetropizing stimuli encouraged us to investigate whether or not the responsive cells were necessary for growth control or merely indicators of activity in growth-control mechanisms. The simplest way to test this was first to see whether Fos expression in the activated cells was necessary for ocular growth control. This was done by chronically blocking Fos expression with daily injections of AODN under conditions in which eyes would otherwise grow to be emmetropic or myopic.

In order to determine the optimum dose of antisense oligodeoxynucleotides to block Fos (AODN-Fos), goggled chicks were injected with various doses of AODN-Fos and then ungoggled 12 hr after injection. Chicks were sacrificed and eyes were harvested 2 hr after ungoggling. Greater doses of AODN-Fos resulted in lower amounts of Fos-LIR to an average 85% reduction in FOS-LIR at the highest dose (fig. 4.1). Data points for 20 and 40 nmol were significantly different from those for control (0 nmol AODN-Fos) at $P<0.05$ with a multiple comparison test. The 20 nmol dose was chosen for all subsequent experiments as its effect on Fos-LIR was not significantly different from that of the 40 nmol dose.

In order to determine the optimum time before ungoggling, and the appropriate frequency of injections, chicks were injected at various lengths of

time prior to two hours of ungoggling. The results showed that the AODN-Fos was maximally effective 18 to 24 hr after ungoggling and that daily injections should be sufficient to reduce Fos protein levels for growth experiments (fig. 4.2).

For all subsequent experiments chicks were treated with 20 nmol daily injections. Figure 4.3 shows that treatment with 20 nmol AODN-Fos 18 hrs prior to goggle removal resulted in an average 85% reduction in Fos-LIR as compared to control.

Chronic administration of AODN-Fos blocks Fos protein expression and results in excessive ocular growth in open eyes.

Eyes treated with saline at P7 and then saline from P14-P21 developed as normal emmetropic eyes with appropriate ocular size and weight, and no net refractive error. Eyes treated with saline at P7 and then AODN-Fos from P14-21 were significantly larger, heavier, and more myopic than their respective controls (significant at $P<0.01$ with a Student's independent t-test). Eyes treated with QA at P7 and then saline from P14-P21 developed emmetropically with appropriate ocular size and weight, and no net refractive error. The eyes treated with QA at P7 and then AODN-Fos from P14-21 were significantly larger and more myopic than their control group ($p< 0.01$ with an independent Student's t-test) (fig. 4.4 a,b,c). In addition, the eyes pretreated with QA at P7 prior to AODN-Fos treatment were much larger, heavier, and more myopic than the group treated with saline at P7 and AODN-Fos from P14 –21. AODN-Fos competently blocked Fos expression to the completion of the experiment as shown by reduced Fos-LIR at P21 (fig. 4.5).

The experiment was repeated using scrambled ODN-Fos probes. All eyes treated with scrambled probes developed emmetropically with appropriate ocular weight and length, as did their controls (fig. 4.6 a,b,c). Scrambled probes did not result in decreased Fos expression at the completion of the experiment (fig. 4.5c). Thus chronically blocking Fos expression in open eyes with AODN-Fos caused myopia to develop in eyes that would otherwise have been emmetropic.

Chronic administration of AODN-Fos does not affect the development of FDM in goggled chicks

Eyes treated with saline or QA at P7, goggled and treated with saline from P14-21 developed significant myopic refraction with increased weight and axial length as expected. Groups treated with either QA or saline at P7, goggled at P14, and treated with AODN-Fos from P14-21 developed similar amounts of refractive error to their control groups. Student's t-tests showed no significant difference between the AODN-Fos treated groups and their controls for refractive error, axial length, or weight (fig. 4.7 a,b,c). Thus, treatment with AODN-Fos does not affect the development of FDM.

Chronic administration of AODN-Fra-2 blocks Fra-2 protein expression and results in excessive ocular growth in open eyes

Since the main Fos isoform induced by goggle switching is Fra-2, AODN specific for Fra-2 may be expected to cause excessive growth in open eyes similar to the effect of AODN-Fos. The effects of AODN-Fra-2 on ocular growth control were comparable to those of AODN-Fos. Daily application to open eyes resulted in increased ocular growth. Daily application to open QA-treated eyes

resulted in very large and myopic eyes. The result was very much the same for each of the two AODN-Fra-2 probes designed to target different regions of the *fra-2* transcript (fig. 4.8 a,b,c,). Fra-2 expression remained attenuated at the conclusion of the experiment as shown by reduced Fra-2-LIR at P21 (fig. 4.9). Daily application of scrambled probes consisting of the same base composition as either AODN-Fra-2^A or AODN-Fra-2^B did not result in the development of myopia, increased axial length or increased weight (fig. 4.10 a,b,c). Scrambled probes did not result in decreased Fra-2 expression at the completion of the experiment (fig. 4.9c).

Chronic administration of AODN-c-Fos blocks c-Fos expression and results in excessive ocular growth in open eyes treated with QA.

Daily application of AODN-c-Fos^A or AODN-c-Fos^B did not produce any myopia in eyes treated with saline at P7. Daily application of AODN-c-Fos into eyes treated with QA at P7 produced negative refractive error, increased axial length and increased weight (significant at $p<0.01$ with an independent Student's t-test) (fig. 4.11 a,b,c). The changes in refractive error, length and weight in response to AODN-c-Fos application were significantly less than those achieved with application of AODN-Fos or AODN-Fra-2 (significant at $p<0.01$ with an independent Student's t-test). c-Fos expression remained attenuated at the conclusion of the experiment as shown by reduced c-Fos-LIR at P21 (fig. 4.12). Daily application of scrambled probes consisting of the same base composition of either AODN-c-Fos^A or AODN-c-Fos^B did not result in the development of myopia, increased length or weight (fig. 4.13, a,b,c). In addition, scrambled

probes did not result in decreased c-Fos expression at the completion of the experiment (fig. 4.12c).

Figure 4.1: Dose-response function for amount of injected AODN-Fos versus the number of Fos-LIR nuclei per field of view (mean $\pm$ SD). Counts of Fos-LIR nuclei were taken from eyes harvested 12 hours after injection and 2 hr after ungoggling. Increasing amounts of injected AODN-Fos resulted in decreasing amounts of Fos-LIR nuclei. Cell counts were obtained by subjectively counting under the microscope. n=6 chicks per group. * * significant at $P < 0.01$.

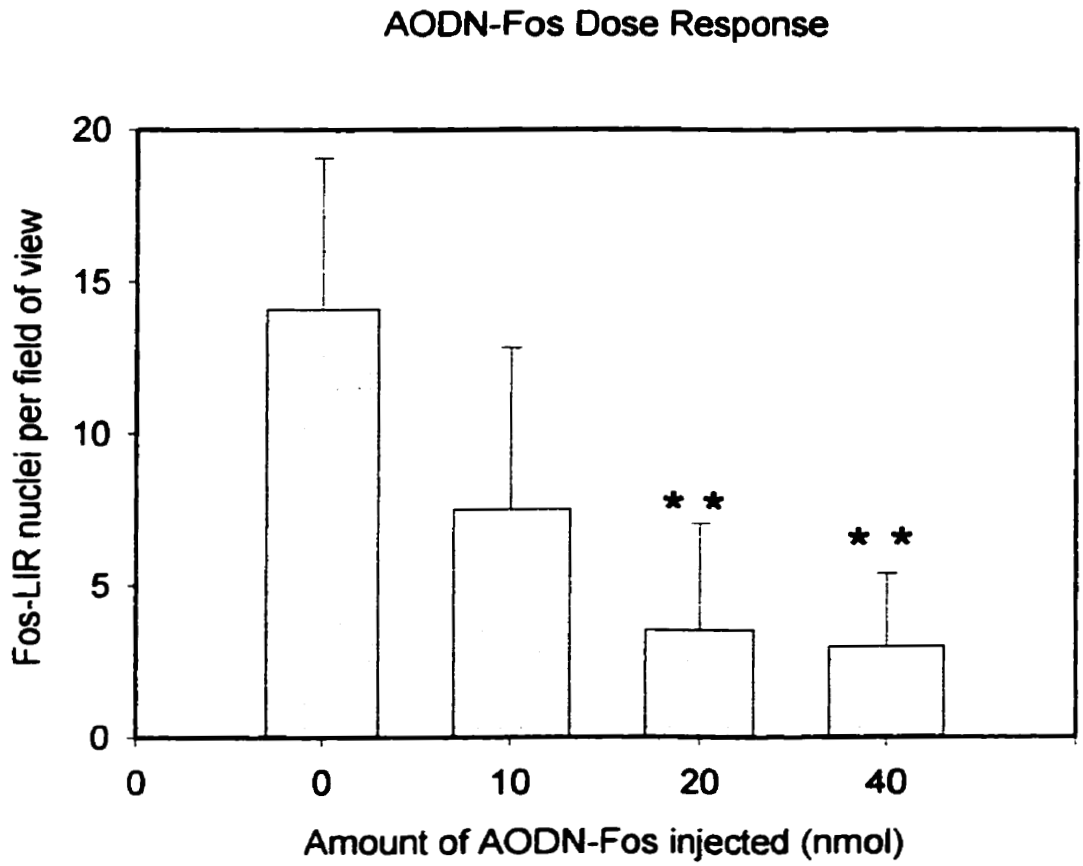

AODN-Fos Dose Response
Fos-LIR nuclei per field of view
20
15
10
5
0
* *
* *
0
0
10
20
40
Amount of AODN-Fos injected (nmol)

Figure 4.2: Time-response function for the number of hours between injection and ungoggling versus the number of Fos-LIR nuclei per field of view (mean $\pm$ SD). In each group 20 nmol of AODN-Fos were injected and chicks were ungoggled for 2hr. A maximal reduction in Fos-LIR resulted from a period of 18 to 24 hr between injection and ungoggling. Cell counts were obtained by subjectively counting under the microscope. n=6 chicks per group. * significant at $P<0.05$.

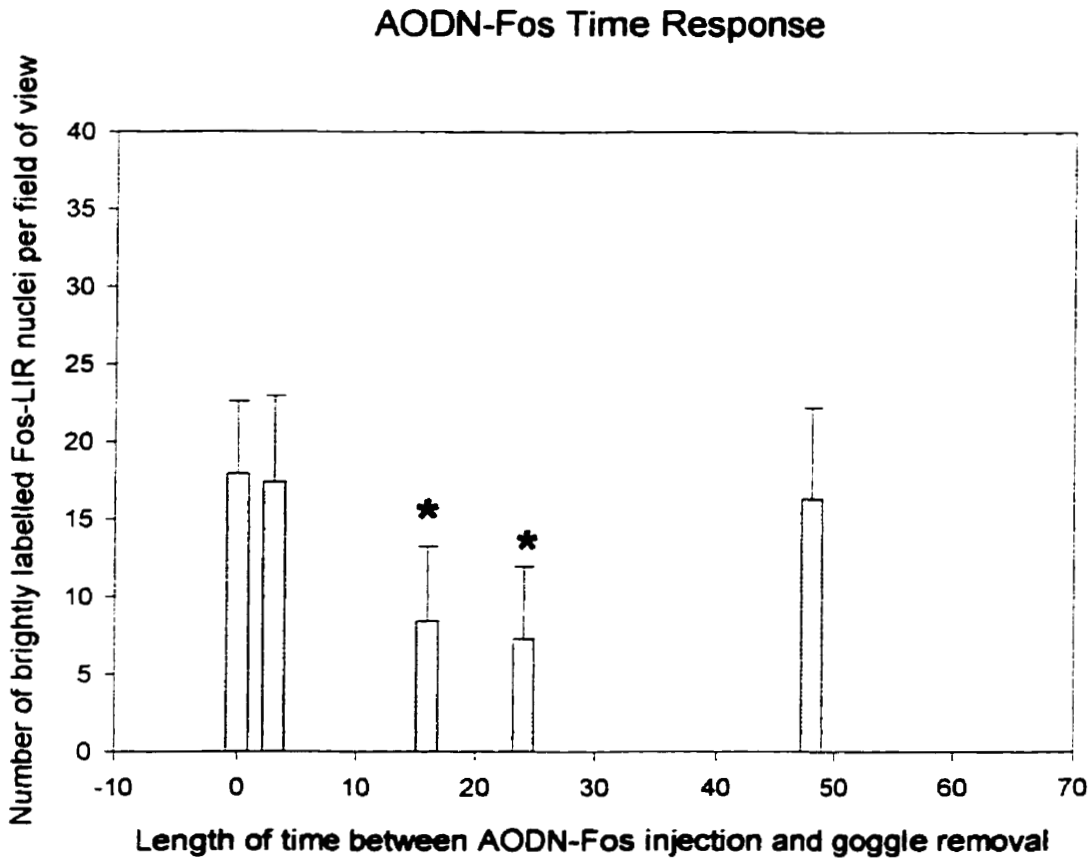

AODN-Fos Time Response
Number of brightly labelled Fos-LIR nuclei per field of view
40
35
30
25
20
15
10
5
0
-10
0
10
20
30
40
50
60
70
*
*
Length of time between AODN-Fos injection and goggle removal

Figure 4.3: Intraocular injections of AODN-Fos block Fos protein expression. a) control: Fos-LIR in the retina of a P8 chick after 24 hr of contrast-degrading goggle wear followed by 2 hr of clear goggle wear. b) Fos-LIR in the retina of a P8 chick after an injection of 20nmol of AODN-Fos followed by 24 hr of contrast-degrading goggle wear followed by 2 hr of clear goggle wear. Scale bar = 50 μm. From top to bottom the arrows indicate the outer border of the inner nuclear layer, inner border of the inner nuclear layer, and the outer border of the ganglion cell layer.

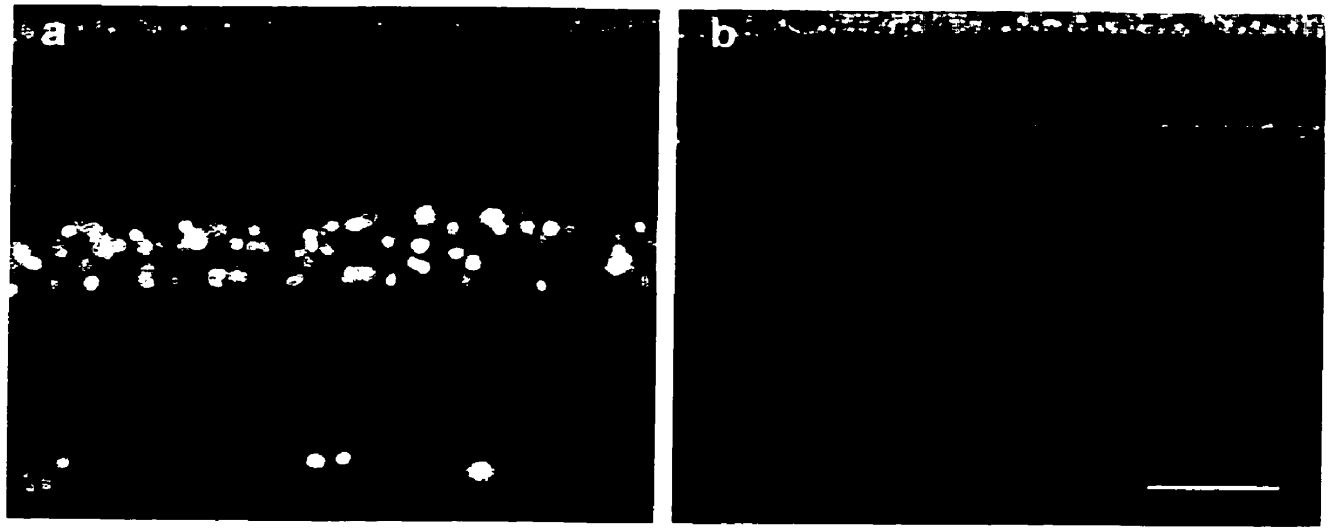
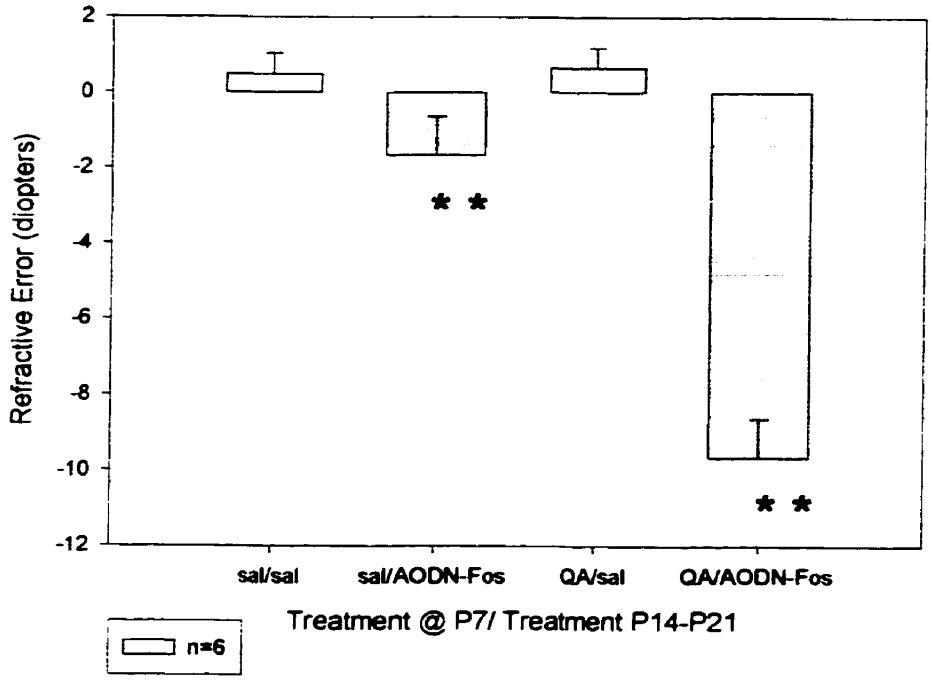

a
b

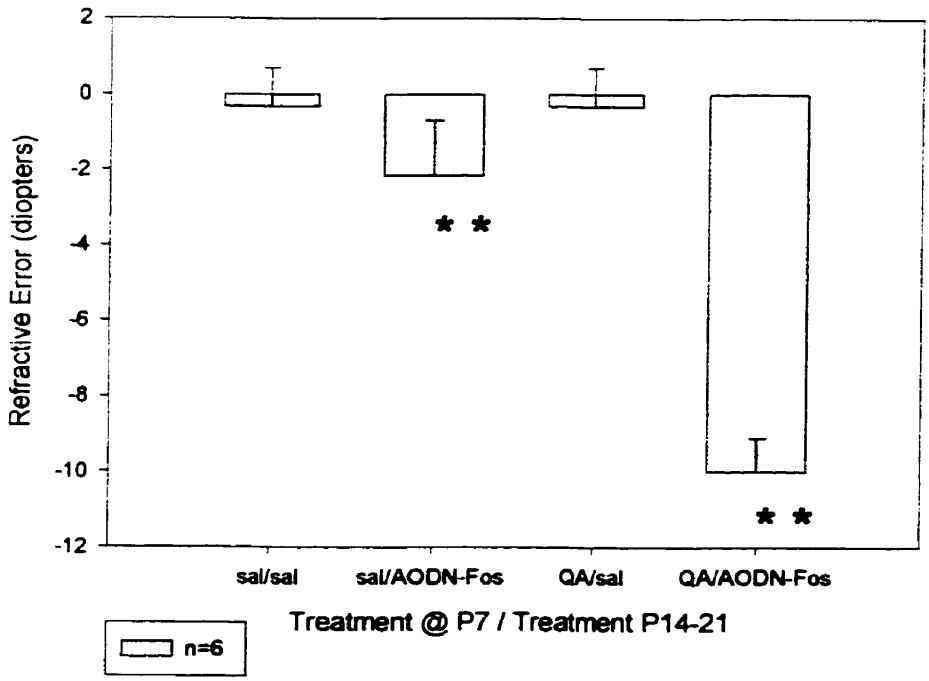
Figure 4.4: Chronic administration of AODN-Fos results in excessive ocular growth in open eyes. A) Refractive error, B) axial length, C) weight of open eyes injected daily with 20nmol AODN-Fos. Groups of chicks were treated with either 0 or 200nmol of QA in 20uL saline at P7. Six daily AODN-Fos injections began at P14. Sal/AODN-Fos treated eyes were more myopic and larger than their respective control, sal/sal treated eyes. QA/AODN -Fos treated eyes were much more myopic and larger than their respective control, QA/sal treated eyes. Bars indicate mean ± SD for each parameter. * * significant at $P< 0.01$, * significant at $P<0.05$.

A) The effect of AODN-Fos on refractive error of open eyes

i) Trial 1

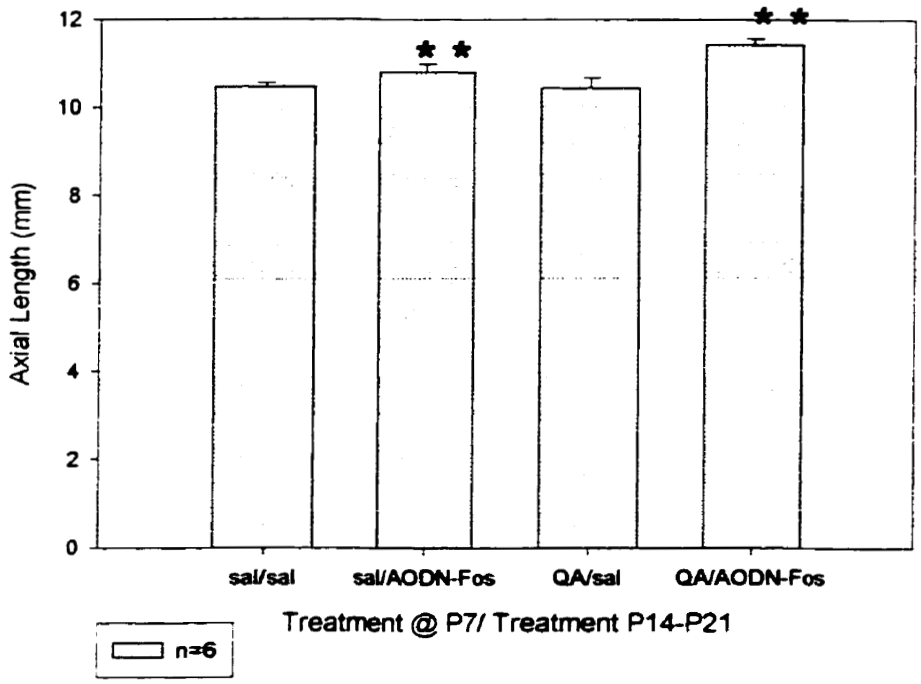


ii) Trial 2

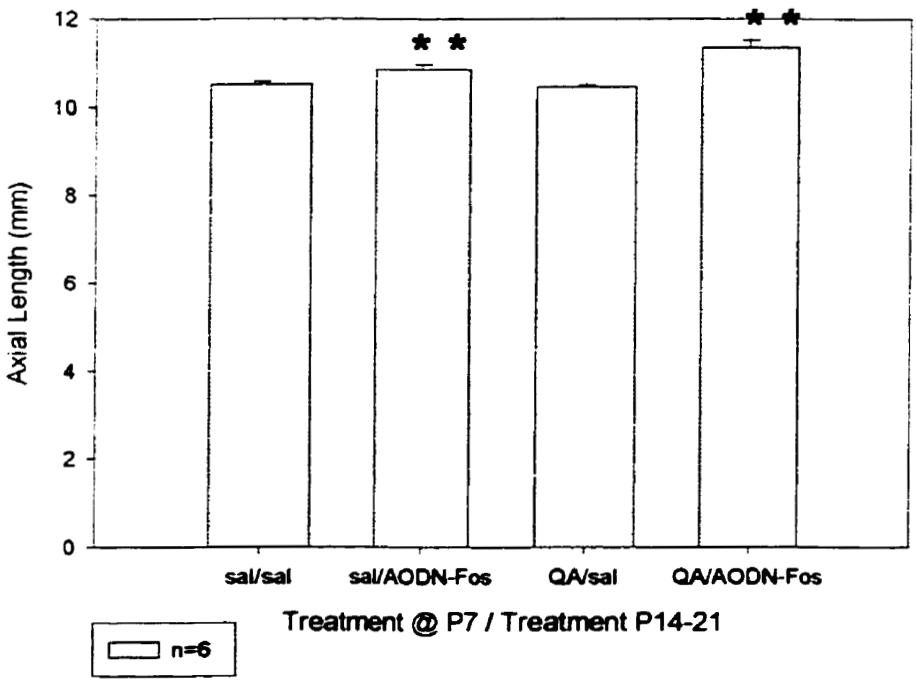


B) The effect of AODN-Fos on axial length of open eyes

i) Trial 1

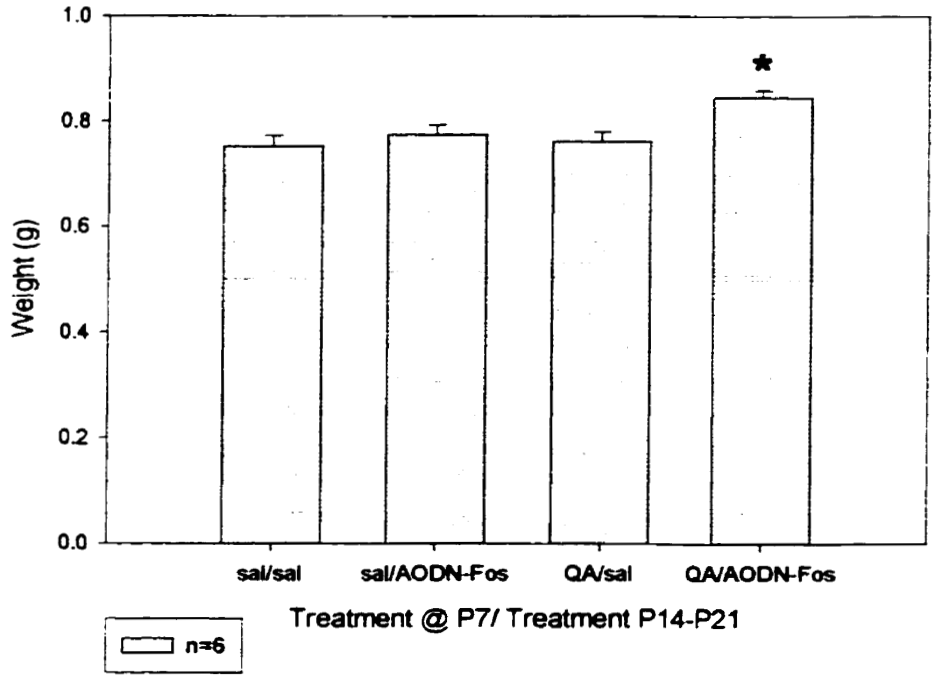


ii) Trial 2



C) The effect of AODN-Fos on weight of open eyes

i) Trial 1



ii) Trial 2

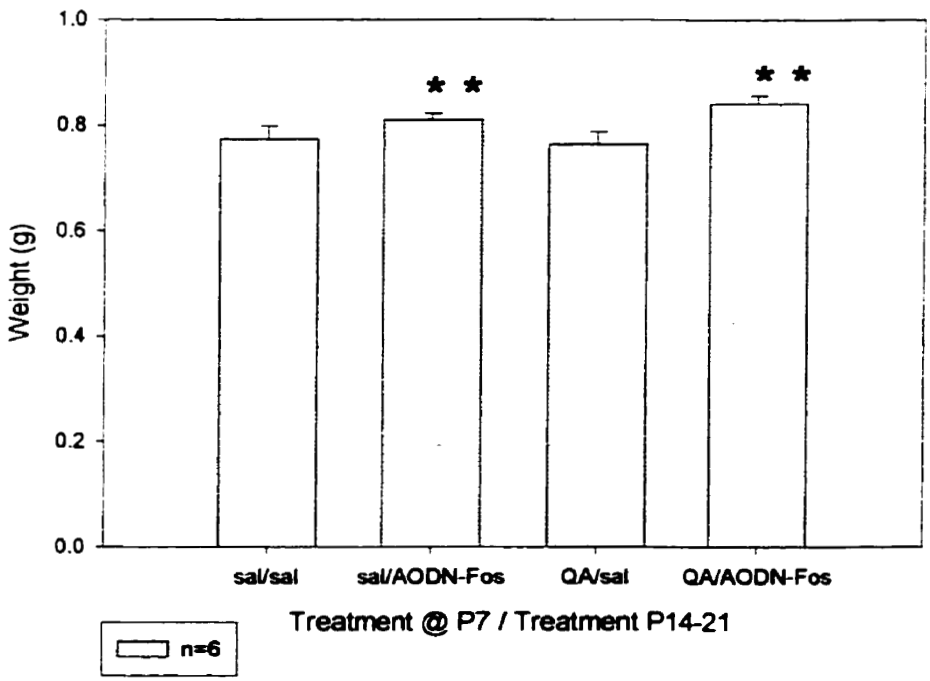


Figure 4.5: AODN-Fos, but not AODN-Fos-SCRAM, competently blocked Fos expression to the completion of the experiment. Fos-LIR at P21 in a) a QA/sal-treated eye, b) QA/ AODN-Fos - treated eye. c) QA/ AODN-Fos-SCRAM treated eye. Scale bar = 50 μm. From top to bottom the arrows indicate the outer border of the inner nuclear layer, inner border of the inner nuclear layer, and the outer border of the ganglion cell layer.

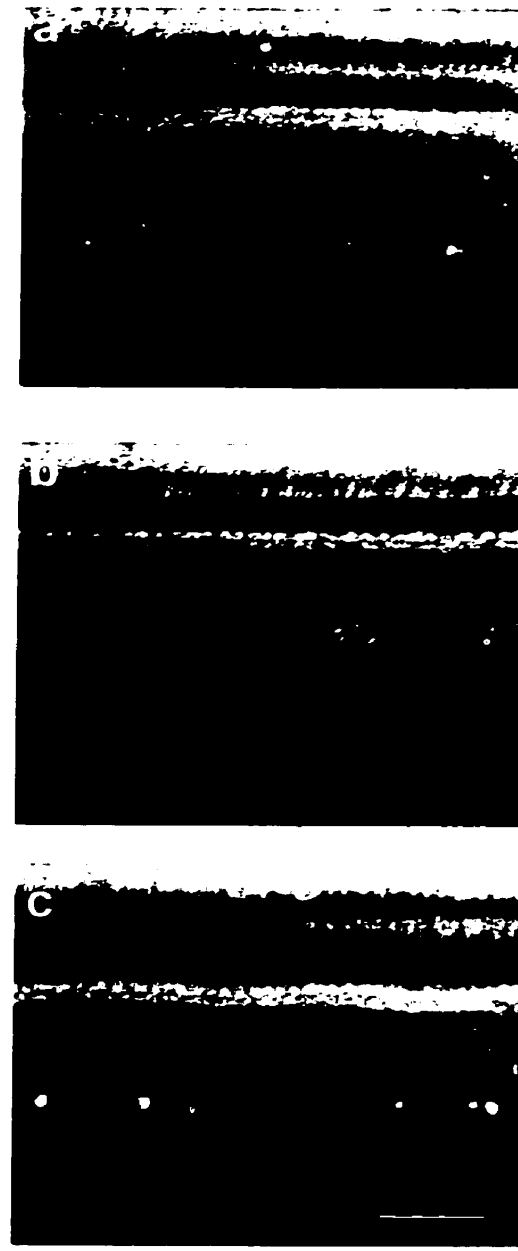
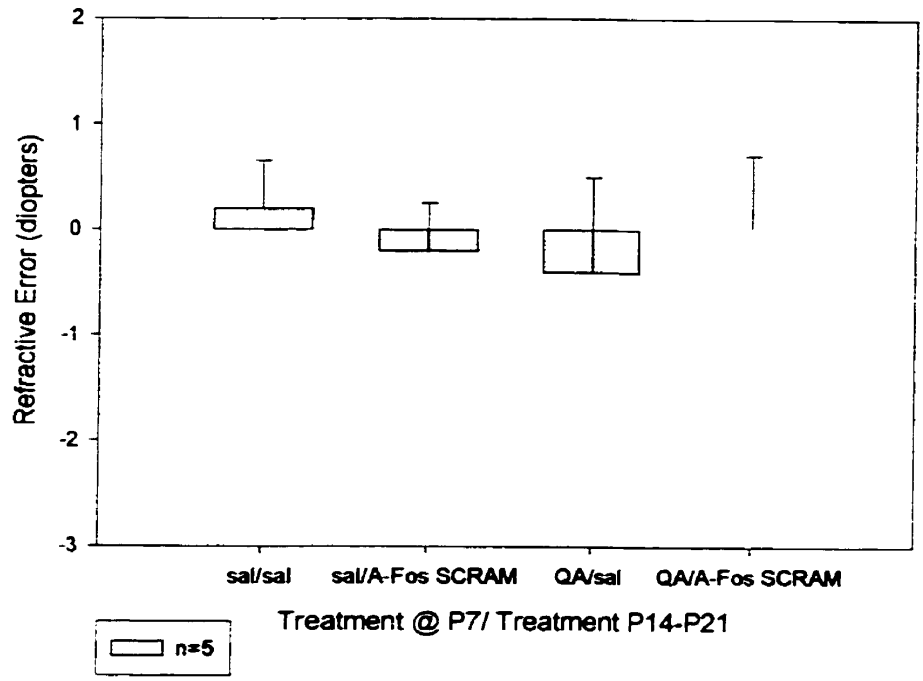

a
b
c

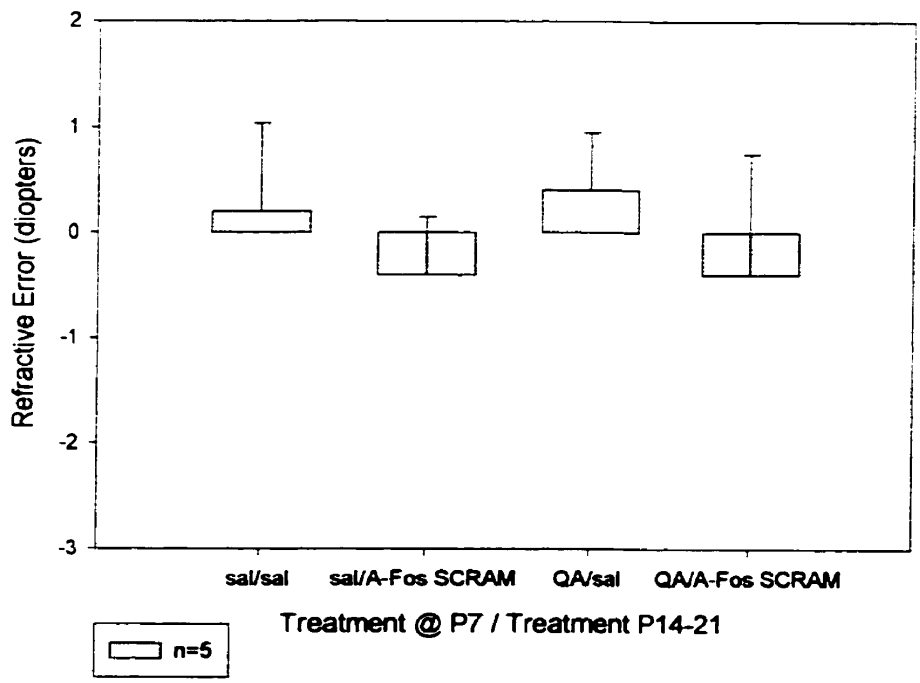
Figure 4.6: Daily injections of AODN-Fos scrambled probes into open eyes (±QA) did not result in enlarged, myopic eyes. A) Refractive error, B) axial length, C) weight of open eyes injected daily with 20 nmol AODN-Fos scrambled probes (A-Fos-SCRAM). Groups of chicks were treated with either 0 or 200 nmol of QA in 20 uL saline at P7. Six daily AODN-Fos scrambled probe injections began at P14. Sal/A-Fos-SCRAM treated eyes were similar in net refractive error to their respective control, sal/sal treated eyes. QA/A-Fos-SCRAM treated eyes were similar in net refractive error to their respective control, QA/sal treated eyes. Bars indicate mean ± SD for each parameter.

A) The effect of AODN-Fos scrambled control on refractive error of open eyes

i) Trial 1

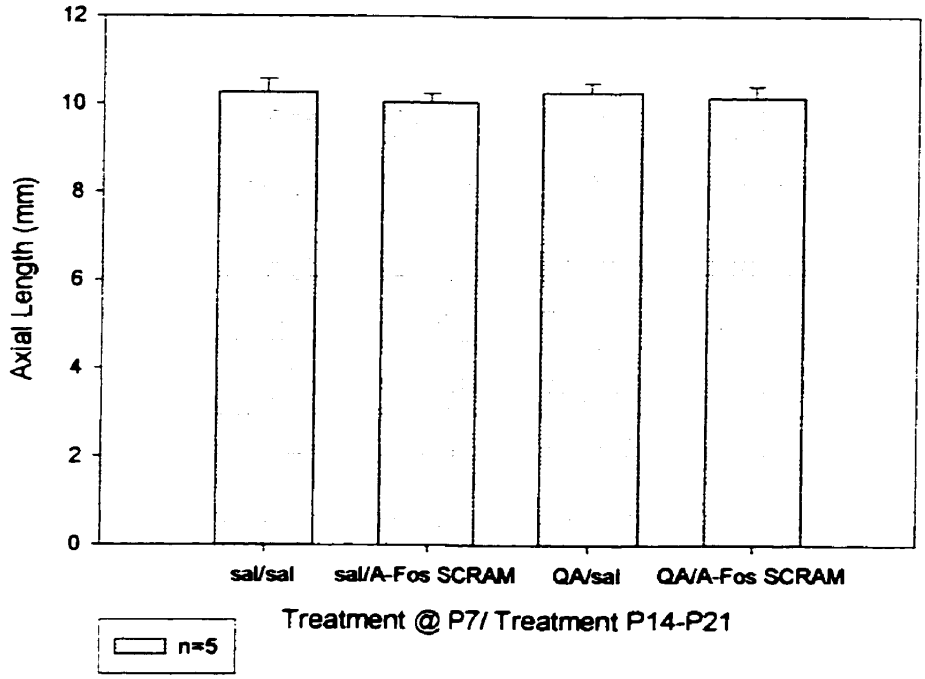


ii) Trial 2

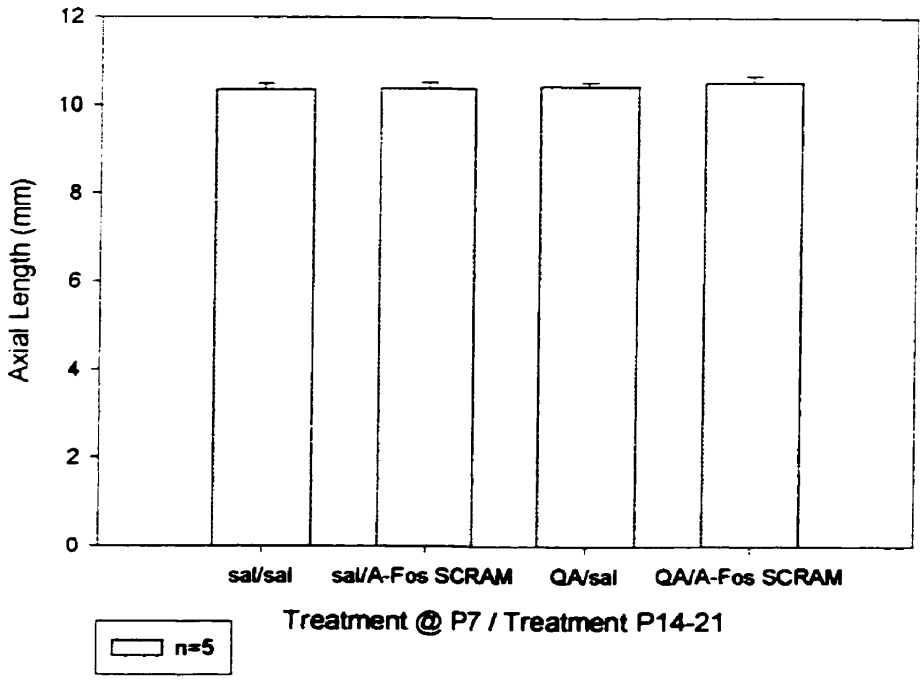


B) The effect of AODN-Fos scrambled control on axial length of open eyes

i) Trial 1

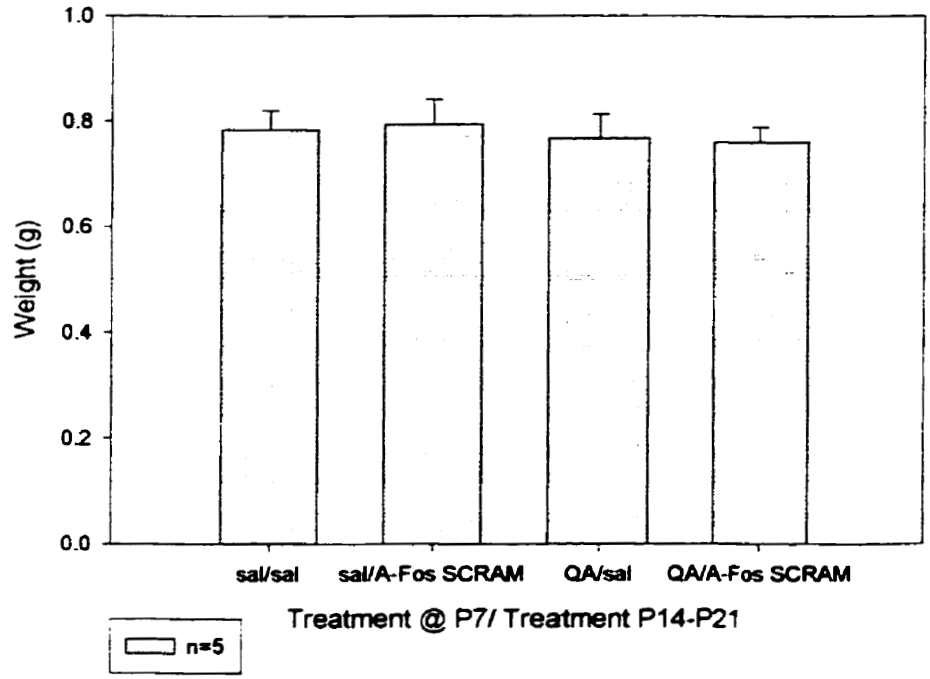


ii) Trial 2



C) The effect of AODN-Fos scrambled control on weight of open eyes

i) Trial 1



ii) Trial 2

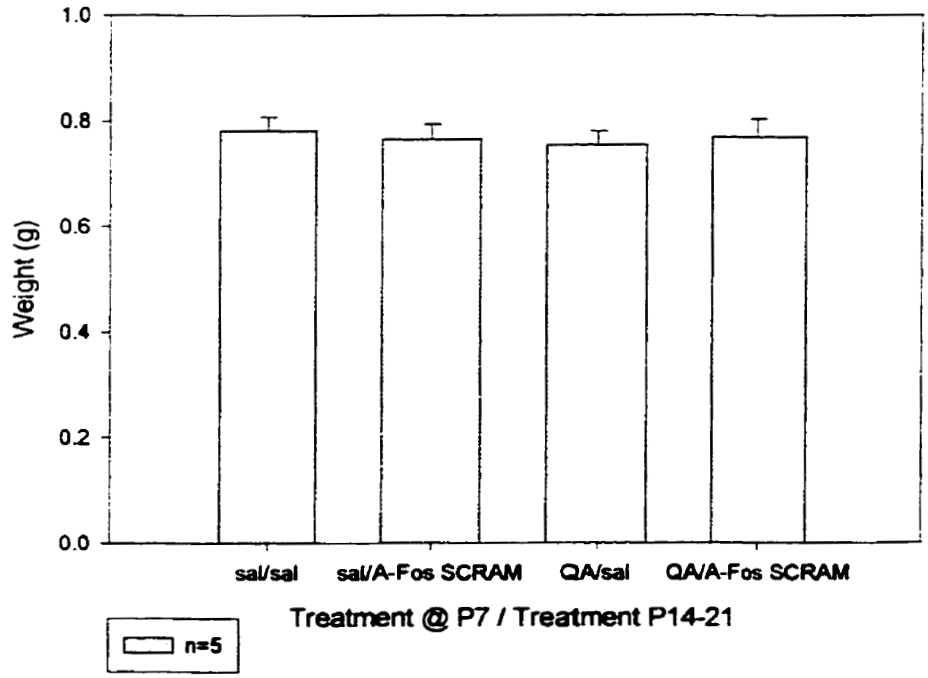
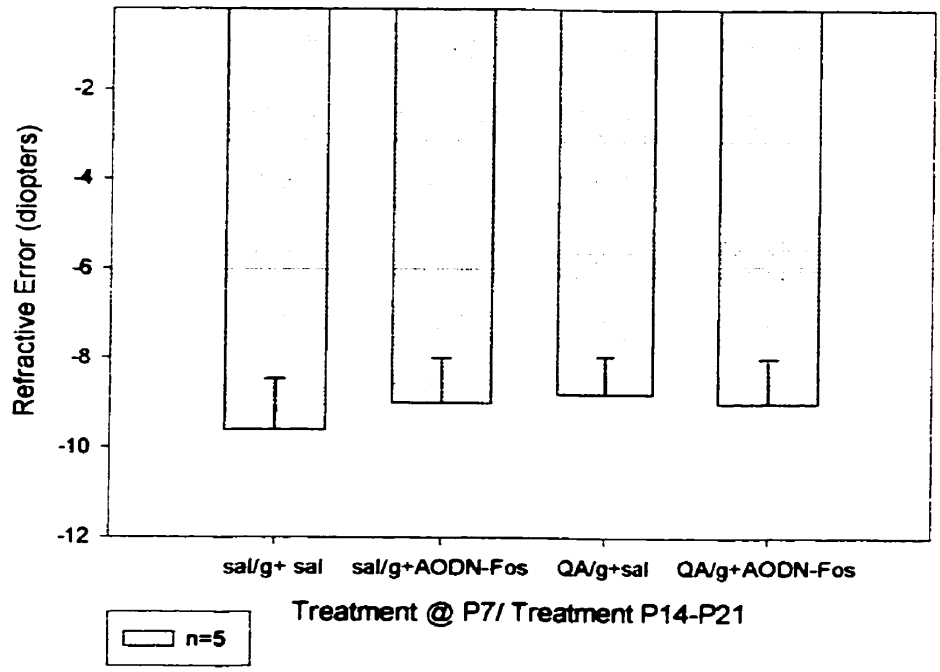


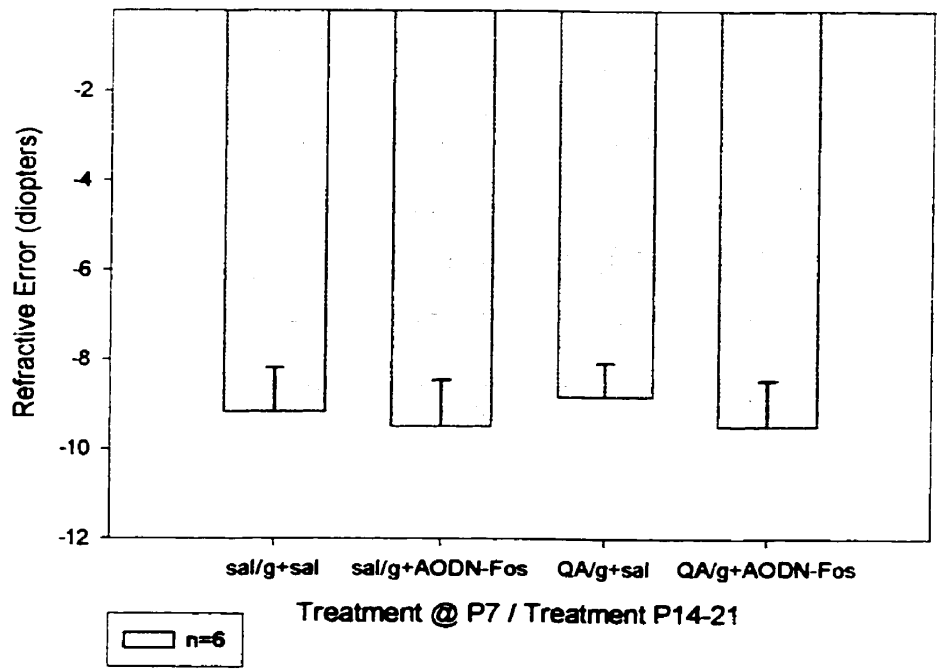
Figure 4.7: Chronic administration of AODN-Fos does not affect the development of FDM in goggled chicks. A) Refractive error, B) axial length, C) weight of goggled eyes injected daily with 20nmol AODN-Fos. Groups of chicks were treated with either 0 or 200nmol of QA in 20uL saline at P7. Chicks were goggled at P7 and then treated with 6 daily injections of AODN-Fos. Sal/g+AODN-Fos treated eyes were similar in net refractive error to their respective control, sal/g+sal treated eyes. QA/g+AODN -Fos treated eyes were similar in net refractive error to their respective control, QA/g+sal treated eyes. Bars indicate mean ± SD for each parameter. * * significant at $P< 0.01$, * significant at $P<0.05$.

A) The effect of AODN-Fos on refractive error of goggled eyes

i) Trial 1

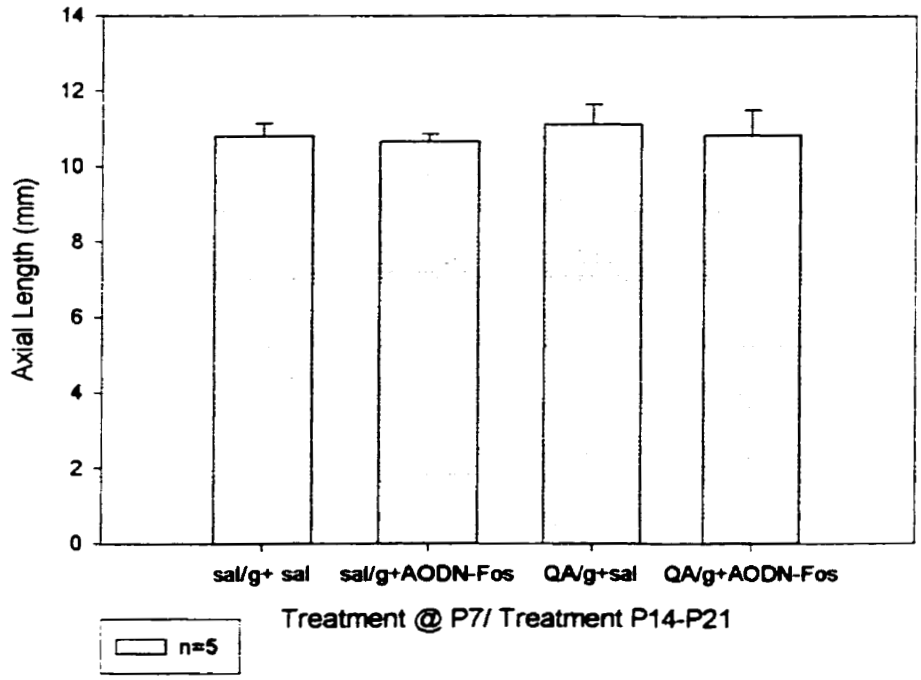


ii) Trial 2

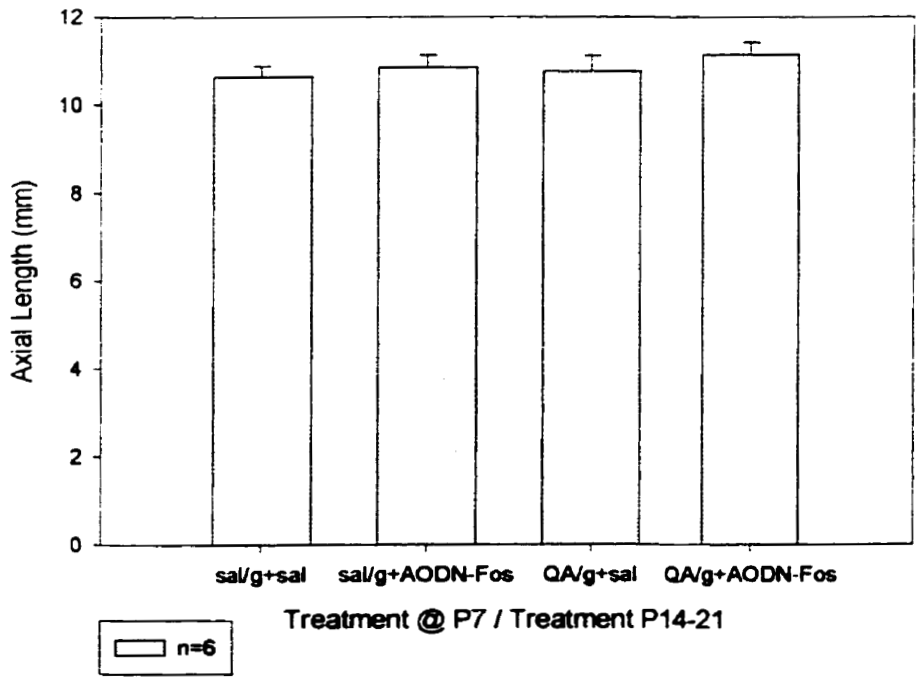


B) The effect of AODN-Fos on axial length of goggled eyes

i) Trial 1

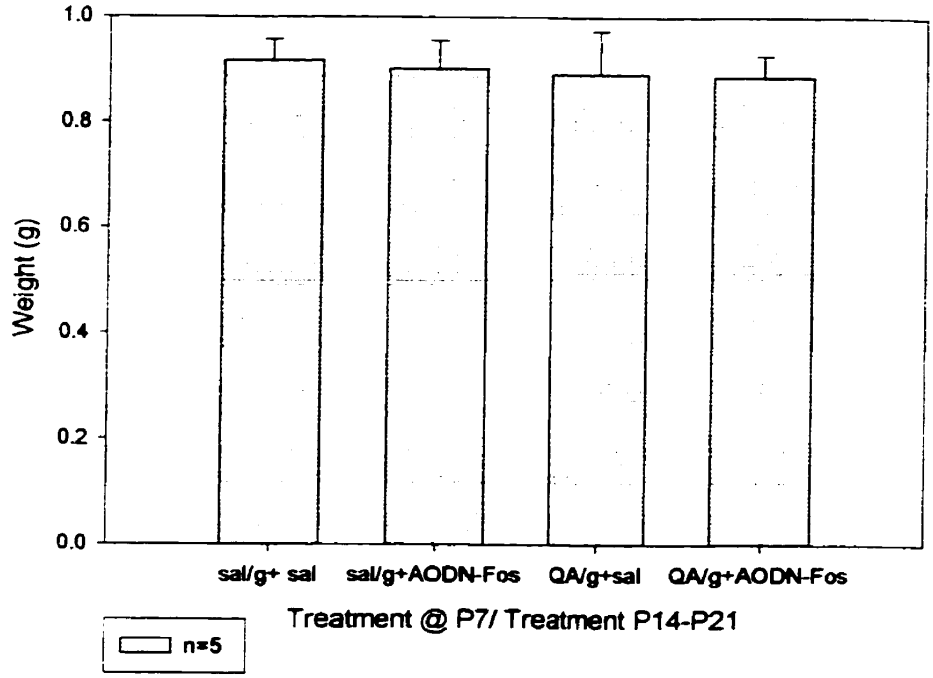


ii) Trial 2



C) The effect of AODN-Fos on weight of goggled eyes

i) Trial 1



ii) Trial 2

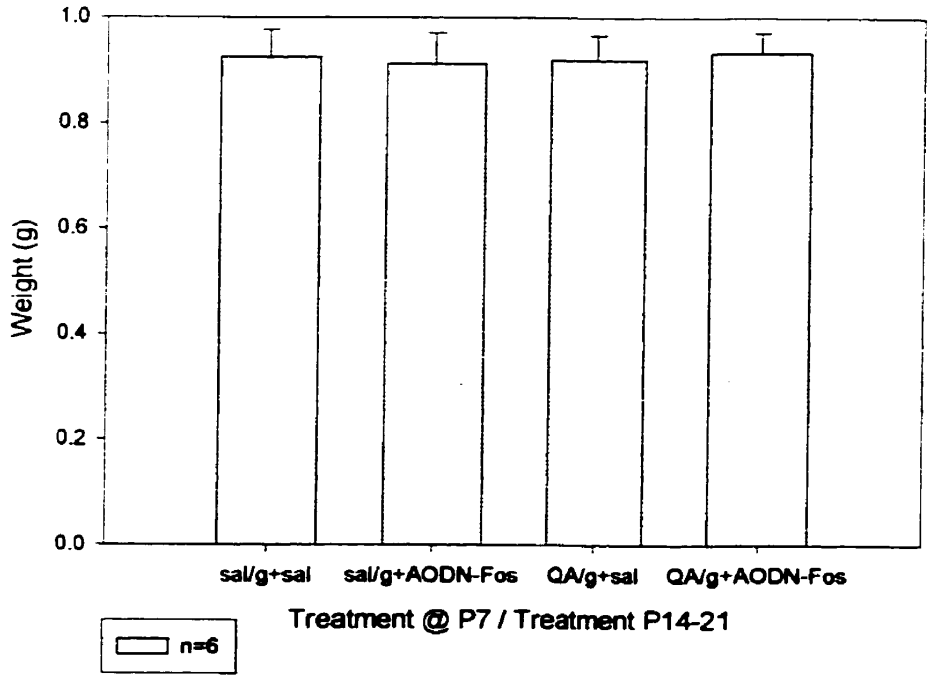
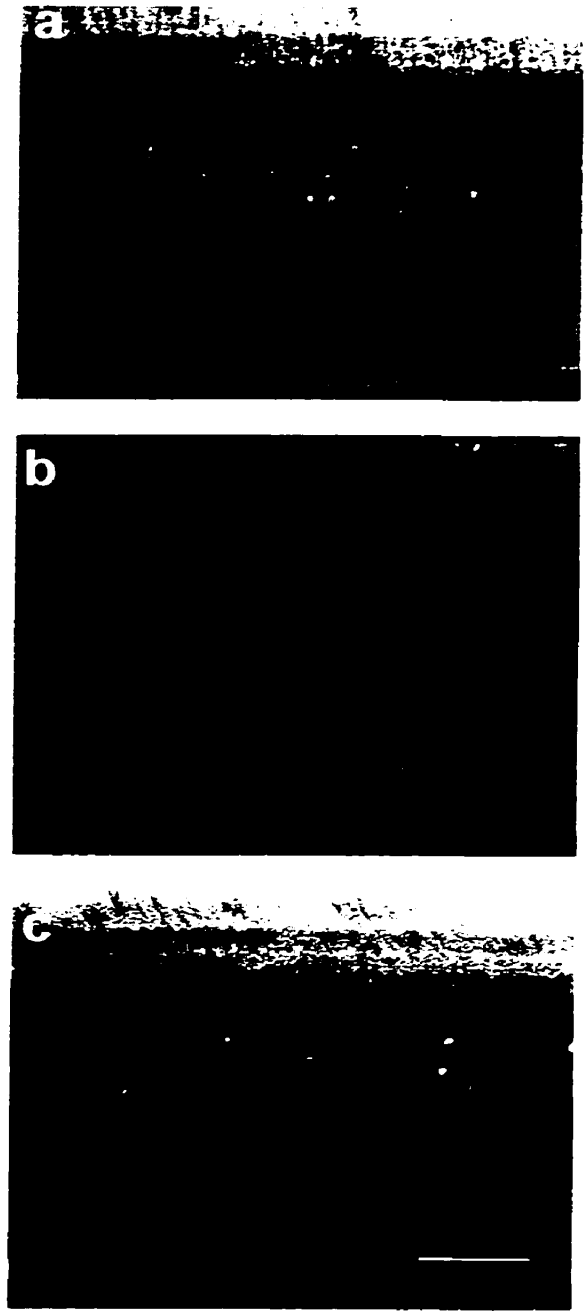
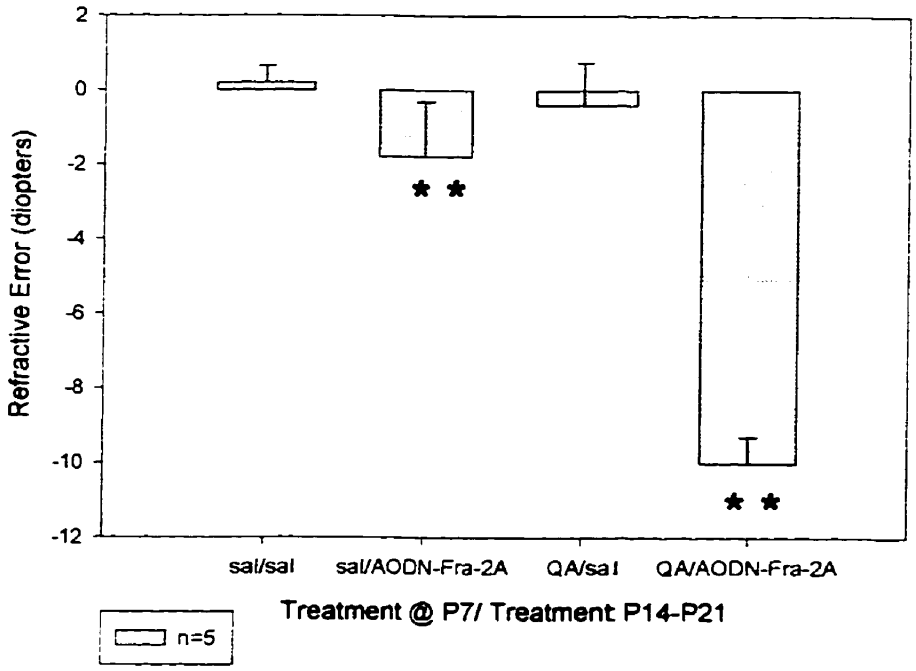


Figure 4.8: Chronic administration of AODN-Fra-2 results in excessive ocular growth in open eyes. A) Refractive error, B) axial length, C) weight of open eyes injected daily with 20 nmol AODN-Fra-2 [A] or [B]. Groups of chicks were treated with either 0 or 200 nmol of QA in 20 uL saline at P7. Six daily AODN-Fra-2 injections began at P14. Sal/AODN-Fra-2 treated eyes were more myopic and larger than their respective control, sal/sal treated eyes. QA/AODN-Fra-2 treated eyes were much more myopic and larger than their respective control, QA/sal treated eyes. Bars indicate mean ± SD for each parameter. * * significant at $P < 0.01$, * significant at $P<0.05$.

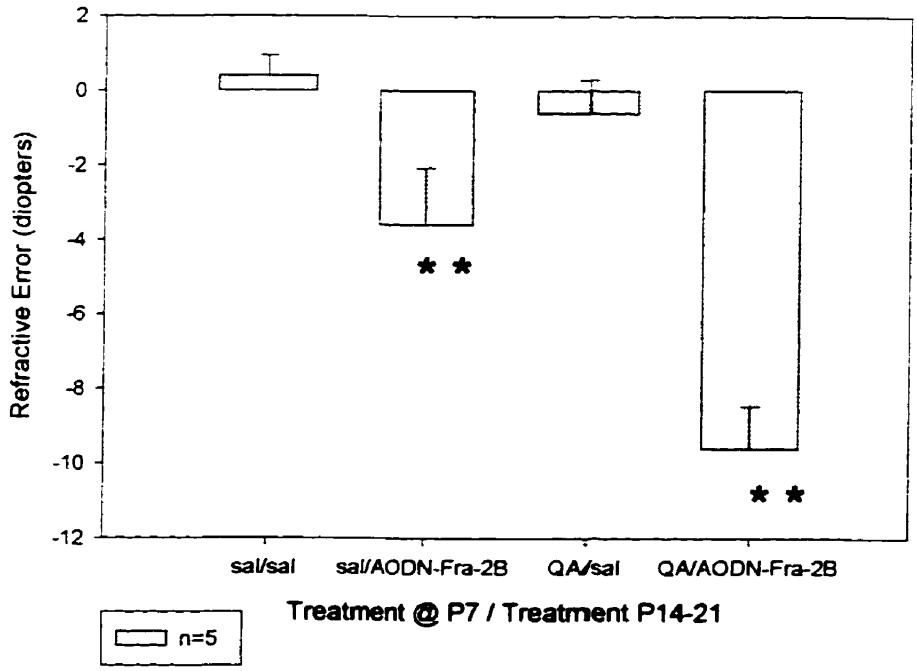

a
b
c

A) The effect of AODN-Fra-2 on refractive error of open eyes

i) AODN-Fra-2[A]

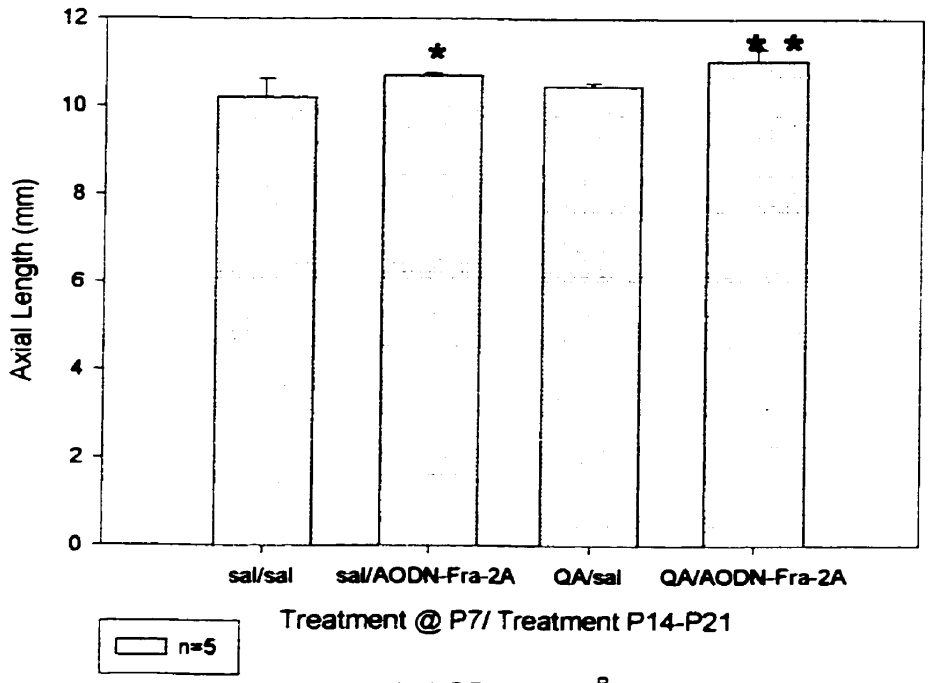


ii) AODN-Fra-2[B]

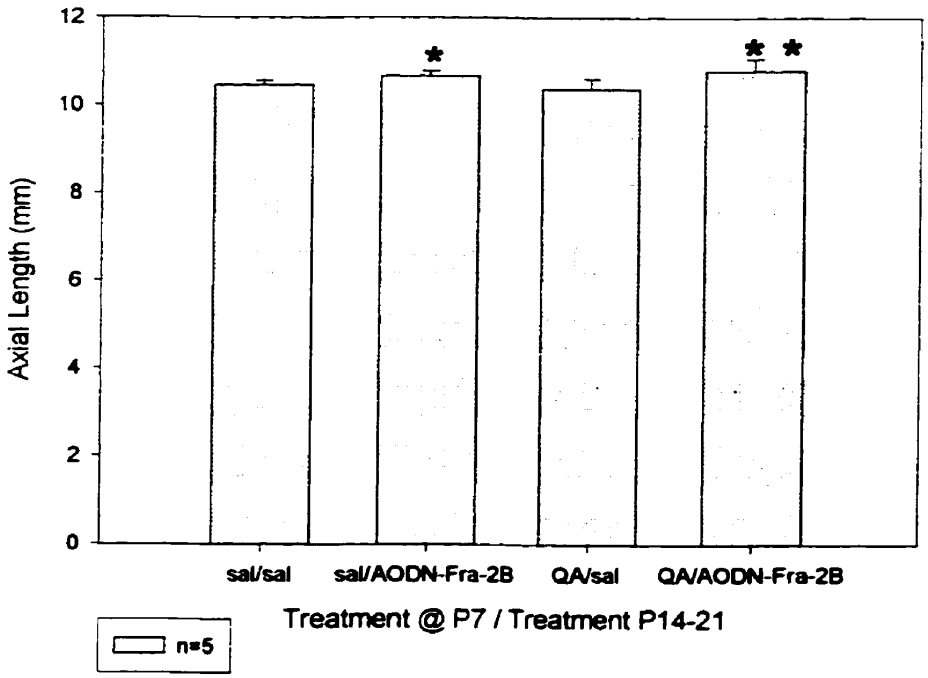


B) The effect of AODN-Fra-2 on axial length in open eyes

i) AODN-Fra-2[A]

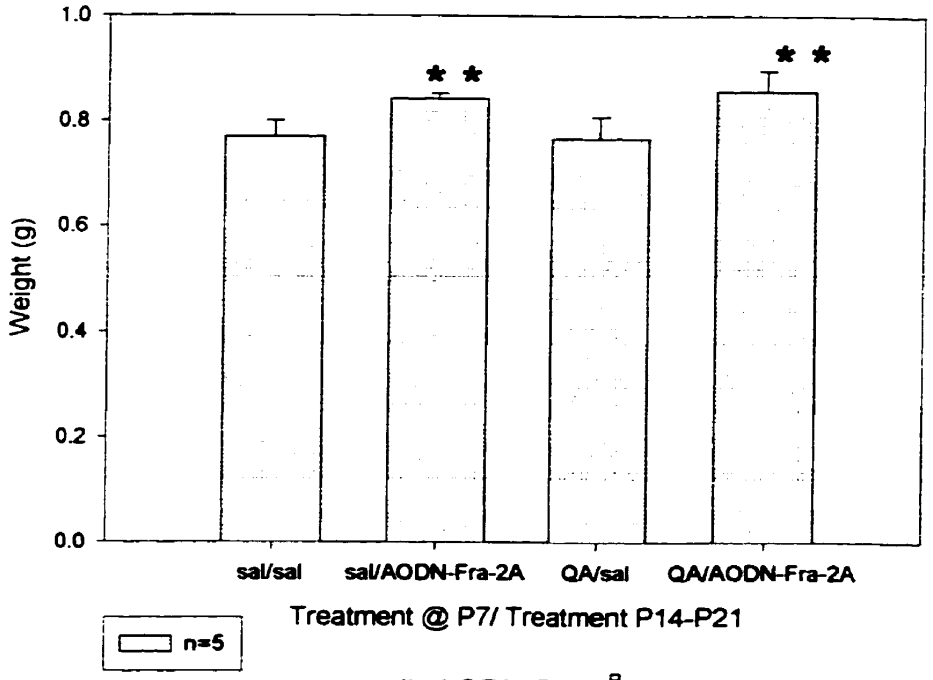


ii) AODN-Fra-2[B]



C) The effect of AODN-Fra-2 on weight of open eyes

i) AODN-Fra-2[A]



ii) AODN-Fra-2[B]

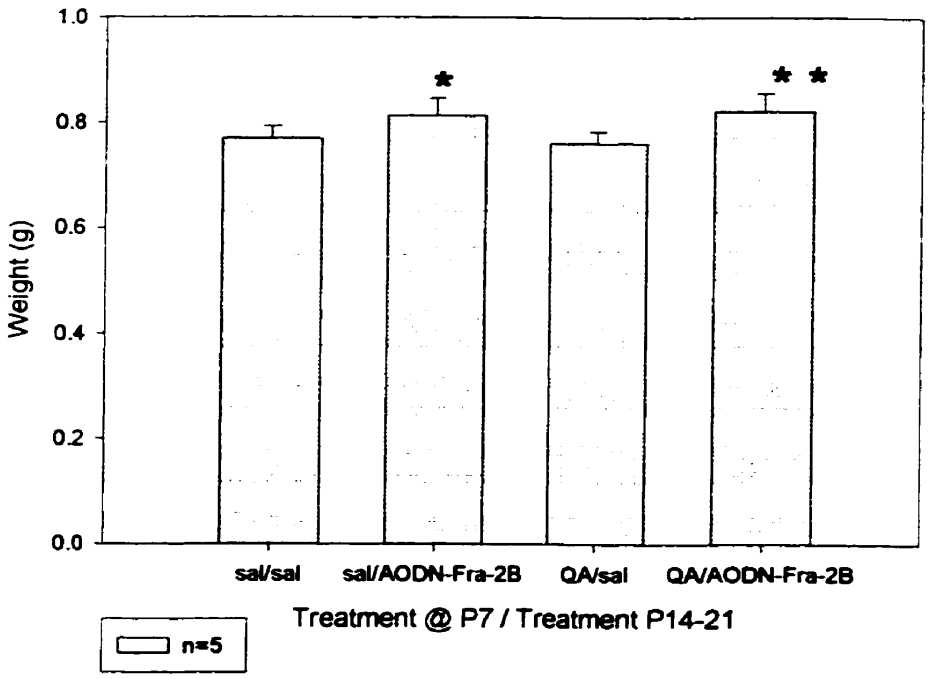


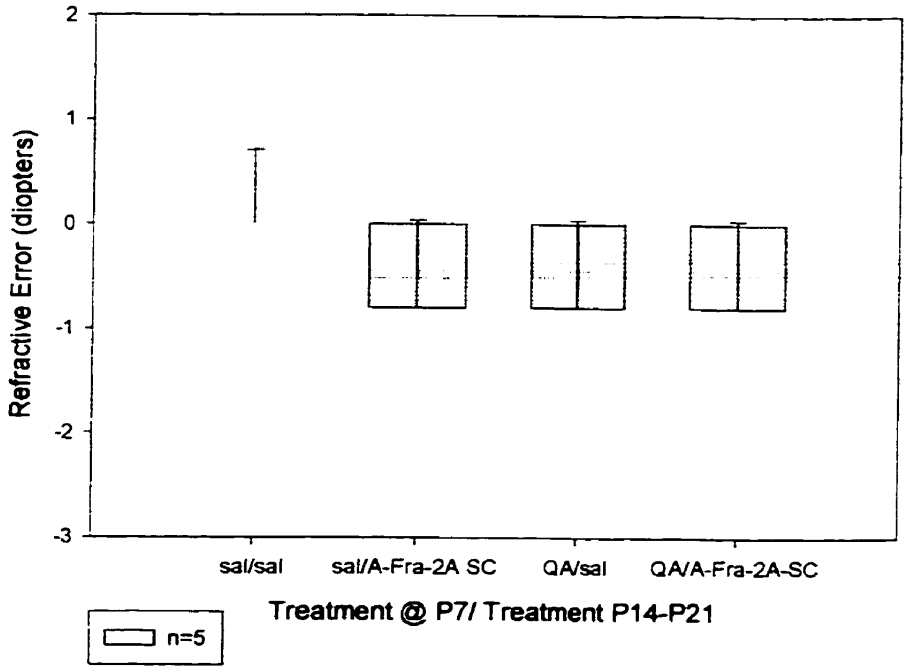
Figure 4.9: AODN-Fra-2, but not AODN-Fra-2 SCRAM competently blocked Fra-2 expression to the completion of the experiment. Fra-2-LIR at P21 in a) a QA/sal- treated chick, b) QA/ AODN-Fra-2^A - treated chick, c) QA/ AODN- Fra-2^A -SCRAM treated chick. Scale bar = 50 μm. From top to bottom the arrows indicate the outer border of the inner nuclear layer, inner border of the inner nuclear layer, and the outer border of the ganglion cell layer.

Figure goes here

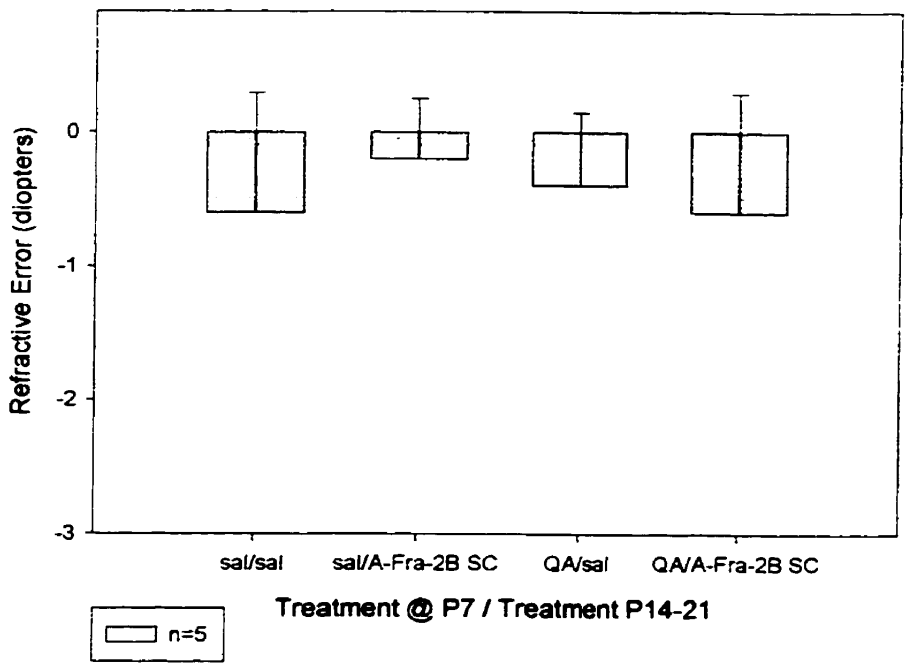
Figure 4.10: Daily injections of AODN-Fra-2 scrambled probes (AODN-Fra-2 SCRAM) into open eyes (±QA) did not result in enlarged, myopic eyes. Refractive error of open eyes injected daily with 20 nmol AODN-Fra-2 scrambled probes. Groups of chicks were treated with either 0 or 200 nmol of QA in 20 uL saline at P7. Six daily AODN-Fra-2-SCRAM injections began at P14. Sal/AODN-Fra-2-SCRAM treated eyes were similar in net refractive error to their respective control, sal/sal treated eyes. QA/AODN-Fra-2-SCRAM treated eyes were similar in net refractive error to their respective control, QA/sal treated eyes. Bars indicate mean ± SD for each parameter.

A) The effect of AODN-Fra-2 scrambled control on refractive error of open eyes

i) AODN-Fra-2^{A} scrambled control

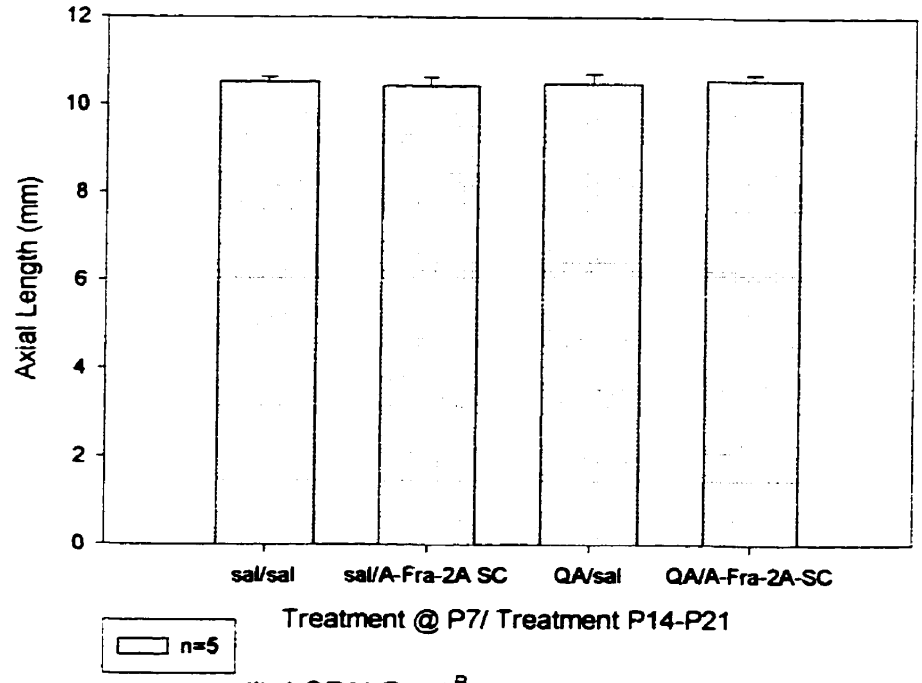


ii) AODN-Fra-2^{B} scrambled control

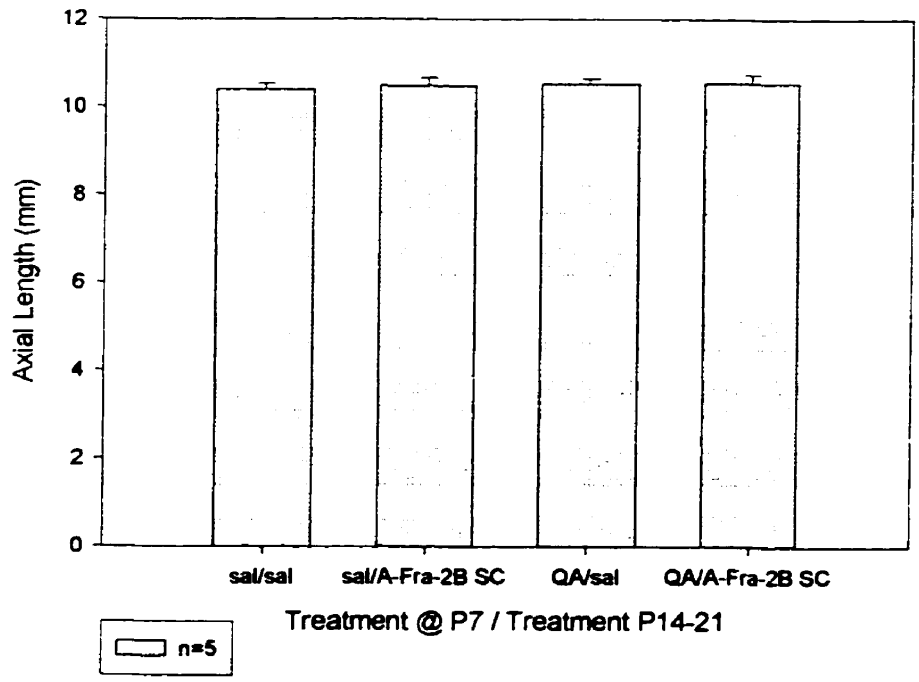


B) The effect of AODN-Fra-2 scrambled control on axial length of open eyes

i) AODN-Fra-2^{A} scrambled control

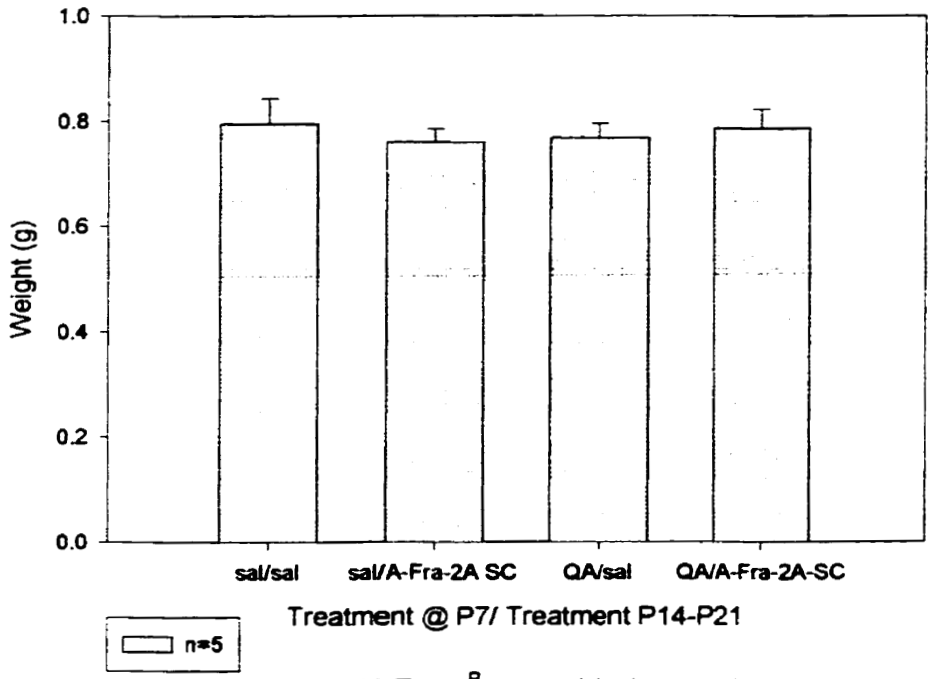


ii) AODN-Fra-2^{B} scrambled control



C) The effect of AODN-Fra-2 scrambled control on weight

i) AODN-Fra-2^{A} scrambled control



ii) AODN-Fra-2^{B} scrambled control

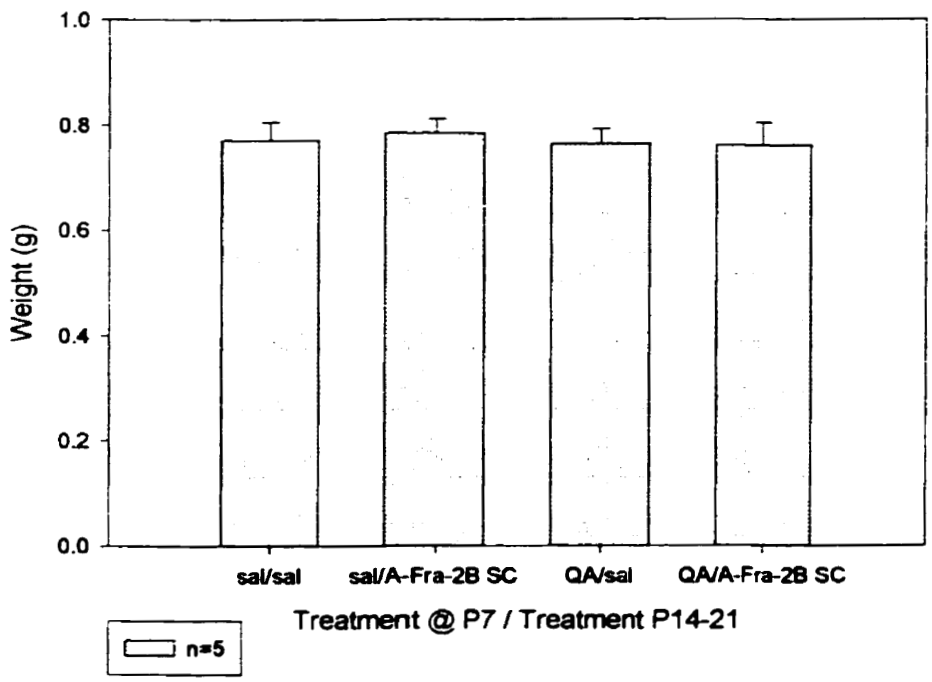
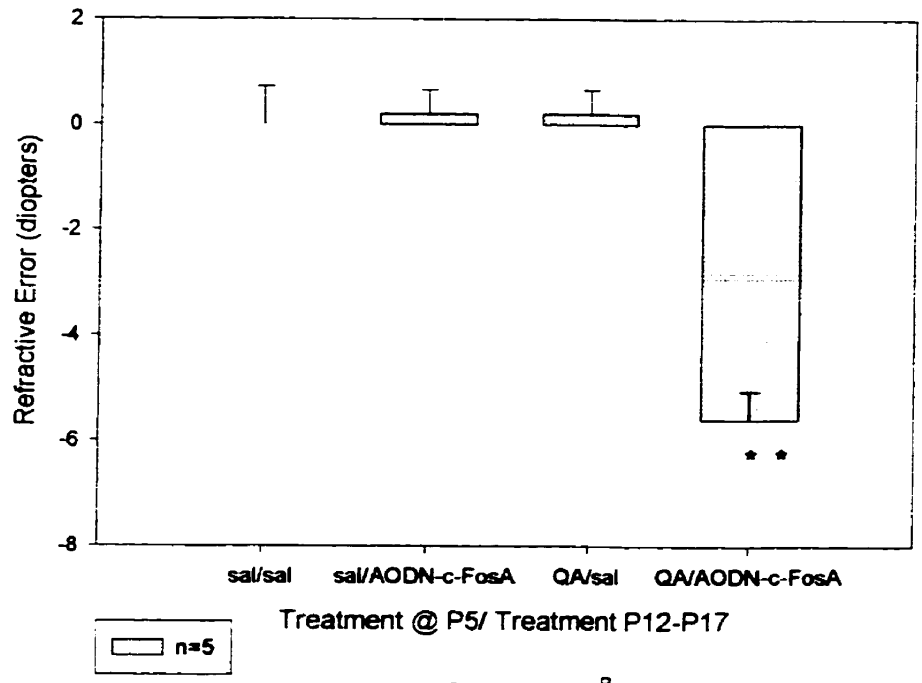


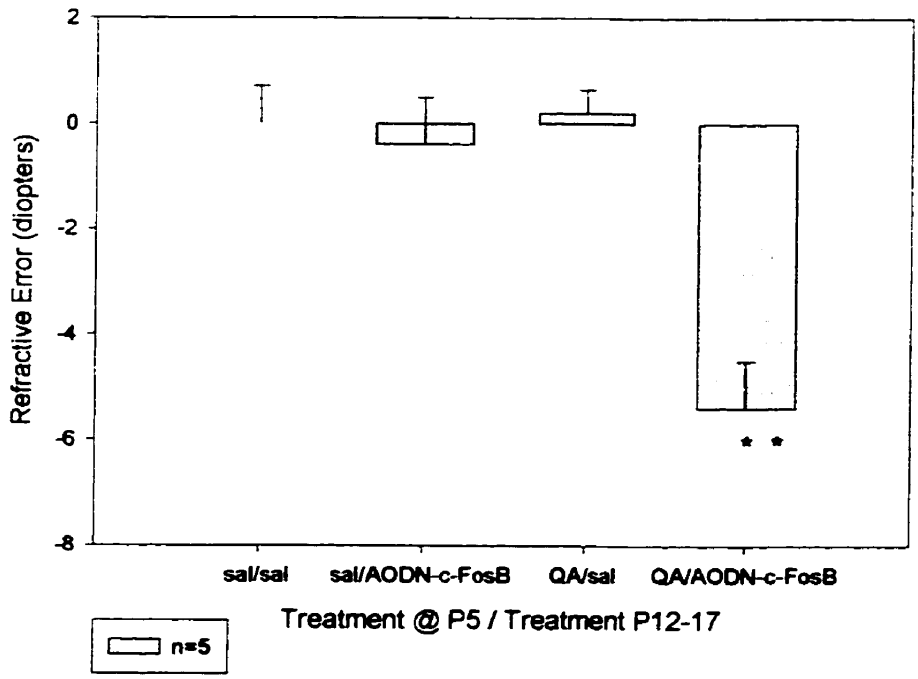
Figure 4.11: Chronic administration of AODN-c-Fos results in excessive ocular growth in open eyes treated with QA. A) Refractive error, B) axial length, C) weight of open eyes injected daily with 20 nmol AODN-c-Fos. Groups of chicks were treated with either 0 or 200 nmol of QA in 20 uL saline at P7. Six daily AODN-c-Fos injections began at P14. Sal/AODN-c-Fos treated eyes were emmetropic and of similar ocular size to their respective control, sal/sal treated eyes. QA/AODN-c-Fos treated eyes were more myopic and larger than their respective control, QA/sal treated eyes. Bars indicate mean ± SD for each parameter. * * significant at $P < 0.01$, * significant at $P<0.05$.

A) The effect of AODN-c-Fos on refractive error in open eyes

i) AODN-c-Fos[A]

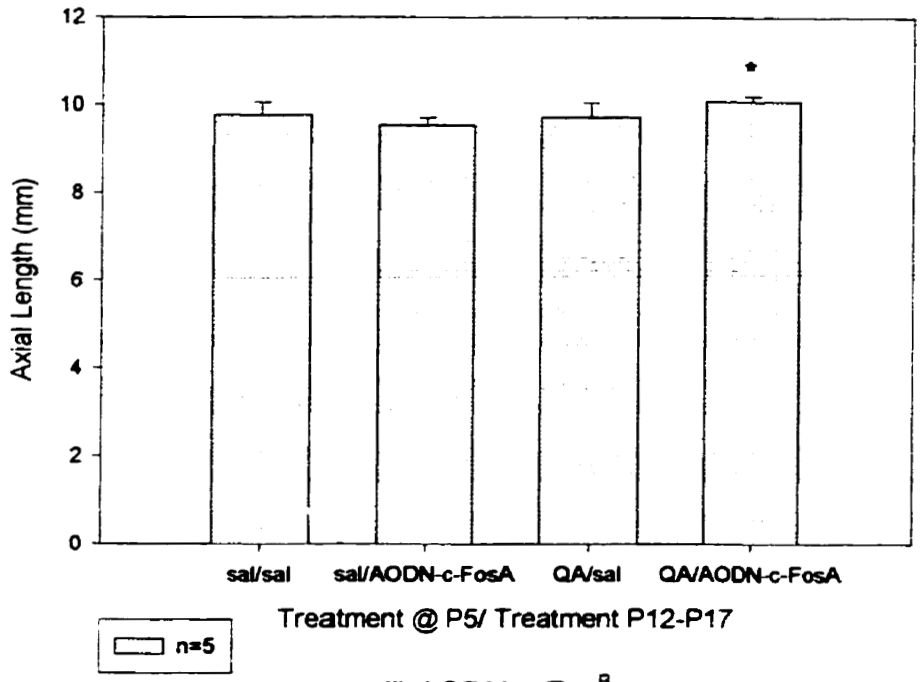


ii) AODN-c-Fos[B]

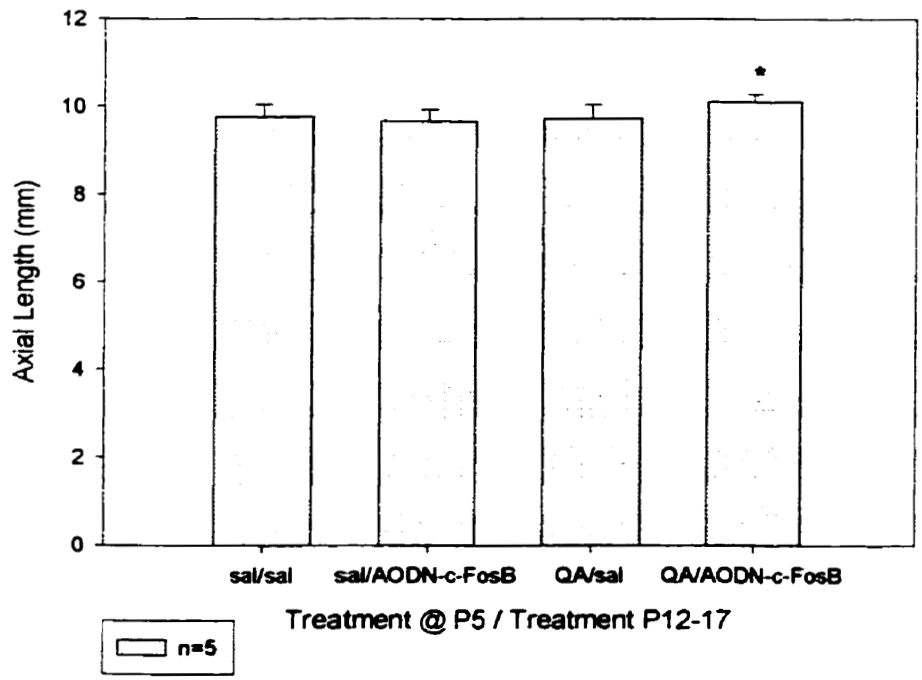


B) The effect of AODN-c-Fos on axial length of open eyes

i) AODN-c-Fos[A]

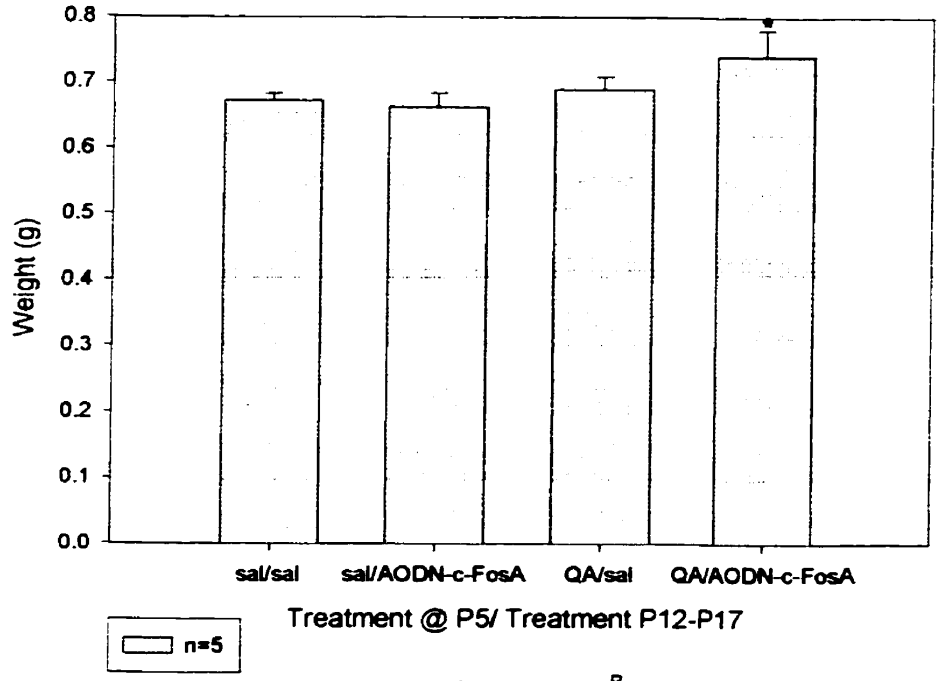


ii) AODN-c-Fos[B]



C) The effect of AODN-c-Fos on weight of open eyes

i) AODN-c-Fos[A]



ii) AODN-c-Fos[B]

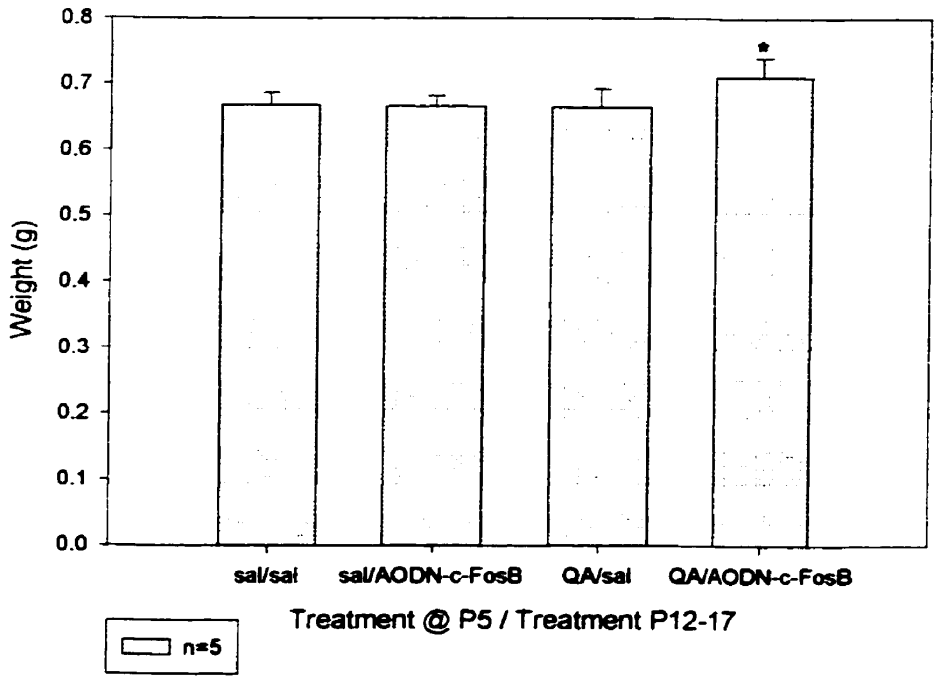


Figure 4.12: AODN-c-Fos but not AODN-c-Fos SCRAM competently blocked c-Fos expression to the completion of the experiment. c-Fos-LIR at P21 in a) a QA/sal- treated eye, b) QA/ AODN-c-Fos^{A} - treated eye, c) QA/AODN-c-Fos-SCRAM treated eye. Scale bar = 50 μm. From top to bottom the arrows indicate the outer border of the inner nuclear layer, inner border of the inner nuclear layer, and the outer border of the ganglion cell layer.

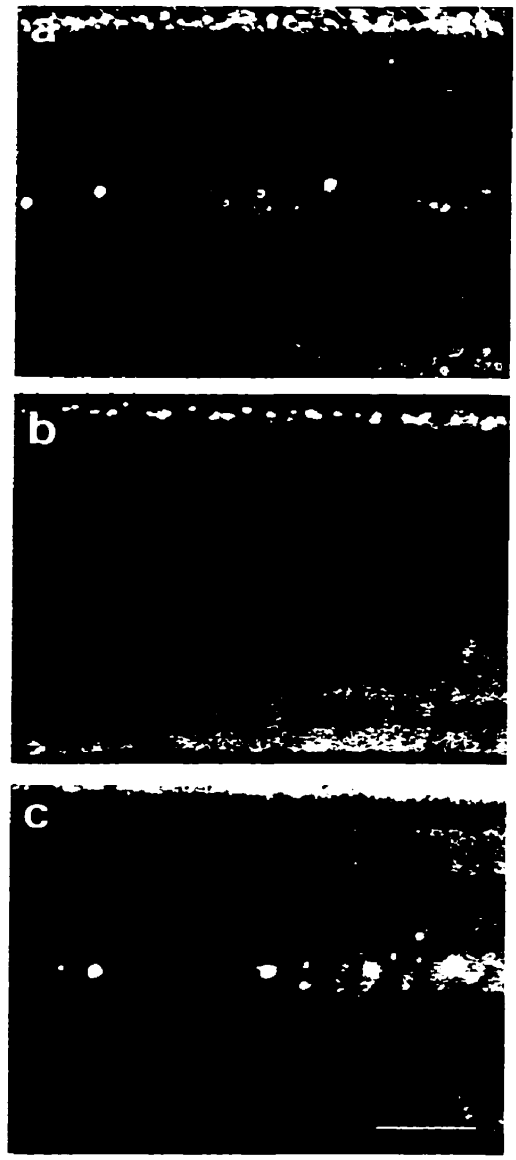
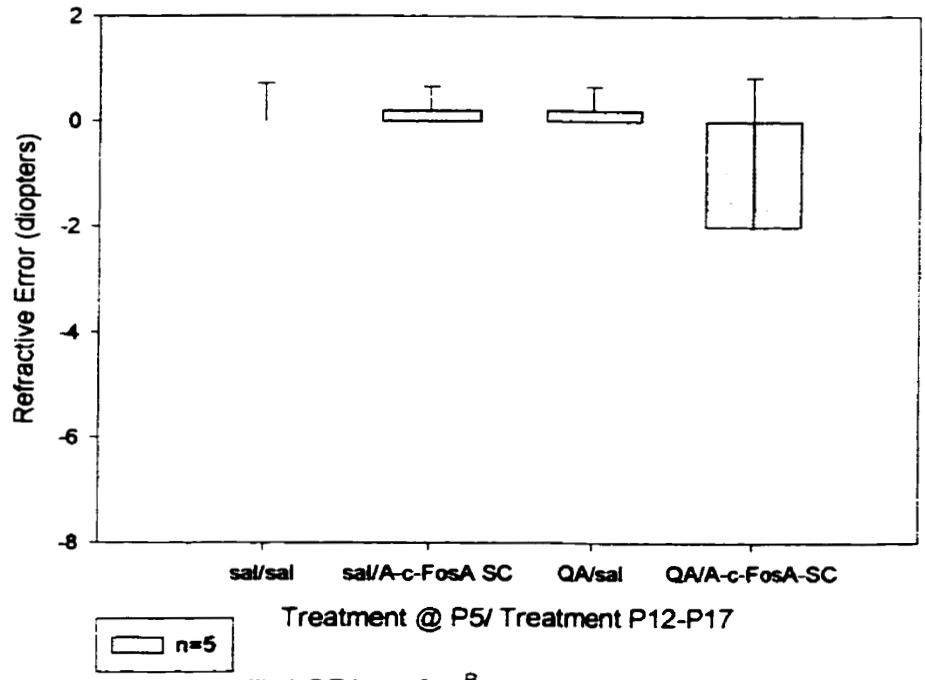

a
b
c

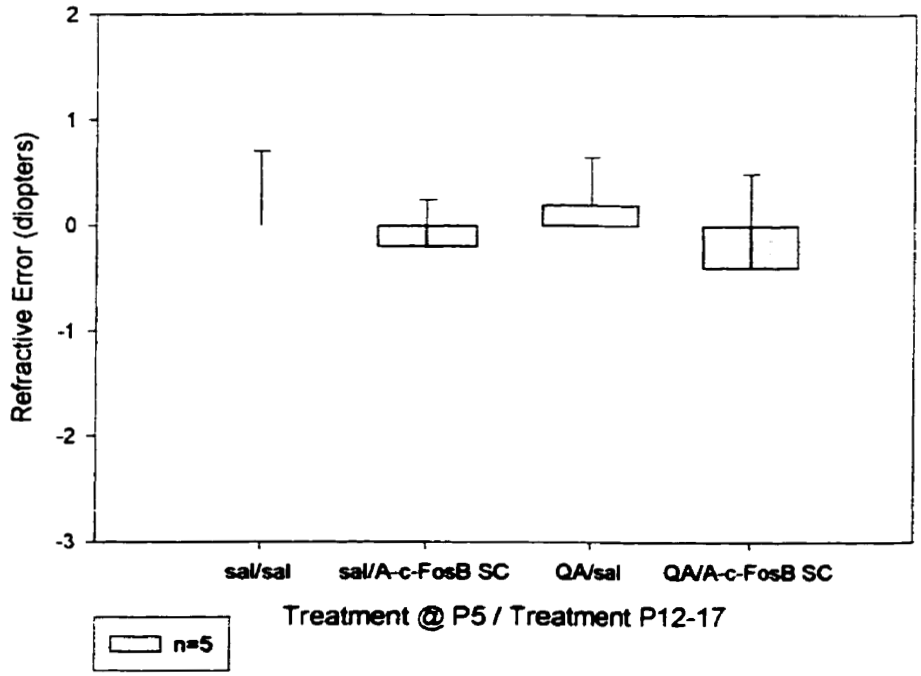
Figure 4.13: Daily injections of AODN-c-Fos scrambled probes (A-Fos-SCRAM) into open eyes (±QA) did not result in enlarged, myopic eyes. Refractive error of open eyes injected daily with 20 nmol A-c-Fos-SCRAM. Groups of chicks were treated with either 0 or 200 nmol of QA in 20 uL saline at P7. Six daily A-c-Fos-SCRAM injections began at P14. Sal/AODN-c-Fos-SCRAM treated eyes were similar in net refractive error to their respective control, sal/sal treated eyes. QA/A-c-Fos-SCRAM treated eyes were similar in net refractive error to their respective control, QA/sal treated eyes. Bars indicate mean ± SD for each parameter.

A) The effect of AODN-c-Fos scrambled control on refractive error of open eyes

i) AODN-c-Fos^{A} scrambled control

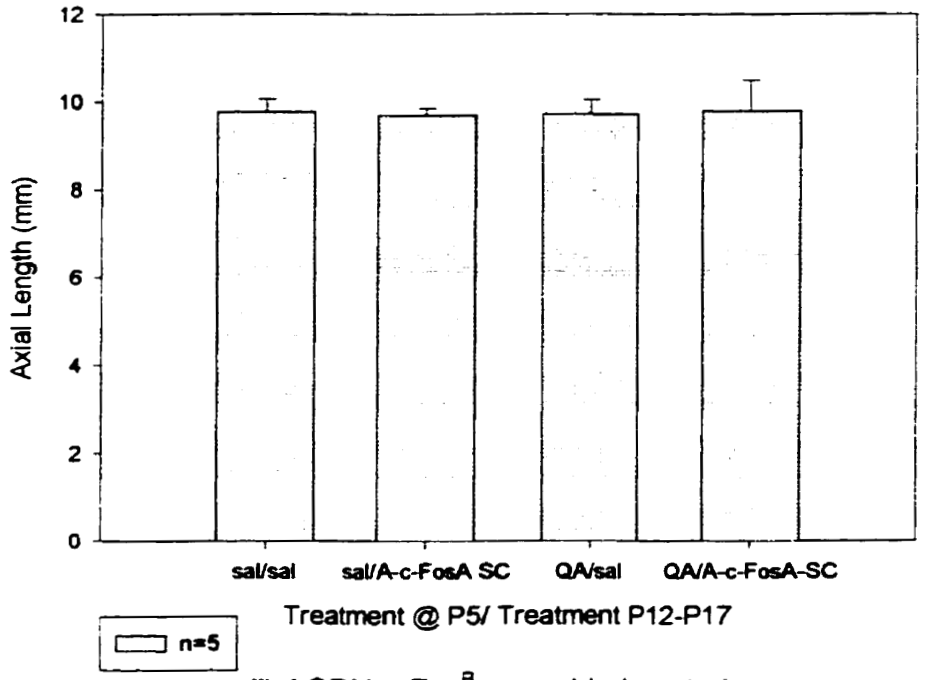


ii) AODN-c-Fos^{B} scrambled control

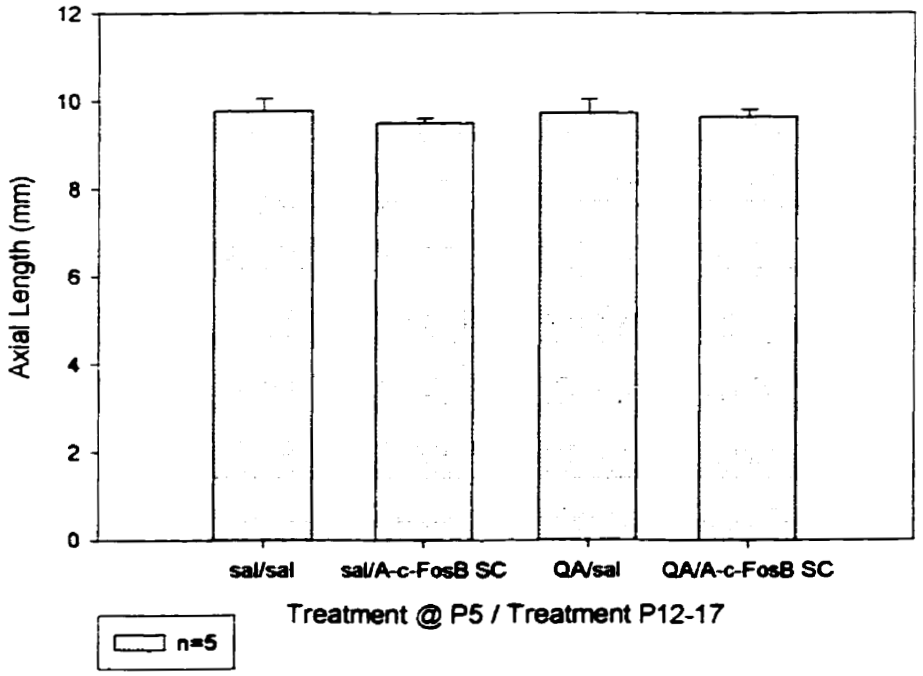


B) The effect of AODN-c-Fos scrambled control on axial length of open eyes

i) AODN-c-Fos^{A} scrambled control

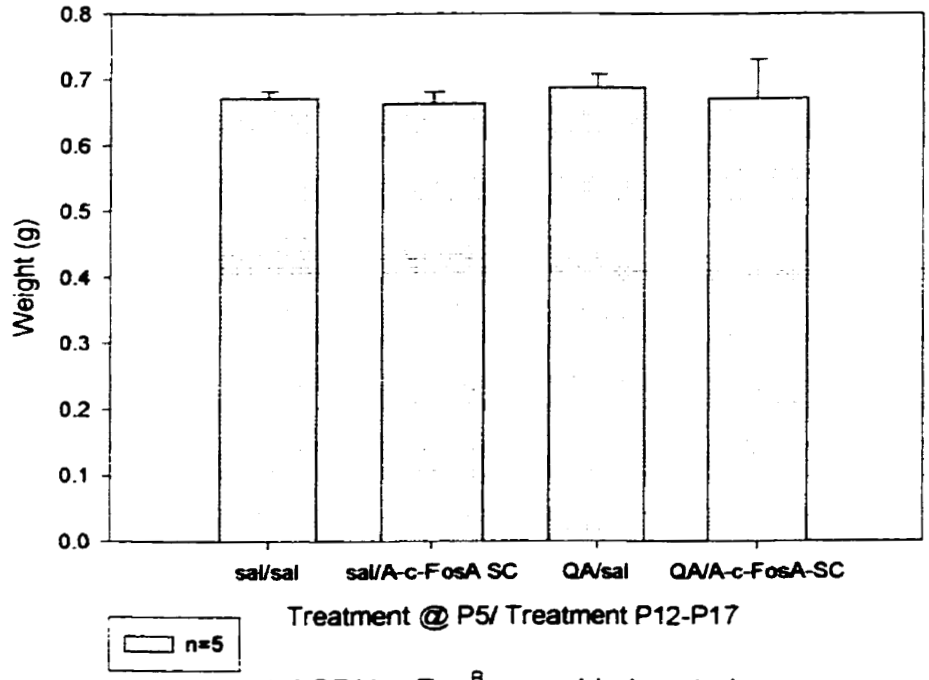


ii) AODN-c-Fos^{B} scrambled control

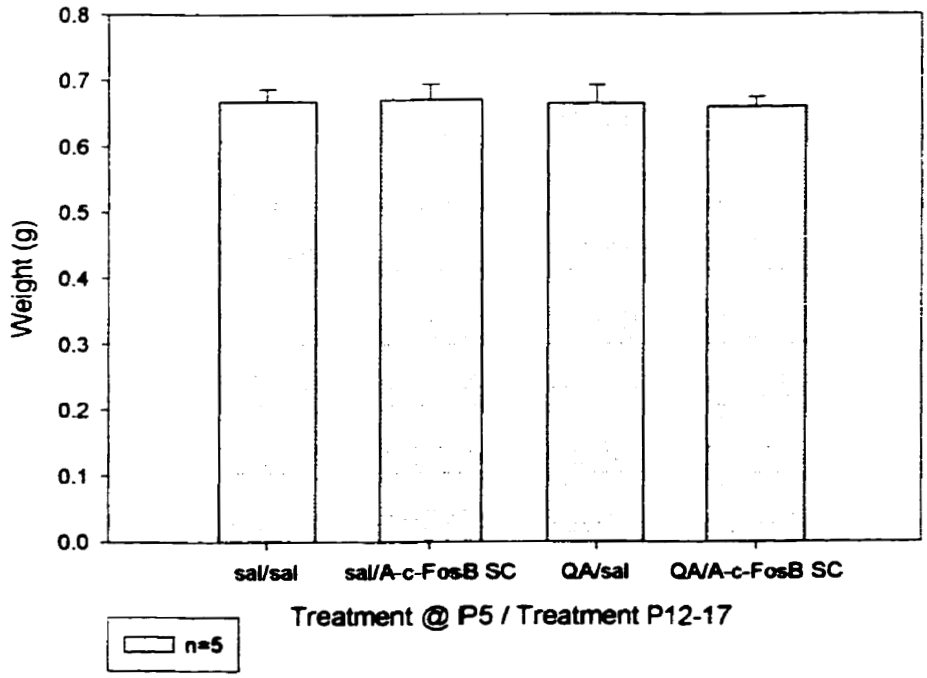


C) The effect of AODN-c-Fos scrambled control on weight of open eyes

i) AODN-c-Fos^{A} scrambled control



ii) AODN-c-Fos^{B} scrambled control



Chapter 5

DISCUSSION

The results of this study provide novel and important insights into retinal pathways that control ocular growth, specifically that they mediate ocular growth restraint. When combined with the work of many other investigators these findings allow a model of ocular growth control, and specifically growth restraint, to emerge. Elucidating the pathways necessary for visually-guided growth may lead to the development of preventive or therapeutic treatments for myopia.

Evaluation of methodology

Much progress has been made by a large community of vision researchers in the understanding of both basic retinal physiology and the development of myopia. It has been difficult to target specific subsets of retinal neurons so as to determine their roles in growth control. For example, pharmacological agents applied *in vivo* to influence the activity of a cell expressing a particular enzyme or receptor often affect other enzymes or receptors, and thus other cell populations. This non-specific effect is even greater when exogenously applied agents are cytotoxic or used at doses out of the physiological range.

In the present study immunocytochemical detection of Fos as an activity indicator circumvents the specificity problems of pharmacology by relying on changes that normally occur in cells to provide the evaluated signal. In addition, these methods have been sensitive enough to detect a response to natural stimuli rather than artificially defined or amplified stimuli.

Using AODN to block Fos expression seems to be sufficiently effective and specific to yield valuable information on the functional role of the activated cell populations. However, like pharmacology, AODN techniques also raise specificity and cytotoxicity issues (Neckers, 1998; Branch, 1998). These concerns have been minimized by choosing AODN probes of optimum length, using unmodified probes, and by repeating experiments with multiple controls and multiple probes targeted to different regions of the *fos* transcripts.

Fos labels a subset of amacrine cells that respond to visual stimuli necessary for emmetropization

The results of this study indicate that Fos is a useful indicator of retinal cell activity. This is not surprising as our lab has previously used Fos to indicate cells activated in response to specific, although more artificial, visual stimulation (stroboscopic illumination) (Rohrer et al., 1995). Several other groups have also used Fos-induction to identify activated retinal neurons (Sagar & Sharp, 1990; Yoshida et al., 1995,1998; Araki & Hamassaki-Britto, 1998). In addition, Fos isoforms and their differential expression have been used in numerous systems to probe for stimulus-induced activation.

More interesting is that Fos indicates at least one subset of amacrine cells that respond to the onset of emmetropizing stimuli (restored form-vision). Rohrer et al. (1995) were unable to identify the cells activated by form-vision with the universal Fos antiserum we used. The discrepancy between Rohrer's results and those presented here might be due to any one of several reasons. Firstly, the immunocytochemical protocol used in Rohrer's study was less sensitive as it

used an FITC-conjugated secondary antibody rather than the Cy3-conjugated secondary antibody used here. Secondly, Rohrer's chicks were form-deprived for 7 days before form-vision was restored. After seven days of form-deprivation chicks would be highly myopic (~ 10D), and goggle-removal would result in myopic defocus rather than clear form-vision. Finally, maximum levels of Fos-labeling in response to restored form-vision occur at time intervals beyond those that Rohrer examined, making the less sensitive detection method even more unlikely to detect the induction of Fos proteins other than c-Fos.

Identification of cells activated by form-vision

The cells that showed an increase in Fos in response to restored form-vision were located in the proximal third of the INL (amacrine cells) and in the ganglion cell layer. It is appropriate that amacrine cells were identified as they are the best candidate cell type for visually-guided growth control regulators. As described in the introduction, amacrine cells are likely to be tuned specifically to the type of visual stimulation that is required for emmetropization. Reasoning that the responsive cells should be amacrines, it follows that the activated cells in the GCL are likely to be displaced amacrine cells. Experiments should be done to confirm this by colocalizing Fos-LIR in the GCL with amacrine cell markers known to be expresed in displaced amacrine cells (GABA, GAD). It is noteworthy that the Fos-expressing cells in the GCL are not ChAT-IR although ChAT does label a subset of displaced amacrine cells. Should Fos colocalize with Thy-1, a marker for ganglion cells, it would imply that the Fos-LIR cells in the GCL are ganglion cells and not amacrine cells.

Because a large number of cells was labeled in response to the onset of form-vision, some retinas were treated with QA to eliminate populations of cells unnecessary for growth control. The smaller population of brightly labeled cells in the INL and GCL that remained were the primary interest for the remainder of the study.

In order to learn more about the cells that were identified by increased Fos expression in response to restored form-vision, we tried to find markers of specific bioactive substances or receptors that would identify the amacrine cells in which Fos was induced. While none of the markers tried was expressed in the Fos-positive cells, many candidate markers remain to be tested. The lack of an identifying marker at this point is disappointing, as we are left with few clues as to what populations of cells are activated, what the downstream signal might be, or what synaptic connections these cells are making. It is interesting, however, that the cells activated by restored form-vision do not express tyrosine hydroxylase (TH), glucagon, vasoactive intestinal peptide, choline acetyltransferase, or somatostatin as these substances or their receptors have been previously implicated as possible mediators of growth-restraint. It is noteworthy that some Fos-LIR cells activated by stroboscopic illumination were TH-IR (Rohrer et al., 1995) and that ZENK-IR cells responding to plus defocus were glucagon-IR (Fischer et al., 1999a). The cells identified by Fos in response to restored form-vision are of a different population from those responding to either stroboscopic illumination or plus defocus, again reaffirming that this strategy of colocalizing the activity marker with the cell specific marker is useful and valid for identifying a

specifically activated cell type. For now, the only information about the cells of interest is that the increase in Fos-LIR in response to restored form-vision can be blocked by the application of MK-801. MK-801 is a non-competitive NMDA receptor antagonist. This implies that the activated cells, or cells upstream, express one or more types of NMDA receptor. Further studies should include using various antisera to NMDA receptors to narrow down the number of possible cell types.

Restoration of form-vision elicited a change in expression of other candidate activity markers. These include antisera that recognize pERK, ZENK, c-Jun, and cGMP. These markers, and others, are being investigated currently or will be studied in the future. Of particular interest is the upregulation of ZENK, a zinc-finger transcription factor, in response to myopic defocus (either plus-lens wear or restoration of form-vision after extended goggle wear). In addition, ZENK is down-regulated in response to hyperopic defocus (minus-lens wear). The defocus-dependent changes in ZENK expression occur specifically in glucagon-expressing amacrine cells (Fischer et al., 1999a). Changes in ZENK expression in response to lens wear are accompanied by similar, though smaller, changes in the contralateral, untreated eye, suggesting that there is an efferent component to the response to defocus. This contralateral effect is also observed in growth and refraction changes. For example, change in growth due to lens wear in the treated eye is paralleled but to a lesser extent by change in growth in the contralateral untreated eye (Wallman, 1993).

Fos expression in amacrine cells is a functional component of the ocular growth-restraining pathway

The stimulus-dependent changes in Fos-expression indicated some cells that were activated in response to form-vision, but did not provide evidence as to whether these cells were involved in or necessary for growth control. It is completely possible that an activity marker be upregulated in response to emmetropizing stimuli without actually being involved in the control of normal eye growth. However, in the absence of further information about the cells that synthesize Fos in response to form-vision, the most straightforward way to manipulate the function of the activated cells was to modulate the expression of Fos itself. Evidence for the role of Fos in the visual regulation of growth came from experiments employing AODN-Fos to chronically block Fos expression. This caused the development of myopia in eyes that would otherwise have emmetropized.

The dose-response and time-response curves were helpful for estimating the amount and interval of AODN-Fos application in order to chronically reduce Fos expression. Despite concerns about AODN non-specificity and toxicity, the eyes of chronically treated chicks had good refractive reflexes and showed normal retinal histology with a significant reduction in Fos-expression.

Chronic knock-down of Fos expression in goggled eyes did not affect the development of form-deprivation myopia. This result is expected because the levels of Fos peak sharply with the addition of a goggle and then decrease to lower than open-eye levels. Thus, there is little Fos synthesis in a goggled eye to

either act as part of a control pathway, or be blocked by AODN-Fos. However, in contrast, chronically blocking Fos synthesis in an open eye, resulted in the development of myopia characterized by increased ocular weight, axial length, and negative refractive error. Eyes treated with QA one week prior to the beginning of AODN-Fos injections developed significantly more myopia than those of a saline-treated control group. The difference between the amount of myopia that develops in QA-treated versus saline-treated groups in response to AODN-Fos application predicts that at least two groups of cells participate in pathways that restrain eye growth: Fos-positive amacrine cells that are insensitive to QA, and a population of Fos-negative amacrine cells that are sensitive to QA. Cell types that are known to be destroyed by QA include those that are immunoreactive for ChAT, VIP, enkephalin, and many of the populations that are immunoreactive for GABA, 5-HT, parvalbumin, AMPA-type glutamate receptors, and nNOS.

The finding that these Fos-expressing cells are involved in growth control is an exciting and important discovery. Prior to this the only exogenously applied agents capable of inducing myopia in the absence of form-deprivation were those that toxically and permanently eliminated many retinal cell types, such as toxic doses of, kainate, NMDA, and colchicine (Wildsoet & Pettigrew, 1988a; Fischer et al., 1997, 1999b). Removal of several populations of amacrine cells allows the role of Fos to be more clearly observed.

Cells expressing Fos isoforms, Fra-2 and c-Fos, participate in the ocular growth-restraining pathway

The antiserum originally used to label activated cells was directed to a region of high homology between all Fos proteins (c-Fos, Fra-1, Fra-2, Fos B) (Nishina et al.). It has been observed that the temporal and spatial response characteristics of Fos proteins vary with the stimulus and with the isoform activated (Nishina et al., 1990). Using antisera specific to each isoform it was possible to determine that Fra-2 and c-Fos were expressed in response to restored form-vision, estimate the duration of their expression, and determine that they were the best candidates for mediating vision-induced growth restraint.

Most of the cells activated by restored form-vision were positive for Fra-2, and at the longer intervals following goggle removal all of the Fos-LIR cells were positive for Fra-2. AODN-Fra-2 treatment of either saline or QA-treated eyes produced results nearly identical to those observed with the AODN-Fos probe. From these results we conclude that it is primarily the Fra-2 isoform that is responsible for the growth-restraining pathway of the Fos-positive amacrine cells. The prolonged time course of Fos induction by termination of form-deprivation is typical of Fra-2 (Cohen & Curran, 1988; Matsui et al., 1990; Nishina et al., 1990). In addition the longer time course of upregulation of Fra-2 as a candidate retinal growth-modulating signal could account, in part, for the very long delay between signal onset and changes in scleral growth rate (Devadas & Morgan, 1996). It has been described that as little as 15 min of daily exposure to form vision can reduce FDM and less than 3 hr of daily exposure to form vision can eliminate FDM (Napper et al.,1995, 1997). The long duration of Fra-2 upregulation in

response to the onset of form-vision may account for the minimal amount of time required to inhibit the effects of goggling.

In addition to the dominant, slow, and prolonged increase in the number of Fra-2-LIR cells in response to restored form-vision, there was a rapid and brief increase in the number c-Fos-LIR amacrine cells. AODN-c-Fos also induced myopia in QA-treated retinas but less than AODN-Fos or AODN-Fra-2. AODN-c-Fos had no significant effect in eyes that had not been treated with QA. Although the time course of c-Fos upregulation is not consistent with the lag between signal onset and changes in scleral growth rate, c-Fos may still play a role in the initial, immediate response to form-vision through the activation of late-response genes. It is apparent from results presented here that there are redundant or convergent pathways that act to restrain eye growth. It is not improbable that c-Fos acts in parallel to Fra-2, likely within different cell populations, with a smaller contribution to growth restraint. An interesting follow-up experiment would be to see whether the effects of AODN-c-Fos and AODN-Fra-2 are additive.

Model of ocular growth-control

The results presented here, considered with those of many other ongoing investigations, lead me to the following model of ocular growth control, and specifically growth restraint:

There exists from postnatal day 1 a default "grow" signal, which at first corrects the eye from its initial hyperopic state. This then is attenuated by a "stop" signal so that the size of the eye is appropriate for its refractive characteristics. The "grow" signal persists throughout development so that ocular size increases

with overall body development but it is prevented from causing abnormal elongation by the form-vision dependent "stop" signal(s). Several factors have been identified that are necessary for the default grow signal to be maintained. For example, the application of APB to the retina blocks ON-responses, reduces growth and results in hyperopia (Fujikado, et al., 1996).

Much evidence supports the idea of a vision-dependent "stop" signal. The eye elongates in response to form deprivation, re-emmetropization requires that the eye be exposed to form-rich visual stimuli, and the eye's growth responds to sign of defocus. The results reported in the present study also support the existence of a vision-dependent growth-restraining pathway.

As a visual signal is transduced through the retina the minimum role of the outer retina (photoreceptors, horizontal cells, and bipolar cells) is to transmit sufficient information so that form and contrast can be detected at the amacrine cell layer. Amacrine cells are tuned specifically to the type of visual information necessary for emmetropization. Many types of amacrine cells and neuroactive substances contained within amacrine cells (dopamine, VIP, ACh, enkephalin) have been implicated as having a role in growth regulation. However, because of the apparent presence of redundant and/or convergent pathways the most useful work with respect to amacrine cells has been that which eliminates groups of cells not required for normal growth-control (Fischer, et al., 1998b).

The results of the present study demonstrate that the expression of Fos in a population of amacrine cells plays an active role in growth control, and that the action of Fos-synthesizing amacrine cells is combined with that of another signal

arising from QA-sensitive pathways. The model presented here depicts the Fra-2 expressing pathway as providing the strongest signal to restrain growth by reason that blocking Fra-2 alone can elicit some myopia. Removal of the stop signal from the QA pathway enhances the effect seen with AODN-Fra-2. The QA-sensitive pathway provides the second strongest "stop" signal, as removal of this pathway alone is not sufficient to cause the development of myopia. However, when used in combination with AODN-Fos or AODN-Fra-2 it results in a great deal of myopia. The c-Fos stop signal is the weakest as its blockade alone does not elicit any myopia and even in combination with QA it only induces low amounts of myopia.

Glucagon-IR amacrine cells respond to defocus by upregulating expression of ZENK in response to positive defocus and decreasing ZENK expression in response to negative defocus (Fischer et al., 1999). Although the effect of blocking ZENK expression has not been fully investigated, it is likely that the ZENK-expressing amacrines participate in a pathway that discriminates defocus and restrains growth in response to positive defocus. The fact that such a system does not detect and compensate for the defocus imposed by AODN-Fos – induced myopia implies that the Fos and ZENK pathways may have a point of convergence or interdependence. It is possible that ZENK expression in glucagon cells is downstream of Fos expression in amacrine cells and somehow dependent upon the presence of Fos. Another possibility is that ZENK expression in glucagon cells is not dependent on Fos expression but that some downstream event that requires Fos is inhibited and thus the eye does not compensate for

imposed defocus. Experiments should be completed to test whether glucagon cells can respond to defocus by upregulating ZENK in an eye treated with AODN-Fos, or whether Fos expression is changed by manipulating ZENK or glucagon signaling.

An important consideration is the possibility that the increased growth due to blocking Fos expression may be due to blocking the response to general illumination rather than to form-vision. Chicks reared in constant darkness develop enlarged eyes (Gottlieb et al., 1987). Chicks reared in constant light develop hyperopia, and form-deprived chicks reared in constant light develop significantly less myopia than controls (Li, et. al., 1995; Bartmann, et al., 1994). This implies that steady levels of illumination contribute to an enhanced "stop" signal. It is well documented that Fos is upregulated in response to light onset and increased illumination. Thus, it is possible that AODN-Fos blocks a component of a pathway that controls the response to illumination or (cessation of darkness) and thus inhibits the light-dependent "stop" signal. If we are able to find cell-type specific markers to separately identify cells that express Fos in response to light versus form-vision we might be able to target each cell type separately to determine which parameter is being affected by AODN-Fos to cause excessive growth.

Fos expression in amacrine cells may be in direct response to the introduction of form-rich visual stimulation or may be downstream of other pathways that are sensitive to the onset of form-vision. Fos forms a dimer with a member of the Jun family that binds to the AP-1 regulatory region of a late-response gene.

Depending on the Fos-Jun combination, the binding of the dimer can either promote or inhibit transcription of the late-response gene (Hughes & Dragunow, 1995). It is likely that more than one late response gene is activated for each Fra-2/Jun and c-Fos/Jun dimer. The product(s) of these late-response genes continue the form-vision-responsive signal cascade that eventually results in ocular growth restraint. There may be a relatively large number of steps between the Fos signal in a population of amacrines and the final effect on scleral growth. These intermediate steps must include factors that can initiate signal transduction that traverses the RPE and choroid to reach the sclera (candidates include retinoic acid and TGF-β). Figure 22 summarizes the role of Fos in ocular growth restraint.

Future Directions

The results of this study have provided interesting and useful information about a subset of neurons activated by a particular stimulus and the functional role of these neurons in growth control. While these findings provide clues about the retinal pathways that control growth they do not completely explain the mechanisms of ocular growth control. The most important task is to find cell-type specific markers that identify the cells in which Fos (Fra-2 and/or c-Fos) is induced. This would further facilitate the identification of inputs to Fos-expressing amacrine cells, and provide candidate bioactive substances for downstream signaling. Once the type of the activated cell is identified, it would be useful to examine the specific types of stimuli to which the cell type responds. With defined stimuli one could test the receptive field characteristics and preferences for specific temporal or spatial frequencies of the cell type of interest. This would

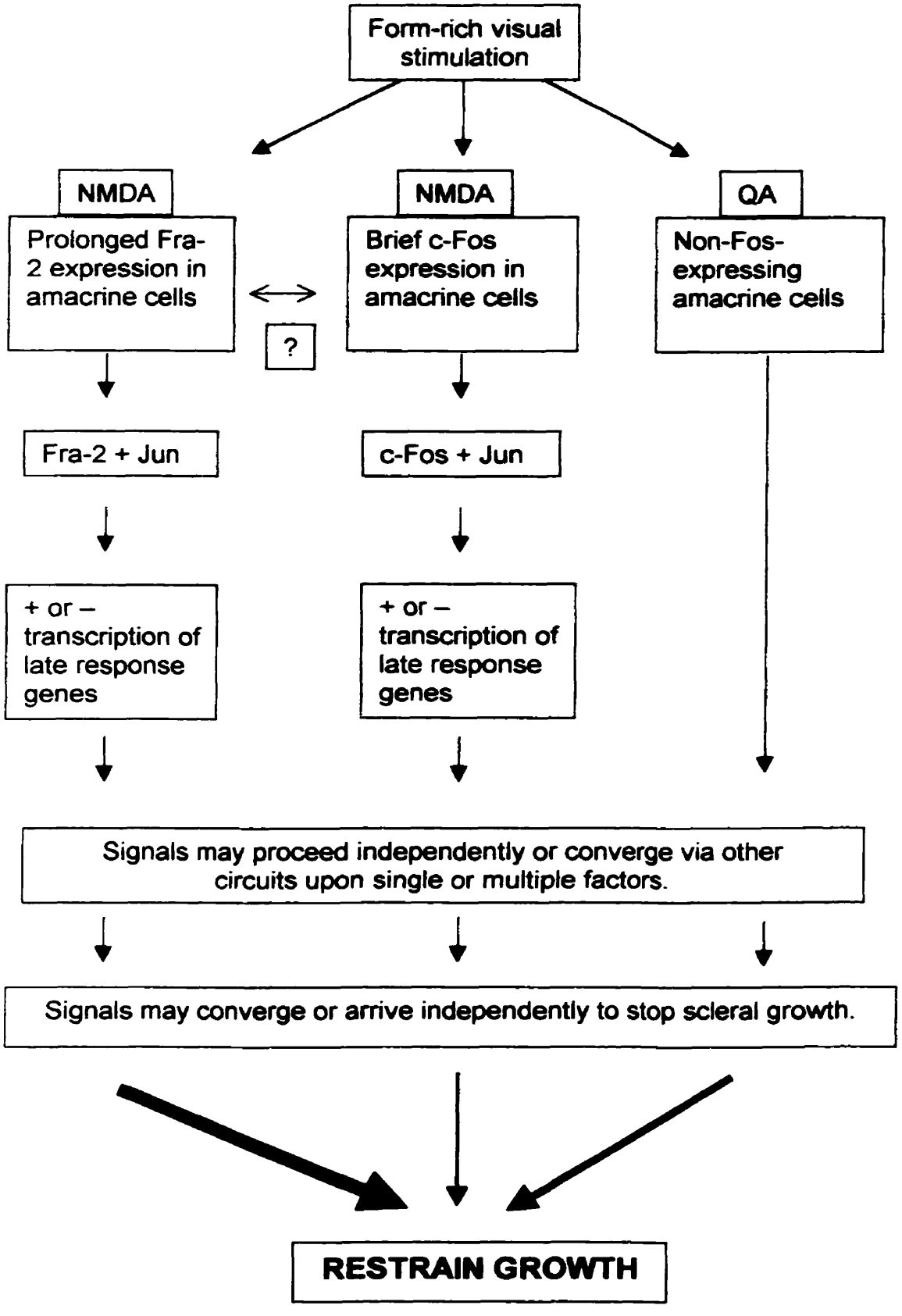
further define the cell type as well as provide valuable information about the visual stimuli that specifically activate a "stop" signal. To further investigate the use of AODN to modulate retinal activities, it would be useful first to identify the minimum effective dose of each AODN to induce growth changes. In addition, it would be useful to try AODN with techniques for more efficient drug delivery or sustained drug delivery (nanospheres or PVA coated pellets (Gogolak, et al., 1999; Sakurai, et al., 1999)) To advance knowledge of pathways that contribute to normal ocular growth control or myopia, important further studies include testing whether overexpression of Fos can block developmental myopia. Limited support for this comes from studies showing that transgenic overexpression of Fra-2 in mice results in microphthalmia (Matthaei, 1998). It would also be useful to try to identify the QA-sensitive cells that act to restrain growth.

Conclusion

In conclusion, the results reported here demonstrate that activity indicators, such as Fos, are valuable tools for identifying retinal pathways that respond to specific stimuli. In addition this project has provided new information about pathways that act to restrict ocular growth. Such knowledge about pathways of emmetropization will help to fully understand the components of growth control and may lead to treatments for developmental myopia.

Figure 5.1: Role of Fos in Ocular Growth Restraint

The results support a functional role for Fos expression in amacrine cells in ocular growth restraint. A population of QA-resistant amacrine cells expresses Fos when the retina is exposed to form-rich visual stimulation. Fos expression in amacrine cells may be in direct response to the introduction of form-rich visual stimulation or may be downstream of other pathways that are sensitive to the onset of form-vision. Fos forms a dimer with a member of the Jun family that binds to the AP-1 regulatory region of late-response gene(s). Depending on the Fos-Jun combination the binding of the dimer can either promote or inhibit transcription of the late-response genes. One product of the late-response genes continues the form-vision-responsive pathway that eventually results in ocular growth restraint. In addition to the Fra-2 and c-Fos-mediated pathways there is also a QA-sensitive pathway that gives rise to a growth-restraining signal.


Form-rich visual stimulation
NMDA
Prolonged Fra-2 expression in amacrine cells
?
NMDA
Brief c-Fos expression in amacrine cells
QA
Non-Fos-expressing amacrine cells
Fra-2 + Jun
c-Fos + Jun
+ or – transcription of late response genes
+ or – transcription of late response genes
Signals may proceed independently or converge via other circuits upon single or multiple factors.
Signals may converge or arrive independently to stop scleral growth.
RESTRAIN GROWTH

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