THE UNIVERSITY OF CALGARY

Muscarinic Mechanisms in Myopia and Ocular Growth

by

Andy J. Fischer

A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF NEUROSCIENCE

CALGARY, ALBERTA

DECEMBER, 1998

Abstract

Abnormal ocular growth results in the common vision disorders myopia (near-sightedness) or hyperopia (far-sightedness). Ocular growth must be precisely regulated to match the combined refractive power of the lens and cornea to the distance between the lens and retina so that distant objects are focussed upon the retina with neutral accommodation. This regulation of ocular growth is a function of the clarity of images to the eye and of processing of visual information by the retina. Several subtypes of retinal amacrine cells are believed to be required to properly guide the growth of the eye and promote excessive growth during deprivation of patterned images. Myopia usually results from excessive axial elongation of the eye, can be induced by form-deprivation, and can be prevented by chronic administration of muscarinic antagonists in several species, including humans. The focus of this dissertation is to investigate the role of cholinergic amacrine cells and muscarinic receptors in visually guided ocular growth. The paradigm of form-deprivation myopia (FDM) is studied commonly in chicks, and, accordingly, was used in the current work.

Three different isoforms of muscarinic acetylcholine receptor (mAChR) were localized to amacrine, bipolar and ganglion cells in the retina. These mAChRs were also detected in the retinal pigmented epithelium, choroid, and ciliary body of the chick eye. The distribution of mAChRs is consistent with a role for cholinergic pathways in visually guided ocular growth. I characterized the effects of neurotoxins upon different populations of retinal cells (cholinergic amacrine cells in particular) and upon normal and

form-deprivation-induced ocular growth. I tested whether the muscarinic antagonist atropine could suppress FDM in toxin-treated eyes. The selective cholinotoxin, ethylcholine mustard aziridinium (ECMA) and a less-selective excitotoxin, quisqualic acid (QA), destroyed most (all in the case of QA) cholinergic amacrine cells, yet did not affect normal ocular growth or FDM. Furthermore, atropine retained its ability to suppress FDM in both ECMA and QA-treated eyes. In conclusion, it is unlikely that cholinergic amacrine cells and mAChRs in the retina are required for visually guided ocular growth, and that atropine suppresses FDM by acting at extraretinal sites or at non-muscarinic receptors.

Acknowledgments

I wish to thank above all Bill Stell for the supervision and friendship offered through the course of my graduate career. Bill was a great supervisor and I owe much of my interest and success in science to him.

For their helpful suggestions which have contributed to the final form of this thesis, I wish to thank the members of my supervisory and examination committees, Drs Steven Barnes, Andy Bulloch, Quentin Pittman, Torben Bech-Hansen, and my external examiner Dr. Josh Wallman. Special thanks and appreciation are also extended to my collaborators and co-authors, including: Drs Neil M. Nathanson, Lise A. McKinnon, Ruth L.P. Seltner, Ian G. Morgan, and Pat Miethke; and journal reviewers, for their contributions to the papers that form Chapters 2, 3 and 4 of this disseration. Thanks also to members of the "Stell" lab for their friendship, comradery, and video-game adversarialism, which made "working in the lab" seem more like "playing in the mud".

I also wish to extend a special thanks to my wife Sheri and daughter Annaya who may have deprived me of sleep, but in turn offered me a family to keep me a well-rounded person (I think?). I also wish to thank my parents for their endless supplies of love and support.

Table of Contents

List of Tables

List of Figures

List of Figures (continued)

Abbreviations and Symbols

ACh	Acetylcholine
AChR	Acetylcholine receptor
AMPA	Aminohydroxy methylisoxazole propionic acid
ANOVA	Analysis of variance
APB	amino-4-phosphonobutyrate
BSA	Bovine Serum Albumin
ChAT	Choline acetyltransferase
cGMP	cyclic guanadine monophosphate
cm2	Chick muscarinic acetylcholine receptor subtype 2
cm3	Chick muscarinic acetylcholine receptor subtype 3
cm4	Chick muscarinic acetylcholine receptor subtype 4
cm5	Chick muscarinic acetylcholine receptor subtype 5
Cy3	Cyanin fluorophore
°C	Degrees Celsius
Da	Dalton
dCTP	Deoxycytosine triphosphate
DNA	Deoxyribonucleic acid
ECMA	Ethylcholine mustard aziridinium
EDTA	Ethylene diamine tetra-acetic acid
ENK	Enkephalin

ENSLI	Enkephalin, neurtensin, somatostatin-like immunoreactive
ETCs	Efferent target cells
EtOH	Ethanol
FDM	Form-deprivation myopia
FITC	Fluorescein isothiocyanate
G	Gravitational force on earth at sea level
GABA	γ-amino butyric acid
$GABA_A$	γ-amino butyric acid receptor subtype A
GAD	Glutamic acid decarboxylase
GAT-1	GABA transporter subtype 1
GC	guanylyl cyclase
GCL	Ganglion cell layer
GluR1	Glutamate receptor subunit 1
GluR2	Glutamate receptor subunit 2
GluR3	Glutamate receptor subunit 3
GST	Glutathione-S-transferase
GTP	Guanidine triphosphate
5-HT	5-hydroxy tryptamine (serotonin)
IC_{50}	50% inhibition concentration
IgG	Immunoglobulin G
INL	Inner nuclear layer
IPL	Inner plexiform layer

kDa	KiloDalton
µg	Microgram
µl	Microliter
µm	Micrometer
M	Molar
ml	Milliliter
mM	Millimolar
MeOH	Methanol
mAChR	Muscarinic acetylcholine receptor
mGluR6	Metabotrophic glutamate receptor subtype 6
mRNA	Messenger ribonucleic acid
NAA	Neurofilament-associated antigen
nAChR	Nicotinic acetylcholine receptor
NCM	Nitrocellulose membrane
NGS	Normal goat serum
nm	Nanometer
nmol	Nanomole
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
OFL	Optic fiber layer
OPL	Outer plexiform layer

PB	Phosphate buffer
PBS	Phosphate buffered saline
PDA	cis-2,3-piperidine-dicarboxylic acid
pH	Log_{10} proton concentration
PKC	Protein kinase C
PrBCM	Propylbenzilylcholine mustard
QA	Quisqualic acid
QNB	Quinuclidinyl benzylate
RA	Retinoic acid
RPE	Retinal pigmented epithelium
rpm	Rotations per minute
SDS	Sodium dodecylsulfate
SNP	Sodium nitroprusside
TH	Tyrosine hydroxylase
Tx-100	Triton X-100
V	Volt
VIP	Vasoactive intestinal polypeptide
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

CHAPTER ONE

Muscarinic Mechanisms of Visually Guided Ocular Growth

Introduction

What is visually guided ocular growth?:

Normal vision, emmetropia, occurs when distant objects are projected onto the retina in focus with the accommodative mechanisms of the eye at rest. At birth, the eyes of most vertebrates are ametropic, but grow towards emmetropia during the post-natal or juvenile period. To achieve emmetropia there must be a precise regulation of ocular growth to match the axial length and physical contours of the posterior segment of the eye to the refractive power of the lens and cornea. This process is referred to as emmetropization. If the vitreous chamber of the eye grows too much, the image of distant objects will be focused in front of the retina, resulting in myopia or near-sightedness, whereas if there is too little growth the image will be focused behind the retina, resulting in hyperopia or far-sightedness.

Human myopia is a common disorder, affecting a significant portion of the world's population (Curtin, 1985). Myopia typically develops early in childhood, progresses through adolescence and early adulthood, and imposes a life-long need for corrective spectacles or corneal surgery. The commoness and burdens that myopia imposes upon day-to-day life and health care expenses warrant that the mechanisms underlying its development be investigated and that practical, alternative therapies be developed.

During growth of the eye, emmetropization is mediated by retinal activity that is driven by the clarity of the incident images. The retina must receive and respond correctly to clearly focused images (reviewed by Wallman, 1993). Investigations have demonstrated that the blockade of electrical signals leaving or coming to the retina, by either optic nerve section (Troilo et al., 1987; Weisel and Raviola, 1977; Wildsoet and Pettigrew, 1988) or tetrodotoxin-mediated blockade of action potentials along axons of ganglion cells (Norton et al., 1994; McBrien et al., 1995), has no effect on the development of experimentally-induced myopia. Furthermore, ocular growth can be modulated in local regions of the eye by sectorial deprivation (Wallman et al., 1987; Wallman, 1990; Norton & Siegwart, 1991) and by imposition of heterogeneous focus or defocus (Hodos & Kuenzel, 1984; Gottlieb et al., 1985, 1987; Hodos & Erichsen, 1990; Miles & Wallman, 1990; Irving et al., 1992). These studies suggest that local retinal activity directly controls the growth of adjacent regions of sclera. Therefore, the feedback control system which regulates visually guided ocular growth is at least in part intrinsic to the eye, and is orchestrated by retinal activity.

Emmetropization can be perturbed by lid-suturing, applying translucent goggles, or plus or minus lenses over the eye, all of which deprive the eye of patterned images or form vision. Form-deprivation has been shown to cause myopia in tree shrews (Sherman et al., 1977), monkeys (Wiesel and Raviola, 1977), and chicks (Wallman et al., 1978). This form-deprivation myopia (FDM) is thought to result from the absence of retinal responses to a narrow range of spatial frequencies and contrast (Wallman, 1993; Bartmann and Schaeffel, 1994; Schmidt and Wildsoet, 1996). Emmetropization from a

form-deprived state can be promoted by limiting daily visual experience to a particular range of temporal (Gottlieb & Wallman, 1987; Wallman, 1990; Vingrys et al., 1991) or spatial frequencies (Schmid & Wildsoet, 1993, 1996). Processing of spatial and temporal image parameters is a primary function of the inner plexiform layer (IPL) of the retina (Sakuranaga & Naka, 1985 a, b; Sakai & Naka, 1987), suggesting that circuits formed between amacrine cells or by their inputs to ganglion cells are required for visual guideance of ocular growth.

Retinal cells involved in the regulation of ocular growth:

Form-deprivation in chicks results in increased axial length, equatorial circumference, eye weight, and negative refractive error (Wallman et al., 1978; Pickett-Seltner et al., 1988). These increases in refractive error and ocular size ultimately result from the growth and remodelling of the sclera, the outer connective tissue sheath of the eye. Several signalling pathways between the retina and sclera are thought to participate in the development of FDM. These pathways may include cells which are influenced by dopamine agonists (Stone et al., 1989; Rohrer et al., 1993), opioid compounds (Seltner et al., 1994; Seltner et al., 1997), basic fibroblast growth factor (Rohrer & Stell, 1994), antagonists to acetylcholine (ACh; Stone et al., 1991; McBrien et al., 1993), vasoactive intestinal polypeptide (VIP; Stone et al., 1988; Seltner et al., 1995a, b), nitric oxide synthase (NOS; Fujikado et al., 1997), neurotensin (Seltner, personal communication), and N-methyl-*D*-aspartate-receptors (NMDA; Seltner et al., 1996; Fischer et al., 1998g). Treatment of form-deprived eyes with agents related to the factors listed above suppresses

excessive ocular growth and myopia. The results of these pharmacological studies, accordingly, have suggested roles in myopia for particular subsets of amacrine cells that have been characterized immunocytochemically including those that contain tyrosine hydroxylase (TH), VIP, choline acetyltransferase (ChAT), nitric oxide synthase (NOS), enkephalin (ENK) and neurotensin. Additionally, form-deprivation alters ocular levels of dopamine and its metabolite dihydroxyphenylacetic acid (DOPAC; Stone et al., 1989), VIP (Stone et al., 1988), ENK (Ali et al., 1993; Megaw et al., 1994), inducible NOS (iNOS; Fujii et al., 1998), and metabolites of nitric oxide (Fujikado et al., 1997). However, it remains uncertain whether these vision-dependent changes in transmitter levels and the enzyme iNOS are causal to accelerated rates of ocular growth, or are merely a few of many changes in cell activity or function caused by deprivation of form-vision and do not have anything to do with increased rates of ocular growth.

Recently, serious doubt has been cast as to the specificity of pharmacological agents and, consequently, the conclusions drawn from studies wherein FDM was suppressed by a drug. For example, evidence supporting a role for ENK-containing amacrine cells in the development of FDM in the chick is controversial. Naloxone, best known as an opiate receptor antagonist, prevents FDM in a dose-dependent manner (Seltner et al., 1994, 1997). However, several puzzles remain unresolved regarding the effect of naloxone on FDM and the involvement of the opiate system in the control of ocular growth. First, the K_d for naloxone at opiate receptors is in the nanomolar range (for review see Jaffe & Martin, 1985), while the calculated ED_{50} for the FDM-blocking effect of naloxone was in the picomolar range (Seltner et al., 1997). Second, naloxone

has been reported to bind preferentially at μ and δ receptors (Martin & Sloan, 1985), whereas the κ receptor is the predominant type in chick retina (Slaughter et al., 1985). Third, the κ antagonist nor-BNI only suppressed FDM by a maximum of 50%, whereas the κ agonist U50,488 suppressed FDM (Seltner et al., 1997). Finally, both opiate-active and inactive enantiomers of morphine-like compounds (levorphanol and detrorphanol, and L- and D-naloxone) are equally effective in blocking FDM (Fischer et al., 1998g), and naloxone and other opiate antagonists have been reported to act at NMDA receptors (Wong & Kemp, 1991; Shulka & Lemaire, 1994). For these reasons, naloxone is not likely to suppress FDM by blocking the actions of enkephalin at opiate receptors, and since NMDA-receptor antagonists prevent FDM (Fischer et al., 1998g), naloxone is likely to prevent FDM by acting at NMDA-receptors. However, in some mammalian systems naloxone does not bind to the NMDA receptor (Ebert et al., 1998).

Of all pathways implicated in ocular growth control, cholinergic mechanisms are of particular interest as they hold the greatest potential as the target of pharmacological therapies for human ametropias. Antagonists to muscarinic acetylcholine receptors (mAChR) reduce the development of myopia in man (Parsons, 1923; Luedde, 1932; Gostin, 1962; Bedrossian, 1971, 1979; Goss, 1982), one species of monkey (Raviola and Weisel, 1985), tree shrew (McKanna and Casagrande, 1981), and chick (Stone et al., 1991; McBrien et al., 1993; Leech et al., 1995). In the chick, treatment of form-deprived eyes with atropine (a non-specific mAChR-antagonist) or pirenzepine (a mammalian M1-specific antagonist) has been shown to prevent FDM (Stone et al., 1991; McBrien et al., 1993; Leech et al., 1995). Stone et al., (1991) reported that atropine or pirenzepine

rescued FDM at lower doses when applied to the eye subconjunctivally when compared to the effective doses of intravitreal applications of these compounds by other investigators. In comparison, McBrien et al. (1993) and Leech et al. (1995) found that intravitreal applications of atropine and pirenzepine were more potent than subconjunctival applications in preventing FDM. These conflicting results might be explained by different dose regimens or perhaps by different abilities of atropine and pirenzepine to penetrate tissues.

It remains uncertain whether retinal or choroidal sources of ACh regulate ocular growth. If choroidal sources of ACh regulate ocular growth, then denervation of cholinergic afferents to the choroid should suppress FDM just as chronic application of mAChR antagonists does. However, the refractive error of open or form-deprived eyes is only reduced slightly when cholinergic innervation to the choroid and ciliary body has been removed (Wallman et al., 1981; Schaeffel et al., 1988, 1990; Troilo, 1990; Lin and Stone 1991; Troilo and Wallman 1991; Wildosoet et al., 1993). It should also be noted that sectioning of the choroidal nerves may result in massive degeneration of the temporal retina (Reiner et al., 1995), which in itself should abolish retinal influence upon ocular growth. In addition, the sectioning, degeneration and removal of the distal segments of lesioned choroidal and ciliary nerves could liberate a number of cellular signals (such as bradykinins, prostaglandins, and trophic factors) and possibly the release of transmitters (such as somatostatin, neurotensin, VIP and norepinephrine) which might affect the normal progression of FDM. Taken together, these results suggest that the cholinergic innervation to the choroid and ciliary body does not contribute to the progression of

FDM. Therefore, the possibility remains, and some evidence suggests (McBrien et al., 1993; Leech et al., 1995), that retinal sources of ACh and mAChRs participate in visually guided ocular growth.

Toxin-mediated changes in ocular growth:

Another approach by which to examine the role of cholinergic pathways in the regulation of ocular growth may be to characterize the effects of neurotoxins upon amacrine cells and visually guided ocular growth. If glutamatergic retinal pathways contribute to the regulation of ocular growth, then the destruction of glutamate-sensitive cells should affect emmetropization and FDM. Indeed, many studies have shown that the application of a single toxic dose of selective glutamate receptor agonists prevents both emmetropization and FDM.

The most thoroughly investigated excitotoxin is kainic acid, an analogue of glutamate that preferentially activates ionotropic non-NMDA glutamate receptors. Wildsoet and Pettigrew (1988a) showed that a single intravitreal dose of kainic acid caused age- and dose-dependent changes in ocular growth; 200 nmoles increased axial eye length, flattened the cornea, and caused myopic refraction. Other studies have confirmed that excitotoxic damage elicited by kainic acid increases eye size (but mainly equatorial diameter) (Barrington et al., 1989; Ehlrich et al., 1990). In contrast, Lauber and Oishi (1990) found that kainic acid had no effect on eye weight or corneal curvature, but suppressed lid-suture myopia and enhanced the enlargement induced by both constant light and cyclical dim light. Ehrlich et al. (1990) also reported that kainic acid reduced

the increases in axial length normally induced by a black occluder.

Aminohydroxy methylisoxazole propionic acid (AMPA) and quisqualic acid (QA) are glutamate analogues, with preference for different ionotropic non-NMDA glutamate receptors, subsets different from those that prefer kainic acid. QA also acts at some metabotropic glutamate receptors (Mody & MacDonald, 1995). The effects of these excitotoxins differ substantially from those of kainic acid. QA has been reported to increase anterior chamber depth and decrease equatorial diameter (Barrington et al., 1989; Ehrlich et al., 1990), with no effect upon the enhancement of ocular growth and negative refractive error caused by form-deprivation (Ehrlich et al., 1990). In contrast, AMPA reduced anterior chamber depth and reduced the enlargement of the vitreous chamber normally caused by form-deprivation or negative lenses (Wildsoet et al., 1995).

NMDA has also been shown to affect both emmetropization and FDM (Fischer et al., 1997b, 1998g). The application of a single toxic dose of NMDA to the chick eye resulted in a transient increase in the rate of ocular growth, myopia that persisted for at least 35 days after treatment, and left eyes non-responsive to form-deprivation (Fischer et al., 1997b, 1998g). This transient increase in rate of growth occured within 7 days of exposure to NMDA, during which time it is likely that certain types of growth regulating-cells were damaged or destroyed (Sattayasai & Ehrlich, 1987; Ehrlich et al., 1990).

The destruction of cells via excitotoxicity:

The specific subsets of retinal cells affected by kainic acid, NMDA, and QA have not been characterized, but may include those implicated as regulators of ocular growth

by pharmacological studies. Identification of the specific types of cells destroyed by these toxins might implicate (or exclude) different transmitters and retinal circuits in the regulation of ocular growth. Therefore, it is important to understand the mechanisms underlying excitoxicity and identify not only those cells that are destroyed, but also those that are damaged and no longer functional following exposure to different growth-modulating excitotoxins. Furthermore, it is important to identify the cells that survive exposure to toxins and continue to maintain visual guidance of ocular growth, as these cells will remain likely candidates as modulators of growth.

Excitotoxicity occurs when an agent causes excessive cellular depolarization, increased influxes of Na^+, Ca^{2+}, and Cl^-, and , consequently, cytolysis. Excitotoxicity can be driven by the over-activation of ionotropic glutamate receptors. Glutamate receptors activated by N-methyl-*D*-aspartate (NMDA) are permeable to Ca^{2+} during strong depolarization and have been shown to be instrumental in some forms of neural excitotoxicity (Choi et al., 1988; Ankarcrona et al., 1995; Romano et al., 1995). Similarly, the over-activation of non-NMDA glutamate receptors by quisqualic acid (QA) can result in excessive depolarization and subsequent cellular dysplasia (Zeevalk et al., 1989). The destruction of neurons by excitotoxicity occurs through necrotic (acute) and apoptotic (delayed) cell death (Olney, 1986; Choi, 1988, 1992; Simonian et al.,1996).

Necrosis and apoptosis are two distinct forms of cell death which proceed at different rates after an insult. Necrotic and apoptotic cells take on different appearances, undergo different types of metabolic and biochemical disintegration, and affect surrounding tissues in different ways. Necrosis is passive, occurs soon after an insult, and

is characterized by swelling of cells and organelles, accompanied by the spillage of intracellular contents. In contrast, apoptosis is thought to be an active, organized process, is often delayed, and is characterized by cell shrinkage, relocation and compaction of organelles, condensation and fragmentation of chromatin, and production of vesicular particles containing cytoplasmic materials known as "apoptotic bodies" (Kerr et al., 1972; Wyllie et al., 1980; Arends and Wyllie, 1991). Necrotic cell death is Na^+ and Cl^--dependent, but Ca^{2+}-independent (Olney et al.,1986), while apoptotic cell death is Ca^{2+}-dependent and is often associated with activation of the NMDA-receptor (Choi, 1985; Rothman et al.,1987). The destruction of retinal cells exposed to excitotoxins is thought to occur predominantly via apoptosis (Romano et al., 1995).

Glutamate receptor-specific agonists have been used to identify retinal cells that putatively express glutamate receptors and are susceptible to excitotoxic insult (Coyle et al., 1978; Morgan and Ingham, 1981; Gibson and Reif-Lehrer, 1984; Lopez-Colome and Somohano, 1984; Kleinschmidt et al., 1986 a,b; Zeevalk and Nicklas, 1990). Kainic acid, at doses that affect ocular growth, has been reported to: (i) destroy specific types of bipolar cells (presumably most, if not all OFF-bipolars); (ii) destroy many amacrine cells; and (iii) damage some ganglion, photoreceptor, and horizontal cells (Ingham and Morgan, 1983; Barrington et al., 1989; Ehrlich et al., 1990; Tung et al., 1990). Amacrine cells affected by kainic acid include those that contain acetylcholine, GABA, enkephalin, VIP, serotonin, and substance P (Schwarcz & Coyle, 1977; Imperato et al., 1981; Morgan & Ingham, 1981; Morgan, 1983; Golcich et al., 1990; Tung et al., 1990), while those containing dopamine probably survive (Ingham & Morgan, 1983: Morgan, 1983).

Because of wide-spread destruction and damage to bipolar, amacrine and ganglion cells, it is impossible to determine which subsets of cells affected by kainic acid are responsible for increased rates of ocular growth or unresponsiveness to form-deprivation.

The administration of a large dose of NMDA or QA to the chick retina has been shown to leave photoreceptor, bipolar, and ganglion cells intact, but to destroy many amacrine cells (Morgan, 1987; Barrington et al., 1989; Ehrlich et al., 1990; Tung et al., 1990; Sheppard et al., 1991). However, the specific types of amacrine cells which are sensitive to NMDA or QA have not been identified. Since NMDA- and QA-induced excitotoxicity both affect primarily amacrine cells and have different affects upon ocular growth and the development of FDM, it is expected that these toxins destroy different subsets of amacrine cells. NMDA should destroy or disable cells required for visual guidance of ocular growth, while QA should not affect them. It is possible that toxic doses of NMDA or QA have different effects upon cholinergic amacrine cells. If cholinergic retinal amacrine cells are needed to regulate ocular growth, then one might expect that NMDA destroys these cells while QA has little effect upon them.

Hypotheses and strategies:

Hypothesis I: Different isoforms of the chick mAChR are distributed in different tissues throughout the eye.

Strategy: I will characterize the distribution of mAChRs in chick retina, RPE, choroid, ciliary body and ciliary ganglion using affinity-purified type-specific antibodies raised against 3 different chick mAChR isoforms. Since any immunolabelling of mAChRs in

the RPE may be masked by its dense pigmentation, I will isolate the RPE and use Western blotting to detect mAChRs.

Hypothesis II: QA-induced excitotoxicity does not destroy any retinal cells that are instrumental in emmetropization or FDM.

Strategy: QA causes increased corneal curvature and consequently myopia, which can then be enhanced by form-deprivation (Ehrlich et al., 1990). Therefore, it is unlikely that amacrine cells required for emmetropization and FDM are affected by QA-induced excitotoxicity. I will use immunocytochemistry to identify different subsets of retinal cells and then determine whether these immunoreactivities are affected by exposure to QA. I will also use a modified 3' nick-end labelling (TUNEL) technique to identify cells containing fragmented DNA in QA-treated retinas, as chromosomal fragmentation is a hallmark symptom of apoptosis (programmed cell death). The distribution and abundance of TUNEL-positive cells will serve as an indication of the degree of toxin-induced damage that might otherwise go undetected using immunocytochemistry. Immunolabelling may not identify all subsets of cells affected by excitoxicity.

Hypothesis III: NMDA-induced excitotoxicity destroys retinal amacrine cells that participate in emmetropization and FDM.

Strategy: NMDA-induced excitotoxicity causes mild myopia and renders eyes unresponsive to form-deprivation (Fischer et al., 1997b, 1998g). Therefore, it is likely that amacrine cells that are required for emmetropization and FDM are damaged or

destroyed by NMDA-induced excitotoxicity. I will use immunocytochemistry to identify different subsets of retinal cells and then determine whether these immunoreactivities are affected by exposure to NMDA. I will also use TUNEL to identify cells containing fragmented DNA in NMDA-treated retinas, as chromosomal fragmentation is a hallmark symptom of apoptosis (programmed cell death). The distribution and abundance of TUNEL-positive cells will serve as an indication of the degree of toxin-induced damage that might otherwise go undetected using immunocytochemistry.

Hypothesis IV: Atropine rescues FDM by blocking the activity of ACh at mAChRs in the retina.

Strategy: Atropine may suppress FDM by antagonising mAChRs in the retina, RPE, and/or possibly choroid. Atropine, acting as an antagonist, should block FDM by preventing the actions of an endogenous source of ACh. Therefore, atropine should lose its ability to prevent FDM if endogenous sources of ACh are removed. Ethylcholine mustard aziridinium (ECMA) and QA will be used to eliminate retinal souces of ACh. I will then test whether toxin-treated eyes grow normally, retain the ability to emmetropize and grow excessively in response to form-deprivation, and whether atropine can suppress FDM in the absence of retinal sources of ACh. If atropine suppresses FDM in toxin-treated eyes, then it will be concluded that cholinergic amacrine cells are not required for excessive ocular growth to develop in form-deprived eyes. If atropine fails to suppress FDM in ECMA or QA-treated eyes, then it will be concluded that antagonism of cholinergic activity in the retina is required to prevent FDM.

The results presented in this dissertation indicate that three different isoforms of mAChR are expressed by different cell types throughout the retina, RPE, and choroid of the chick eye. This distribution of mAChRs is consistent with their putative role in modulating ocular growth. However, I also show that the toxin-mediated destruction of all cholinergic amacrine cells and most mAChRs in the retina has no effect upon emmetropization, FDM, or the atropine-mediated suppression of FDM. Furthermore, the destruction of other subsets of amacrine cells that were previously implicated as modulators of ocular growth, including those that contain VIP or enkephalin, has no effect upon normal and form-deprivation-induced ocular growth. These studies are the first to investigate directly the retinal circuitry involved in controlling ocular growth, and provide the first direct evidence against roles for specific retinal cells and transmitters that were previously believed to be crucial for the visual regulation of ocular growth. This work also suggests new candidate cell-types, and adds support in favour of the role of a former candidate cell-type (the dopaminergic amacrine cell), as being required for visually guided ocular growth.

CHAPTER TWO

Identification and localization of muscarinic acetylcholine receptors in the ocular tissues of the chick

Introduction:

Cholinergic innervation to the eye is found in all vertebrate classes. Acetylcholine (ACh) is released from autonomic axon terminals on vascular smooth muscle and iris and ciliary muscles, as well as from intrinsic interneurons that make synapses within the retina. Cholinergic innervation to the choroid, iris and ciliary muscles arises mainly from distinct populations of parasympathetic neurons located in the ciliary ganglion. Parasympathetic activity in the eye results in dilation of choroidal blood vessels, constriction of the pupil, and contraction of the ciliary muscle. In the retina, cholinergic neurons have been identified as amacrine cells with somata located at the proximal margin of the inner nuclear layer (INL) and displaced to the ganglion cell layer (GCL), and processes confined to two discrete strata in the inner plexiform layer (IPL; Eckenstein and Thoenen, 1982; Eckenstein et al., 1983; Famiglietti, 1983; Ma and Grant, 1984; Tauchi and Masland, 1984; Tumosa et al., 1984; Millar et al., 1985, 1987b; Conley et al., 1986; Voigt, 1986; Spira et al., 1987); in the avian retina at least one additional subtype of cholinergic amacrine cells has been identified (Baughman and Bader, 1977; Millar et al., 1987b. It is currently believed that these cells modulate receptive field properties of ganglion cells (Ariel and Daw, 1982a, b; Masland et al., 1984; Schmidt and

Vijayaraghavan., 1992; Kittila and Massey, 1997). To better understand the functions of ocular cholinergic systems, it is necessary to characterize the post-synaptic targets for cholinergic innervation and to identify the specific ACh receptors (AChRs) that mediate specific post-synaptic responses.

AChRs can be segregated into two categories: (1) ionotropic receptors that are selectively activated by nicotine-like ligands (nAChR); and (2) metabotropic receptors that are selectively activated by muscarine-like ligands (mAChR). Muscarinic AChRs belong to a family of receptors that contain seven transmembrane domains and elicit cellular responses via interactions with GTP-binding proteins (reviewed by Nathanson, 1987). In mammals, five distinct mAChR isoforms, representing the products of five separate genes, have been characterized by both molecular biology (m1 to m5; Bonner et al., 1987, 1988; Peralta et al., 1987, 1988) and pharmacology (M1 to M5; Buckley et al., 1989; Dorje et al., 1991). Autoradiographic studies, using [^{3}H]quinuclidinyl benzylate (QNB) or [^{3}H]propylbenzilylcholine mustard (PrBCM), revealed muscarinic binding sites mainly in the IPL of human retina (Hutchins and Hollyfield, 1985; Zarbin et al., 1986), salamanders (Polans et al., 1985; Townes-Anderson and Vogt, 1989), ferrets (Hutchins, 1994), and monkeys (Zarbin et al., 1986).

In the chick, very little is known about the distribution of mAChR subtypes in the different tissues of the eye. Four avian mAChR subtypes have been characterized and named according to sequence homology with their mammalian counterparts: cm2 (Tietje and Nathanson, 1991), cm3 (Gadbut and Galper, 1994), cm4 (Tietje et al., 1990), and cm5 (Creason et al., 1995). Autoradiographic studies have revealed muscarinic binding

sites in the IPL (Sugiyama et al., 1977; Large et al., 1985), but the neurons that express these receptors remain unknown. In addition, cm2, cm3, and cm4 receptor mRNAs and proteins have been detected by using solution hybridization, immunoblotting, and immunoprecipitation techniques in retinal tissue extracts from embryonic and hatched chicks (McKinnon and Nathanson, 1995), but the precise anatomical distribution of each isoform remains unknown. Pharmacological techniques have been used to detect muscarinic binding sites in the chick ciliary ganglion (Schmidt and Vijayarghauan, 1992; Sorimachi, 1993; Furukawa et al., 1994), iris muscles, ciliary body (Pilar et al., 1987), and choroid (Marwitt et al., 1971; Meriney and Pilar, 1987). Futhermore, pharmacological studies have implicated mAChRs in the metabolism of retinal pigment epithelium (RPE) in humans (Osborne et al., 1991) and rats (Salceda, 1994), and in the visual regulation of ocular growth in humans (Bedrossian, 1971, 1979), monkeys (Raviola and Weisel, 1985), tree shrews (McKanna and Casagrande, 1981), and chicks (Stone et al., 1991; McBrien et al., 1993; Leech et al., 1995).

Therefore, to better understand the roles of ocular mAChRs, we have characterized their distribution in the retina, RPE, choroid, ciliary body and ciliary ganglion of hatched chicks by using type-specific antibodies directed against cm2, cm3, and cm4. We found mAChRs distributed throughout the eye, with unique patterns of distribution in each tissue.

Methods and Materials:

Animals:

Newly-hatched male leghorn chicks (*Gallus gallus domesticus*) were obtained from a local hatchery (Lillydale Hatchery, Linden, Alta) and kept on a cycle of 12 hours light, 12 hours dark with light onset at 0700. Illumination was provided by 100W incandescent light bulbs, resulting in illuminance levels ≤ 0.8 cd/m^2, depending on the direction of gaze. Chicks were held for one week in a stainless steel brooder, at a temperature of approximately 25°C. Thereafter chicks were kept in groups of ≤ 7 animals in clear Nalgene™ cages with stainless steel mesh covers. Chicks were fed water and Purina™ chick starter *ad libitum*. The use of animals was according to the Guide to the Care and Use of Experimental Animals as set out by the Canadian Council on Animal Care.

Antisera:

Antisera and their working dilutions included: anti-cm2 at 1:200, anti-cm3 at 1:1000, and anti-cm4 at 1:500 (affinity-purified rabbit polyclonal IgGs; McKinnon and Nathanson, 1995); anti-somatostatin at 1:300 (rat monoclonal IgG #S-10; gift from Dr. A. Buchan, University of British Columbia); anti-vasoactive intestinal polypeptide at 1:80 (rat monoclonal IgG #VP31; gift from Dr. A. Buchan, University of British Columbia); anti-tyrosine hydroxylase at 1:50 (mouse monoclonal IgG; Developmental Studies Hybridoma Bank, University of Iowa); anti-ChAT at 1:1000 (rabbit polyclonal #1465; gift from Dr. M. Epstein, University of Wisconsin); and anti-protein kinase C (α

and β isoforms) at 1:50 (mouse monoclonal #RPN 536; Amersham). Antisera directed against the chick mAChRs were the same as used previously by McKinnon and Nathanson (1995). Crude sera from immunized rabbits were affinity-purified, first through a recombinant glutathione-S-transferase (GST)-conjugated affinity column and then through a recombinant GST-mAChR fusion protein affinity column. The fractions that bound to GST alone have been shown to produce no immunolabelling of cultured retinal cells from the chick embryo (McKinnon and Nathanson, unpublished result). Controls were treated identically, except that the primary antiserum was excluded from the second incubation step. The specificity of the antisera raised against the chick mAChRs was tested by preabsorption with recombinant GST overnight at 4°C. Preabsorption of mAChR antibodies with the immunizing fusion proteins was not performed as these antisera already had been affinity-purified by binding to their respective immunizing proteins.

Immunocytochemistry:

Between 7 and 14 days after hatching, chicks were killed by chloroform inhalation. Eyes were enucleated, hemi-sectioned equatorially, gel vitreous removed, and tissues placed into fixative (4% paraformaldehyde, 3% sucrose in 0.1 M phosphate buffer pH 7.4) at room temperature for 30 minutes. Tissues were washed 3 times in PBS (phosphate buffered saline; 0.05 M phosphate buffer plus 195 mM NaCl pH 7.4), and cryoprotected in PBS plus 30% sucrose for at least 24 hours. Samples were soaked in embedding medium (O.C.T. Compound; Tissue-TEK, Miles Inc.) for 30 minutes, frozen

in liquid nitrogen, and mounted onto sectioning blocks. Sections 10 to 14 µm thick were melted onto gelatin-coated slides, air-dried, ringed with rubber cement, and stored at -20°C until use. Each slide was washed 3 times in PBS and covered with 150 µl 2% bovine serum albumin (BSA), 2% glycine, plus 0.3% Triton X-100 (TX-100) in PBS for at least one hour at room temperature. This solution was then aspirated and the primary antibody solution applied (100 to 150 µl of antibody diluted in PBS, 0.3% TX-100, plus 0.01% NaN_3) for 24 hours at room temperature in a humidified chamber. Slides were then washed 3 times in PBS and covered with the secondary antibody solution, 100 to 150 µl of either 1:80 FITC-conjugated goat-anti-rabbit IgG or anti-mouse IgG (Sigma) or 1:1000 Cy3-conjugated goat-anti-rabbit IgG or anti-mouse IgG (Biological Detection Systems Inc.), diluted in PBS plus 0.3% TX-100, for 2 hours at room temperature in a humidified chamber. The slides were washed in PBS, mounted in 4:1 (v/v) glycerol to water, and coverslips added for observation under an epifluorescence microscope. Photographs were taken on TMAX 400 black and white film (Kodak), and developed in TMAX Developer (Kodak).

Tissue dissection for immunoblots:

The eyes from 4 chicks (8 days after hatching) were enucleated, hemi-sectioned equatorially, gel-vitreous removed, and the remaining eye-cup placed into ice-cold PBS. The lens, iris, and ciliary body were removed together from the front of the eye and placed into ice-cold extraction buffer (0.05 M Tris-HCl plus 1% (w/v) sodium dodecylsulfate; SDS). The pecten was then cut out of the eye-cup and the retina was

removed with RPE still attached, leaving the choroid adherent to the sclera. The choroid was then dissected out and placed into 1.5 ml of ice-cold extraction buffer. The RPE was pulled away from the retina using fine forceps, and the isolated retina placed into extraction buffer. The fragments of RPE were gathered with a pasteur pipette into a conical centrifuge tube. The RPE suspension was then centrifuged for 5 minutes at 1000 X G, the supernatant aspirated, and the fragments of RPE resuspended in 1 ml of extraction buffer. Samples were homogenized using a Tissue-Tearor (Biospec Products), sonicated for 15 min., centrifuged at 4300 X G, the supernatant decanted and the pellet discarded. The supernatant was stored at -20°C until use.

Polyacrylamide gel electrophoresis:

Tissue extracts were assessed for protein content using a modified Bradford assay. In short, 2 µl of serially diluted (1:2) tissue extract was blotted and dried on filter paper in parallel with 2 µl of bovine serum albumin standards (1.0, 0.1, 0.01, and 0.001 µg/µl). The filter paper was stained in Coomassie blue (0.1 % w/v Coomassie blue R-250; Bio-Rad; 40% methanol and 10% acetic acid in dH_2O) for about 5 minutes on a rotary shaker. The filter paper was then destained (in 40% methanol and 10% acetic acid). The staining intensities of samples and standards were compared by visual inspection to estimate the total protein content of samples to the nearest standard. Tissue extracts were diluted in sample buffer (0.6 M Tris-HCl pH 6.8, 4.8% v/v β-mercaptoethanol, 9.6% v/v glycerol, 1.9% w/v SDS, 0.0024% w/v bromophenol blue) to 1 µg/µl to allow equal amounts of protein per sample to be loaded onto a gel. Finally, tubes containing diluted samples were bathed in boiling water for 5 minutes prior to loading onto a gel.

20 µl of sample (equivalent to 20 µg of protein) per lane were loaded and run through a stacking gel (3.9% acrylamide pH 6.8) at 100 V and a separating gel (7.5% acrylamide pH 8.8) at 80 V. Prestained molecular weight standards (Bio-Rad) were run in lanes adjacent to tissue samples. The contents of the gel were then blotted onto nitrocellulose membrane (NCM; Bio-Rad) overnight at 30 V. Membranes were washed three times in PBS pH 7.4, soaked in 5% (w/v) skim milk powder in buffer B (2.5 mM Tris-HCl, 14 mM NaCl pH 7.4) for 1 hour at room temperature, and cut into separate lanes. The NCM lanes were added to the primary antibody solution (700 µl 1:200 anti-cm2, 1:1000 anti-cm3, or 1:500 anti-cm4 diluted in buffer B) and incubated overnight in a humidified chamber at room temperature. The membranes were washed three times in buffer B plus 1% BSA, followed by one wash in buffer B, incubated in the secondary antibody solution (5 ml of 1:1000 alkaline phosphatase-conjugated goat-anti-rabbit IgG, Sigma) for at least 1 hour at room temperature, and then washed once in buffer B, once in 1% (v/v) TX-100 and 5 mM EDTA in buffer B, and twice in buffer B. The membranes were covered with detection solution (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$ pH 9.5 plus 0.033% (w/v) nitroblue tetrazolium, 0.017% (w/v) 3-bromo-4-chloro-indolyl phosphate, and 0.69% (v/v) N,N-dimethylformamide) and reacted for 5 to 30 minutes. The colour reaction was stopped by washing the NCM lanes in PBS.

Statistics and measurements:

All sample errors are given as the standard deviation of the mean. To express the distribution of cm3-immunoreactive amacrine cells, an index of regularity was calculated as the sample mean of the distance to the nearest neighbour divided by the standard

deviation (Wässle and Riemann, 1978). The higher this ratio the more regular the pattern of distribution (values greater than 7 represent a highly regular distribution, while values less than 3 represent a random distribution). The distance between cells was measured as the distance from the centre of one cell to the centre of the next nearest cell, in horizontal sections (parallel to the plane of the the retina). Sections were thick enough to include all cells of the type being analysed. Percentage IPL depth was calculated as the distance from the IPL/INL border divided by the total thickness of the IPL, multiplied by 100. All measurements were made from photomicrographs.

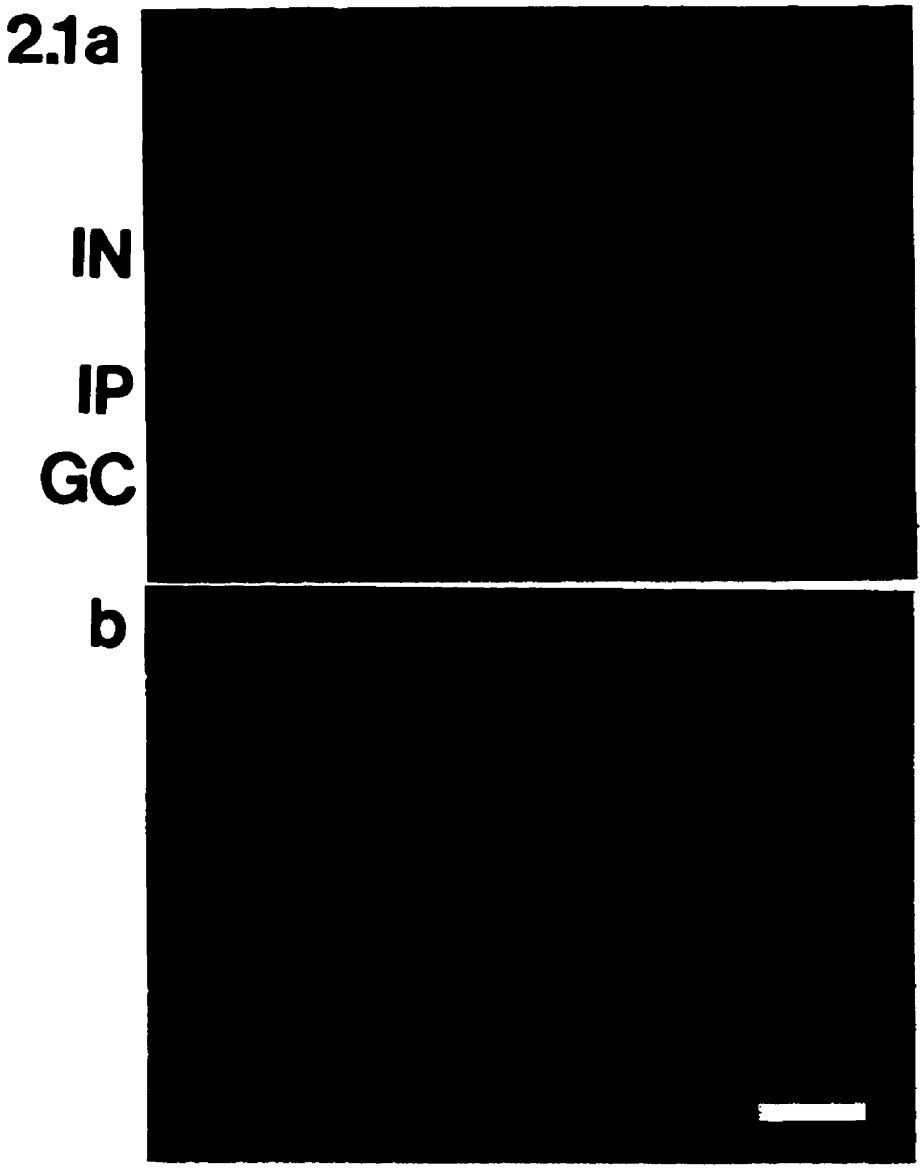
Results:

Localization of mAChRs in the chick eye:

Anti-cm2 weakly labelled cells in the amacrine cell layer of the inner nuclear layer (INL) and in the ganglion cell layer (GCL) (Fig. 2.1a). Cm2-immunoreactivity was localized to the outer plexiform layer (OPL) and four thick strata in the IPL (Fig. 2.1a), at 0 to 15%, 40 to 60%, 70 to 85%, and 95 to 100% IPL depth (Fig. 2.2). Superimposed upon the stratum at 40 to 60% IPL depth was a more intensely labelled layer at about 50% IPL depth (Fig. 2.2). No detectable staining was produced by the anti-cm2 in ciliary body, choroid, or ciliary ganglion.

Cm3-immunoreactivity was localized to about two-thirds of all bipolar cells (i.e. cells between amacrine and horizontal cell layers), sparsely distributed amacrine cells, and a few cells in the GCL (Fig. 2.1b, 2.3a and 2.3b). The amacrine cells labelled with anti-cm3 in the central retina were 6.7 ± 0.2 μm (N=20) in horizontal diameter, and

Figure 2.1: Vertical sections of retina stained with antibodies to (**a**) cm2, (**b**) cm3, (**c**) cm4, and (**d**) ChAT. Abbreviations: IP-inner plexiform layer; IN-inner nuclear layer: GC-ganglion cell layer. Calibration = 50 μm.


2.1a
IN
IP
GC
b

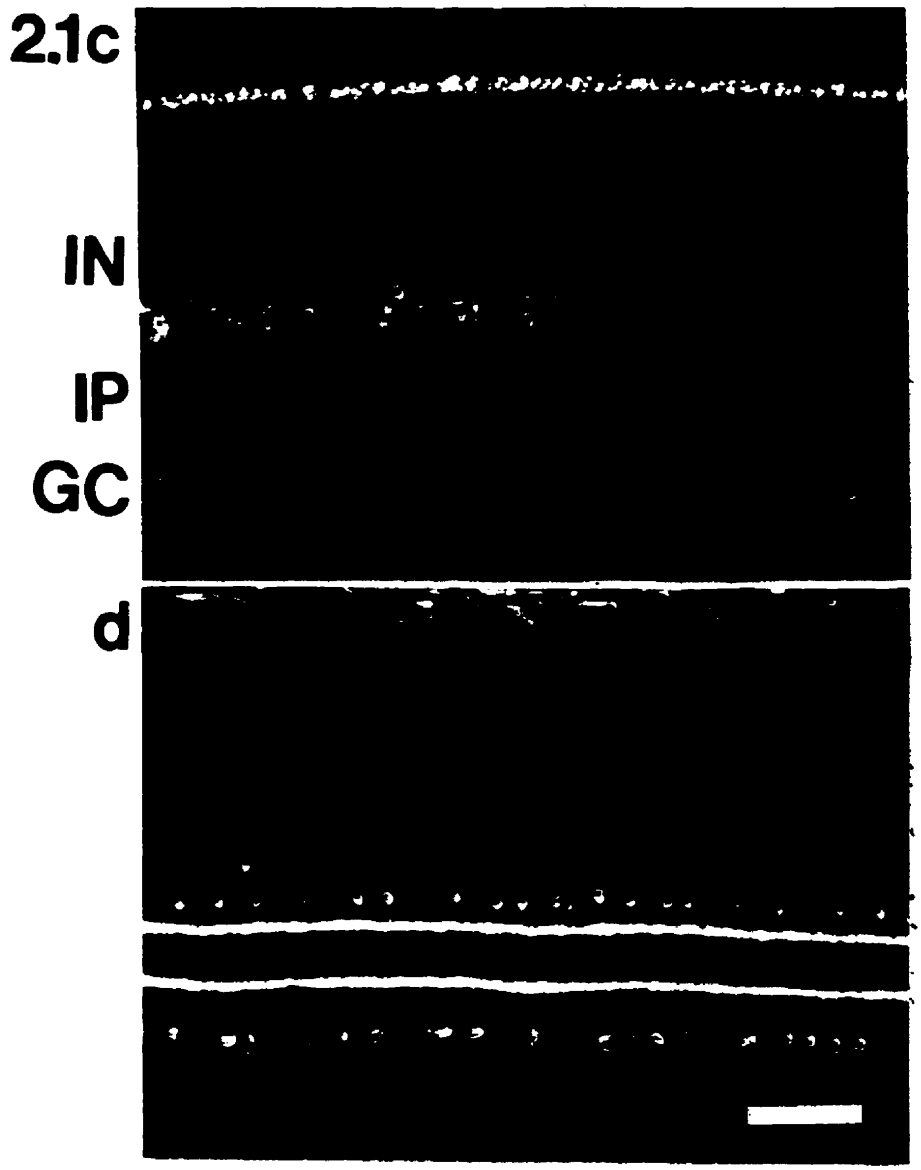
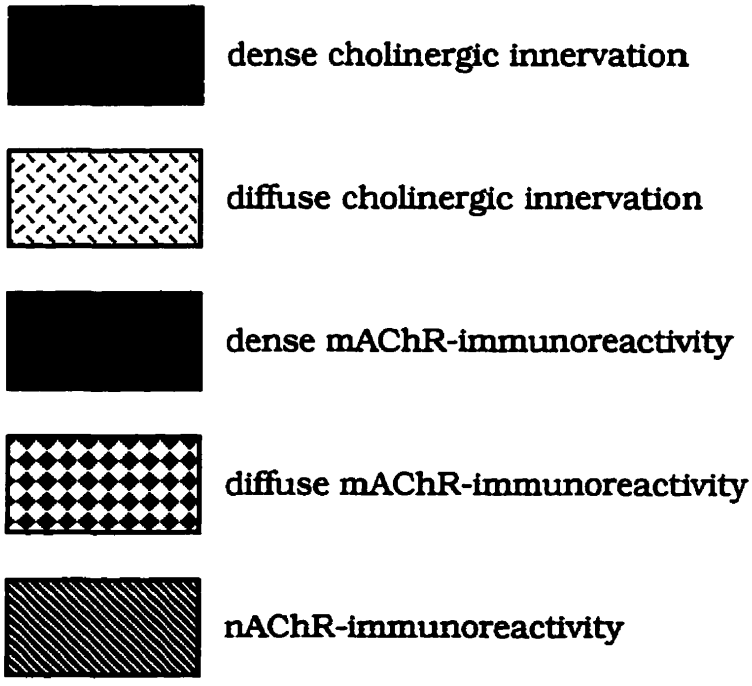
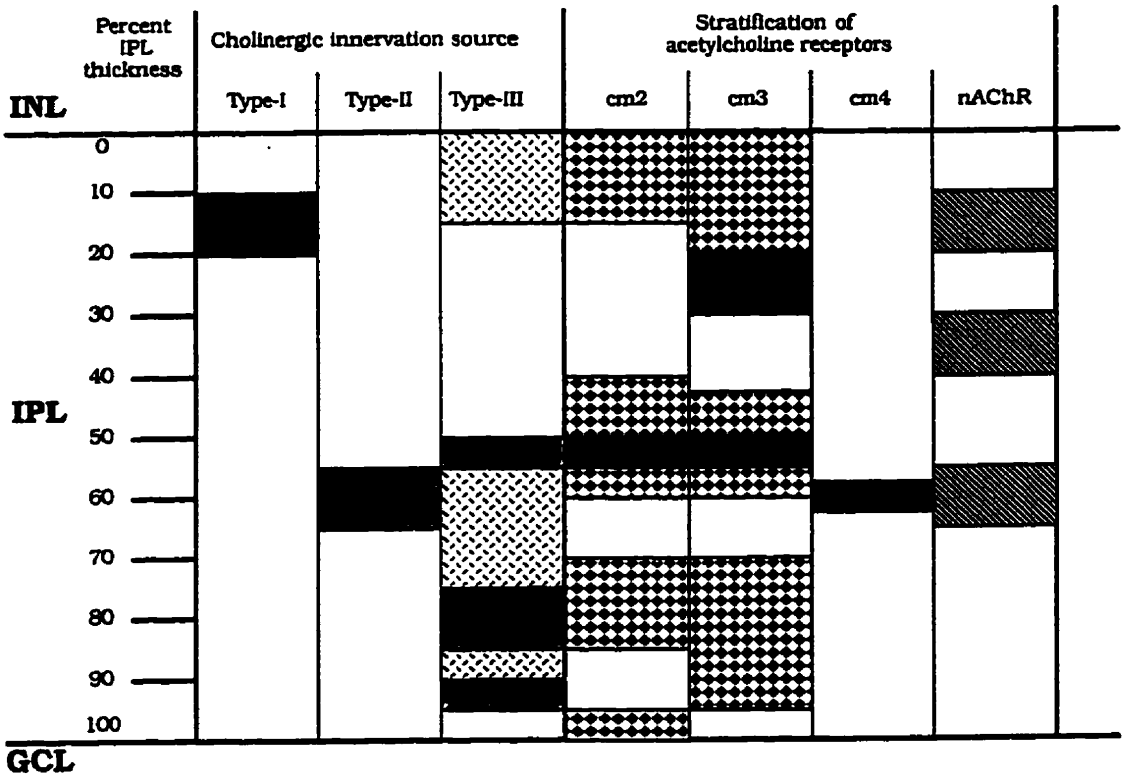

2.1c
IN
IP
GC
d

Figure 2.2: Schematic diagram of the retinal distribution of cholinergic innervation (Millar et al., 1987a), mAChR (muscarinic acetylcholine receptors), and nAChR (nicotinic acetylcholine receptors; Hamasaki-Britto et al ., 1994). Abbreviations: IPL- inner plexiform layer; INL-inner nuclear layer; GCL-ganglion cell layer.




Percent IPL thickness
Cholinergic innervation source
Stratification of acetylcholine receptors
INL
Type-I
Type-II
Type-III
cm2
cm3
cm4
nAChR
0
10
20
30
40
IPL
50
60
70
80
90
100
GCL

distributed at about 1000 cells per mm^2 with a regularity index of 3.88. These cells appeared to contribute their processes to an intensely stained stratum at 20 to 30% IPL depth. The cells immunoreactive for cm3 in the GCL appeared to be displaced amacrine cells, as their somata were relatively small (6.9 ± 0.5 µm in diameter; N=10). They were distributed randomly, with a regularity index of only 2.31. Anti-cm3 produced punctate labelling in the OPL, but did not stain horizontal or photoreceptor cells. Anti-cm3 also labelled three strata at 0 to 30%, 45 to 60%, and 70 to 95% depth of the IPL (Fig. 2.2). Superimposed upon the two distal strata were two more intensely stained strata at 20 to 30% and 50 to 55% IPL depth (Fig. 2.2). In addition, anti-cm3 diffusely labelled the muscles and epithelium of the ciliary body (2.4), and the RPE (Fig. 2.5a), and robustly labelled the walls of most blood vessels in the choroid (Fig. 2.6a). No cm3-immunoreactivity was seen in the ciliary ganglion.

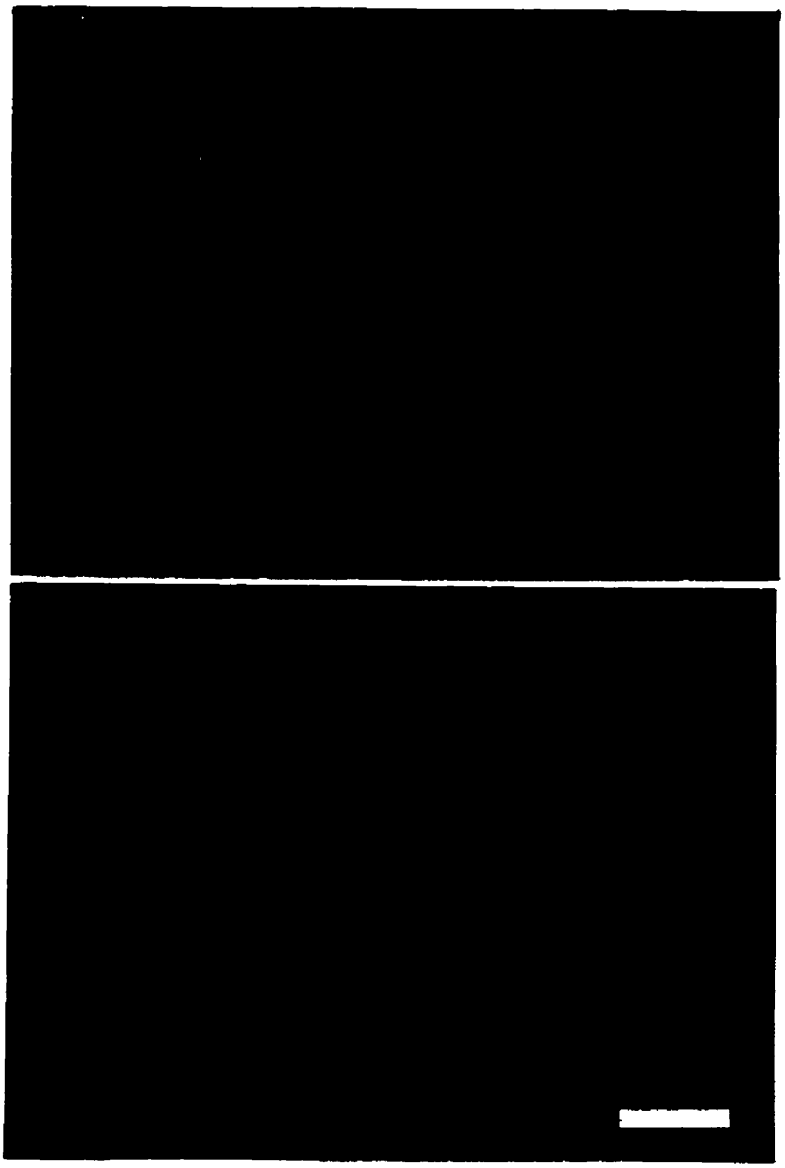
Anti-cm4 robustly labelled most, if not all, amacrine cells in the INL and most cells in the GCL, as well as one thin stratum at about 60% IPL depth (Figs. 2.1c and 2.2). In addition, immunoreactive cm4 was detected in the ciliary epithelium (Fig. 2.4), RPE (Fig. 2.5b), and walls of most choroidal blood vessels (Fig. 2.6b). Anti-cm4 was the only antiserum that labelled cell bodies in the ciliary ganglion (Fig. 2.7).

Double-labelling of retinal neurons:

Anti-cm2 was not used in double-labelling experiments, since its weak labelling of cell bodies would have made it difficult to identify doubly-labelled cells. Double-labelling experiments were performed to characterize further the cm3- and

Figure 2.3: Horizontal sections of retina at the level of (**a**) amacrine cells in the inner plexiform layer and (**b**) ganglion cell layer, stained with antibody directed against cm3. Calibration = 50 μm.

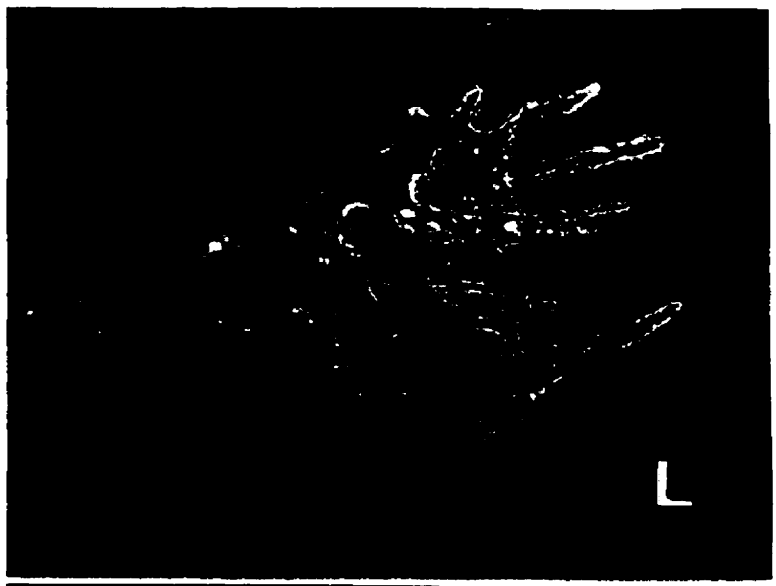
2.3a



b

Figure 2.4: Transverse sections through the ciliary body, (**a** and **c**) at the lens and (**b** and **d**) just lateral to the lens. These sections have been stained for (**a** and **b**) cm3-immunoreactivity and (**c** and **d**) cm4-immunoreactivity. Abbreviation: L = lens. Calibration = 100 μm.

2.4a



b

2.4c

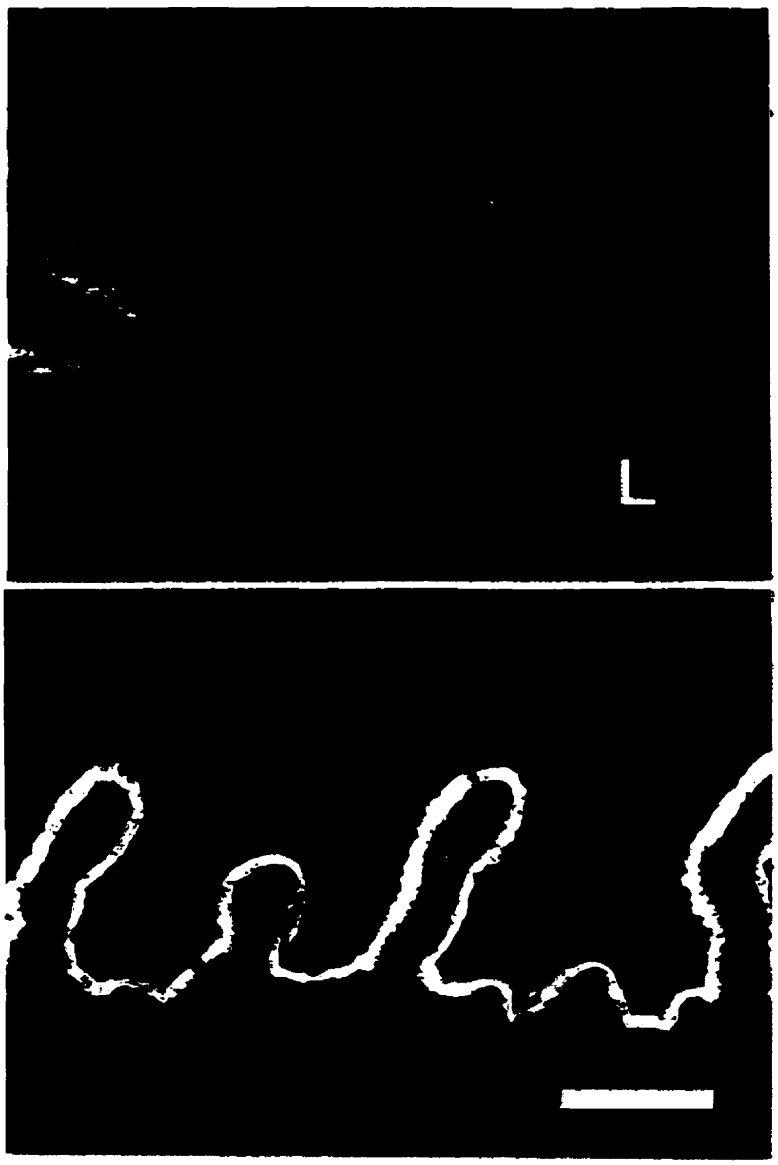
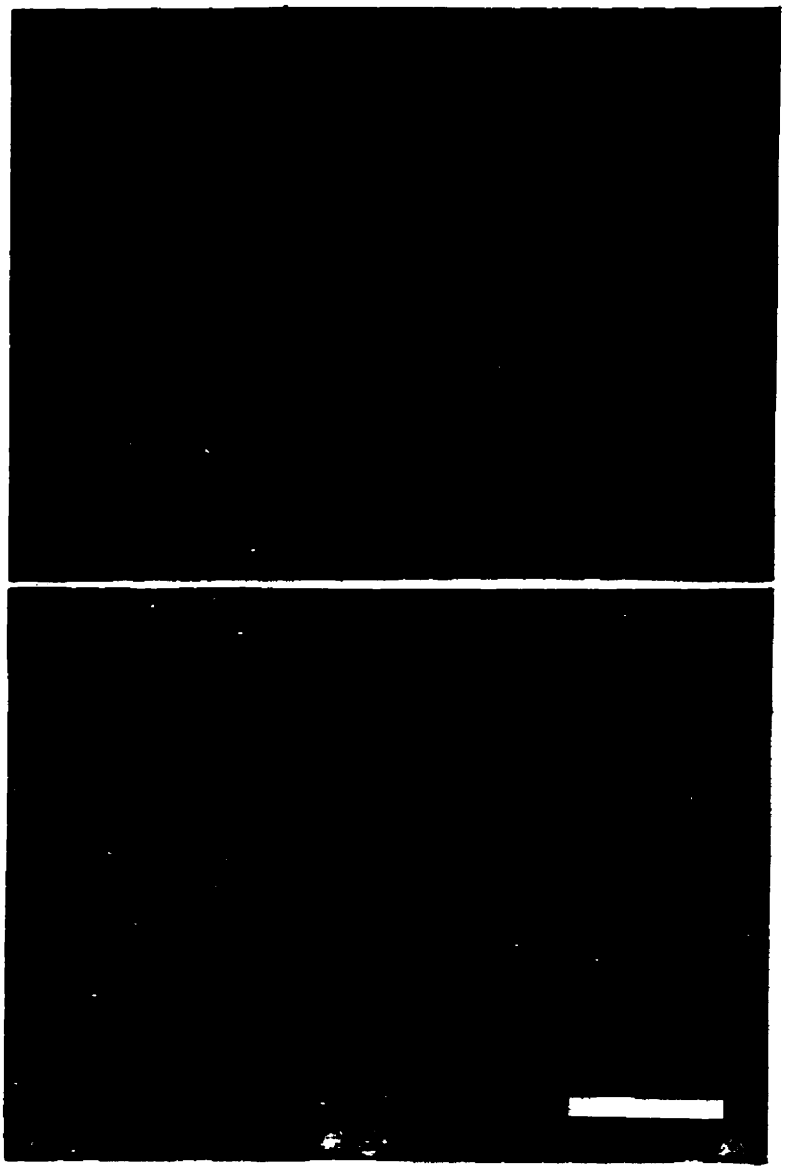


Figure 2.5: Horizontal sections through the RPE which have been stained for (**a**) cm3-immunoreactivity and (**b**) cm4-immunoreactivity. Calibration = 50 μm.

2.5a



b

Figure 2.6: Cross sections through the choroid stained with antibody directed to (**a**) cm3 and (**b**) cm4. Abbreviation: L = lumen of blood vessel. Calibration = 50 μm.

2.6a

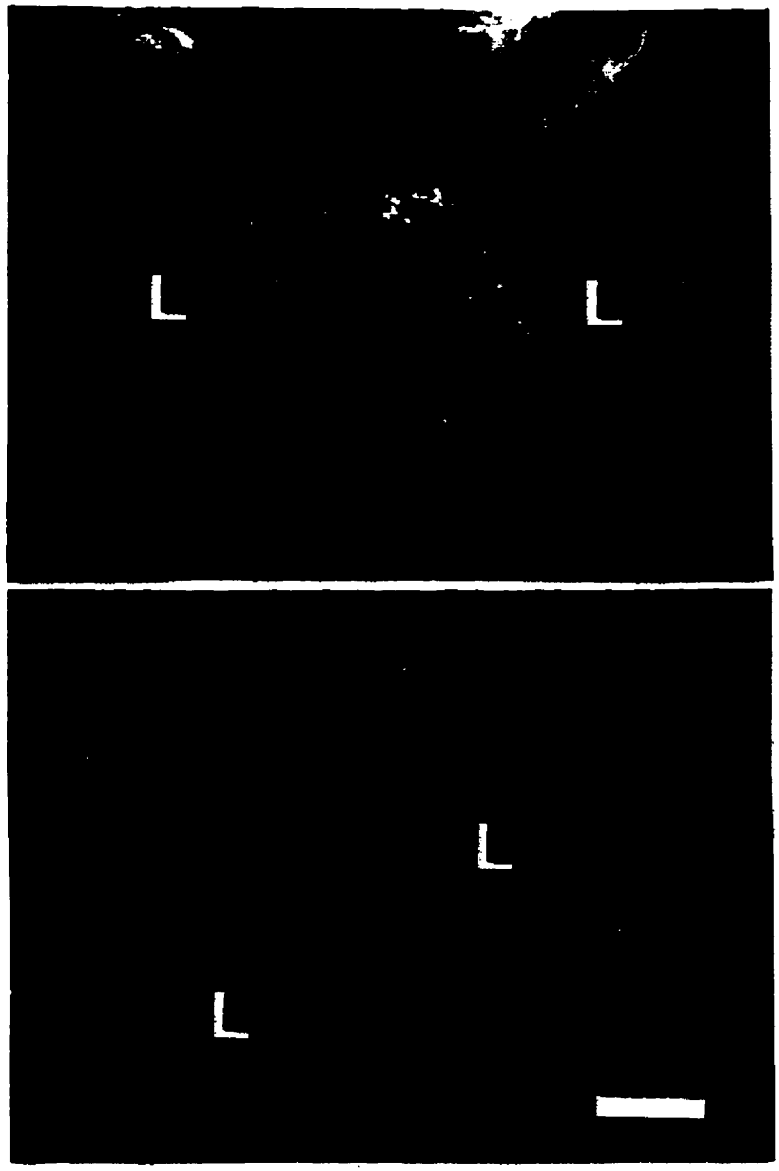
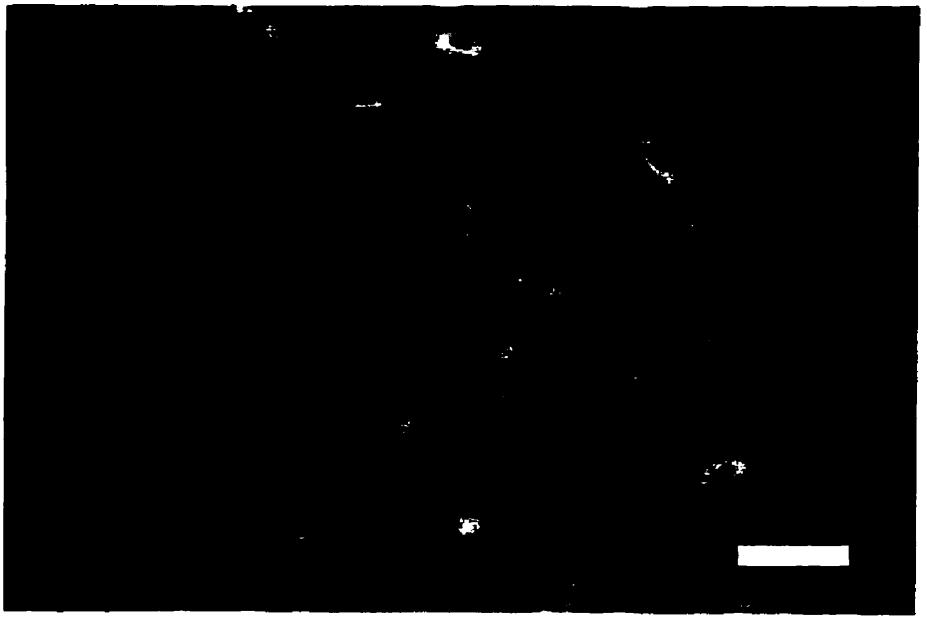


Figure 2.7: Longitudinal section through the ciliary ganglion stained with antibody to cm4. Calibration = 50 μm.

2.7



cm4-immunoreactive cell, using antisera known to label well characterized subsets of amacrine and bipolar cells that might be involved in the regulation of ocular growth. The bipolar cells labelled by anti-cm3 did not contain PKC-immunoreactivity (Fig. 2.8). The subset of amacrine cells labelled with anti-cm3 did not express any of the other markers for which we probed.

Some amacrine cells labelled by anti-cm4 were also doubly-labelled with other antibodies. Cm4-immunoreactivity was found in all TH-immunoreactive amacrine cells (N=75), as well as all amacrine cells containing somatostatin-immunoreactivity (N=134), and VIP-immunoreactivity (N=100).

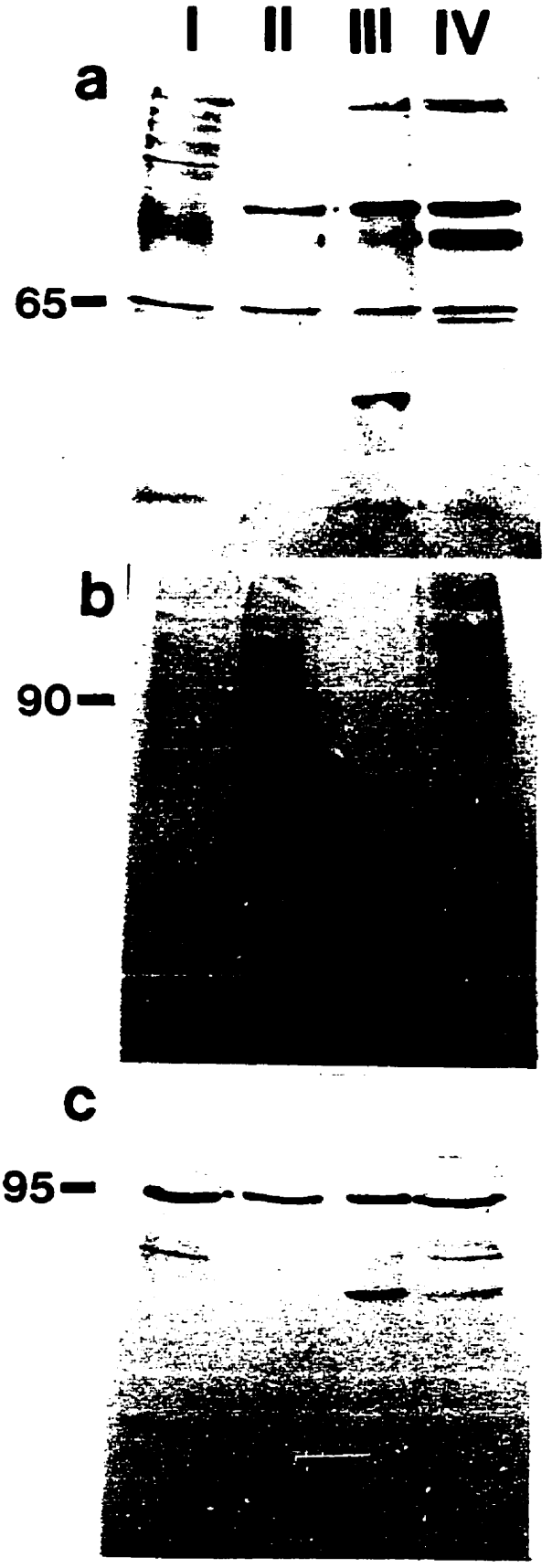
Immunoblot analysis of mAChRs in the ocular tissues of the chick:

Cm2-immunoreactivity was detected in tissue extracts of retina, RPE, choroid, and ciliary body at an apparent molecular mass of about 65 kDa (Fig. 2.9a). Cm3-immunoreactivity was detected in the retina, RPE, and ciliary body at an apparent molecular mass of about 90 kDa (Fig. 2.9b), but in the choroid at about 60 and 70 kDa (Fig. 2.9b). Immunoreactive-cm4, with an apparent molecular mass of about 95 kDa, was detected in the retina, RPE, choroid, and ciliary body (Fig. 2.9c).

Figure 2.8: Horizontal section of the retina at the level of bipolar cells in the inner plexiform layer which has been doubly-stained for cm3-immunoreactivity (in red) and PKC-immunoreactivity (in green). The negatives of separate images were digitized, assigned colour, enhanced for contrast, and overlayed by aligning fiduciaries using Adobe Photoshop 4.0. The absence of yellow structures (red + green) indicates that cm3 and PKC immunoreactivities were not colocalized. Calibration = 50 μm.

2.8

Figure 2.9: Protein blots labelled with antibodies to (**a**) cm2, (**b**) cm3, and (**c**) cm4. Lanes were loaded with 20 μg of protein extracted from (**I**) retina, (**II**) retinal pigment epithelium, (**III**) choroid, and (**IV**) ciliary body. The numbers on the right of each blot indicate the molecular mass of putative mAChR-immunoreactive bands as calculated from molecular mass standards run on the same gel.


I
II
III
IV
a
65
b
90
c
95

Discussion:

Labelling of cell bodies with antibodies directed against mAChRs:

The antibodies directed against mAChRs labelled cell bodies in both the INL and GCL. Similarly, antibodies raised to different subunits of the nAChR (Keyser et al., 1988; Hamassaki-Britto et al., 1991, 1994) and the $GABA_A$ receptor (Hughes et al., 1989) also have been reported to label cell bodies in addition to processes in synaptic layers of the retina. The significance of receptor-immunoreactivity in neural somata remains uncertain. Such immunoreactivity may represent: (i) labelling of cytoplasmic non-receptor proteins sharing homology with antigenic epitopes of the receptor; (ii) newly synthesized receptor molecules en route to peripheral synapses; (iii) degradation products or internalized receptors being recycled or down-regulated; or (iv) functionally expressed receptors at the surface of the cell body. The fact that some muscarinic binding sites have been detected in the INL (Sugiyama et al., 1977) suggests that functional mAChRs might be expressed at the surface of the cell body.

Muscarinic AChRs have been detected on isolated retinal cells, including ganglion, amacrine, bipolar and horizontal cells, from the tiger salamander (Townes-Anderson and Vogt, 1989). Similarly, we have shown that all of these cell types, except horizontal cells, express mAChR-immunoreactive proteins in the chick retina. *In situ* hybridization of mAChR mRNAs would be required to confirm that these cells do synthesize mAChRs.

Distribution of muscarinic receptors in the IPL and OPL:

Previous reports of muscarinic ligand-binding within the retina of chicks described three distinct bands of mAChRs within the IPL (Sugiyama et al., 1977). The results presented here indicate that cm2 and cm3 are distributed into three broad bands within the IPL, with cm4-like immunoreactivity contributing to the most proximal stratum. The composite distribution of all three immunoreactive mAChR subtypes in the IPL falls into 3 broad bands at 0 to 30%, 40 to 60%, and 70 to 95% IPL depth (Fig. 2.2). Since the binding affinity of [^{3}H]QNB for each isoform of chicken mAChR is roughly equivalent (Tietje et al., 1990; Tietje and Nathanson, 1991; Gadbut and Galper, 1994), it is likely that the autoradiographic localization of mAChRs within the IPL represents binding to at least these three different isoforms. Furthermore, the distribution of mAChRs within the IPL closely matches cholinergic innervation. In the chick retina, cholinergic innervation of the IPL is supplied by four different subtypes of cholinergic amacrine cell. Millar et al., (1987) identified three subtypes of ChAT-immunoreactive amacrine cells in the chick retina: type-I cholinergic amacrine cells, with cell bodies at the IPL/INL border and neurites stratified at 10 to 20% IPL depth; type-II cholinergic amacrine cells, with cell bodies in the GCL and processes at 55 to 65% IPL depth; and type-III cholinergic amacrine cells, with cell bodies near the middle of the INL and processes diffusely distributed at 0 to 10% and 50 to 95% IPL depth (Fig. 2.2). Furthermore, type-III cholinergic amacrine cells can be roughly divided in half into 2 subtypes: type-IIIa including cells which contain enkephalin, neurotensin, and somatostatin-like immunoreactivity, and type-IIIb cells which do not (Chapter 3; Fischer

et al., 1998f). In addition, type-III cholinergic amacrine cells ramify more densely in the proximal IPL at 50 to 55%, 75 to 85%, and 90 to 95% depth (Fig. 2.2; Millar et al, 1987b; Watt and Florack, 1994; Chapter 3; Fischer et al., 1998f).

Assuming that cholinergic synaptic transmission occurs within well-defined IPL strata where neurites of cholinergic amacrine cells ramify, then several conclusions can be drawn by comparing the distributions of mAChRs and cholinergic innervation in the IPL. First, cm3 is the only mAChR subtype that can be directly post-synaptic to type-I cholinergic amacrine cells, whereas cm2, cm3 and cm4 are all potentially post synaptic to type-II cholinergic amacrine cells. Second, in the distal IPL, type-III cholinergic amacrine cells can be presynaptic only to the cm2 and cm3 isoforms of mAChR, whereas in the proximal IPL, type-III cells could form synapses involving all three types of mAChR. However, it is also likely that some ACh escapes hydrolysis by ACh esterase, and diffuses away from its point of release to activate ectopic mAChRs.

The significance of cm3-like immunoreactivity in the OPL remains uncertain. This localization recalls that nAChRs have been detected in bipolar cells and in the OPL of the chick retina (Yazulla and Schmidt, 1977; Hamassaki-Britto et al., 1994). However, no source of ACh in the OPL is known, as ChAT has not been detected in this layer or in photoreceptors, horizontal or bipolar cells. While some evidence suggests that photoreceptors in turtle retina might synthesize acetylcholine (Lam, 1972; Ross and McDougal, 1976; Sarthy and Lam, 1979), possibly to modulate the activity of bipolar cells (James and Klein, 1985), there is no evidence that outer retinal neurons release ACh in the chick. Therefore, cm3-immunoreactivity in the OPL may represent: (i) functional

non-synaptic receptors that are activated at a distance by ACh released from cholinergic amacrine cells; (ii) synaptic ACh receptors that are activated by an unidentified local source of ACh or other unknown mAChR ligand; (iii) non-functional receptors which have been transported or otherwise allocated to non-cholinoceptive sites; or (iv) a cm3-cross-reactive protein which is not a receptor. With regard to point (ii), it has been proposed that dopamine released from neurites in the IPL may escape reuptake and diffuse, in substantial amounts, through the INL to activate receptors in the OPL (Witkovsky and Schütte, 1991). The diffusion of ACh out of the IPL may be largely prevented by ACh esterase, but the distributions of ChAT and ACh esterase in the IPL do not entirely overlap (Millar et al., 1985). Therefore, if ChAT-immunoreactive amacrine cells are the only retinal source of ACh, then activation of mAChRs in the OPL might require ectopic release or transport of ACh into areas devoid of ACh esterase, or pharmacologically significant amounts of ACh to escape hydrolysis and diffuse across the retina. Alternatively, ACh may be produced by a ChAT-independent process in outer retinal neurons, and released from them to act upon AchRs nearby in the OPL.

Significance of mAChRs in RPE, choroid and ciliary body:

In the choroid, cm3-immunoreactivity was detected at a lower molecular weight than in other tissues in protein blots and appeared as intense staining of the walls of blood vessels in sections of the choroid. Cm3 is 639 amino acids long (Gabut and Galper, 1994), which is equivalent to about 70 kDa. Therefore, the cm3-IR band in blotted RPE and choroidal extracts (Fig. 9b) may represent a functional but non-glycosylated form of

the receptor. Pharmacological evidence suggests that mAChRs are expressed in the RPE of rats (Salceda, 1994) and humans (Osborne et al., 1991), but to the best of our knowledge there have been no prior reports of mAChR-proteins, mRNAs, or mAChR-mediated functions in the RPE of chickens.

It has been reported that mAChR-antagonists do not interfere with the normal functioning of the accommodative mechanisms in the anterior of the chick eye, and that only nAChRs participate in accommodation (McBrien et al., 1993). Therefore, it is likely that mAChRs in the ciliary body have some function other than evoking the contraction of ciliary and iris muscles. It has also been reported that mAChRs participate in the development of form-deprivation myopia in chicks (Stone et al., 1991; McBrien et al., 1993; Leech et al., 1995). However, the location of the mAChRs that participate in growth-regulating pathways in the eye remains unknown. It is possible that one or more mAChR isoforms in the retina, RPE, choroid, or ciliary body may be involved in form-deprivation myopia and the visual regulation of ocular growth.

Interpretation of immunoblots:

The apparent molecular masses of the chick mAChRs closely match those revealed in retina by previous affinity alkylation and immunoblotting analyses (Large et al., 1985; McKinnon and Nathanson, 1995). We detected immunoreactive cm2 in tissue extracts of RPE, choroid and ciliary body despite being unable to detect it in tissue sections. This likely resulted in part from the greater inherent sensitivity of the immunoblot assay and from having concentrated the antigen through the tissue extraction

and blotting procedures used in this study. We also detected additional immunoreactive bands in blotted extracts of choroid, RPE and ciliary body. These bands may represent proteins that contain mAChR-like epitopes, or, in some cases, the degradation products of proteolytic cleavage. It is also possible that these bands resulted from non-specific binding of antibody with proteins that were present in high amounts in these concentrated, whole-tissue extracts. It is noteworthy that the specificity of these antibodies has been tested previously by McKinnon and Nathanson (1995), who only reported immunoblots for extracts of membrane from retina, but not choroid, RPE or ciliary body, while in the present study whole cell extracts were used. Differences between the apparent molecular masses of chick mAChRs and banding patterns reported previously, and those detected in our experiments, were likely due to variations in procedures for electrophoresis and detection of blotted proteins. Despite, the presence of extraneous bands on immunoblots, it can still be concluded that immunoreactive mAChRs are present at the appropriate molecular masses in extracts of several ocular tissues.

Previous reports, in which mAChRs were assayed by PrBCM binding, have also described a shift in the relative abundance of mAChR isoforms in the retina between embryonic and adult chicks. Large et al., (1985) reported that the lower molecular mass mAChR isoform (likely to be cm2) was less abundant than the larger isoforms (likely to be the sum of cm3 and cm4) in the retina of embryonic chicks, while the smaller mAChR isoform was more abundant than the larger isoform in hatched chicks. In contrast, McKinnon and Nathanson (1995) reported that cm4 mRNA and protein were more

abundant than those of other isoforms in both embryonic and hatched chick retina, and that expression levels decreased as development progressed. However, we detected substantial amounts of cm4 protein in the retina of hatched chicks. This discrepancy may have resulted from differences in tissue extraction, antigen concentration, and immunoblotting techniques employed in these two studies. In addition, both cm2 and cm3 mRNAs in the retina were expressed at low levels early in development, but at higher levels after hatching (McKinnon and Nathanson, 1995). These findings suggest that the higher molecular weight isoforms of mAChRs are more abundant than the lower molecular weight isoform in the retina of hatched chicks. This is in agreement with our immunocytochemical findings in retina of hatched chicks, that the apparent amounts of both higher molecular-mass mAChR isoforms are greater than the apparent amount of the lower-mass form. The discrepancy between the results of these two methods for receptor localization may have been due to differences in the relative binding affinity of PrBCM or antibodies for each mAChR subtype, or the presence and developmental regulation of additional mAChRs for which we do not yet have antisera.

Summary and conclusions:

We have reported here the localization of three different subtypes of mAChRs in the retina, choroid, ciliary body, RPE, and ciliary ganglion of the chick. The results indicate that all three isoforms of chick mAChR for which we were able to test are present in the RPE, ciliary body, and retina. Although different mAChR isoforms may be present in one ocular tissue, their distribution within that tissue is variable. For example

in the retina, immunoreactivity for different mAChR subtypes appeared in unique layers of the IPL. In addition, cm3 was expressed by distinct subsets of amacrine, ganglion, and bipolar cells, while cm2 and cm4 appeared in the vast majority of amacrine and ganglion cells, but were absent from bipolar cells. The expression of different mAChR isoforms is tissue-specific, since cm3 was not detectable in the choroid and neither cm3 nor cm2 was detected in the ciliary ganglion, while cm4 was found in all tissues tested.

CHAPTER THREE

Immunocytochemical characterization of quisqualic acid- and NMDA-induced excitotoxicity in the retina of chicks

Introduction:

Ocular growth is regulated precisely by visual experience. Myopia (near-sightedness) resulting from excessive ocular growth is commonly studied in chicks and can be induced by depriving the eye of patterned images; this is called "form-deprivation myopia" (FDM; reviewed by Wallman, 1993). Myopia can also be induced by excitotoxins, including kainate, quisqualic acid (QA) and N-methyl-*D*-aspartate (NMDA) (Wildsoet and Pettigrew, 1988; Ehrlich et al., 1990; Fischer et al., 1997b; 1998g). Both form-deprivation and excitotoxins are thought to elicit their growth-altering effects by disrupting the activity of retinal neurons. It is currently believed that retinal modulation of ocular growth is mediated by sub-populations of amacrine cells, including those which release or respond to acetylcholine, dopamine, vasoactive intestinal polypeptide (VIP), and enkephalin (ENK) (Wallman, 1993; Seltner and Stell, 1995a; Seltner et al., 1997). The administration of a large dose of NMDA or QA to the chick retina has been shown to leave photoreceptor, bipolar, and ganglion cells intact, but to destroy many amacrine cells (Morgan, 1987; Barrington et al., 1989; Ehrlich et al., 1990; Tung et al., 1990; Sheppard et al., 1991). The specific subsets of amacrine cells that are sensitive to NMDA or QA have not been identified. It is possible that excessive ocular growth resulting from retinal exposure to excitotoxins is mediated by the differential destruction of amacrine cells that

contribute to growth-regulating retinal pathways. Although the degree of myopia caused by excitotoxins is small (about -2 dioptres) in comparison to that caused by form-deprivation (about -10 dioptres), excitotoxins have different effects upon the growth-promoting pathways in the retina that are actived by form-deprivation. Growth-promoting mechanisms are disrupted in NMDA-intoxicated retinas, since myopia can no longer be enhanced by form-deprivation (Fischer et al., 1997b, 1998g). In contrast, at least some growth-promoting mechanisms are intact in QA-intoxicated retinas, since myopia can be enhanced by form-deprivation (Erhlich et al., 1990; Chapter 4; Fischer et al., 1998c, d). Therefore, it is expected that NMDA and QA might destroy different sub-types of amacrine cells involved in ocular growth regulation, thereby differentially affecting growth-enhancing pathways that are activated by visual form-deprivation.

The purpose of this study was to identify subsets of amacrine cells that might be involved in the retinal control of ocular growth and the pathogenesis of myopia. To do this, we characterized the effects of NMDA or QA upon cells believed to participate in the regulation of post-natal ocular growth and FDM by using antisera raised against different cytological markers (either transmitters, enzymes, transporters, or receptors). We also characterized the time course of cell damage in retinal neurons treated with NMDA or QA by probing *in situ* for DNA fragmentation. We showed that most amacrine cells previously implicated in growth regulation, including those immunoreactive for ENK, choline acetyltransferase (ChAT) and VIP, are destroyed by QA as well as NMDA, thereby making unlikely the participation of these cells in the progression of FDM. However, dopaminergic amacrine cells survive exposure to both

NMDA and QA, and therefore these, along with other unidentified subsets of amacrine cells, remain as candidates for growth-modulators.

Methods and materials:

Animals:

Animals were maintained as described in Chapter 2.

Intraocular injections:

Chicks were anesthetized with 1.5% halothane in 50% N_2O and 50% O_2 prior to injection. Injections were made into the vitreous chamber using a 25 µl Hamilton syringe with a 26 gauge needle. Penetration of the needle was consistently made into the dorsal quadrant of the eye. The left eye (control) was injected with 20 µl of sterile saline and the right eye (treated) was injected with 20 µl of 0.1 M (2,000 nmol) NMDA or 20 µl of 0.01 M (200 nmol) QA dissolved in sterile saline. Doses of NMDA and QA were similar to those used in prior studies in chick eyes (Sattayasai and Ehrlich, 1987; Ehrlich et al., 1990; Tung et al., 1990; Sheppard et al., 1991). Assuming that the volume of liquid vitreous within an eye was 150 µl, the initial maximum concentration of NMDA presented to the retina was about 11.7 mM and that of QA about 1.17 mM. All drugs were obtained from Research Biochemicals International, Natick, MA.

Tissue dissection, fixation, and sectioning:

Tissues were fixed and sectioned as described in Chapter 2, with the following

exceptions. Samples to be labelled with antisera to serotonin, γ-amino butyric acid (GABA) or glutamic acid decarboxylase (GAD) which were fixed for 24 hours at 4°C. Sections from control and treated eyes from the same individual were placed together in pairs on each slide to ensure equal exposure to reagents.

Immunocytochemistry:

Immunocytochemistry was performed as descibed in Chapter 2. Antibodies used in this section are listed in Table 3.1.

Histology:

Slides were warmed to 20°C, washed three times in PBS, and incubated under 200 µl 0.1% (w/v) toluidine blue for about 2 minutes. The stain was drained, slides were washed three times in PBS and mounted, as described above, for microscopy in transmitted white light.

Labelling of fragmented DNA:

Retinal sections were obtained as described above from chicks 1, 3, and 7 days after NMDA or QA-treatment. Slides were warmed to 20°C and washed once in PBS, followed by one wash in PBS plus 0.3% Triton X-100, and two more washes in normal PBS. Sections were then covered with 100 µl of incubation medium (0.5 nmol Cy3-conjugated dCTP, 20 units of 3'-terminal deoxynucleotidyl transferase (Amersham), 100 mM sodium cacodylate, 2 mM $CoCl_2$, and 0.25 mM β-mercaptoethanol, in sterile saline at pH 7.2) and incubated for 1 hour in a humidified chamber at 37°C. The sections were

Table 3.1. List of antisera, their antigens, sources, and working dilutions

Table 3.1

Antiserum and Antigen	Species/Type	Source	Working Dilution
1465	rabbit polyclonal	Dr.M.Epstein	1:1000
choline acetyltransferase (ChAT)		(U. of Wisconsin)	
α-GABA	rabbit polyclonal	Chemicon	1:100
γ-aminobutyric acid (GABA)		(Temecula, CA)	
R24	rabbit polyclonal	Drs. R. Jahn & L. Edelman	1:1000
GABA transporter (GAT-1)		(Yale)	
8305034	rabbit polyclonal	Dr. J. Walsh	1:1000
glucagon		(UCLA)	
634	rabbit polyclonal	Dr. C. Brandon	1:1000
glutamic acid decarboxylase (GAD-65)		(Chicago School of Medicine)	
9T	rabbit polyclonal	Chemicon	1:50
glutamate receptor 1 (GluR1)			
25-7	rabbit polyclonal	Chemicon	1:50
glutamate receptor 2/3 (GluR2/3)			
LEP 100	mouse monoclonal	Hybridoma Bank	1:50
lysosomal membrane glycoprotein		(U. Of Iowa)	
1473	rabbit polyclonal	Dr.J.Walsh	1:1000
Met-enkephalin (ENK)			
3A10	mouse monoclonal	Hybridoma Bank	1:50
neurofilament-associated antigen (NAA)			
αPA	mouse monoclonal	Sigma	1:1600
parvalbumin		(Mississauga, Ontario)	
RPN536	mouse monoclonal	Amersham	1:50
protein kinase C (PKC) α and β isoforms		(Oakville, Ontario)	
5-HT	rabbit polyclonal	Inc Star	1:800
serotonin (5-HT)		(Stillwater, MN)	
S-10	rat monoclonal	Dr.A.Buchan	1:400
somatostatin		(U. of British Columbia)	
#16	rabbit polyclonal	Dr.W.Tank	1:1000
tyrosine hydoxylase (TH)		(U. of Rochester)	
VP31	rat monoclonal	Dr.A.Buchan	1:80
vasoactive intestinal polypeptide (VIP)			

then washed three times in PBS, mounted in 4:1 (v/v) glycerol to water, and coverslips added were for observation by epifluorescence with a rhodamine filter combination.

Measurements, cell counts, and statistical analyses:

Errors were calculated as the standard deviation of each sample which was comprised of at least six individuals per group. To compare data from treated and control eyes, statistical significance was assessed by using a paired two-tailed Student *t*-test. Percentage inner plexiform layer (IPL) depth was calculated as the distance from the border of the IPL and inner nuclear layer (INL) divided by the total IPL thickness, multiplied by 100. All thickness measurements were made from photomicrographs of central retina, while all cell counts were made from central retina under the microscope of at least 100 cells per individual on at least 4 different sections.

Results:

Histology:

The appearance and thickness of retinal layers did not change significantly in control eyes over the course of the study. In contrast, the appearance and thickness of retinal layers treated with QA or NMDA varied dramatically. One day after treatment with NMDA there was a large increase in the thickness of the IPL, whereas the thickness of other retinal layers was unchanged (Table 3.2; Fig. 3.1b). Three days after NMDA-treatment, there were areas of reduced thickness in the IPL accompanied by folding and detachment of the retina, between which the retina remained attached and the thickness of the IPL was not significantly different from that in the control (Table 3.2; Fig. 3.1c). In contrast, in areas of retinal folding the thickness of the INL was not significantly affected, but between folds the thickness of the INL was reduced (Table 3.2; Fig. 3.1c). Seven days after NMDA-treatment the thicknesses of both the IPL and INL were severely reduced, while that of the optic fibre layer (OFL) was increased (Table 3.2; Fig. 3.1d).

One day after retinal exposure to QA the thickness of the IPL was significantly increased, while that of the INL was reduced (Table 3.2; Fig. 3.1b). Three days after exposure to QA, the thickness of the IPL remained slightly increased, and the thickness of the INL remained reduced (Table 3.2; Fig. 3.1c). Seven days after QA-treatment, the thicknesses of both the IPL and INL were substantially reduced (Table 3.2; Fig. 3.1d), whereas the thickness of the OFL was nearly double that of the control (Table 3.2; Fig. 3.1d). Twenty-one days after exposure to NMDA or QA the reduction in thickness of the

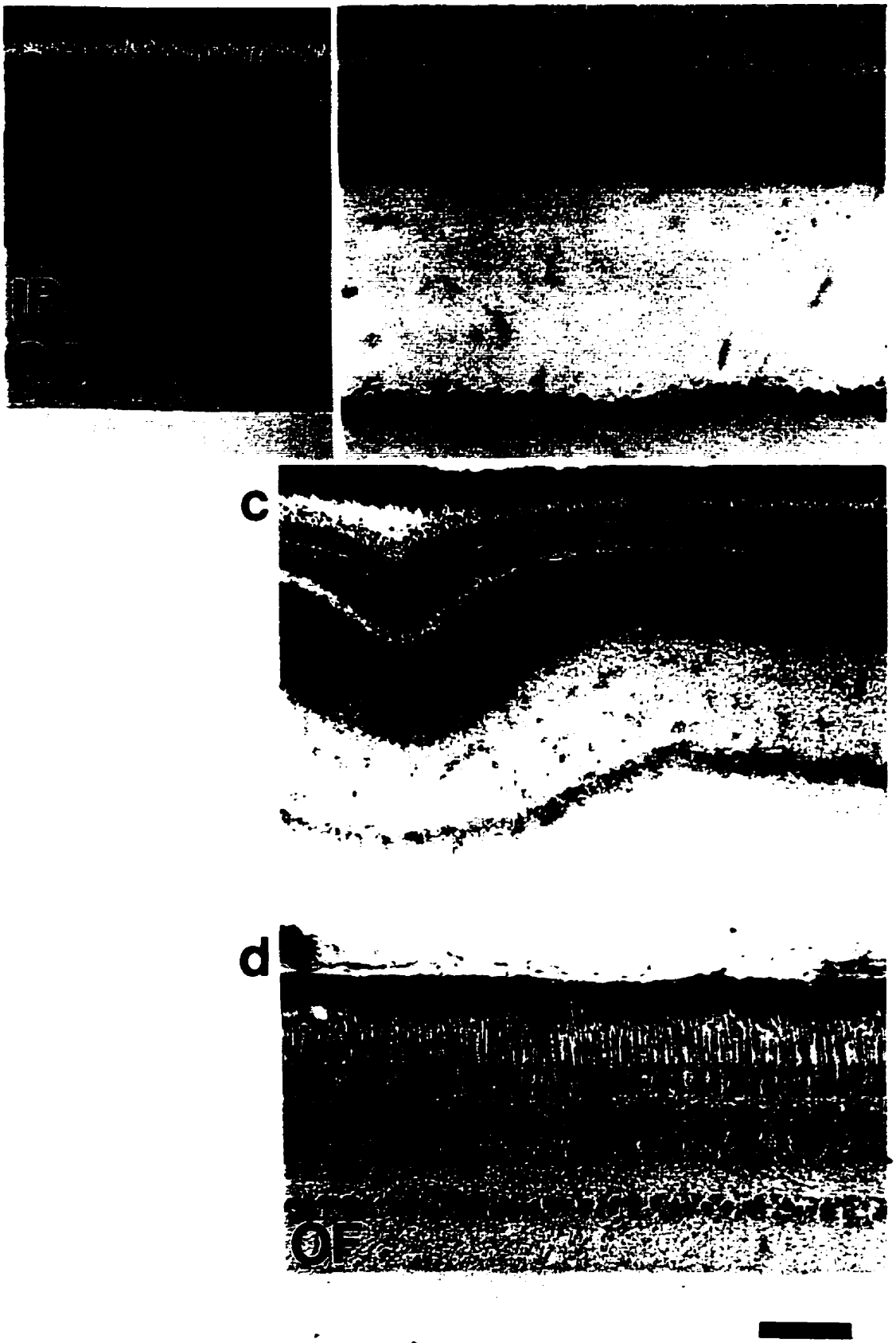
Table 3.2: Percent thickness of the control of the IPL, INL and OFL, 1, 3 and 7 days after treatment with NMDA or QA. The percent thickness was derived by dividing the treated by control, and multiplying by 100. Each sample is comprised of measurements from 6 individuals.

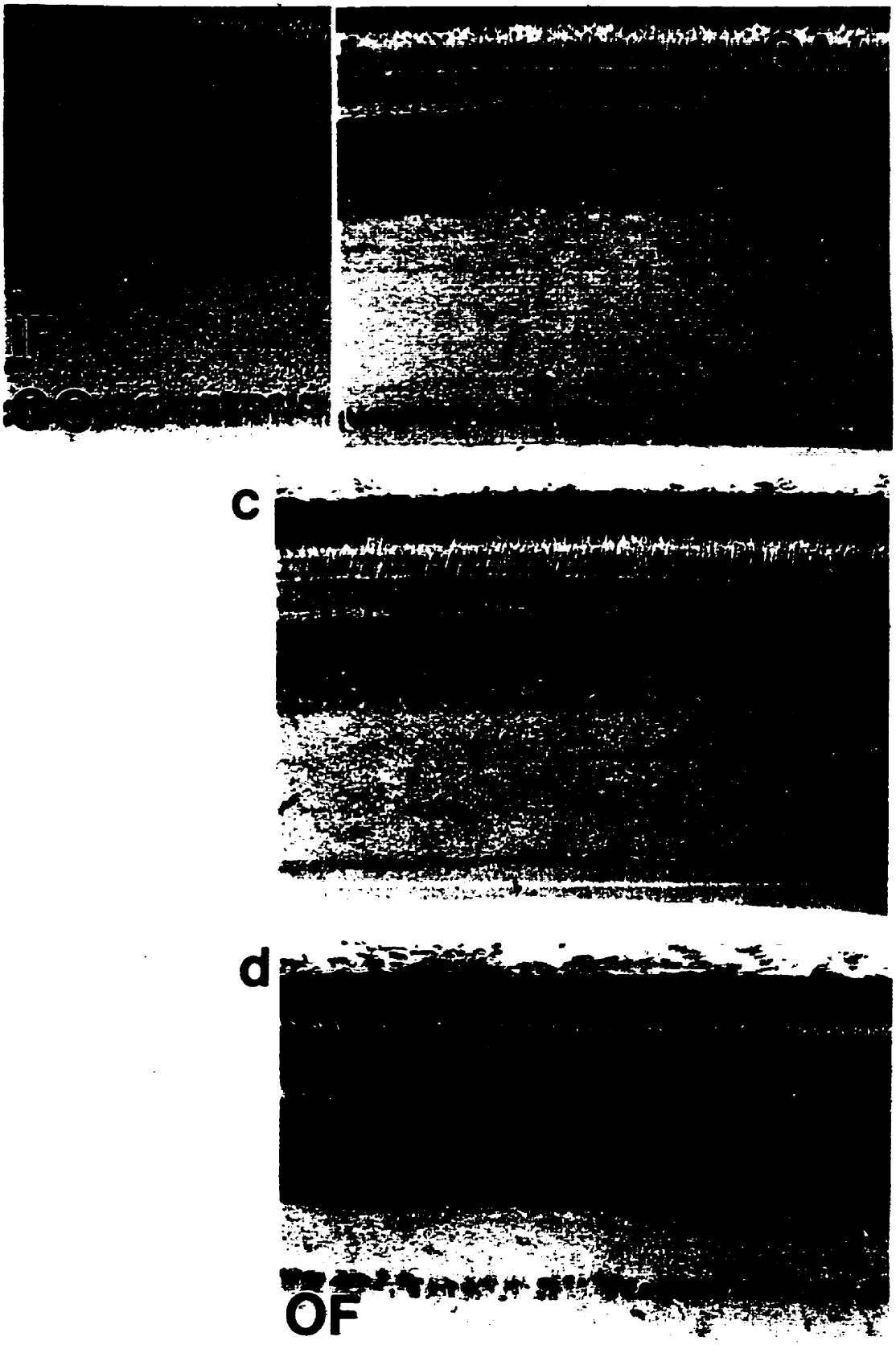
Table 3.2

Treatment	Retinal Layer	Time after treatment (days) 1	3	7
NMDA	IPL	174.5 ± 26.8****	105.3 ± 6.3 #66.0 ± 11.2****	37.4 ± 7.0****
	INL	94.9 ± 4.0*	68.6 ± 12.0**** #94.8 ± 12.6	74.2 ± 8.4****
	OFL	94.5 ± 19.3	102.4 ± 32.9	207.7 ± 33.2****
QA	IPL	169.1 ± 8.0****	123.4 ± 9.5***	55.8 ± 4.8****
	INL	81.8 ± 5.6****	84.8 ± 8.4**	82.7 ± 3.2****
	OFL	124.5 ± 21.8*	109.9 ± 21.0	194.9 ± 30.5****

(# retinal fold; $\bar{x}$ ± SD; *p < 0.05; **p < 0.005; ***p < 0.0005; **** p < 0.0001; significance assessed using a one-tailed Student *t*-test to compare differences between treated and control conditions at each time interval and for each treatment; Abbreviations: IPL- inner plexiform layer; INL - inner nuclear layer; OFL - optic fibre layer; NMDA - N-methyl-D-apartate; QA - quisqualic acid)

Figure 3.1: Vertical sections of retina stained with toluidine blue, taken from chicks at different times after treatment with N-methyl-D-aspartate (NMDA) or quisqualic acid (QA): (**a**) before injection; (**b**) one day after treatment; (**c**) three days after treatment; and (**d**) seven days after treatment. There was no apparent difference between control retinas 1, 3, and 7 days after treatment in comparison to the pre-injection control (**a**). Abbreviations: IP - inner plexiform layer; IN - inner nuclear layer; GC - ganglion cell layer; OF - optic fibre layer. Scale bar = 50 μm.


c
d


c
d
OF

IPL and INL remained unchanged from 7 days after treatment, while the thickness of the OFL approached that of control retinas (results not shown).

In all cases, QA or NMDA-induced thinning of the INL appeared to result from reduction in numbers of amacrine cells in the proximal INL. Amacrine cells were distinguished from other cells in the INL by their relatively intense staining with toluidine and the known location of amacrine cells in the proximal half of the INL. In addition, the number and density of cell bodies in the ganglion cell layer (GCL) appeared to be reduced by both QA and NMDA-treatments. The loss of amacrine and ganglion cell bodies was not uniform across the retina. The loss of amacrine cells, in particular, was greatest in the nasal half of the retina near the site of the injection. In addition, 1 and 3 days after QA or NMDA-treatment, significant numbers of irregularly shaped cell bodies were scattered throughout the IPL, which was practically acellular in control retinas (Fig. 3.1).

NMDA and QA-induced changes in ENK, ChAT, VIP and TH immunoreactivity:

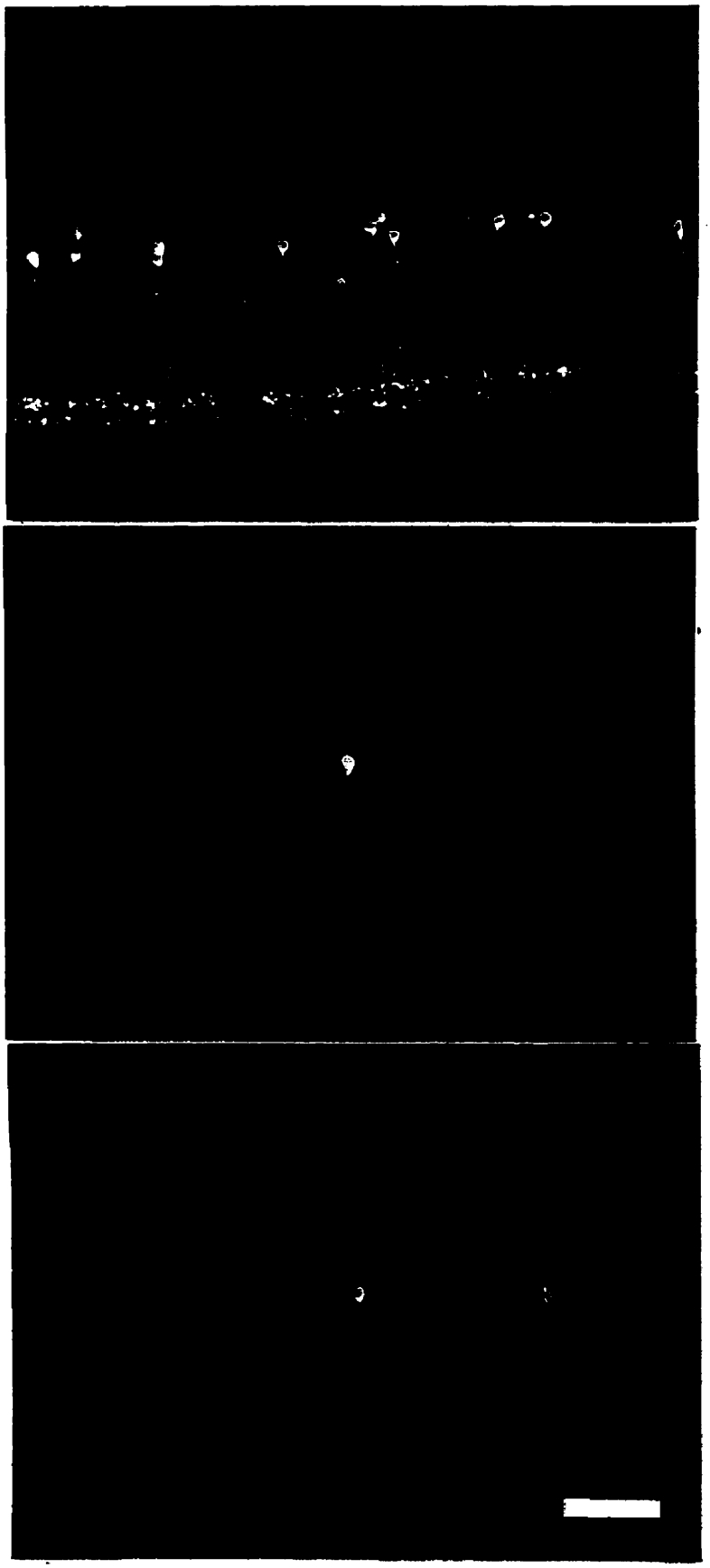
Immunohistochemical changes in retinas were tested only 7 days after exposure to either NMDA or QA, as excitotoxin-induced lesions have been shown to become stabilized within 1 week after treatment (Dvorak and Morgan, 1983).

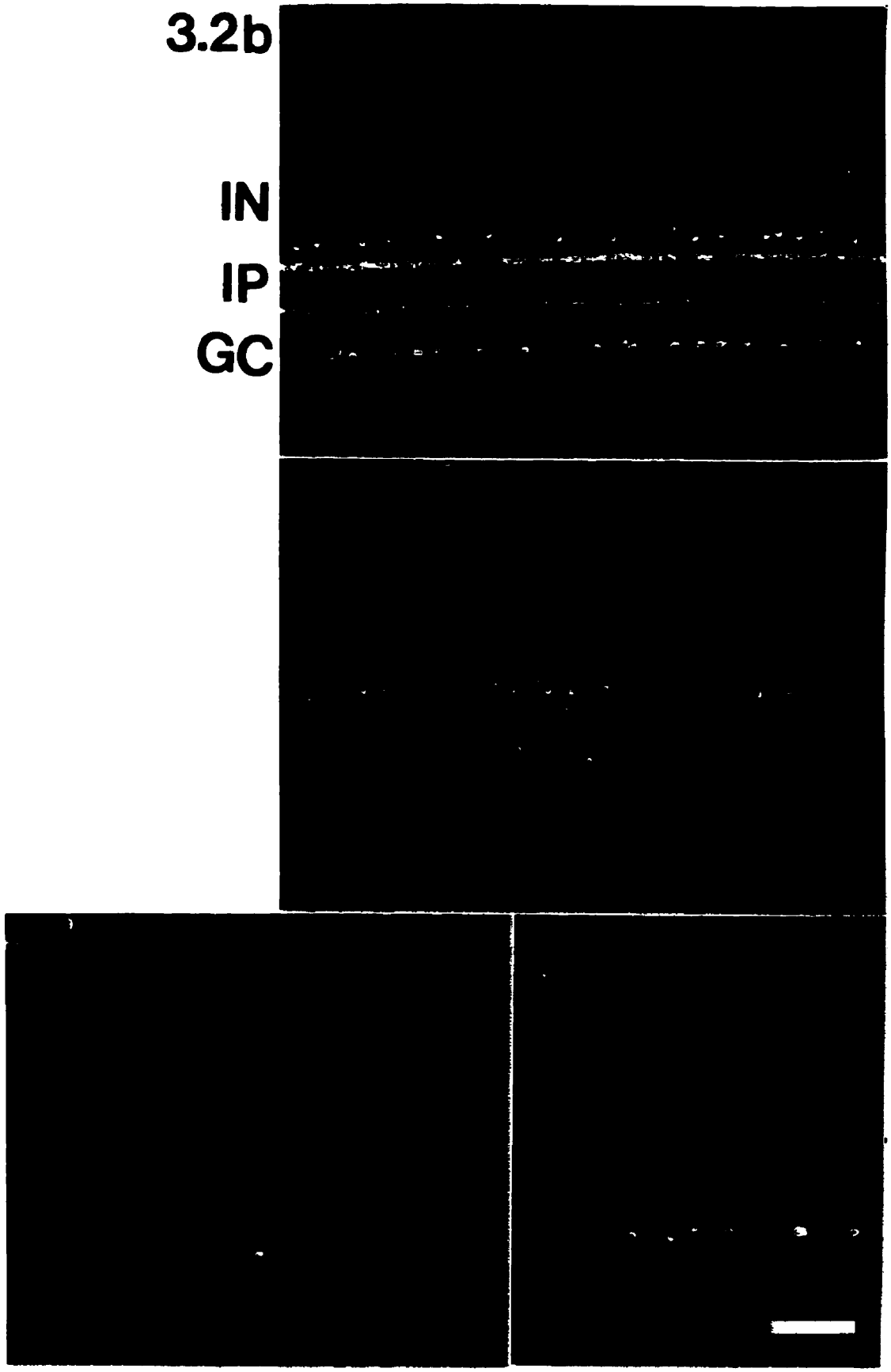
(a) Enkephalin:

Antiserum to ENK labelled many cells having somata near the middle of the INL, and dendrites in several distinct sublaminae of the IPL (Fig. 3.2a). These cells are the enkephalin-neurotensin-somatostatin-like immunoreactive (ENSLI) amacrine cells which have been described previously (Brecha et al., 1979; Watt et al., 1985; Morgan et al.,

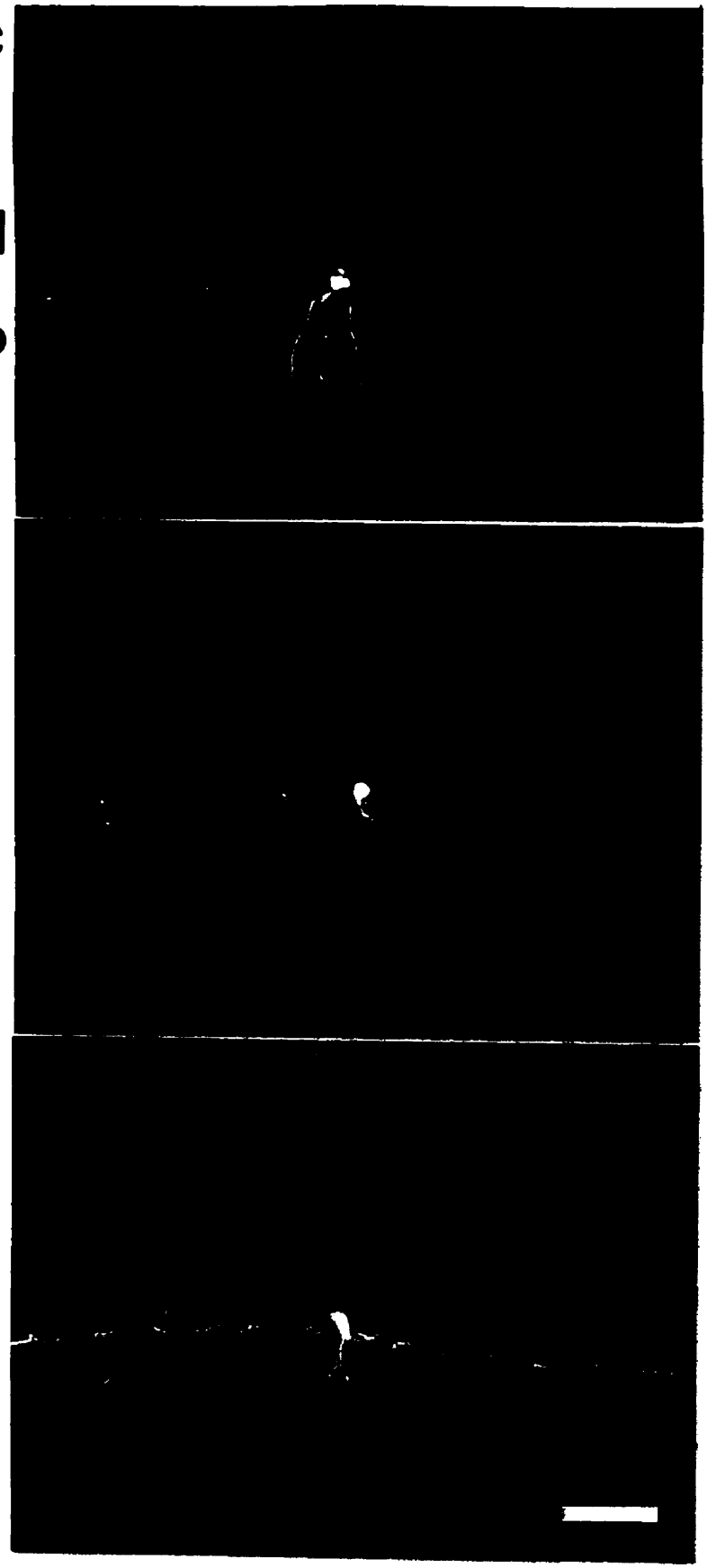
Figure 3.2: Vertical sections of retina obtained from eyes 7 days after treament with saline (top), NMDA (middle), or QA (bottom). These sections have been labelled with antisera raised against (**a**) Met-enkephalin (ENK), (**b**) choline acetyltransferase (ChAT), (**c**) tyrosine hydroxylase (TH), and (**d**)vasoactive intestinal polypeptide (VIP). Figure **3.2b** (bottom) shows QA-treated retina at the area centralis (left) and temporal region (right). Abbreviations: IP - inner plexiform layer; IN - inner nuclear layer; GC - ganglion cell layer. Scale bar = 50 μm.

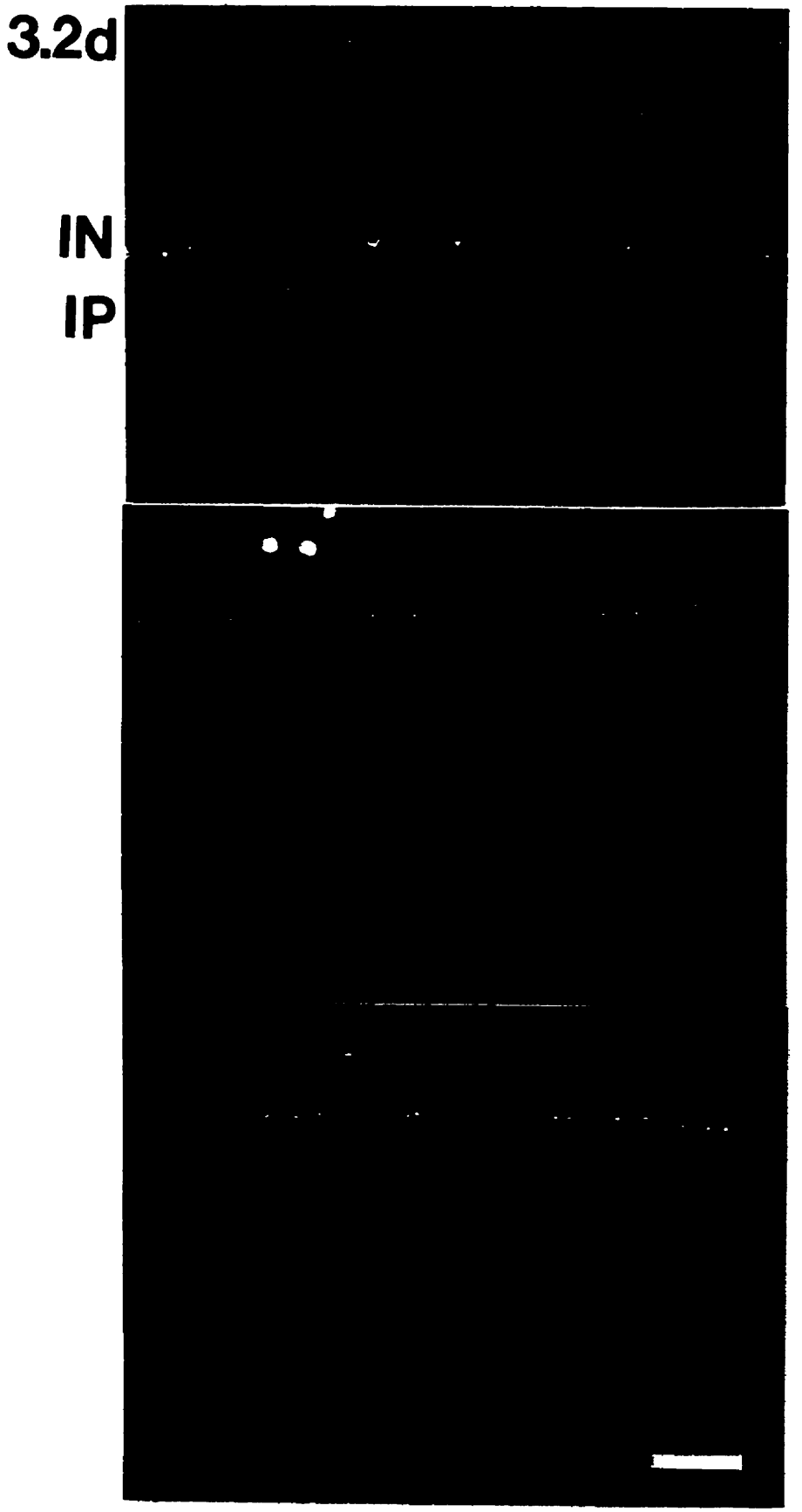
3.2a




3.2b
IN
IP
GC

3.2c




3.2d
IN
IP

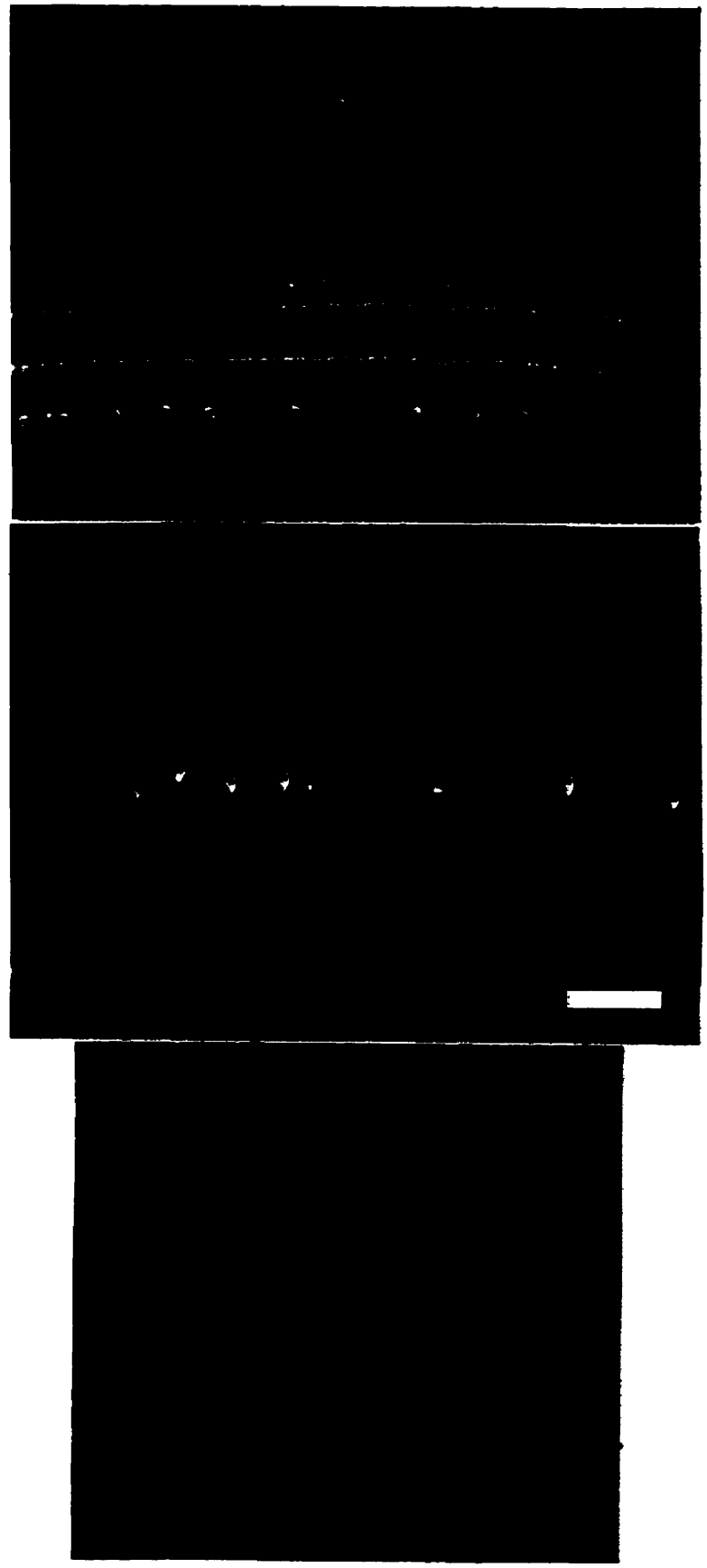
1994; Watt and Florack, 1994. After NMDA-treatment, ENSLI cells disappeared almost entirely, with only 13.2 ± 7.4% (n=6) of their cell bodies remaining detectable. ENK-immunoreactive (IR) neurites in the IPL were almost entirely disintegrated, with remnants no longer clearly stratified and forming only short, narrow arbors extending into the proximal IPL (Fig. 3.2a). After retinal exposure to QA fewer than 1% of these ENK-IR amacrine cells remained (Fig. 3.2a). In all cases of QA-treatment (N=6), a small patch of ENK-IR amacrine cells (5 to 10 cells per section) remained near the central retina, medial to the optic nerve head. The dendritic arbors of these residual cells were narrow, but retained their stratification in the IPL (Fig. 3.2a).

(b) ChAT:

Antiserum directed against ChAT revealed numerous cells in the INL and GCL, as well as 2 robustly-labelled strata in the IPL (Fig. 3.2b), exactly as described previously. Millar (et al., 1987b) identified 3 subtypes of ChAT-IR amacrine cells in the chick retina, which included: type-I cholinergic amacrine cells, with cell bodies at the IPL/INL border and processes in stratum 2 (15 to 25% depth) of the IPL; type-II cholinergic amacrine cells, with cell bodies in the GCL and processes in stratum 4 (65 to 75% depth) of the IPL; and type-III cholinergic amacrine cells with cell bodies near the middle of the INL and processes diffusely distributed in strata 1 (0 to 10% depth) and 3-5 (45 to 85% depth) of the IPL. In the present study, we found that nearly half (46.2% ± 9.1%; 937 cells counted from 7 individuals) of the type-III ChAT-IR amacrine cells were also immunoreactive for somatostatin, i.e. ENSLI cells (Fig. 3.3), and that all ENSLI cells (N=433) also were ChAT-IR. ENSLI and non-ENSLI type-III cholinergic amacrine cells

Figure 3.3: Vertical section of retina from an untreated eye which has been doubly-labelled with antisera raised against (**a**) ChAT and (**b**) somatostatin. Arrow heads in figure **3.3a** indicate cells that contain both ChAT and somatostatin-immunoreactivities. Figure **3.3c** is a psuedocolour composite image in which the negatives of the images in figures **3.3a** and **3.3b** were digitized, enhanced for contrast, assigned colour (green for ChAT-immunoreactivity and red for somatostatin-immunoreactivity), and overlayed using Adobe Photoshop 4.0. Scale bar = 50 μm.

3.3



could be distinguished further by structural and immunoreactive criteria. The type-III_{NE} cholinergic amacrine cells (non-ENSLI, ChAT immunoreactivity only) were weakly immunoreactive for ChAT and had smaller, irregularly shaped cell bodies, with a thin layer of cytoplasm surrounding the nucleus, and a slender primary neurite. In contrast, the type-III_E cholinergic amacrine cells (ENSLI) were strongly immunoreactive for ChAT and had larger, rounder cell bodies, a larger cytoplasmic volume, and a thick primary neurite. These findings indicate that ENSLI amacrine cells as well as a fourth, unidentified type of amacrine cell, may also utilize acetylcholine as a neurotransmitter.

After NMDA-treatment, there was a significant reduction in retinal ChAT immunoreactivity. In all cases (N=7) type-II, type-III_{NE} and type-III_E cholinergic amacrine cell bodies and neurites were almost entirely destroyed. In contrast, type-I cholinergic amacrine cells appeared to be only moderately affected (Table 3.3; Fig. 3.2b). ChAT-IR neurites in the proximal IPL were nearly non-existent, while those arising from type-I cells in the distal IPL appeared only moderately less abundant when compared to those in control retinas (Fig. 3.2b).

Following exposure to QA nearly all type-I cholinergic amacrine cells were destroyed or entirely depleted of ChAT immunoreactivity (Table 3.3; Fig. 3.2b). Similarly, all type-III_{NE} cholinergic amacrine cells, and most of the type-III_E cells were no longer detectable (Table 3.3; Fig. 3.2b). In all cases (N=7), most type-II cholinergic amacrine cells were lost; the residual type-II cells were scattered across the retina (Table 3.3; Fig. 3.2b), except in the temporal retina (region farthest from the site of injection) where they were more abundant and their processes in the IPL appeared nearly normal

Table 3.3: Percentage of ChAT-IR amacrine cells remaining 7 days after retinal exposure to NMDA or QA.

Table 3.3

Type of cholinergic cell	NMDA-treated	QA-treated
Type -I	78.8 ± 13.9*	3.8 ± 5.9**
Type-II	27.4 ± 26.3**	C: 12.4 ± 7.8** P: 94.2 ± 4.8**
Type-III (a + b)	8.4 ± 5.9**	3.2 ± 4.1**

(Significance of difference from control retinas: *$p < 0.01$, **$p < 0.0005$, C=central retina, P=peripheral retina; ChAT-IR - choline acetyltransferase-immunoreactive)

(Table 3.3; Fig. 3.2b).

(c) TH:

Antiserum to TH labelled sparsely distributed cell bodies with neurites in 3 different strata in the IPL, at 0 to 10%, 35%, and 75% depth (Fig. 3.2c), exactly as described previously (Su and Watt, 1987). In all cases (N=8), treatment with NMDA caused little or no loss of TH-IR amacrine cells and some depletion TH-IR neurites in the distal IPL, while neurites in the more proximal layers were less abundant and were compressed into the remaining IPL (Fig. 3.2c). Following exposure to QA there was some loss of TH-IR neurites and cells, mostly from the central retina (Fig. 3.2c).

(d) VIP:

Antiserum to VIP weakly labelled many cell bodies near the border of the INL and IPL, in addition to sparsely distributed neurites at 0 to 10%, 35%, and 70% IPL depth, as described previously (Fig. 3.2d; Brecha, 1983; Seltner and Stell, 1995). After NMDA-treatment, more than 90% of the VIP-IR amacrine cells were no longer detectable, and the residual neurites in the IPL were almost entirely disintegrated (Fig. 3.2d). Similarly, VIP-IR amacrine cells in the INL and neurites in the IPL were no longer detectable after retinal exposure to QA (Fig. 3.2d). Depletion of ChAT, ENK, and VIP immunoreactivities was evident as soon as 24 hours after treatment with NMDA or QA (results not shown).

NMDA and QA-induced changes in other amacrine cell markers:

(a) GABA:

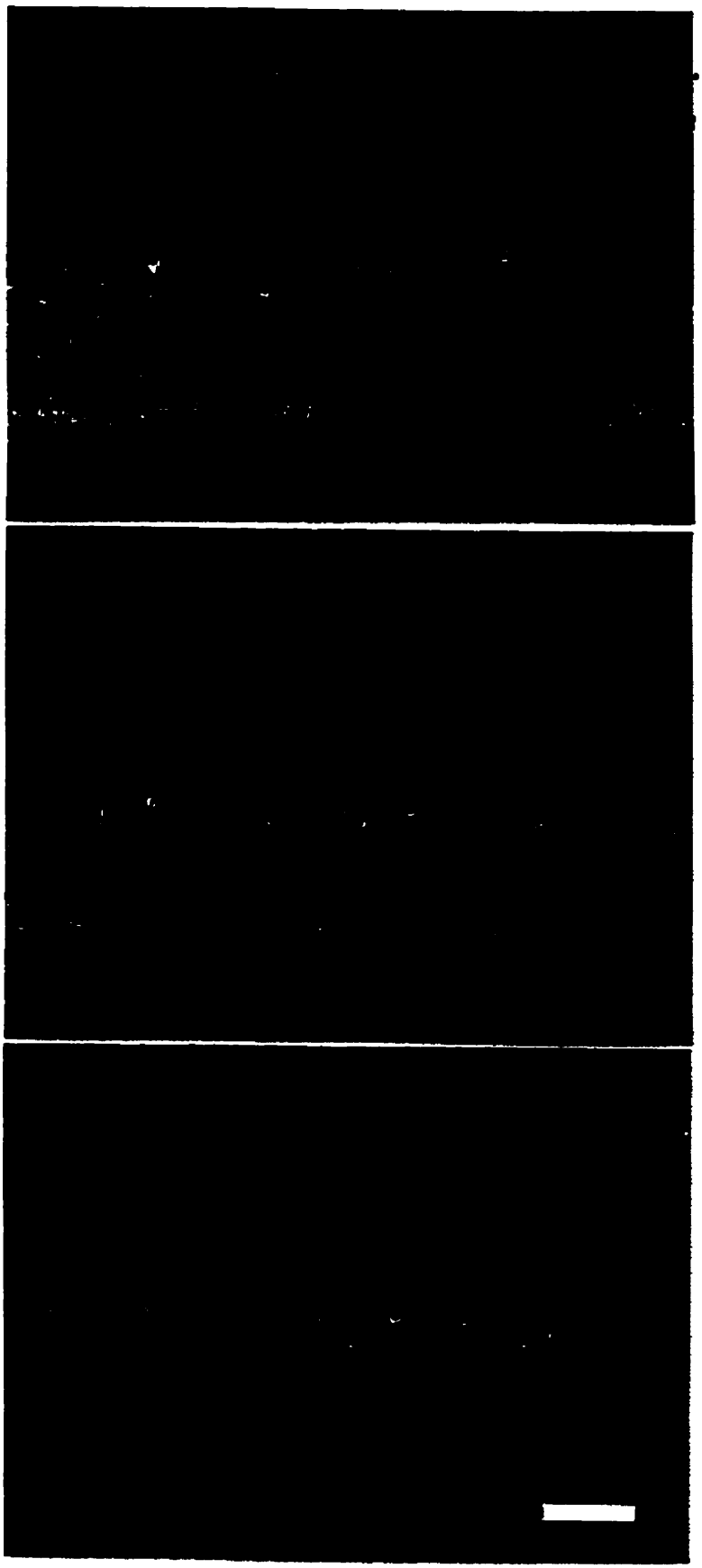
Numerous GABA-IR cell bodies were present in the amacrine and horizontal cell layers of the INL, and in the GCL (Fig. 3.4a). Immunoreactive GABA was also detected in neurites throughout the IPL, in sparsely distributed processes in the outer plexiform layer (OPL), and in the nerve fibers extending vertically through the INL to the OPL (Fig. 3.4a). NMDA-treatment destroyed many GABAergic amacrine cells in the INL, while GABA-immunoreactivity in the OPL, horizontal cells, and cells in the GCL appeared unaffected (Fig. 3.4a). Although the labelling intensity of GABA-IR dendrites in the IPL was unchanged by exposure to NMDA, stratification was no longer evident (Fig. 3.4a). Similarly, after retinal exposure to QA there was a substantial loss of GABA-IR amacrine cells and cells in the GCL, no apparent loss of GABA-IR horizontal cells, and a significant depletion of GABA immunoreactivity in the IPL from 0 to 85% depth (Fig. 3.4a).

(b) GAD:

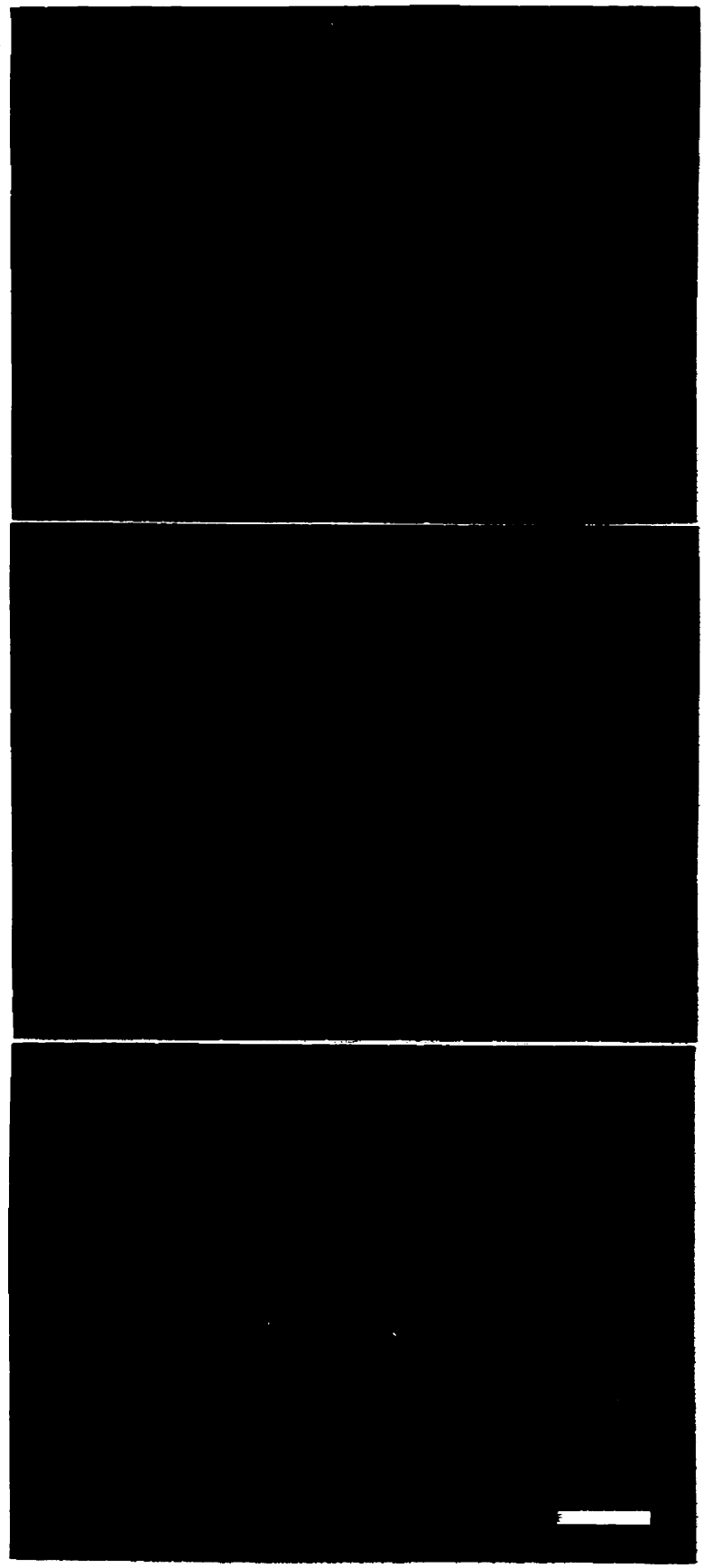
Some amacrine cells, many cells in the GCL, and strata at 0 to 20%, 35 to 60%, and 70 to 100% IPL depth were GAD-IR (Fig. 3.4b). NMDA-treatment greatly reduced numbers of GAD-IR amacrine cell bodies and abolished most GAD immunoreactivity in the IPL (Fig. 3.4b). In contrast, there was little loss of GAD-IR amacrine cells in QA-treated retinas, and only partial loss of GAD-IR neurites, mostly in the 2 proximal strata of the IPL (Fig. 3.4b)

Figure 3.4: Vertical sections of retina obtained 7 days after treatment with saline (top), NMDA (middle), and QA (bottom). The sections have been labelled with antisera directed against the following: (**a**) γ-aminobutyric acid (GABA); (**b**) glutamic acid decarboxylase (GAD); (**c**) GABA transporter (GAT-1); (**d**) parvalbumin. Scale bar = 50 μm.

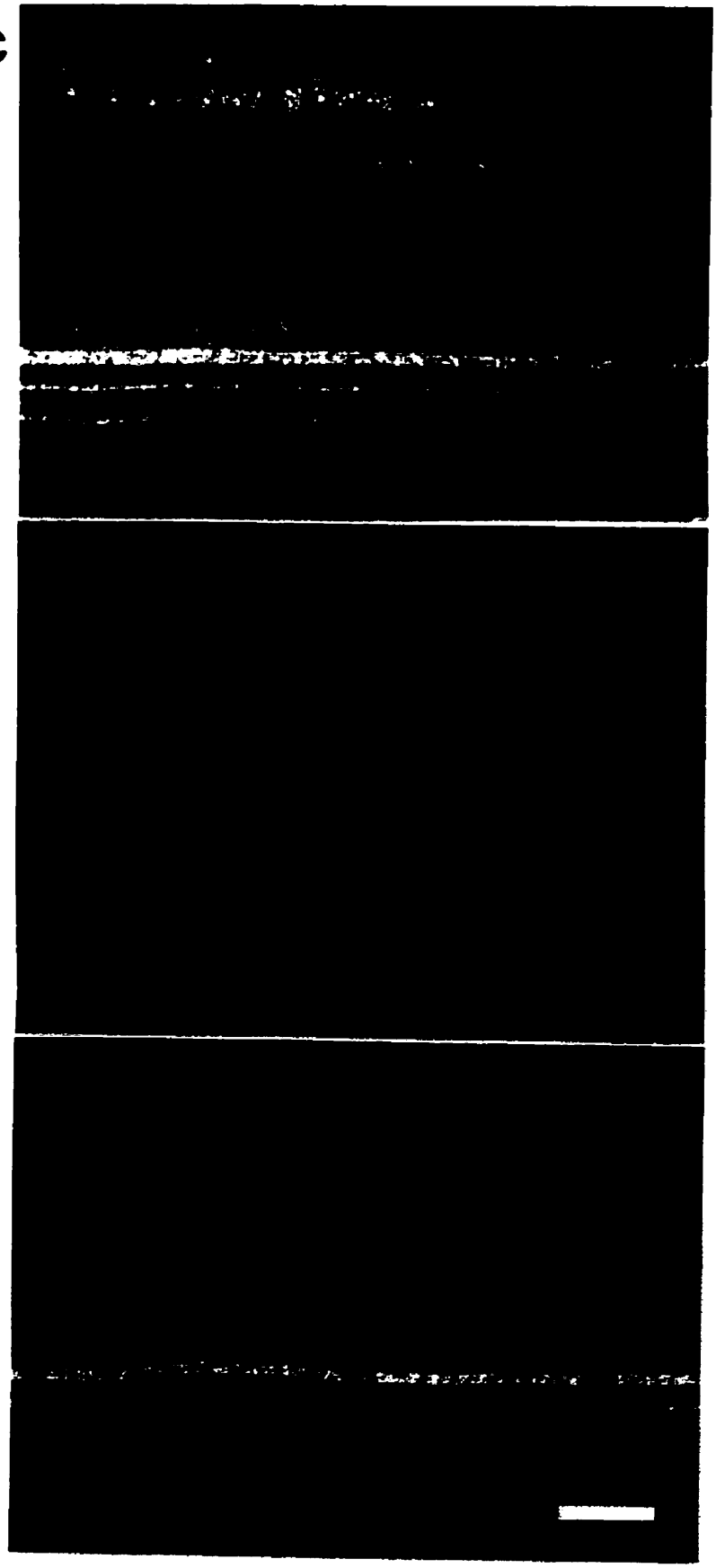
3.4a



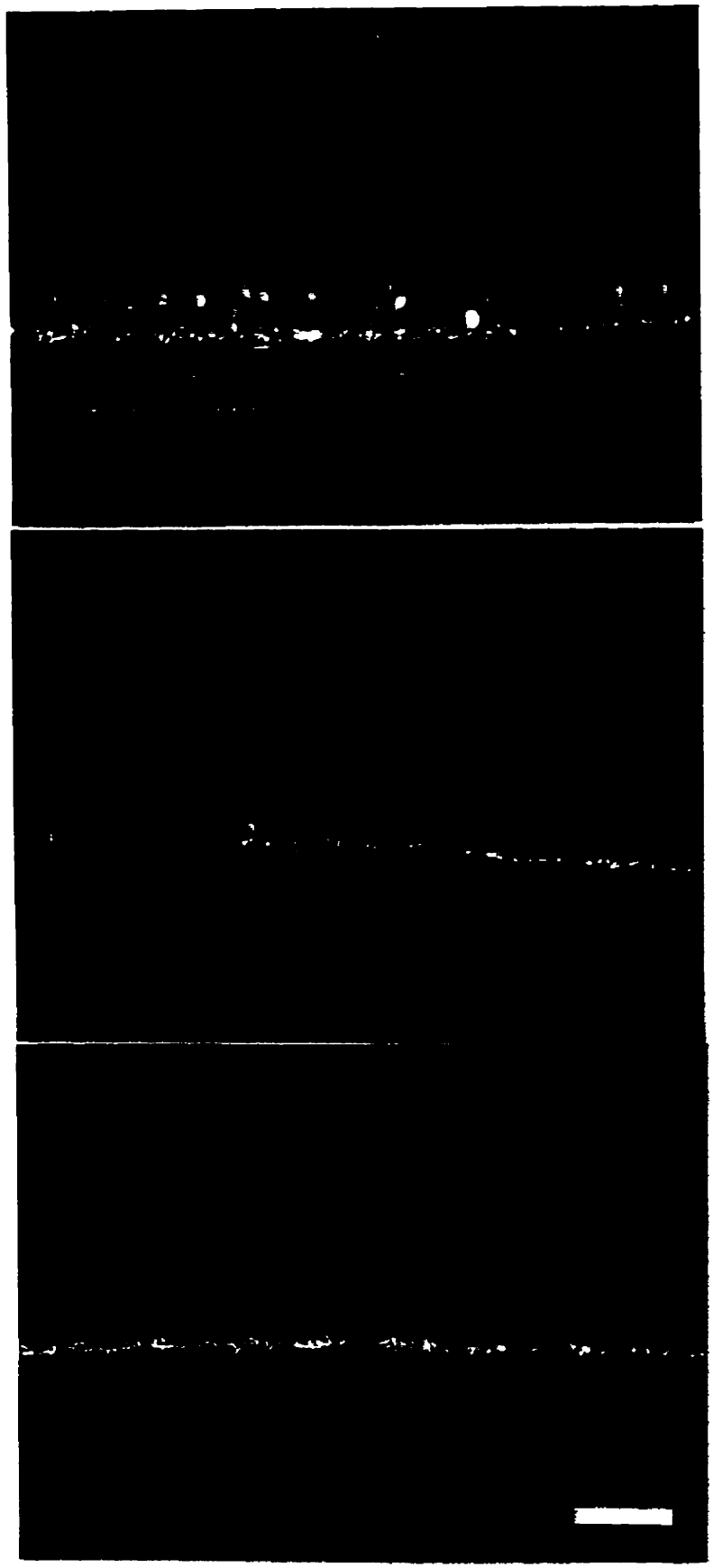
3.4b



3.4c



3.4d



(c) GAT-1:

Immunoreactivity for GAT-1 was seen in many amacrine cells, in some cells in the GCL, and in 2 broad strata at 20 to 35% and 50 to 55% IPL depth (Fig. 3.4c). Following treatment with NMDA, little GAT-1 immunoreactivity remained in the IPL and most GAT-1-IR amacrine cells were no longer detectable (Fig. 3.4c). After treatment with QA, GAT-1-IR strata remained in the IPL, while many GAT-1-IR amacrine cells were lost and those remaining appeared hyper-immunoreactive (Fig. 3.4c).

(d) Parvalbumin:

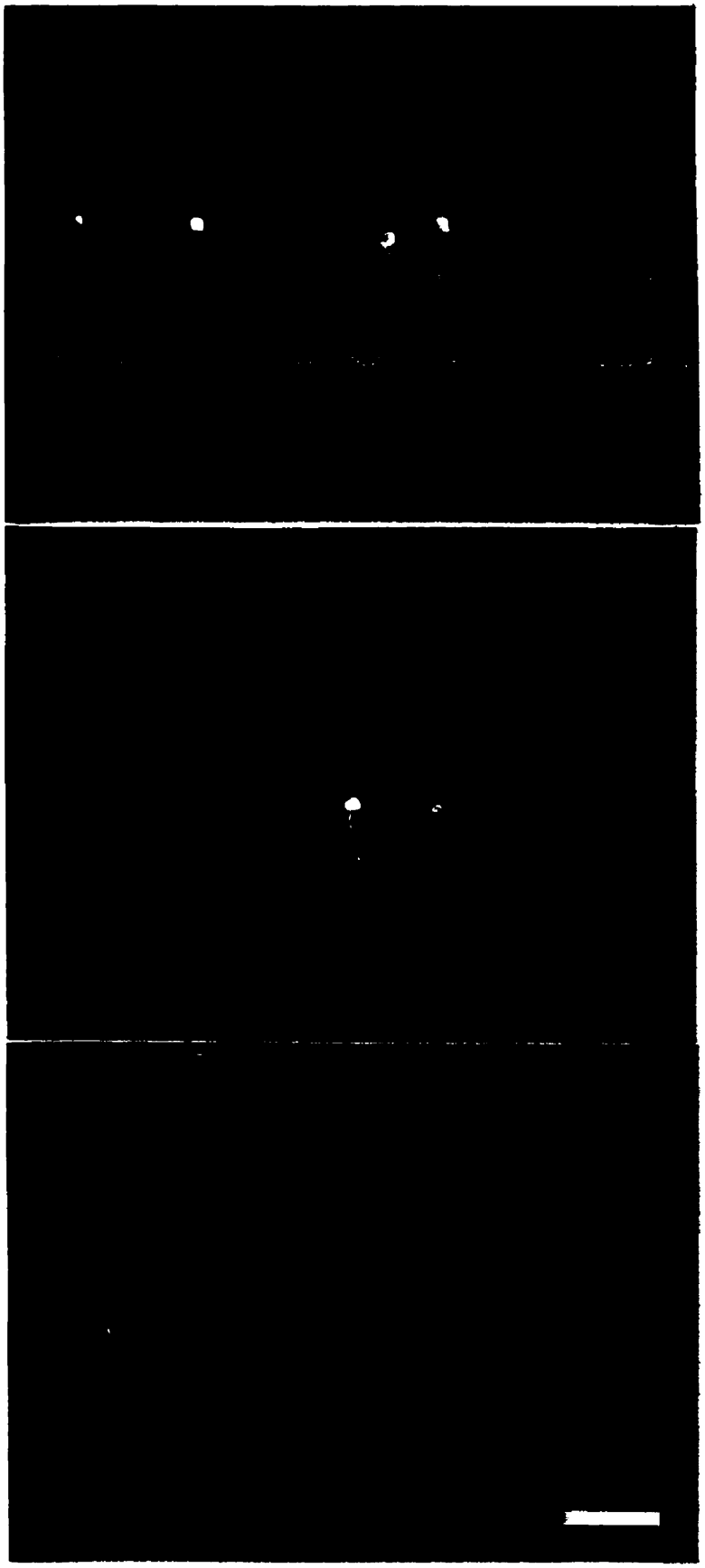
Parvalbumin immunoreactivity was present in numerous amacrine cells, densely innervated strata at 0 to 10% and 70% IPL depth, and a weakly innervated stratum at about 40% IPL depth (Fig. 3.4d). NMDA-treatment resulted in the destruction of many (43.0% ± 14.7%; N=6) parvalbumin-IR amacrine cells and most neurites in the proximal IPL, but left processes in the distal IPL intact (Fig. 3.4d). After QA-treatment, parvalbumin was detected in only a few (<1%) weakly immunoreactive amacrine cells and in neurites at the IPL/INL border (Fig. 3.4d). These neurites were also immunoreactive for neurofilament-associated antigen (results not shown), and therefore likely originated from displaced ganglion cells.

(e) Serotonin:

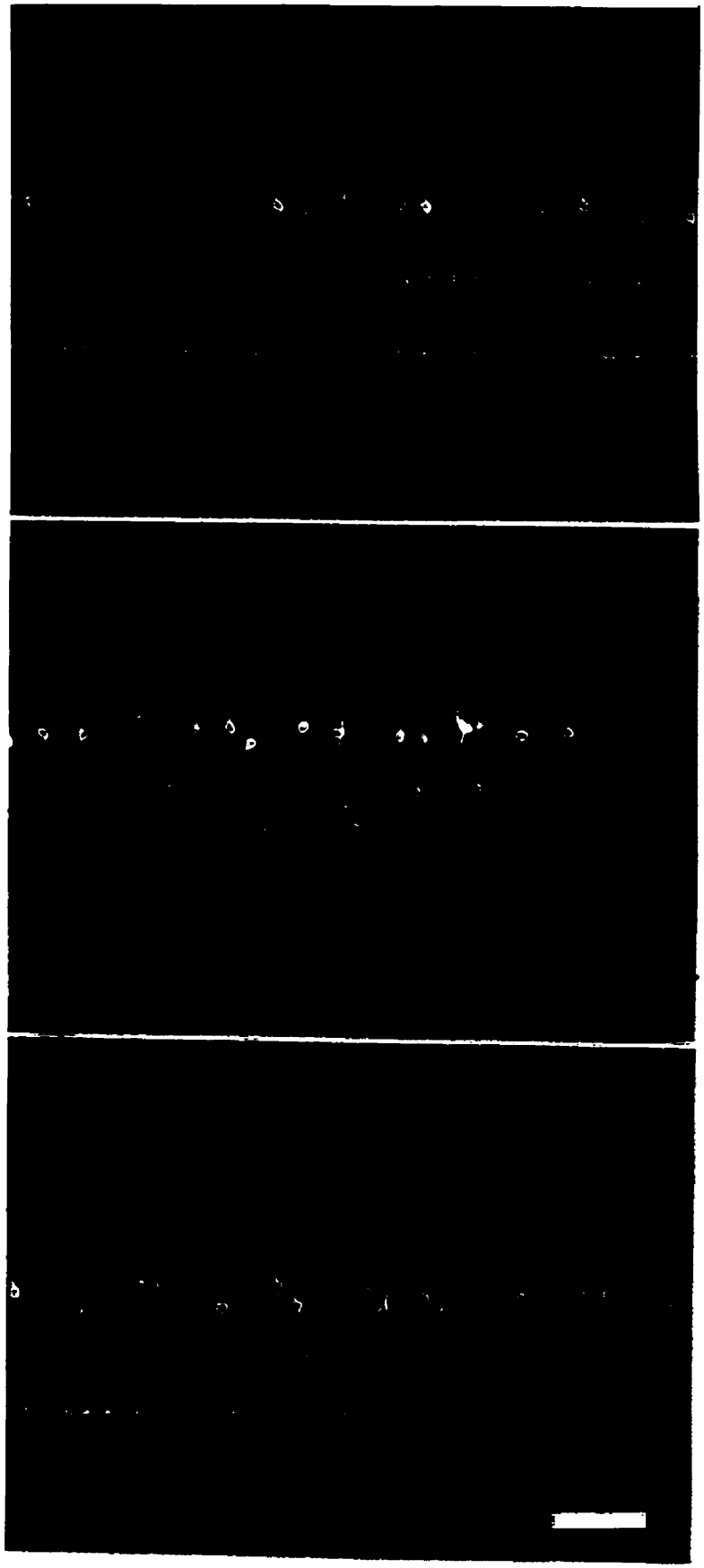
Serotonin immunoreactivity was present in sparsely distributed amacrine cells, with cell bodies located near the middle of the INL and neurites ramifying in strata at 0 to 15% and 65 to 85% IPL depth, as well as some weakly immunoreactive bipolar cells (Fig. 3.4e), as described previously (Millar et al., 1988). After exposure to NMDA, most

Figure 3.4: (Continued) Vertical sections of retina obtained 7 days after treatment with saline (top), NMDA (middle), and QA (bottom). The sections have been labelled with antisera directed against the following (**e**) serotonin; (**f**) protein kinase C (PKC); (**g**) glutamate receptor 1 (GluR1); (**h**) GluR2-3; and (**i**) glucagon. Scale bar = 50 μm.

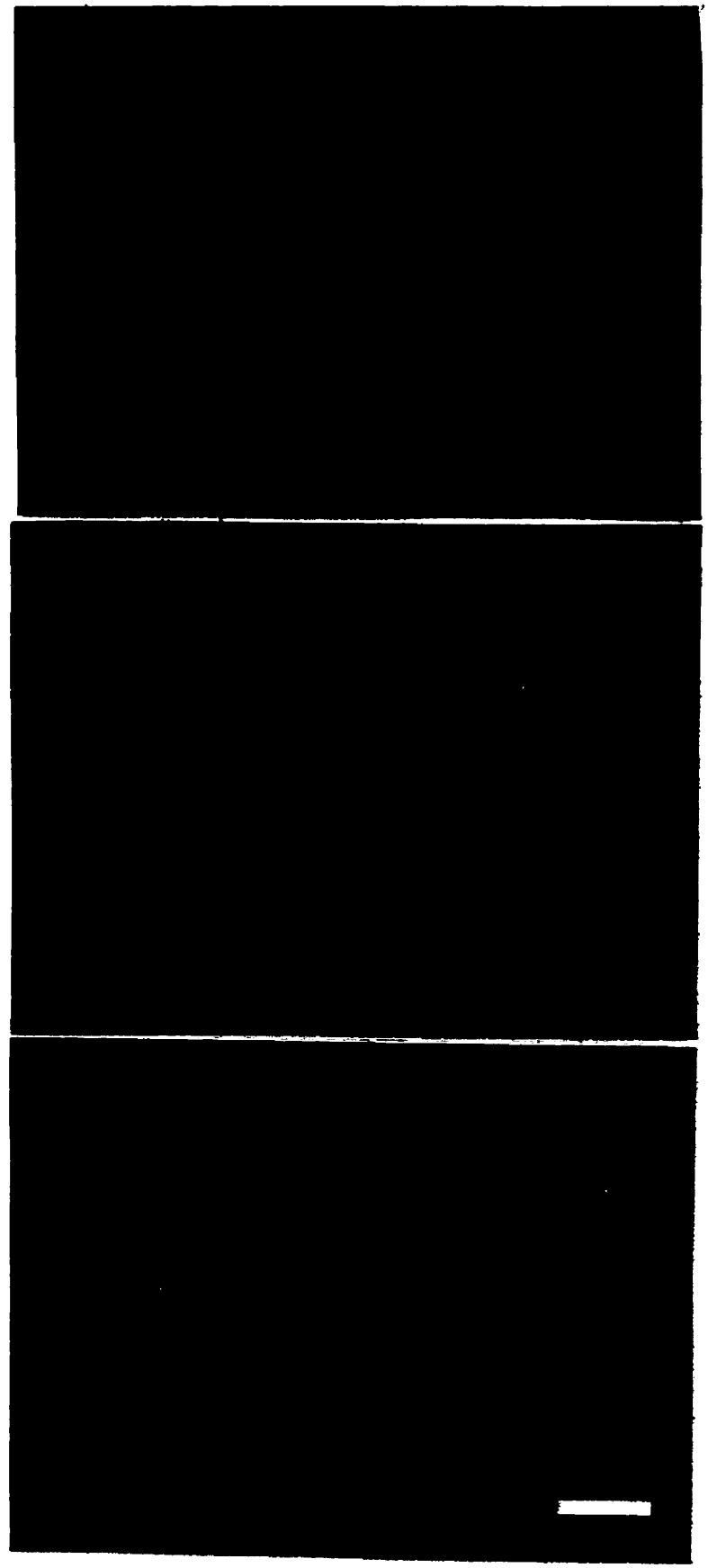
3.4e



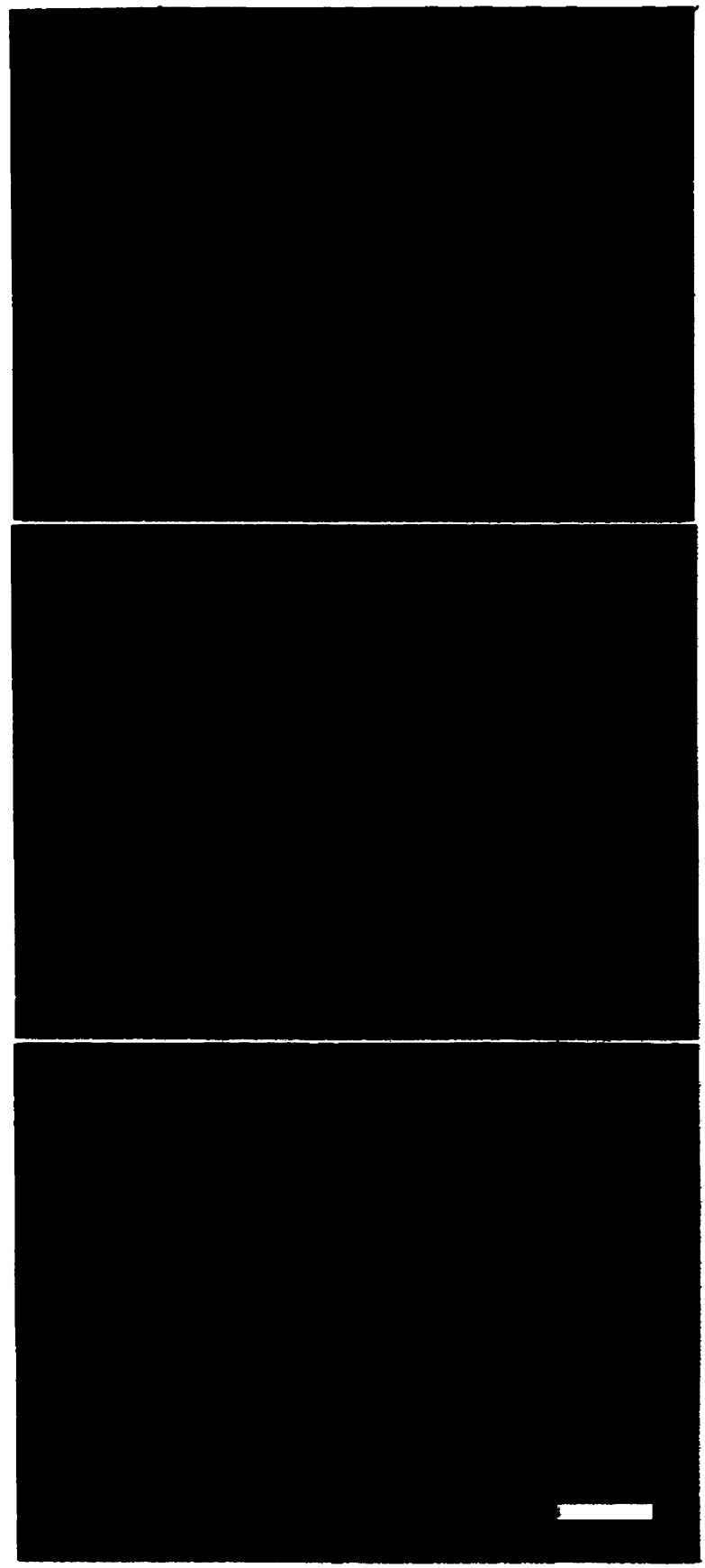
3.4f



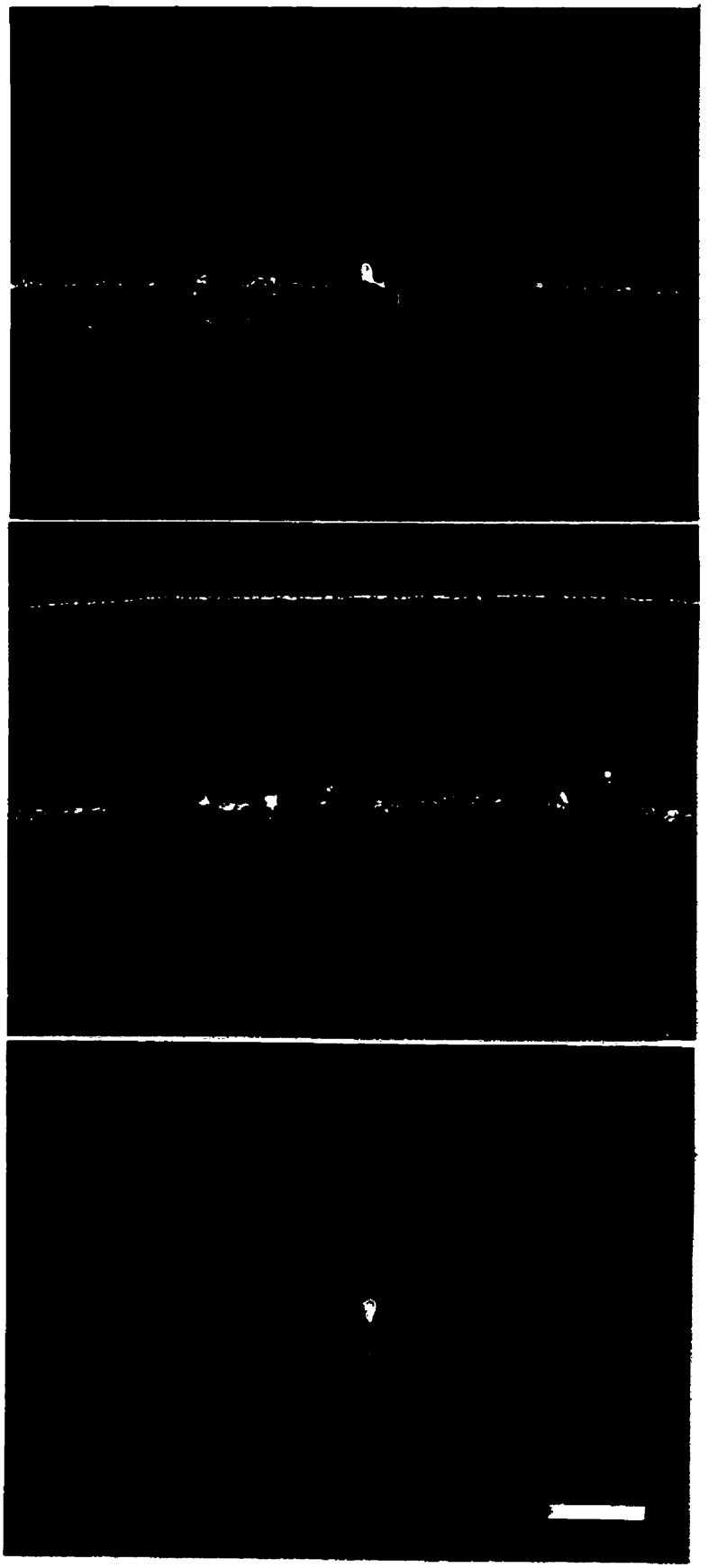
3.4g



3.4h



3.4i



serotonin-IR amacrine cells (73.6 ± 25.0%; N=6) were undetectable, and the density of serotonin-IR processes within the IPL was severely reduced (Fig. 3.4e). After QA, serotonin-IR amacrine cells in the INL and their neurites in the IPL were entirely absent (Fig. 3.4e). Both QA and NMDA had little or no effect upon serotonin-IR bipolar cells.

(f) PKC α/β:

Antiserum to PKC labelled 2 different types of bipolar cells. The first, weakly immunoreactive bipolar cell type had small somata in the distal INL, while the second, strongly immunoreactive bipolar cell type had larger somata near the middle of the INL and sent axon terminals mainly into the distal (0 to 10% depth) and proximal (80 to 90% depth) levels of the IPL (Fig. 3.4f). PKC immunoreactivity was also present in 3 weakly immunoreactive strata at 45%, 65% and 75% IPL depth (Fig 4f). In addition, some amacrine cells were also weakly immunoreactive for PKC (Fig. 3.4f). This distribution closely matches that descibed previously (Negishi et al., 1988). NMDA and QA-treatments did not significantly affect the abundance and distribution of either type of PKC-IR bipolar cell, except as a consequence of the reduction in INL and IPL thickness (Fig. 3.4f). However, following exposure to NMDA or QA, PKC-IR amacrine cells were less abundant.

(g) AMPA-type glutamate receptors:

Antiserum to GluR1 labelled some ganglion and amacrine cells, and weakly labelled strata at 0 to 15%, 40 to 60%, and 70 to 100% IPL depth (Fig. 3.4g). After treatment with NMDA, immunoreactivity remained in the IPL, but lamination was no longer evident, and GluR1-IR amacrine and ganglion cells both appeared unchanged (Fig.

3.4g). In contrast, QA-treatment abolished most GluR1 immunoreactivity in the retina (Fig. 3.4g).

Antiserum to GluR2/3 labelled most, if not all, amacrine and ganglion cells, and diffusely labelled the IPL with immunoreactivity concentrated at 0 to 10%, 40%, 55%, and 75% IPL depth (Fig. 3.4h). Anti-GluR2/3 also weakly labelled the OPL and produced punctate labelling in the OFL (Fig. 3.4h). After NMDA treatment stratification of GluR2/3 immunoreactivity in the IPL was lost and many GluR2/3-IR amacrine cells also disappeared from the INL (Fig. 3.4h), while GluR2/3 immunoreactivity remained in the GCL, OPL, and in putative displaced ganglion cells in the INL (Fig. 3.4h). QA-treatment resulted in the loss of many cells immunoreactive for GluR2/3 and the loss of immunoreactive strata from the IPL (Fig. 3.4h).

(h) Glucagon:

Antiserum to glucagon robustly labelled sparsely distributed amacrine cells having large somata at the IPL/INL border, one densely innervated stratum at 0 to 10% IPL depth, and one sparsely innervated stratum at 35% IPL depth (Fig. 3.4i), as described previously (Kiyama et al., 1985). After exposure to NMDA or QA the abundance and labelling intensity of glucagon-IR somata and processes at 0 to 10% level of the IPL were unaffected, while there was a slight reduction in the abundance of glucagon-IR neurites at 35% IPL depth (Fig. 3.4i).

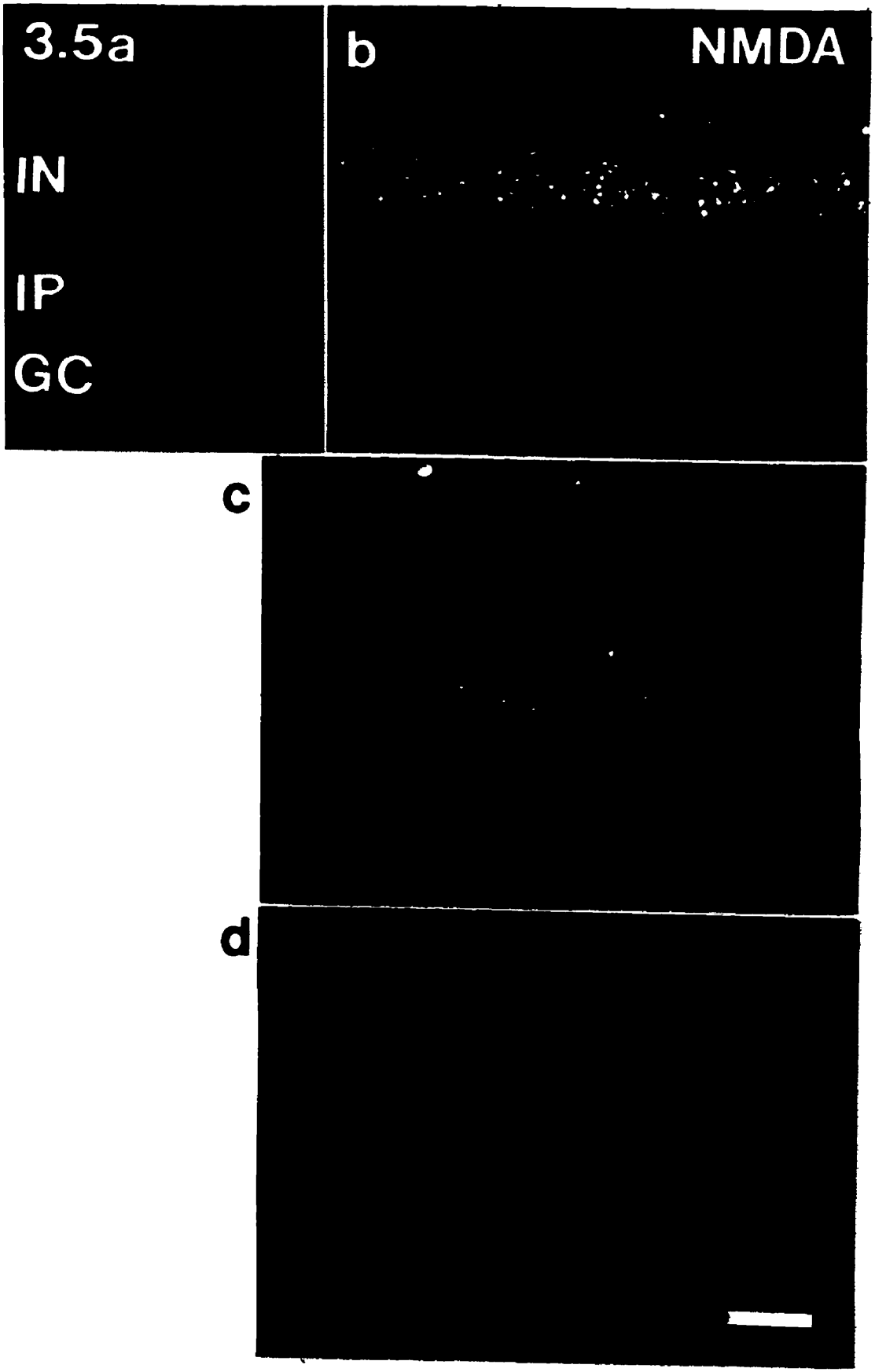
Cells responding to QA or NMDA by fragmentation of nuclear DNA:

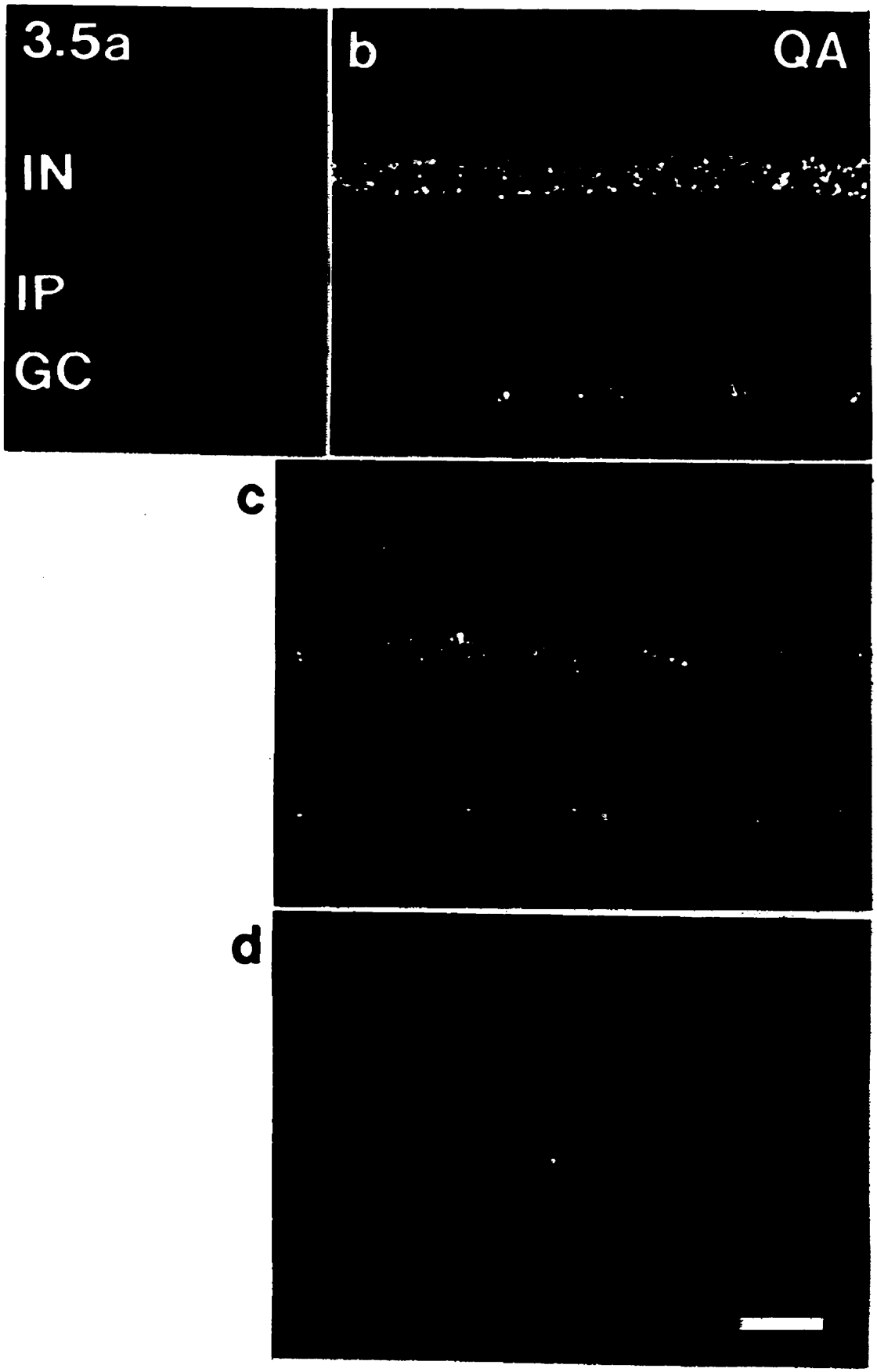
No nuclei were labelled for DNA fragmentation in retinas from control eyes at any

time following treatment (Fig. 3.5a). Four hours after injection of NMDA many scattered nuclei in the INL, mostly in the amacrine cell layer, contained fragmented DNA (results not shown). One day after exposure to NMDA, DNA fragmentation was detected in increased numbers of nuclei in the amacrine cell layer of the INL, as well as few nuclei in both the ganglion and bipolar cell layers (Fig. 3.5b). About 45% of all amacrine cells were nick end-labelled one day after NMDA-treatment, with the greatest density near the site of injection (nasal retina), and lower densities farther from the injection site (toward temporal retina). Three days after NMDA-treatment, DNA fragmentation was still detectable, mostly in amacrine cells. Less than 10% of the remaining amacrine cells, as well as a few cells (< 1%) in the bipolar cell layer of the INL, contained fragmented DNA (Fig. 3.5c). Seven days after NMDA-treatment, fragmented DNA was present in less than 1% of the residual amacrine cells, rarely in bipolar cells, and never in ganglion cells (Fig. 3.5d).

Four hours after exposure to QA, no retinal cells were labelled for DNA fragmentation (results not shown). However, 24 hours after QA-treatment, most nuclei in the amacrine cell layer of the INL and a few nuclei in the GCL were intensely labelled (Fig. 3.5b). Three days after QA-treatment, nuclei of most amacrine cells were weakly labelled, while a few were robustly labelled (Fig. 3.5c). There were also some weakly labelled nuclei in the GCL and bipolar cell layer of the INL (Fig. 3.5c). Very few of the residual amacrine cell nuclei were weakly labelled 7 days after treatment (Fig. 3.5d). Nuclei in the outer nuclear layer were never labelled at any time following retinal exposure to NMDA or QA.

Figure 3.5: Vertical sections of retina which have been labelled for DNA fragmentation in (**a**) control eyes, and (**b**) 1, (**c**) 3 and (**d**) 7 days after treatment with QA or NMDA. Abbreviations: IP - inner plexiform layer; IN - inner nuclear layer; GC - ganglion cell layer. Scale bar = 50 μm

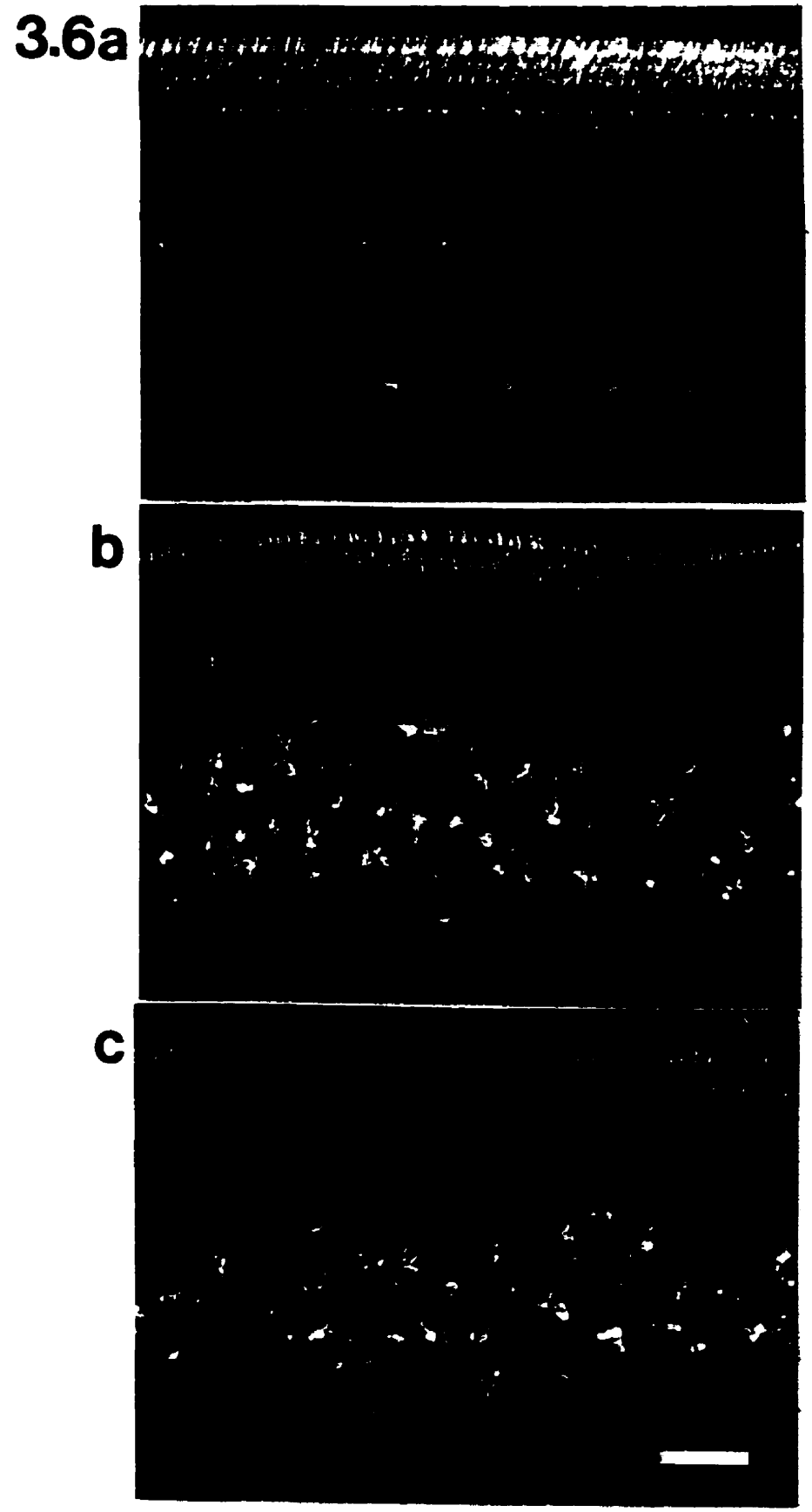

3.5a
b
NMDA
IN
IP
GC
c
d


3.5a
IN
IP
GC
b
QA
c
d

Accumulation of cells immunoreactive for lysosomal membrane in NMDA and QA-treated retinas:

In control eyes, antisera raised against lysosomal membrane (LM, a marker for phagocytes) labelled very thin, sparsely distributed fibers scattered throughout the retina, as well as the apical half of the retinal pigmented epithelium (Fig. 3.6a). Immunoreactivity for LM was dramatically increased in toxin-treated retinas. Three days after exposure to NMDA or QA, LM-immunoreactivity was detected in numerous fibers and cells spread throughout the OFL, GCL, IPL, and amacrine cell layer of the INL (Fig. 3.6). LM-immunoreactivity was detected in several morphologically distinct cell types including: (i) sparsely distributed fusiform cells with somata near the middle of the INL (possibly Müller cells); (ii) amacrine-like cells with cell bodies present in the proximal INL and thin neurites extending into the IPL; (iii) small rod-shaped cells, with little nucleoplasm, found mostly in the IPL (possibly activated microglia); and (iv) larger cells with large nuclei and few peripheral processes found mostly in the IPL (possibly macrophages).

Figure 3.6. Vertical sections of retina which have been labelled for lysosomal membrane in (**a**) control eyes and 3 days after treatment with (**b**) NMDA or (**c**) QA. Scale bar = 50 μm


3.6a
b
c

Discussion:

Destruction of amacrine cells involved in ocular growth-regulation:

NMDA-treated eyes do not grow further in response to form-deprivation (Fischer et al., 1997b, 1998g), while QA-treated eyes retain this ability (Ehrlich et al., 1990; Chapter 4; Fischer et al., 1998c, d). An important goal of the present study was to identify underlying mechanisms that might be responsible for this difference. NMDA-treatment resulted in a broad spectrum of degenerative changes in the chick retina (Table 3.4). Since many immunoreactive strata of the IPL were lost, and the contribution of neurites of many types of amacrine cells to the IPL was severely reduced, it is likely that signal processing by amacrine cells in the IPL was severely disrupted. It is also likely that at least some of the residual amacrine cells had been deprived of normal input from other amacrine cells and therefore were unable to carry out their normal functions. It is not surprising that this might result in the disruption of retina-dependent ocular growth-regulation in NMDA-treated eyes (Fischer et al., 1997b, 1998g), since amacrine cells are likely to play important roles in the spatiotemporal processing of visual information that is crucial for emmetropization (Wallman, 1993). In contrast, QA resulted in more selective retinal damage, with less thinning of retinal layers and greater persistence of immunoreactive strata in the IPL. For example, QA had a lesser impact than NMDA upon amacrine cells immunoreactive for GAD-65, GAT-1 and GluR2/3 (Table 3.4). The survival of amacrine cells responsible for growth-regulation would permit QA-treated eyes to respond to form-deprivation (Ehrlich et al., 1990; Chapter 4; Fischer et al., 1998c, d).

Table 3.4. Summary of the effects of NMDA or QA upon different retinal cells.

Table 3.4.

Marker/Cell Type	NMDA	QA
TH/AC	-	+
VIP/AC	+++	++++
Type-I cholinergic AC	+	++++
Type-II cholinergic AC	+++	+++
Type-IIIa cholinergic AC	++++	++++
Type-IIIb cholinergic/ENK/AC	+++	++++
PKC/BP	-	-
PKC/AC	+++	+++
glucagon/AC	-	-
serotonin/AC	+++	++++
parvalbumin/AC	++	++++
GABA/AC	++	+
GAD/AC	++	+
GAT-1/AC	+++	+
GluR1/AC & GC	++	++++
GluR2-3/AC	+++	++

(AC amacrine cell; BP bipolar cell; GC ganglion cell; - little or no effect; + some loss of immunoreactivity; ++ moderate disruption; +++ substantial disruption; ++++ complete ablation)

QA-treatment destroyed amacrine cells that putatively participate in the progression of FDM, including those immunoreactive for ENK, VIP and ChAT. This suggests that amacrine cells that release ENK, VIP or acetylcholine are not required for the progression of FDM, since QA-treated eyes still respond to form-deprivation (Ehrlich et al., 1990; Chapter 4; Fischer et al., 1998c, d). Pharmacological antagonists to opiate receptors, VIP, and muscarinic acetylcholine receptors are capable of suppressing FDM (reviewed by Wallman, 1993; Seltner and Stell 1995a; Seltner et al., 1997). The absence of cells that produce and release VIP, ACh, or ENK in QA-treated eyes might be expected to have consequences similar to those of antagonizing of receptors to these transmitters. Therefore, not only are VIP, ACh, or ENK-containing amacrine cells not needed for the progression of FDM, they likely do not participate in the antagonist-mediated suppression of FDM. If this hypothesis were true, then antagonists to opiate, VIP, or muscarinic receptors should suppress FDM in QA-treated eyes. This hypothesis will be tested for the muscarinic cholinergic system in the following chapter of this dissertation.

Somehow the retinal pathways that mediate form-deprivation-induced ocular growth remain intact after QA-treatment, despite the destruction of numerous sub-types of amacrine cell. It is possible that QA-insensitive amacrine cells, including those immunoreactive for TH, are required for the progression of FDM or prevention of excessive ocular enlargement during normal vision. For example, it has been reported that the destruction of TH-IR cells by 6-hydroxydopamine prevents the progression of FDM in chicks (Li et al., 1992; Schaeffel et al., 1994). Other candidates for growth-regulatory pathways would include GABAergic and glucagon-IR amacrine cells, since a

substantial fraction of these cells appear to be spared by QA-treatment.

Effects of NMDA and QA on subpopulations of amacrine cells:

NMDA and kainate have been shown to stimulate loss of immunoreactive neuroactive substances, including glucagon, neurotensin and ENK, from specific amacrine cell populations in turtle retina *in vitro* (Yaqub and Eldred, 1993). In chick retina we observed that glucagon-IR amacrine cells were unaffected by both NMDA and QA, while ENSLI amacrines were sensitive to them. We found that serotonergic amacrine cells in chick are sensitive to NMDA and QA, whereas serotonergic cells in the turtle retina were found to be affected only by kainate (Yaqub and Eldred, 1993). Thus, serotonergic amacrine cells in chick retina are sensitive to NMDA and QA, while those in turtle retina are not. In contrast, glucagonergic cells in the chick retina are sensitive to NMDA, unlike those in the turtle. It is possible, however, that these apparent differences are due, at least in part, to differences in method (acute, *in vitro* vs chronic, *in situ*) rather than differences in species.

Both QA and NMDA have been shown to evoke the release of GABA and acetylcholine from isolated chick retina (Campochiaro et al., 1985; Zeevalk et al., 1989; Ferreira et al., 1994). Similarly, we have shown that both GABAergic and cholinergic amacrine cells are destroyed in significant numbers following exposure to NMDA or QA. These findings suggest that GABAergic and cholinergic amacrine cells express receptors that are activated by NMDA and QA. However, not all GABAergic and cholinergic amacrine cells were destroyed by NMDA or QA-treatments, suggesting that not all of

these cells are affected by NMDA or QA.

Subtypes of cholinergic amacrine cells differed in sensitivity to NMDA and QA. Type-I cholinergic amacrine cells were the most tolerant to NMDA-treatment, while type-II cells were the most tolerant to QA-treatment. This may have resulted from a difference in the relative abundance of glutamate receptor isoforms expressed by these cell types. For example, type-I cells may express fewer NMDA-receptors, while type-II cells express fewer QA-selective receptors. Alternatively, different cell death programs in these cells may be activated by NMDA or QA-selective glutamate receptors, thereby endowing them with different tolerances to NMDA or QA.

The receptor-specificity of NMDA- and QA-induced cell death is a complicated issue. The high doses of drugs we applied could decrease specificity for receptor subtypes, and the specificity of these drugs for glutamate and non-glutamate receptors in chick tissues is not well characterized. Therefore, the results presented in this study should be considered primarily as examples of excitotoxic rather than receptor-specific effects.

Survival of retinal cells after exposure to QA or NMDA:

Most horizontal cells, ganglion cells, photoreceptors, Müller cells (results not shown), and bipolar cells survived exposure to either QA or NMDA. Similar results have been described in other studies on chick retina using a single dose of NMDA or QA (Sattayasai and Ehrlich, 1987; Barrington et al., 1989; Sheppard et al., 1991). There are several possibilities to explain the tolerance of these cells. First, an absence or low level

of expression of relevant glutamate receptors could render cells insensitive to NMDA or QA. Second, some cells that express NMDA and/or QA-selective receptors may possess a greater ability than others to cope with sustained depolarization and ionic fluctuations. For example, excitatory glutamate receptors, including NMDA receptors are expressed by ganglion cells in the chick retina (Fischer et al., 1998g; and the present work), yet most of these cells survive exposure to toxic levels of kainate or NMDA (Sheppard et al., 1989; Ehrlich et al., 1990; and the present work). It has been suggested that the larger cytosolic volume in ganglion cells might buffer the accumulation of intracellular Ca^{2+}, thereby rendering these cells less susceptible to excitotoxicity than intrinsic retinal interneurons (Sheppard et al., 1989). Ganglion cells might also resist excitotoxicity because they are adapted to large ion fluxes associated with bursts of action potentials, or because voltage-gated calcium channels are absent from their somata.

Changes in the thickness of retinal layers following treatment with NMDA or QA:

Large changes in thickness of the IPL were seen after treatment with NMDA or QA. The swelling of the IPL one day after treatment was likely caused by excessive depolarization of processes, build-up of ions (probably Cl^-), and consequent osmotic swelling. Such swelling of the IPL has been seen in as little as 2 minutes following retinal exposure to kainate (Kleinschmidt et al., 1986a, b), and can be prevented by pre-treatment with chloride-channel blockers (Zeevalk et al., 1989). There was no apparent swelling of the INL, even though swelling was observed in the IPL which contains neurites from cells whose somata are located in the INL. It is possible that by 24 hours

after treatment substantial numbers of amacrine cells became pyknotic, while others conversely became swollen, resulting in little net change in the thickness of the INL. In fact, there was a significant decrease in INL thickness 24 hours after QA-treatment (Fig. 3.3b), suggesting that some cells within the INL had already perished. This thinning of the INL may result from the acute death of some horizontal and amacrine cells. This hypothesis is supported by the findings of Sattayasai and Ehrlich (1987) who reported that QA caused swelling and death of horizontal cells, and thinning of the INL, 2 days after treatment.

Thinning of the IPL 7 days after treatment was probably due to the destruction and removal of neurites of amacrine cells and possibly ganglion cells. This retinal degeneration closely matches qualitative aspects of NMDA or QA-induced IPL degeneration reported previously in the chick eye (Sattaysai and Erhlich, 1987; Barrington et al., 1989; Erhlich et al., 1990; Sheppard et al., 1991). Similarly, Dvorak and Morgan (1983) reported that kainic acid-induced retinal thinning due to the permanent removal of neurons is stabilized one week after treatment.

Accumulation of cells in the IPL following retinal exposure to NMDA or QA:

The identity of the cells that appeared within the IPL 3 days after treatment, and the mechanisms underlying their appearance, remains somewhat uncertain. These cells contain immunoreactivity for LM and therefore likely are phagocytic glial cells (activated microglia) and/or macrophages that have entered the retina to remove cellular debris. Attempts to further characterize and label these cells with antisera directed against glial

fibrillar acidic protein (Sigma), vimentin (H5, AMF-17b and 40E-C; Hybridoma Bank), quail haematopoietic stem cells (QH-1; Hybridoma Bank; Cuadros et al., 1992), NN-1 and NN-2 (Dr. P.Raymond), human lysozyme, human CD68 (DAKO Corporation), and H386F and H381B4B5 (Dr. N. Tumosa), were unsuccessful, perhaps because of species-specific differences in epitopes for these mostly mammalian microglial markers.

Regional retinal sensitivity to QA and NMDA:

It remains unclear why less DNA fragmentation and more type-II cholinergic amacrine cells were detected in temporal than in other regions in treated retinas. It is possible that lesser amounts of QA and NMDA reached the temporal retina because of greater dilution at a distance from the nasodorsal injection site. It is also possible that temporal retinal regions are simply less susceptible to excitotoxicity, for unknown reasons. Indeed, Zeevalk et al., (1989) reported that temporal regions of isolated embryonic chick retina were affected less than nasal regions by exposure to kainate *in vitro*, suggesting that intrinsic rather than microenvironmental factors could be responsible for these regional differences.

QA and NMDA-induced DNA fragmentation:

Most of the amacrine cells destroyed by NMDA or QA appear to undergo apoptosis, assuming that the DNA fragmentation detected in our study is representative of programmed cell death. The number of retinal cells showing fragmentation of DNA was maximal at 24 hrs and was completed 3 to 7 days after treatment with either NMDA or

QA. In cultured cerebellar granule cells, excitotoxin-induced apoptosis has been shown to produce DNA fragmentation that is maximal at 4 hours and completed by 12 hours after treatment (Simonian et al., 1996), whereas apoptosis caused by low extracellular potassium is maximal at 24 hours and completed by 4 days after treatment (D'Mello et al., 1993). These results suggest that excitotoxin-induced apoptosis in cerebellar granule cells and cell death in retinal amacrine cells occurs at different rates, and possibly by different mechanisms. In addition, many retinal cells contained fragmented DNA only 4 hours after exposure to NMDA, whereas QA elicited no such effect. This suggests that different cell death pathways, in which DNA fragmentation begins soon after insult, are activated by exposure to NMDA than QA in some populations of retinal neurons.

DNA fragmentation was detected in the vast majority, if not all, amacrine cells following exposure to QA. However, clearly not all amacrine cells were destroyed, because the thickness of the amacrine cell layer was reduced by only 60% after QA. Similarly, although many bipolar cells contained fragmented DNA following exposure to NMDA, there was no detectable loss of PKC-IR bipolar cells. These results suggest that some cells damaged by QA or NMDA, although labelled by the 3' nick end-labelling technique, were not committed to apoptosis. These non-apoptotic cells that contained fragmented chromatin may have sustained limited DNA damage (not enough to become committed to apoptosis) or may ultimately have been rescued by DNA repair.

Conclusions

NMDA-induced excitotoxicity resulted in widespread disruption of many sub-populations of retinal amacrine cells, accompanied by general disruption of the IPL,

whereas QA-induced excitotoxicity resulted in more specific destruction of restricted subsets of retinal amacrine cells with less loss of neurites from the IPL. Damage within the QA-treated IPL appeared to be restricted enough to allow FDM to develop, whereas damage within the NMDA-treated IPL was either too widespread, or directed to critical cell types involved in the regulation of ocular growth. These results support the hypothesis that retinal control of ocular growth is dependent upon signal processing involving amacrine cells in the IPL. We propose that amacrine cells containing acetylcholine, ENK, or VIP are not required for the progression of FDM, while those containing dopamine, GABA and glucagon may be. We observed that DNA fragmentation is evident in many amacrine cells and some bipolar cells exposed to QA or NMDA, but conclude that it may not be an accurate criterion for commitment to apoptosis.

CHAPTER FOUR

Cholinergic amacrine cells are not required for the progression and atropine-mediated suppression of form-deprivation myopia

Introduction:

Visual experience is required to properly guide ocular growth. Image features are processed and translated by retinal neurons into biochemical signals that precisely control the growth of the sclera (the outer, shape-determining sheath of the eye) so that the length of the vitreous chamber is matched to the combined refractive powers of the cornea and lens. Normally, visual processing regulates ocular growth so that with neutral accommodation distant objects are focussed upon the retina. This state is referred to as emmetropia. The process of emmetropization can be perturbed by attenuating contrast and high spatial frequencies with light diffusing goggles or eye lid-suture, causing elongation of the vitreous chamber of the eye and myopia (near-sightedness; Wallman, 1993; Bartmann & Schaeffel, 1994). For example, myopia can be induced early in life (while the eye is growing) by blurring images incident to the retina with translucent goggles or suturing the eye-lids together. This experimental paradigm is commonly studied in chicks and has been termed form-deprivation myopia (FDM).

In chicks, FDM results from the disruption of visual processing and growth-controlling pathways that are intrinsic to the eye (Wallman, 1987). Regulation of ocular growth requires spatiotemporal processing, probably at the level of the amacrine cells (Wallman, 1990, 1993). Pharmacological studies have implicated several signalling

pathways in the progression of FDM. These pathways include cells that release or respond to dopamine (Stone et al., 1989), vasoactive intestinal polypeptide (VIP; Stone et al., 1988; Seltner & Stell, 1995a), basic fibroblast growth factor (Rohrer & Stell, 1994), enkephalin (Seltner et al., 1997), N-methyl-*D*-aspartate (Seltner & Stell, 1996, Fischer et al., 1998g), and acetylcholine (ACh; Stone et al., 1991). The contribution of cholinergic pathways to the regulation of ocular growth is of particular interest, as it holds the greatest potential as the focus of drug therapies aimed to correct ametropias. Topical application of the muscarinic antagonist atropine has been used for decades to retard the progression of human myopia (Parsons, 1923; Luedde, 1932; Gostin, 1962; Bedrossian, 1971, 1979; Gimbel, 1973; Dyer, 1979; Goss, 1982). Antagonists to muscarinic acetylcholine receptors (mAChRs) have also been shown to suppress FDM in one species of monkey (Raviola & Weisel, 1985), tree shrews (McKanna & Casagrande, 1981), and chicks (Stone et al., 1991), and US Patent protection has been granted for the treatment of human myopia with muscarinic agents (Laties and Stone, 1992).

The source(s) of ACh and subtypes of muscarinic receptors that participate in the regulation of ocular growth remain unknown. Cholinergic systems in the eye comprise both intrinsic retinal amacrine cells and the parasympathetic nervous system. Cholinergic innervation to the choroid, iris sphincter, and ciliary body arises primarily from post-ganglionic parasympathetic neurons in the ciliary ganglion (Meriney & Pilar, 1987). Millar et al. (1987b) identified 3 subtypes of ChAT-immunoreactive amacrine cells in the chick retina, which included: type-I cholinergic amacrine cells, with cell bodies at the proximal border of the inner nuclear layer (INL) and neurites in sublamina 2 of the inner

plexiform layer (IPL); type-II cholinergic amacrine cells, with cell bodies in the ganglion cell layer (GCL) and neurites in sublamina 4 of the IPL; and type-III cholinergic amacrine cells, with cell bodies near the middle of the INL and neurites distributed in sublaminae 1 and 3 to 5 of the IPL. In addition, type-III cholinergic amacrine cells can be further segregated into type-III_{NE} that contain only ChAT-immunoreactivity, and type-III_{E} (ENSLI cells) that also contain enkephalin, neurotensin and somatostatin immunoreactivities (Watt & Florack, 1994; Chapter 3; Fischer et al., 1998f). Furthermore, at least 3 different isoforms of mAChRs are expressed in the retina, retinal pigmented epithelium, and choroid (McKinnon & Nathanson, 1995; Chapter 2; Fischer et al., 1997a; 1998b).

The purpose of these studies was to test whether retinal sources of acetylcholine and their muscarinic targets contribute to normal ocular growth, the progression of FDM, and the suppression of FDM by muscarinic antagonists. We ablated cholinergic amacrine cells with ethylcholine mustard aziridinium (ECMA) or QA and tested whether treated eyes grew normally, became myopic in response to form-deprivation, and were prevented from becoming myopic by treatment with atropine. The results presented here suggest that cholinergic amacrine cells and mAChRs in the retina do not contribute to visually guided ocular growth, and that they are not responsible for the enhanced rates of growth caused by form-deprivation or the prevention of FDM by atropine.

Methods and materials:

Animals:

Animals were maintained as described in Chapter 2, with the following exception. Australian animals (White leghorn-black australorp cross) were used for the ChAT-activity assays, and were treated identically to the Canadian experimental animals. They were also used for a less complete set of studies on eye growth. Similar results were obtained in the Australian and Canadian experiments.

Preparation of ECMA:

ECMA was prepared from acetylethylcholine mustard-HCl (Research Biochemical International) as described by Millar et al. (1987a). In short, 5 mg of acetylethylcholine mustard-HCl was dissolved in 0.1 M phosphate buffer (PB), pH 11.7, and de-acetylated for 1 hour at 20°C. The pH of this solution was adjusted to 7.4 with 4M HCl, and the volume adjusted to a working dilution with PB pH 7.4, and left for 1 hour at 20°C to form a solution of activated ethylcholine mustard aziridinium ion. The doses injected are expressed in terms of the starting material, since rates of conversion to ECMA are difficult to determine (Barlow & Marchbanks, 1984).

Intraocular injections:

Intraocular injections were made as described in Chapter 3. At 6 days of age, under common fluorescent lights at mid-day, the left eye (control) was injected with 20 µl of vehicle (sterile saline for QA-treated chicks or 0.1 M phosphate buffer pH 7.4 for

ECMA-treated chicks) and the right eye (treated) was injected with 20 µl of sterile saline, 20 µl of 1.2 mM (25 nmol or 5.8 µg) ECMA, or 20 µl of 10 mM (200 nmol or 37.8 µg) QA. We tested the effects of atropine and form-deprivation on chicks treated at only one dose of QA, which I have found to have devasting effects upon cholinergic amacrine cells (Chapter 3; Fischer et al., 1998f), but still leaves eyes responsive to form-deprivation (Ehrlich et al., 1990). We tested the effects of atropine and form-deprivation on only one dose of ECMA, as previous reports (Millar et al., 1987a) and preliminary dose-response studies (Stell et al., 1997) suggested that a dose in this range would destroy substantial numbers of cholinergic amacrine cells without damaging non-cholinergic cells. Six days after exposure to ECMA or QA, treated eyes were covered with a translucent goggle and left alone or injected every 24 hours for the next 6 days with either 20 µl of saline or 20 µl of 1.95 mM (45.3 nmol or 40 ug) atropine sulphate (Sigma) dissolved in saline. Assuming that the volume of liquid-vitreous within an eye was about 150 µl, the initial maximum vitreal concentration of QA was about 1.17 mM, that of ECMA about 0.16 mM, and that of atropine about 0.26 mM. After 6 days of form-deprivation and daily injections, i.e. at 19 days of age, eyes were refracted (to the nearest dioptre) by using a streak retinoscope and trial lenses. Chicks were then killed by chloroform inhalation, eyes removed from the orbit, and most of the attached connective tissues and muscles trimmed away. Equatorial diameter (at the most narrow part of the eye) and axial length (at the apex of the cornea to the base of the posterior eye) were measured using digital calipers.

Fixation and sectioning:

Tissues were fixed and sections as described in Chapter 2.

Immunocytochemistry:

Immunocytochemistry was performed as described in Chapter 2, with the exception of some antibodies that were applied. The antibodies that were used in this section are listed in Table 4.1

Choline acetyltransferase activity assay:

Eyes of chicks were injected with different doses of ECMA or 200 nmol QA. Seven days later, chicks were killed by ether inhalation, decapitated, and eyes were enucleated. Eyes were bisected equatorially, vitreous removed, and the remaining tissues of the eye dissected into retina, RPE plus choroid, and anterior segment (iris plus ciliary body). Dissected tissues were homogenized in dH_2O and incubated with [^{14}C]acetyl-CoA and choline chloride for the determination of ChAT activity (Haywood et al., 1975). The radioactivity of extracts was measured using a scintillation counter and enzyme activity expressed as nmol of ACh formed per eye per hour.

Table 4.1: List of antisera, their antigens, sources, and working dilutions

Table 4.1

Antibody and Antigen	Isotype/Species	Source	Working Dilution
1465	IgG - rabbit polyclonal	Dr. M. Epstein	1:1000
Choline acetyltransferase (ChAT)			
cm2	IgG - rabbit polyclonal	Dr. N. Nathanson	1:200
Chick muscarinic acetylcholine receptor (m2 isoform)			
cm3	IgG - rabbit polyclonal	Dr. N. Nathanson	1:1000
Chick muscarinic acetylcholine receptor (m3 isoform)			
cm4	IgG - rabbit polyclonal	Dr. N. Nathanson	1:500
Chick muscarinic acetylcholine receptor (m4 isoform)			
8305034	IgG-rabbit polyclonal	Dr.J.Walsh	1:1000
Glucagon			
MC5	IgG - mouse monoclonal	Amersham	1:50
Protein kinase C (α/β isoforms)			
5-HT	IgG - rabbit polyclonal	Inc Star	1:800
Serotonin			
S-10	IgG - rat monoclonal	Dr. A. Buchan	1:400
Somatostatin			
#16	IgG - rabbit polyclonal	Dr. W. Tank	1:1000
Tyrosine hydroxylase (TH)			
VP31	IgG - rat monoclonal	Dr. A. Buchan	1:80
Vasoactive intestinal polypeptide (VIP)			

Labelling of fragmented DNA:

Retinal sections were obtained as described above, 1, 3, and 7 days after ECMA or QA-treatment. Fragmented DNA was detected *in situ* as described in Chapter 3.

Measurements, cell counts, and statistical analyses:

Errors bars were calculated as the standard deviation of each sample. To compare data between treated and control eyes from the same experimental group, statistical significance was assessed using a paired, two-tailed Student *t*-test. To compare data between experimental conditions, statistical significance was assessed using a two-way ANOVA. All cell counts were made from central retina under the microscope.

Results:

Survival of cholinergic amacrine cells following exposure to ECMA or QA:

Antiserum directed against ChAT labelled numerous cells in the INL and GCL, as well as 2 prominent strata in the IPL (Fig. 4.1a), exactly as described previously (Millar et al., 1987b; Chapter 3; Fischer et al., 1998f). Cholinergic amacrine cells were assessed immunocytochemically only in ECMA- or QA-treated eyes that had been form-deprived and not injected, or form-deprived and injected with atropine. In all cases (n=20), after exposure to ECMA all type-I and most type-II cholinergic amacrine cells were destroyed or entirely depleted of detectable ChAT-immunoreactivity, while most type-III_{NE} and type-III_{E} cells appeared intact (Table 4.2; Fig. 4.1b). Only rarely (2 of 20 retinas) more than 30% of the type-II cells survived exposure to ECMA. ECMA-treatment did not

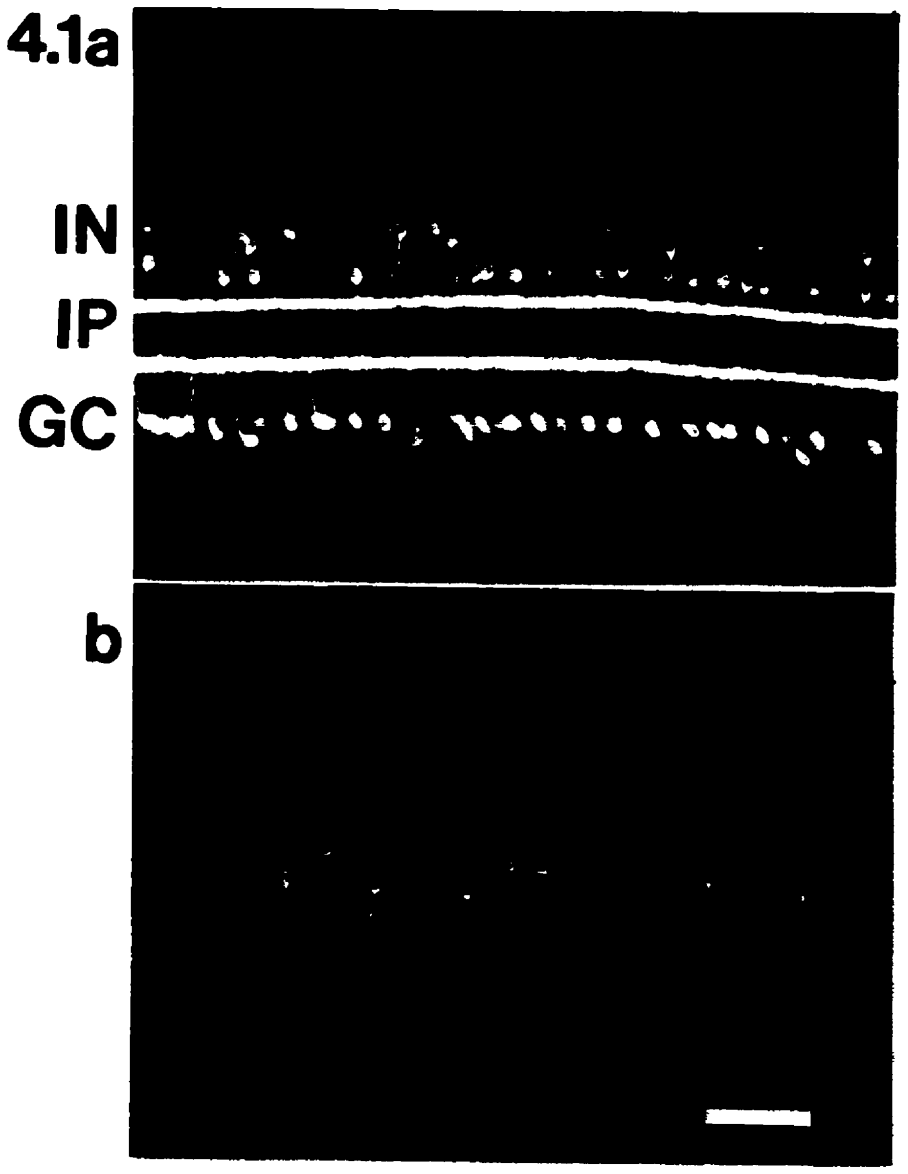
Table 4.2: Effects of ECMA or QA and form-deprivation upon the abundance of different types of cholinergic amacrine cells and refractive error.

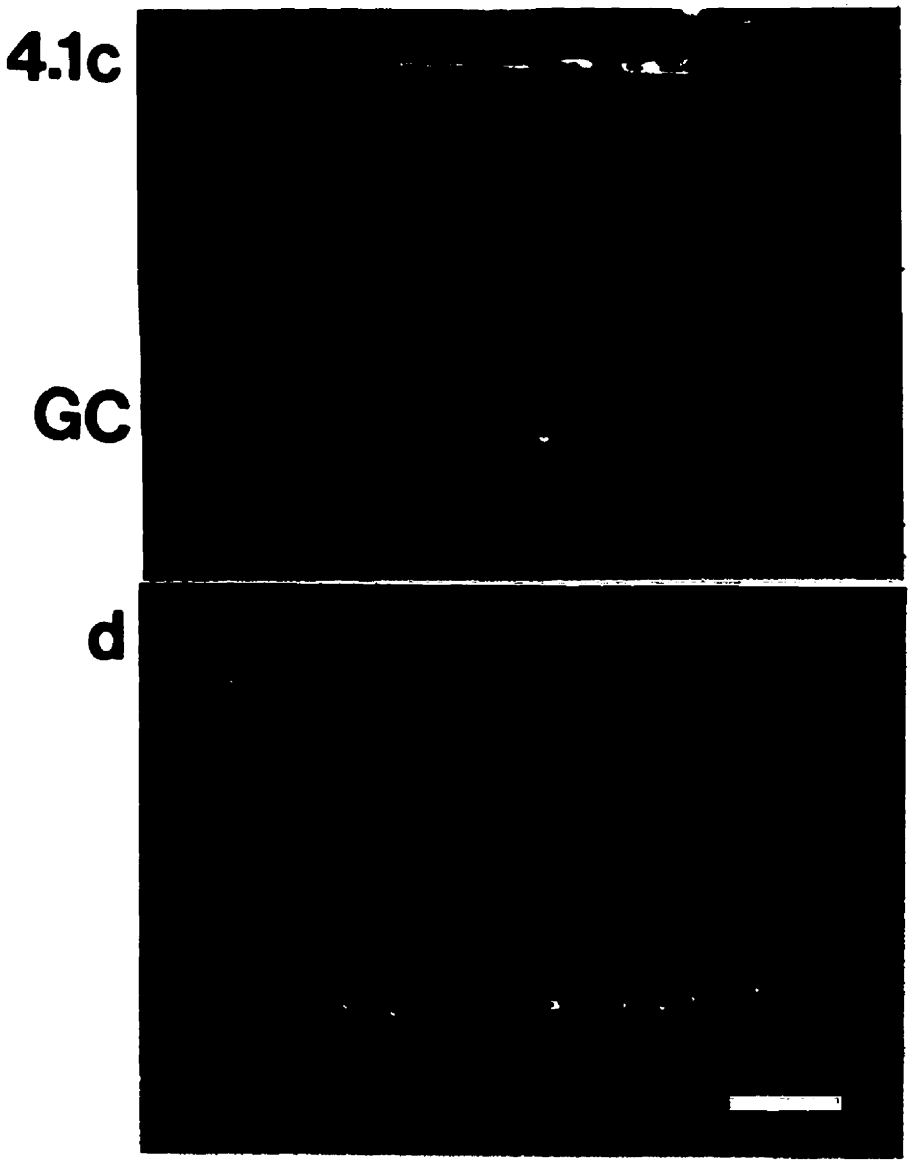
Table 4.2

Treatment	Percentage of remaining cells type-I	type-II	type-III	Refractive error	Sample size
ECMA + no injection	0 ± 0	14.7 ± 25.0	93.1 ± 17.2	-4.4 ± 1.3	7
ECMA + atropine	0 ± 0	18.1 ± 28.8	103.1 ± 10.4	0 ± 0	7
QA + no injection	3.8 ± 5.9	12.4 ± 7.8	3.2 ± 4.1	-5.4 ± 1.0	7
QA + atropine	0.7 ± 1.1	4.3 ± 2.5	1.3 ±1.1	-0.8 ± 1.0	6

(percentage values given as mean ± standard deviation)

Figure 4.1: Vertical sections of retina and choroid labelled with antiserum to ChAT, from eyes treated with (**a**) saline, (**b**) ECMA, (**c**) QA (central retina), and (**d**) QA (peripheral retina). Abbreviations: IP - inner plexiform layer; IN -inner nuclear layer; GC - ganglion cell layer. Scale bar = 50 μm.


4.1a
IN
IP
GC
b


4.1c
GC
d

affect subsets of amacrine or bipolar cells immunoreactive for PKC, VIP, somatostatin (ChAT type-III_E), TH, glucagon, or serotonin (results not shown).

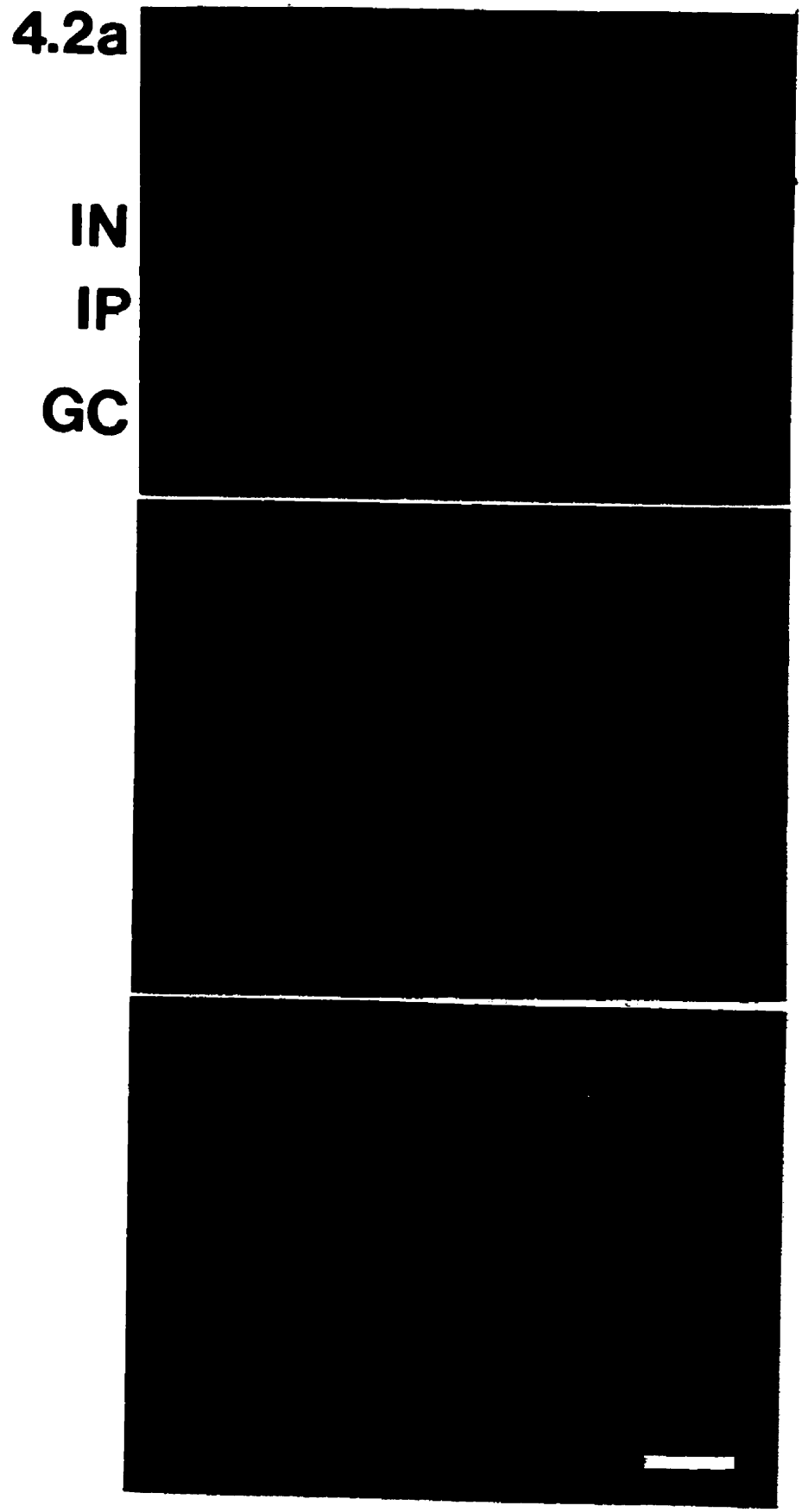
After exposure to QA nearly all type-I cholinergic amacrine cells were destroyed or entirely depleted of ChAT-immunoreactivity (Table 4.2; Fig. 4.1c). Similarly, all type-III_{NE} cholinergic amacrine cells, and most of the type-III_E cells were no longer detectable (Table 4.2; Fig. 4.1c). In all cases (n=20), residual type-II cholinergic amacrine cells with abnormal, narrow dendritic arbours were scattered across the retina (Table 4.2; Fig. 4.1c), except in the peripheral retina where these cells were more abundant and their processes in the IPL appeared nearly normal (Fig. 4.1d). This confirms previous reports of QA-induced depletion of ChAT-immunoreactive amacrine cells in the chick retina (Chapter 3; Fischer et al,. 1998f).

The distribution, abundance, and staining intensity of ChAT-immunoreactive fibres in the choroid appeared unchanged after exposure to ECMA or QA (Fig. 4.1).

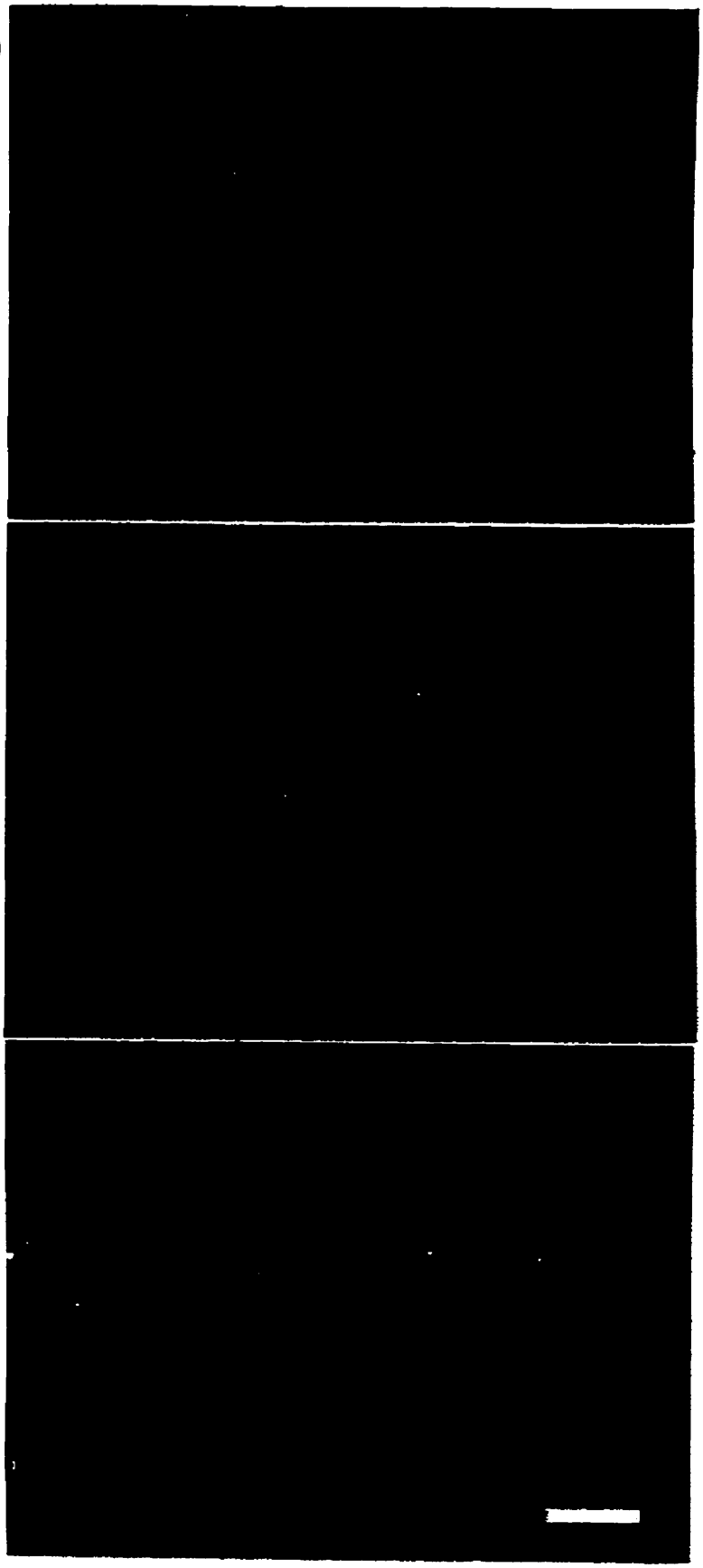
mAChR-immunoreactivity remaining after treatment with ECMA or QA:

Anti-cm2 weakly labelled cells in the amacrine cell layer of the INL and GCL (Fig. 4.2a). Cm2-immunoreactivity was localized to 4 strata in the IPL, at 0 to 15%, 40 to 60%, 70 to 85%, and 95 to 100% IPL depth (Fig. 4.2a). Cm3-immunoreactivity was localized to many bipolar cells, sparsely distributed amacrine cells, a few cells in the GCL, as well as 3 strata at 0 to 30%, 45 to 60%, and 70 to 95% depth of the IPL (Fig. 4.2b). Superimposed upon the two distal strata were two more intensely stained strata at 20 to 30% and 50 to 55% IPL depth (Fig. 4.2b). Anti-cm4 robustly labelled most

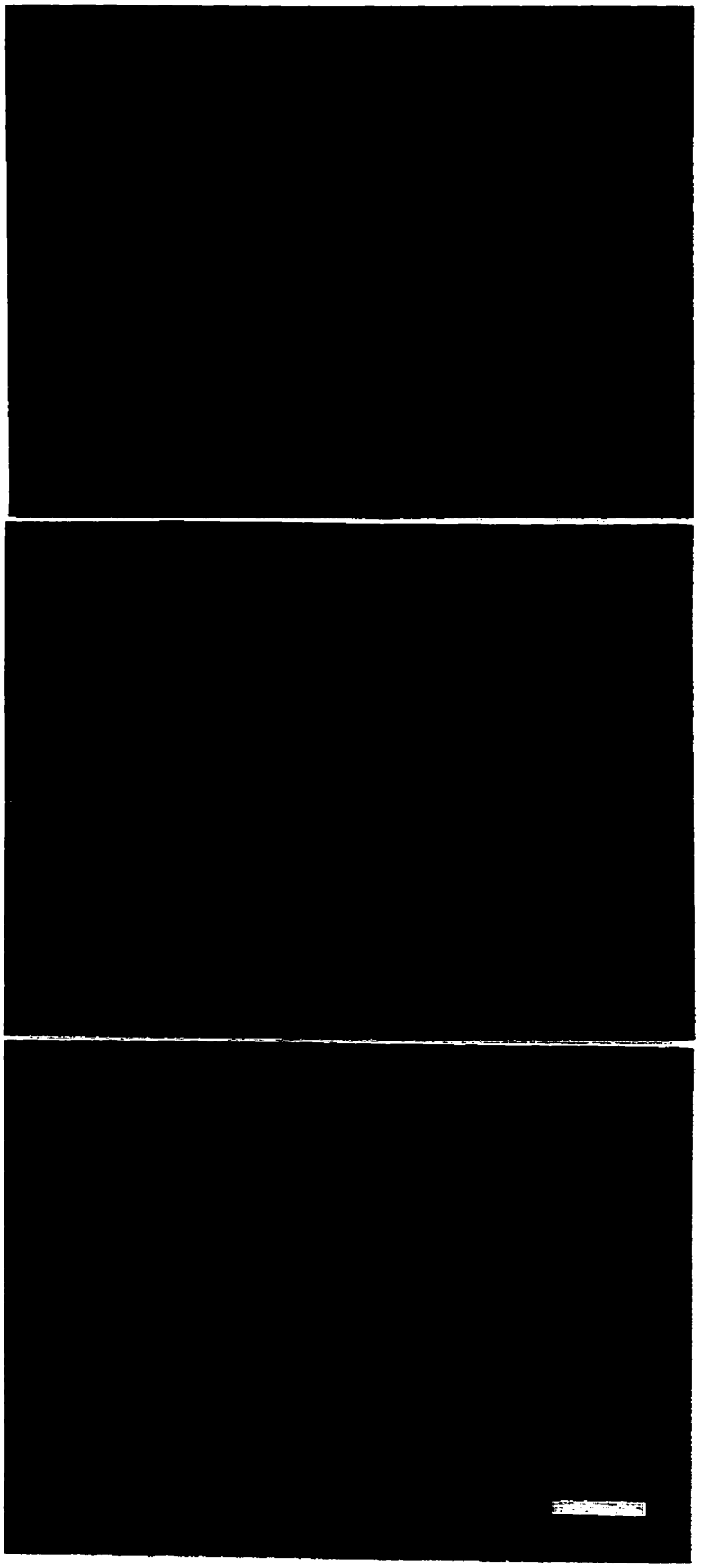
Figure 4.2: Vertical sections of control (top), ECMA (middle), and QA-treated retinas (bottom) labelled with antisera raised against (**a**) cm2 , (**b**) cm3, and (**c**) cm4. Abbreviations: IP - inner plexiform layer; IN -inner nuclear layer; GC - ganglion cell layer. Scale bar = 50 μm.


4.2a
IN
IP
GC

4.2b



4.2c

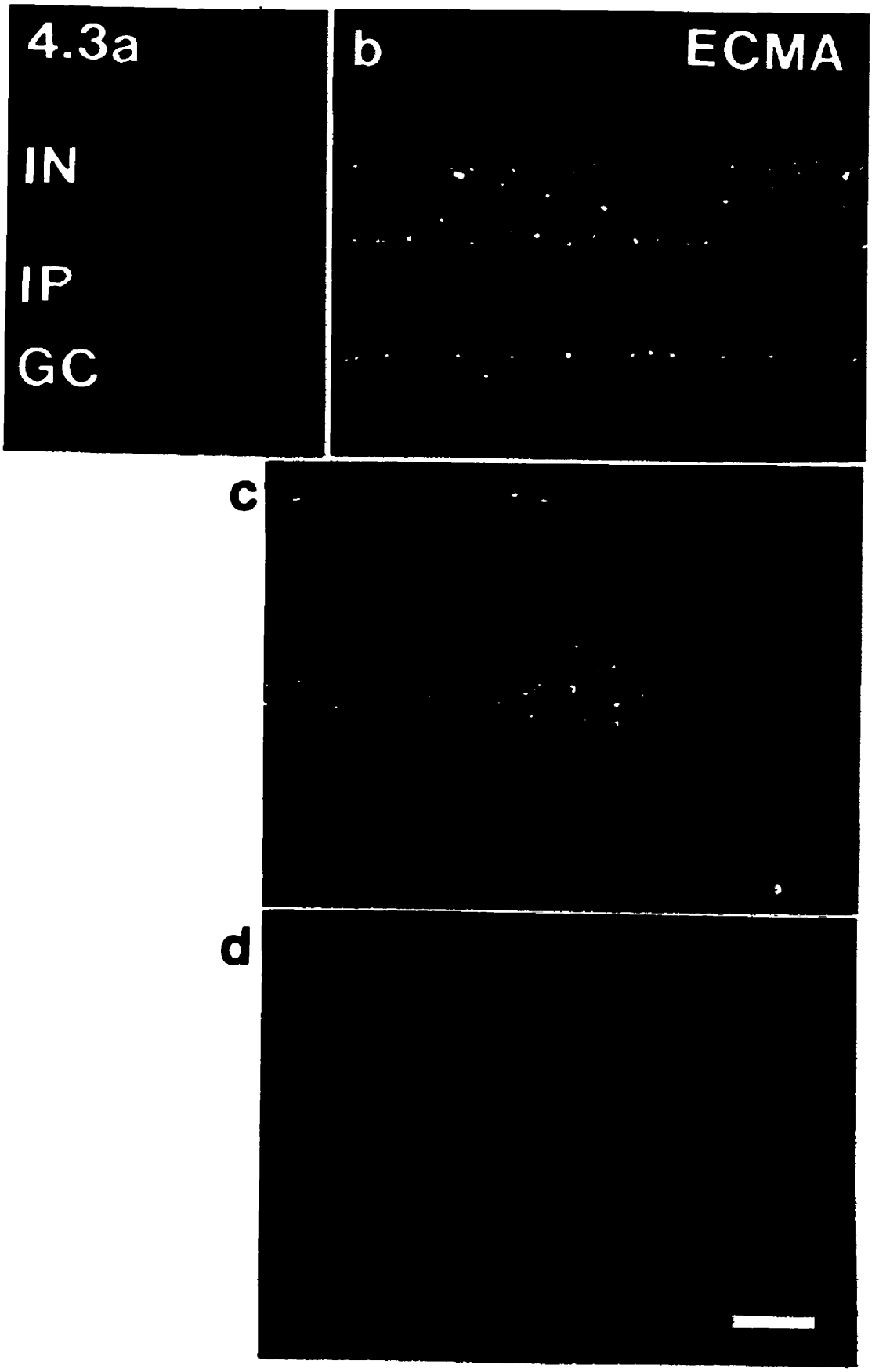


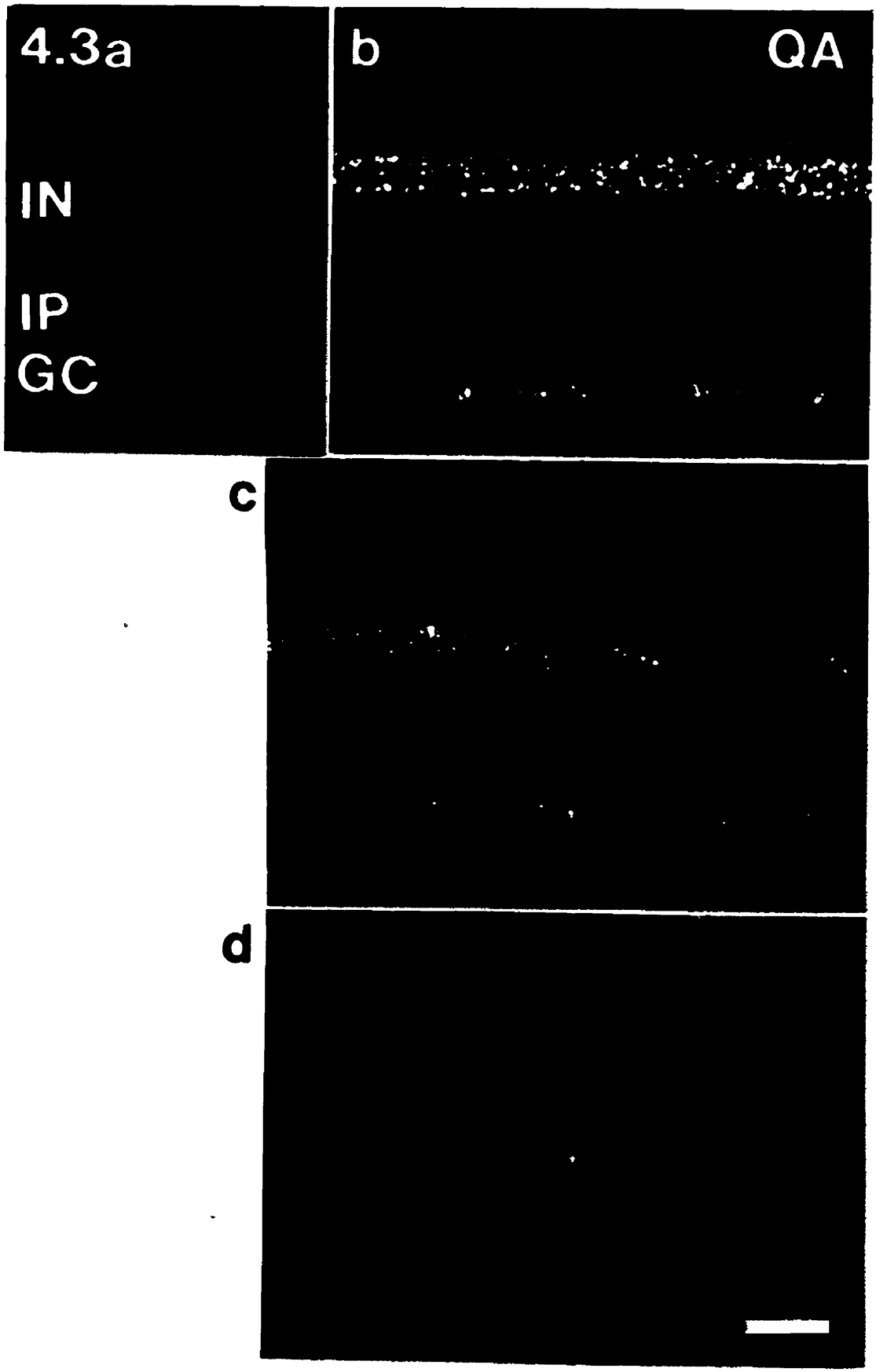
amacrine cells in the INL and GCL, as well as one thin stratum at about 60% IPL depth (Fig. 4.2c). The distribution of cm2, cm3 and cm4 immunoreactivities was exactly as I have described previously (Chapter 2; Fischer et al., 1997a, 1998b). There was little change in the distribution and abundance of mAChRs in ECMA-treated retinas. After exposure to EMCA, both cm2 and cm3 immunoreactivities appeared unchanged (Figs. 4.2a and 4.2b), while the cm4-immunoreactive stratum at 60% IPL depth was absent (Fig. 4.2c). After treatment with QA, the expression of mAChRs was severely disrupted. Many cm2-immunoreactive cells in the INL and GCL and most cm2-immunoreactivity in the IPL were destroyed by QA (Fig. 4.2a). QA had little or no effect upon cm3-immunoreactive bipolar cells, while many amacrine cells and cells in the GCL became hyperimmunoreactive and most cm3-immunoreactivity in the IPL was absent (Fig. 4.2b). QA-treatment destroyed many cm4-immunoreactive cells in the GCL and in the amacrine cell layer of the INL, and ablated the cm4-immunoreactive stratum at 60% IPL depth (Fig. 4.2c).

ECMA and QA-induced fragmentation of nuclear DNA:

Fragmented DNA was not detected in saline-treated retinas at any time after treatment. Twenty-four hours after ECMA-treatment, many nuclei of retinal neurons contained fragmented DNA. The majority of labelled nuclei were those of amacrine cells (in the INL and displaced to the GCL), but there was also labelling of bipolar, and possibly ganglion cell nuclei (Fig. 4.3b). Three days after exposure to ECMA, fragmented DNA was detected in the nuclei of some bipolar and amacrine cells, while

Figure 4.3: Vertical sections of retina which have been labelled for DNA fragmentation in (**a**) control eyes, and (**b**) 1, (**c**) 3 and (**d**) 7 days after treatment with ECMA or QA. Abbreviations: IP - inner plexiform layer; IN - inner nuclear layer; GC - ganglion cell layer. Scale bar = 50 μm


4.3a
IN
IP
GC
b
ECMA
c
d


4.3a
b
QA
IN
IP
GC
c
d

very little fragmented DNA was detected in the GCL (Fig. 4.3c). Seven days after ECMA-treatment, fragmented DNA was not detected anywhere in the retina (Fig. 4.3d).

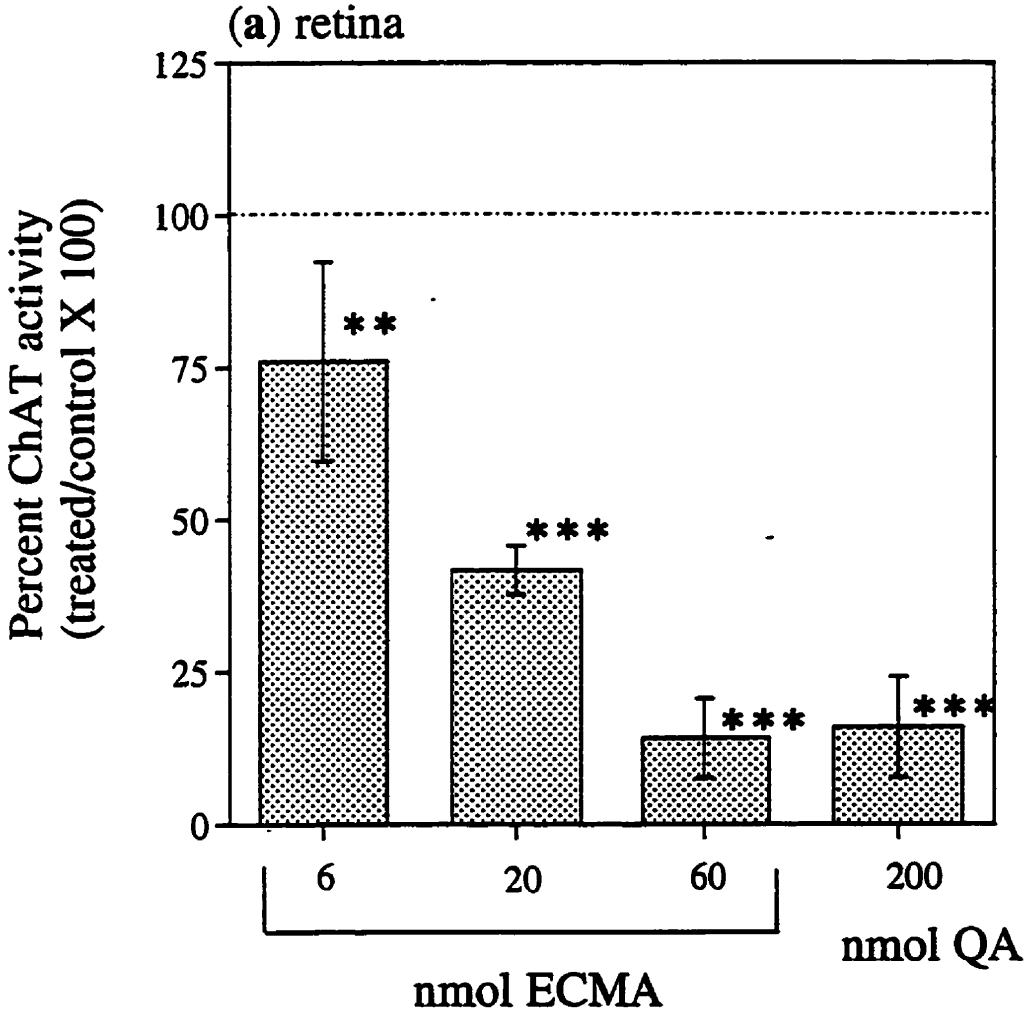
Twenty-four hours after QA-treatment, most nuclei in the amacrine cell layer of the INL and a few nuclei in the GCL were intensely labelled for fragmented DNA (Fig. 4.3b). Three days after treatment, nuclei of most amacrine and some ganglion cells were weakly labelled, while a few were robustly labelled (Fig. 4.3c). There were also some weakly labelled nuclei in the bipolar cell layer of the INL (Fig. 4.3c). Rarely occuring residual amacrine cell nuclei were weakly labelled 7 days after treatment (Fig. 4.3d). These results confirm previous reports of QA-induced DNA fragmentation in the chick retina (Chapter 3; Fischer et al., 1998f).

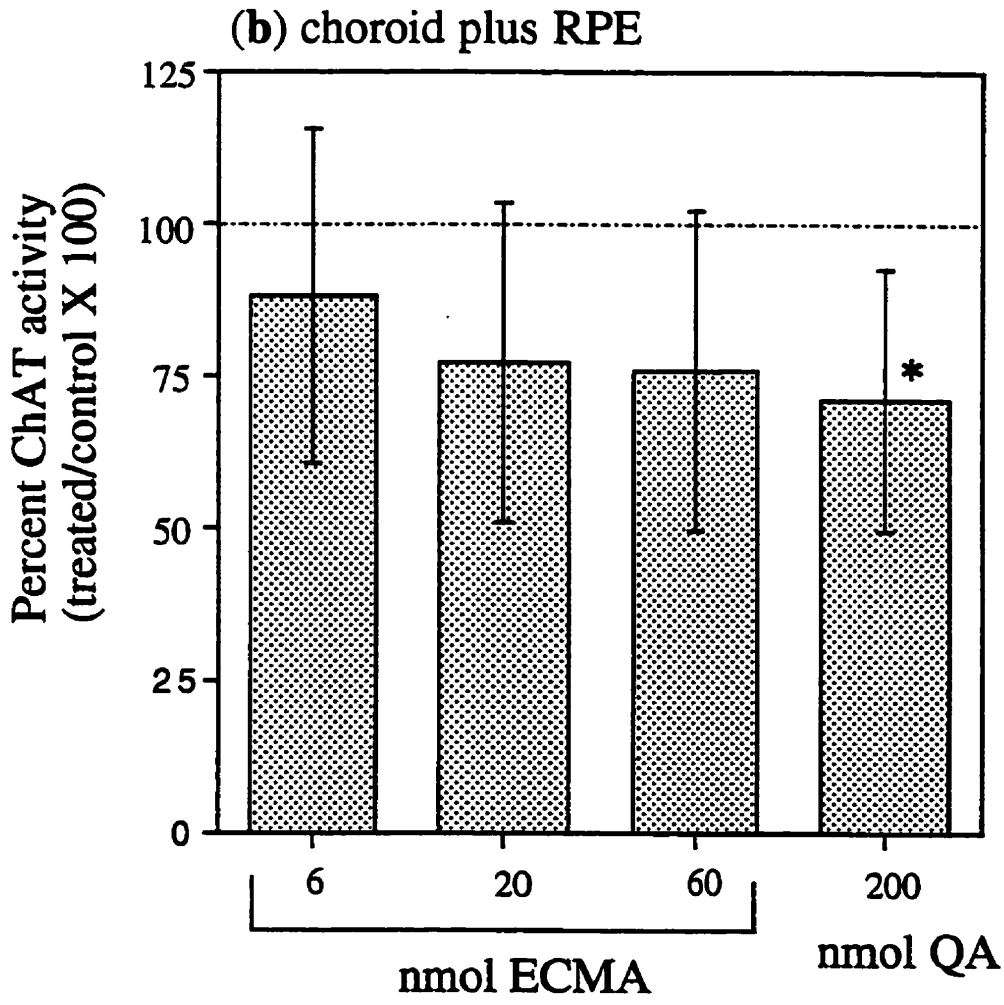
The effects of ECMA and QA upon ChAT activity:

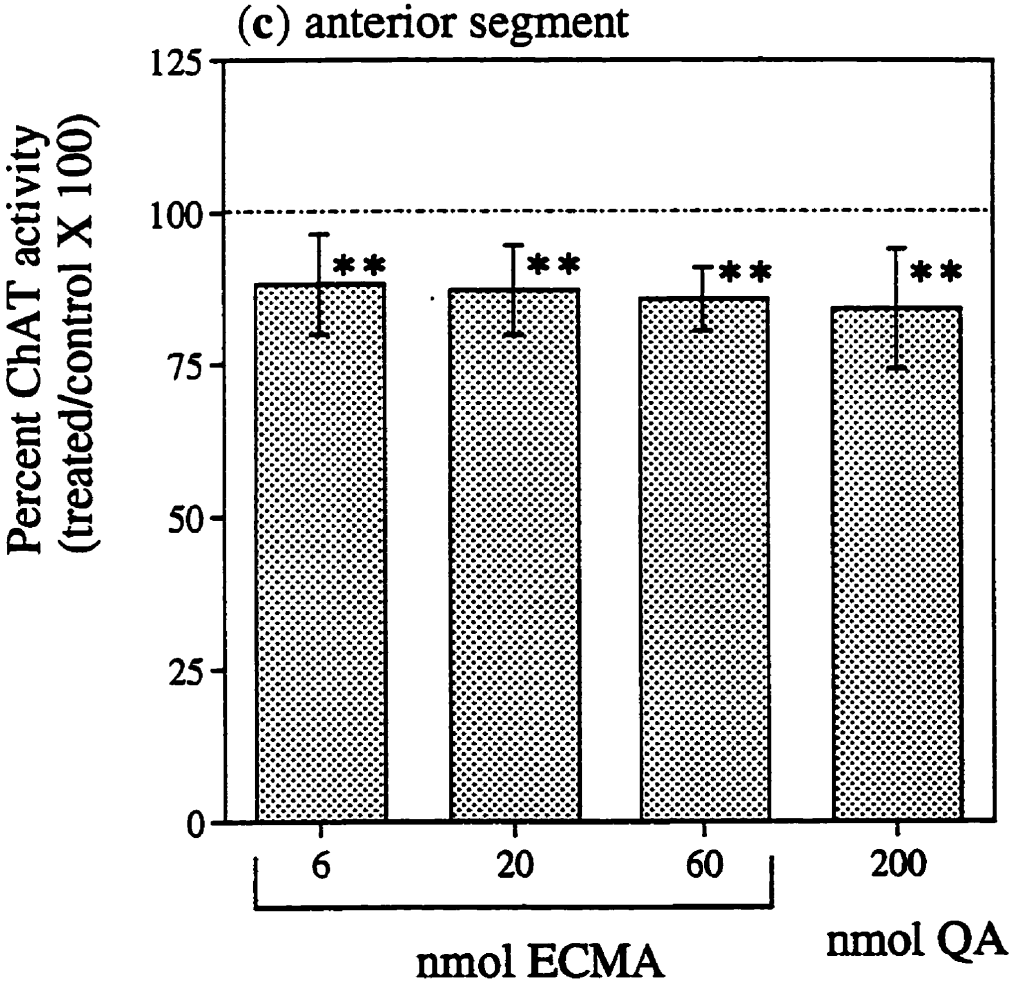
ECMA caused a dose-dependent decrease in ChAT activity of ocular tissues. The ChAT activity in retinas treated (at P6) with 6 nmol ECMA was reduced to about 75% that of controls (Fig. 4.4a). ChAT activity in retinas treated with 20 nmol ECMA was reduced to about 42%, while that of retinas treated with 60 nmol was reduced to 14% (Fig. 4.4a). ECMA had little effect upon ChAT activity in the choroid or anterior segment (Fig. 4.4b and 4.4c). Only at a 600 nmol dose, ECMA significantly decreased ChAT activity in the choroid and anterior segment (data not shown).

The activity of ChAT was severely attenuated in retinas exposure to 200 nmol QA. Only 16% of the ChAT activity remained in treated retinas (Fig. 4.4a). In comparison, QA had only slight effects upon ChAT activity in the choroid or the anterior

Figure 4.4: Activity of ChAT in extracts of (**a**) retina, (**b**) choroid plus RPE, or (**c**) anterior segment that have been treated with ECMA or QA. The control is equal to 100%. Significance (*$p<0.01$; **$p<0.005$; ***$p<0.0001$) between treated samples and the mean of the control was assessed by using a one-tailed Student *t*-test.


(a) retina
125
100
75
50
25
0
Percent ChAT activity
(treated/control X 100)
**
***
***
***
6
20
60
200
nmol QA
nmol ECMA


(b) choroid plus RPE
Percent ChAT activity
(treated/control X 100)
125
100
75
50
25
0
*
6
20
60
200
nmol ECMA
nmol QA


(c) anterior segment
Percent ChAT activity
(treated/control X 100)
125
100
75
50
25
0
**
**
**
**
6
20
60
200
nmol ECMA
nmol QA

segment of the eye. The ChAT activity in the choroid was reduced to about 71%, while that of the anterior segment was reduced to about 82% (Fig. 4.4b and 4.4c).

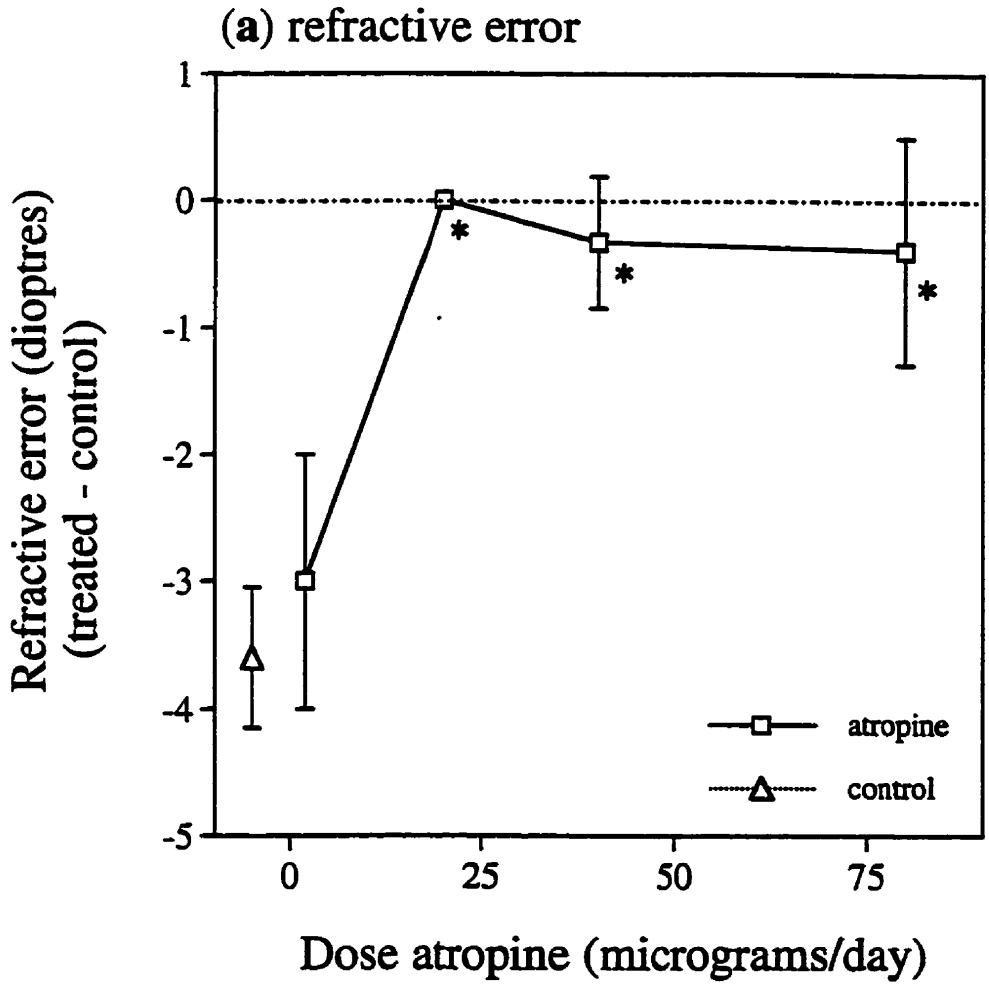
The effect of atropine upon FDM:

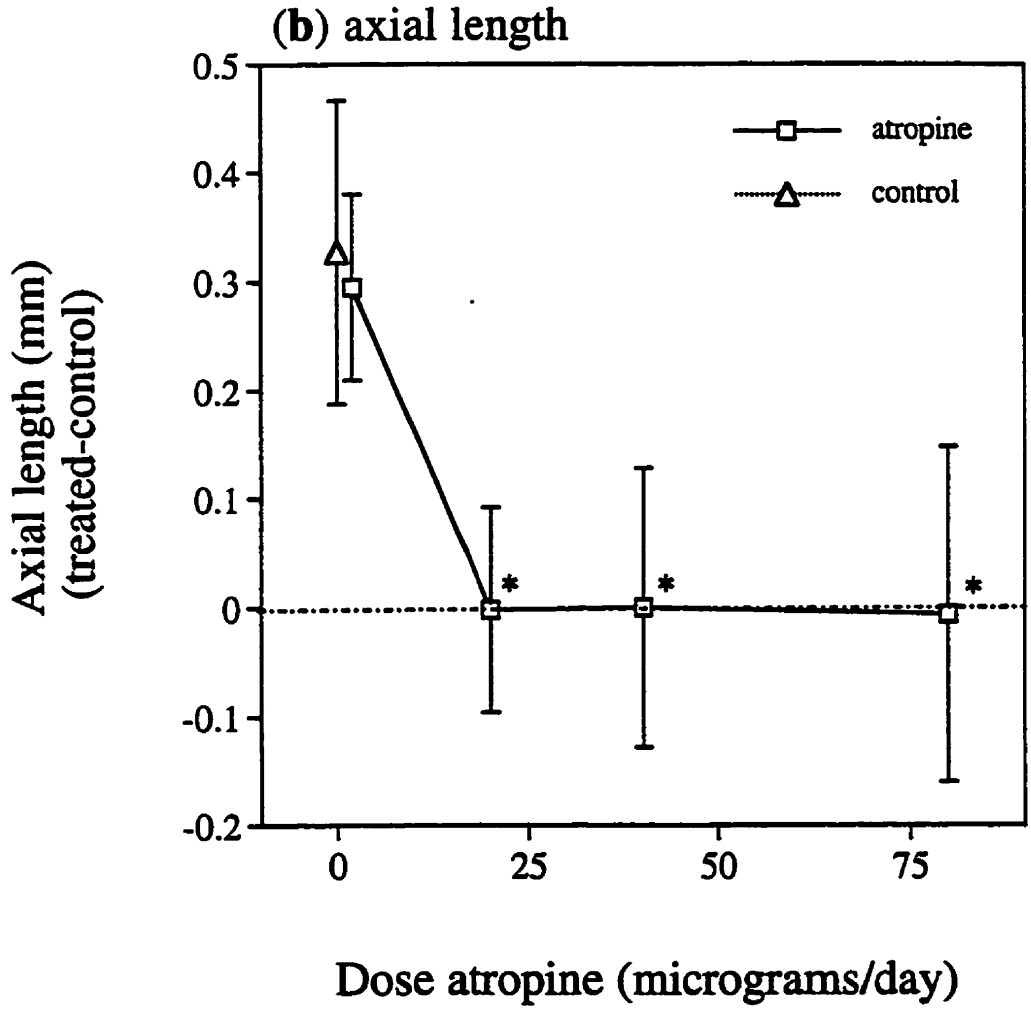
Atropine suppressed FDM in a dose-dependent manner. Daily intravitreal application of atropine at 2.0 μg had no significant effect upon the refractive error, axial length, or equatorial diameter of form-deprived eyes (Fig. 4.5a, b, c respectively). However, atropine at 20 , 40 and 80 μg per day prevented the excessive ocular elongation and negative refractive error that normally resulted from form-deprivation (Fig. 4.5). These results confirm the suppression of FDM by atropine as reported previously (Stone et al., 1991; McBrien et al., 1993).

The effect of atropine and toxin-treatment upon normal and form-deprived eyes:

ECMA and QA had little effect upon normal ocular growth or the excessive growth and myopia caused by form-deprivation. Injections of ECMA, QA, and atropine had no effect on refraction of contralateral saline-treated eyes, which were consistently emmetropic. Twelve days after toxin treatment, ECMA and QA-treated ungoggled eyes remained emmetropic (Fig. 4.6a) and had normal equatorial diameter and axial length (Fig. 4.6b, c respectively), as well as normal wet weight (data not shown). Form-deprived ECMA and QA-treated eyes became significantly myopic ($p<0.0001$; paired *t*-test between control and contralateral treated eyes), but less so than saline-treated form-deprived eyes ($p<0.0001$; ANOVA comparison between groups; Fig. 4.6c). In addition,

Figure 4.5: Dose response functions for the effects of atropine (2, 20, 40, and 80 µg/day) upon the (**a**) refractive error, (**b**) axial length, and (**c**) equatorial diameter of form-deprived eyes. Significance (*$p < 0.005$; $n = 6$) between treated and control (saline-treated) groups was assessed by using a two-way Student *t*-test.


(a) refractive error
Refractive error (dioptres)
(treated - control)
1
0
-1
-2
-3
-4
-5
0
25
50
75
Dose atropine (micrograms/day)
atropine
control
*
*
*


(b) axial length
Axial length (mm)
(treated-control)
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
atropine
control
*
*
*
0
25
50
75
Dose atropine (micrograms/day)

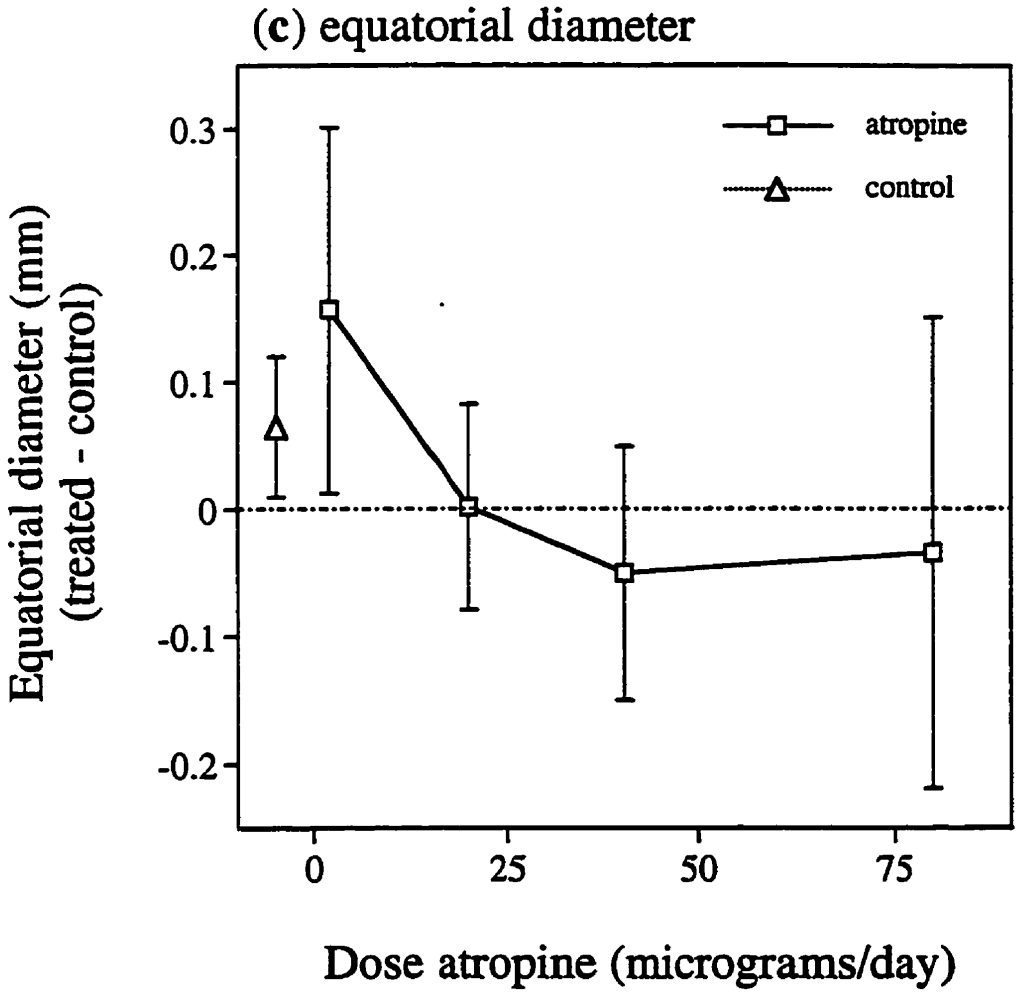
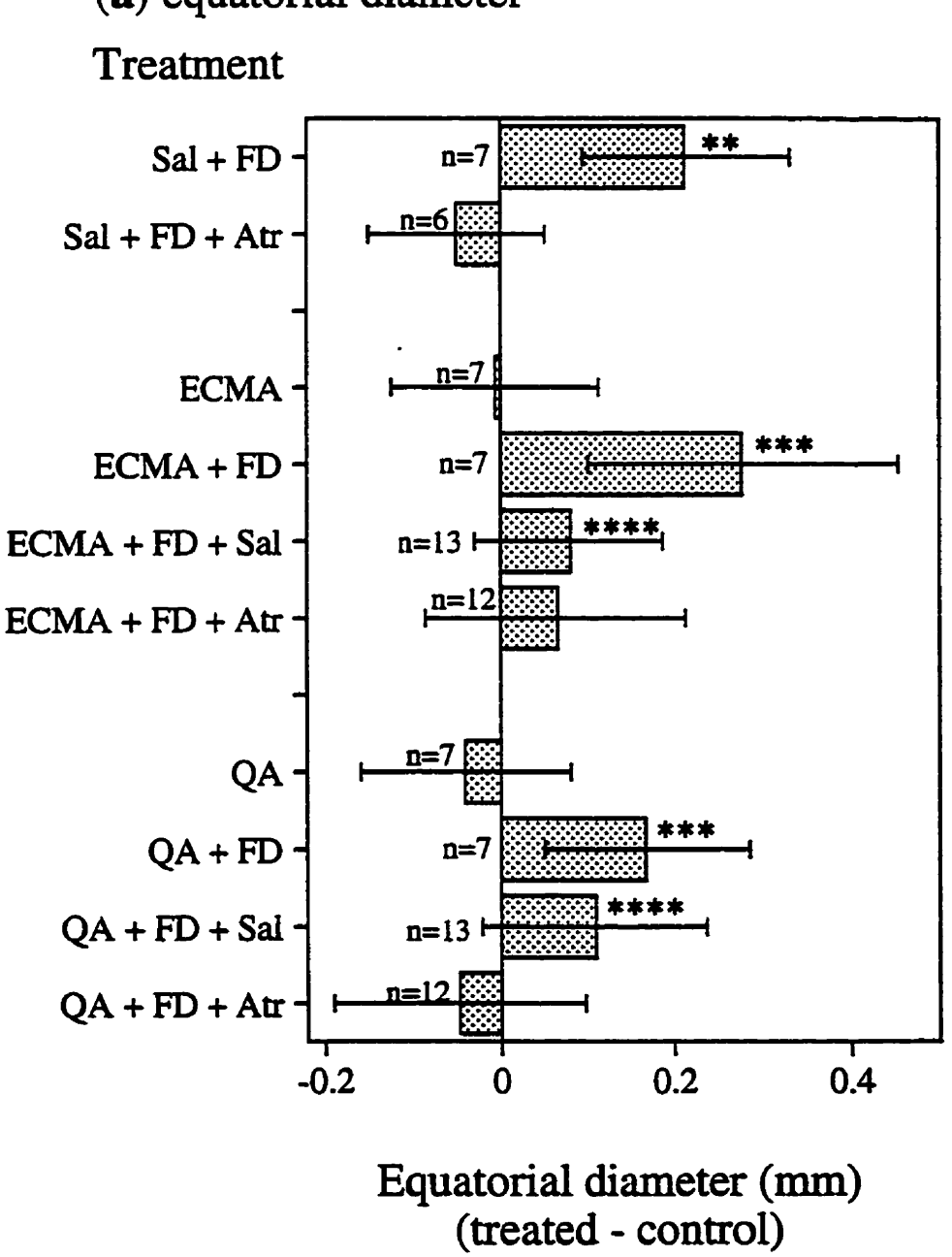

(c) equatorial diameter
atropine
control
0.3
0.2
0.1
0
-0.1
-0.2
Equatorial diameter (mm)
(treated - control)
0
25
50
75
Dose atropine (micrograms/day)

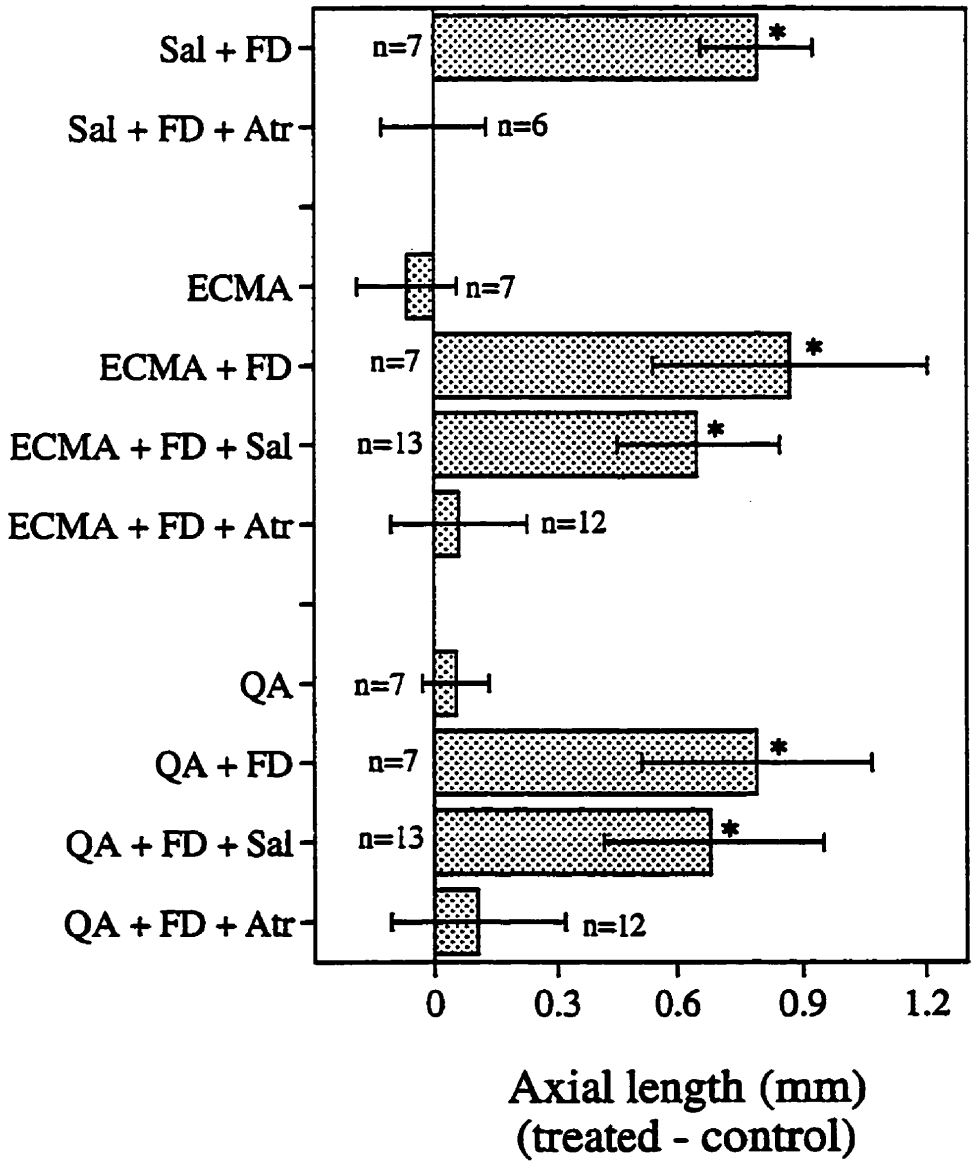
Figure 4.6: The effects of atropine, ECMA, and QA upon (**a**) axial length, (**b**) equatorial diameter, and (**c**) refractive error of form-deprived eyes. The two empty lanes in figure 6a (QA and ECMA alone) represent means and standard deviations equal to zero. Significance (*$p<0.0001$; **$p<0.005$; ***$p<0.01$; ****$p<0.05$) between treated and control eyes from the same experimental group was assessed using a one-way Student *t*-test, while significance of difference between non-injected, saline-injected and atropine-injected eyes was assessed using a two-way ANOVA. Abbreviations: FD - form-deprived; QA - quisqualic acid; ECMA - ethylcholine mustard aziridinium; Atr- atropine; Sal - saline.

(**a**) equatorial diameter

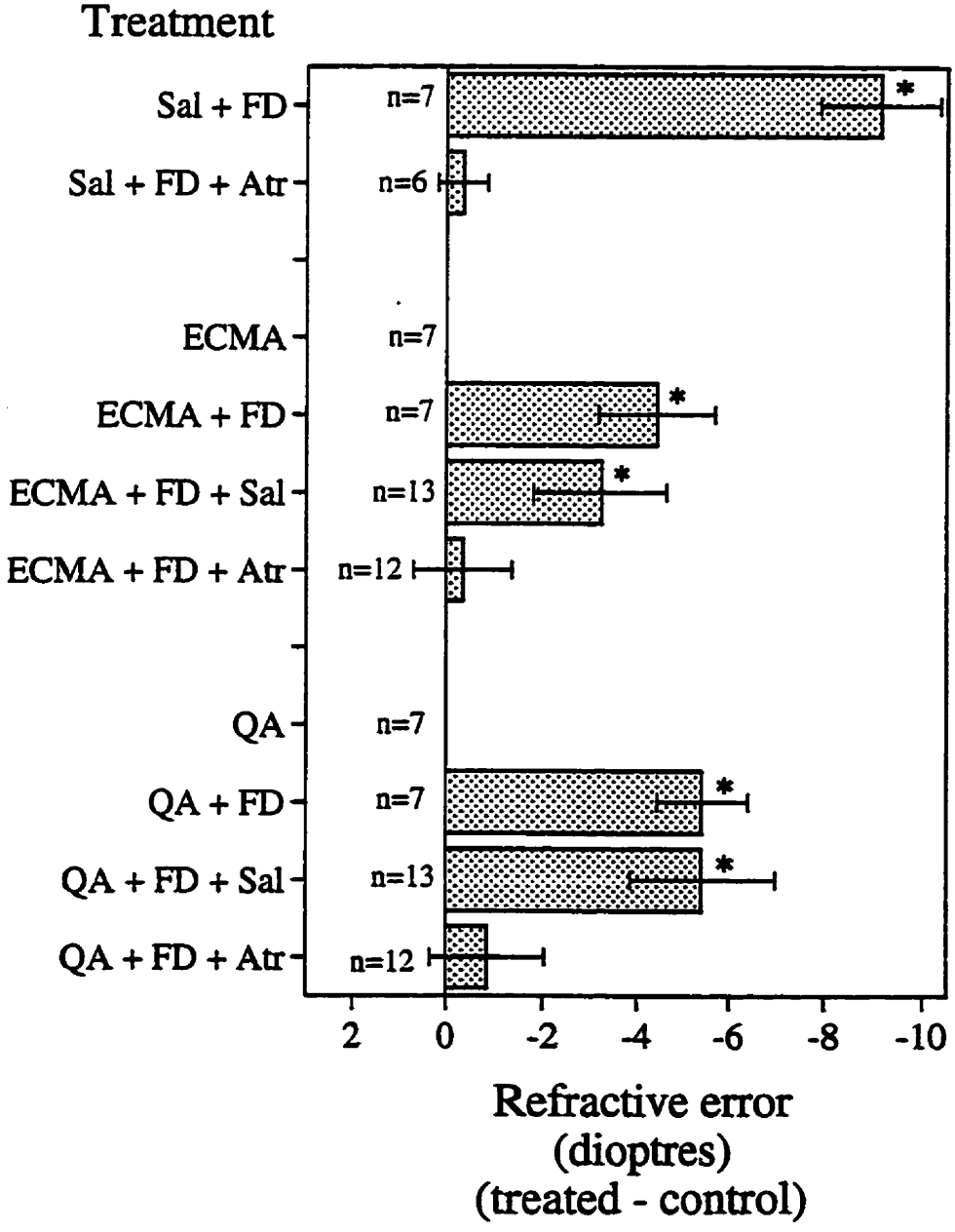


(b) axial length

Treatment



(c) refractive error



form-deprivation of ECMA and QA-treated eyes caused significant increases in axial length ($p<0.0001$; *t*-test between control and treated eyes; Fig. 4.6b). However, the increases in axial length resulting from form-deprivation of saline-, ECMA-, or QA-treated eyes did not differ significantly (Fig. 4.6b). Form-deprivation of eyes treated with saline, ECMA or QA at P6 caused significant increases in equatorial diameter ($p<0.01$;paired *t*-test between treated and contralateral control eyes; Fig. 4.6a). Form-deprivation of eyes treated with ECMA or QA at P6 and daily saline injection caused significant increases in equatorial diameter ($p<0.05$; *t*-test between control and treated eyes), but no significant difference from daily injection with atropine (Fig. 4.6a). Atropine significantly suppressed the refractive error and increases in axial length that normally result from form-deprivation in ECMA-treated eyes ($p<0.0001$; for both *t*-test between control and treated eyes and ANOVA comparison between groups; Fig. 4.6). Atropine also significantly suppressed the refractive error ($p<0.0001$; ANOVA comparison between groups), the increase in axial length ($p<0.0001$; ANOVA comparison between groups), and the increase in equatorial diameter ($p<0.01$; ANOVA comparison between groups) that normally result from form-deprivation in QA-treated eyes.

The difference between the size or refractive state of eyes that received daily injections of saline and those that did not receive daily injections was not significant. There was no correlation between the numbers of type-II cholinergic amacrine cells that survived exposure to QA and the amount of refractive error that resulted from form-deprivation (data not shown). Similarly, there was no correlation between the numbers of

type-II or type-III cholinergic amacrine cells that survived exposure to ECMA and the amount of refractive error that resulted from form-deprivation.

Discussion:

The role of retinal cholinergic pathways in visually guided ocular growth:

ECMA and QA induced massive losses of ChAT-immunoreactive amacrine cells in the chick retina. The majority of cholinergic amacrine cells (except type-III cells) were ablated from (or undetectable in) ECMA-treated retinas, while essentially all cholinergic cells were ablated from the central retina of QA-treated animals, and many non-cholinergic cells were also affected at least transiently (as suggested DNA fragmentation labelling). The residual ChAT activity that was detected in QA-treated retinas likely resulted mainly from the large numbers of type-II cells that survived at the periphery of the retina, as well as (perhaps) immunocytochemically undetectable levels of ChAT in residual, damaged cholinergic cells in central retina. It is unlikely that these residual type-II cholinergic amacrine cells in the retinal periphery contributed to the progression of FDM or atropine-mediated rescue of FDM, as they should be unable to influence the growth of the posterior pole of the eye that is associated with FDM (Wallman, 1993). The remaining type-II cells in central retina that survived exposure to QA were also unlikely to participate in the regulation of ocular growth because of their severely reduced numbers and the considerable damage to their dendritic arbours. Furthermore, QA-induced massive loss of mAChRs, and widespread DNA fragmentation shortly after treatment (this paper), and destruction of amacrine cells immunoreactive for VIP,

serotonin, PKC, GluR1 or parvalbumin (Chapter 3; Fischer et al., 1998f). QA caused little or no loss of cholinergic fibers or ChAT enzyme activity from the choroid or anterior segment of toxin-treated eyes. Nevertheless, toxin-treated eyes remained emmetropic, grew excessively in response to form-deprivation, and were rescued from FDM by atropine. We expected that the QA-treated eyes would grow excessively in response to form-deprivation, because of prior reports that form-deprivation induces increased axial length in QA-intoxicated eyes (Ehrlich et al., 1990). Assuming that atropine acts only as a mAChR antagonist at the doses used in this study, our results indicate that: (i) cholinergic retinal amacrine cells do not contribute to the processes of emmetropization or growth-enhancement that are activated by form-deprivation; (ii) atropine does not suppress FDM by antagonizing the actions of ACh released from cholinergic retinal amacrine cells; (iii) atropine does not suppress FDM by acting upon mAChR's in the retina; and (iv) atropine may suppress FDM by antagonizing ACh released from parasympathetic axon terminals in the choroid or anterior segment of the eye.

Three different isoforms of mAChR have been detected in the retina, RPE, choroid and ciliary body (Chapter 2; Fischer et al., 1997a, Fischer et al., 1998b). Muscarinic AChRs may also be present in scleral chondrocytes, since atropine has been reported to suppress the production of proteoglycans by scleral chondrocytes (Marzani et al., 1994; Lind et al., 1997). Therefore, the activation of mAChRs in the RPE, choroid, or sclera by ACh released from choroidal innervation could promote vitreous chamber elongation during form-deprivation. On the other hand, it has been shown that ablation of

cholinergic innervation to the choroid has little effect upon the FDM (Lin & Stone, 1991; Reiner et al., 1995).

It is unlikely that cholinergic innervation to the anterior segment of the eye participates in the visual modulation of ocular growth. Neither atropine nor the subtype selective antagonist pirenzepine have any detectable effects on pupillary responses and accommodation in chicks (McBrien et al., 1993), and the removal of cholinergic innervation to the ciliary body and iris sphincter has no affect upon emmetropization or FDM (Wallman, et al., 1981; Schaeffel et al., 1990; Wildsoet & Howland, 1991; Wildsoet et al., 1993; Schmid & Wildsoet, 1996).

Alternatively, atropine might influence non-cholinergic growth-modulating pathways in the eye. The only evidence that cholinergic pathways are involved in the regulation of ocular growth comes from studies on the effects of muscarinic antagonists on FDM (Stone et al., 1991; McBrien et al., 1993; Leech et al., 1995) and on the effects of form-deprivation on ChAT activity in the ciliary ganglion and choroid (Pendrak et al., 1995). The results of these studies suggest contradictory roles for cholinergic pathways in the visual regulation of ocular growth. The suppression of FDM by muscarinic antagonists suggests that activation of mAChRs causes excessive growth, and it has been argued that this pathway resides in the retina (Stone et al., 1991; McBrien et al., 1993; Leech et al., 1995). However, Pendrak et al. (1995) reported that ChAT activity was not altered in the retina, but its activity was reduced in the choroid and ciliary ganglion of form-deprived eyes, suggesting that a decrease in cholinergic transmission in the choroid might be responsible for FDM. Both choroidal neurectomy and chemical lesioning of

cholinergic retinal cells do not affect emmetropization or the development of FDM, while reduced ChAT activity in choroid is likely to result from form-deprivation and a reduction in release of ACh from choroidal nerve endings probably causes the reduction in choroidal blood flow observed in form-deprived eyes (Reiner et al., 1995). These findings would seem to suggest that cholinergic pathways in the eye are not required for: (i) growth towards and maintenance of emmetropia; (ii) the excessive ocular growth that results from form-deprivation; and (iii) the atropine-mediated suppression of FDM. Therefore, it remains uncertain where in the eye and through which receptor systems atropine elicits its growth-modulating influence. Further experimentation is required to test whether cholinergic innervation to the choroid plays any role in visually guided ocular growth or atropine-mediated suppression of FDM, and whether atropine and pirenzepine may act via mechanisms other than mAChR antagonism.

QA and ECMA did not cause any changes in the size (axial length and equatorial diameter) of either open or form-deprived eyes (Fig. 4.7). ECMA and QA did, however, cause a greater reduction in FDM (refractive error) than that caused by injection of saline alone. In addition, 12 days after treatment, neither ECMA nor QA caused any detectable change in corneal curvature (by visual inspection), contrary to previous reports that QA causes significant increases in corneal curvature and anterior chamber depth 3 weeks after intravitreal injection (Barrington et al., 1989; Ehrlich et al., 1990). However, it is possible that these QA-induced changes in the anterior chamber occurred between 12 and 21 days after treatment, and that any decreases in the refractive error of form-deprived toxin-treated eyes resulted from undetected changes in the anterior segment.

Persistence of ChAT-immunoreactive fibers and cells following exposure to QA or ECMA:

The cholinergic neurotoxin, ECMA, is an analogue of choline that specifically alkylates and inactivates transporters and enzymes that utilize choline as a substrate (Sandberg et al., 1985; Allen et al., 1988; Hanin 1990, 1996). The primary target of ECMA is the high-affinity choline transporter that is strongly expressed by cholinergic neurons (Fisher et al., 1982; Hanin, 1990, 1996). Previous reports have shown that this toxin is capable of destroying cholinergic amacrine cells, while apparently sparing other retinal neurons (Millar et al., 1987a; Estrada et al., 1988; Gómes-Ramos et al., 1990). This is consistent with our results of immunolabelling, but not of DNA nick-end-labelling. We detected widespread DNA fragmentation, a symptom of apoptosis, 1 and 3 days after retinal exposure to ECMA, while only the loss of type-I and type-II cholinergic amacrine cells was detected. In contrast, QA is an excitotoxin that destroys cells by over-stimulating subsets of ionotrophic non-NMDA glutamate receptors. QA also induces DNA fragmentation in most amacrine cells, while only destroys limited subsets (Chapter 3; Fischer et al., 1998f). These results indicate that both ECMA and QA cause widespread fragmentation of genomic DNA, and that labelled cells are not necessarily committed to apoptosis, but may instead be undergoing DNA repair and recovery.

Moderate doses ($\leq$ 25 nmol) of ECMA preferentially destroyed type-I and type-II cholinergic amacrine cells, leaving the majority of type-III cells intact, and resulted in substantial decreases in retinal ChAT activity (see Fig. 4.5a). This is consistent with

results reported previously. However, our findings are inconsistent in some respects with those of Millar et al. (1987a), who reported substantial amounts of ChAT activity after retinal exposure to high doses (> 500 nmol) of ECMA that were reported to destroy the entire retina. Millar et al. (1987a) also reported that ECMA elicited no effects upon cells immunoreactive for enkephalin, while those immunoreactive for somatostatin were affected. This is not possible, as somatostatin and enkephalin are colocalized in the same sub-set of amacrine cells (Watt & Florack, 1994), which are also immunoreactive for ChAT and comprise about one-half of the type-III cholinergic cells (Chapter 3; Fischer et al., 1988f). This discrepancy likely resulted from the capricious actions of ECMA, differences in sensitivity of immunocytochemical detection of somatostatin and enkephalin, and sampling errors.

Not only are type-III cholinergic amacrine cells less susceptible to ECMA-treatment than other ChAT-immunoreactive cells, but cholinergic fibers in the choroid are also resistant, except as measured by ChAT activity assay at the 600 nmol dose (results not shown). The tolerance to ECMA of cholinergic fibers in the choroid and type-III cells in the retina may have resulted from: (i) an absence or low level of expression of choline transporters; (ii) reduced exposure to ECMA due to diffusion barriers and dilution (applicable only to cholinergic fibers in the choroid); or (iii) differences in transporter kinetics or availability due to differences in light-modulation of neuronal activity. Similarly, cholinergic fibers in choroid and some type-II cholinergic amacrine cells survived exposure to QA. This may have resulted from: (i) an absence or low level of expression of QA-sensitive glutamate receptors (particularly in the case of choroidal

fibers); (ii) increased tolerance to excitotoxicity; or (iii) relative exposure to QA from a distant source due to diffusion and dilution (applicable only to cholinergic fibers in the choroid).

ECMA-induced reduction of cm4 levels in the retina:

No loss of cm2 or cm3 was detected in retinas treated with ECMA. However, cm4-immunoreactivity was obviously lost from ECMA-treated retinas. After exposure to ECMA, cm4-immunoreactivity was absent from the IPL stratum coincident with the neurites of type-II cholinergic amacrine cells. Since there was no apparent destruction of amacrine cells other than type-I and type-II cholinergic cells, and of ChAT-immunoreactive amacrines only type-II cells send neurites to sublamina 4 of the IPL, then it is likely that the ECMA-induced depletion of cm4-immunoreactivity in the IPL represents the loss of cm4 autoreceptors expressed by type-II cholinergic cells.

Summary and conclusions:

The ablation of most ChAT-immunoreactivity in the retina did not prevent emmetropization or FDM. This suggests that none of the 4 types of cholinergic amacrine cells contributes to visually guided ocular growth. Furthermore, since atropine prevented FDM in retinas depleted of muscarinic receptors and cholinergic cells, cholinergic pathways in the retina are unlikely to participate in atropine-mediated suppression of FDM. These results suggest that cholinergic growth-modulatory pathways are extraretinal, or that atropine prevents FDM through non-cholinergic mechanisms.

CHAPTER FIVE

Summaries and Conclusions

Visually guided ocular growth is a complicated process that requires image processing by the retina. In particular, subsets of retinal amacrine cells are required to process information regarding the contrast and spatial frequency content of images, and translate this information into growth-modulating signals. Several distinct subsets of amacrine cells have been implicated indirectly in this process. These cells include those that release and/or respond to dopamine (Stone et al., 1989; Rohrer et al., 1993), ENK (Seltner et al., 1997), ACh (Stone et al., 1991; McBrien et al., 1993), VIP (Stone et al., 1988; Seltner et al., 1995a, 1995b), nitric oxide (Fujikado et al., 1997), neurotensin (Seltner, personal communication), and glutamate acting at NMDA-receptors (Fischer et al., 1998g). Evidence supporting roles for these cells in growth regulation has been provided mostly by pharmacological studies.

The purpose of this dissertation was to investigate the role of cholinergic pathways in visual guidance of ocular growth. Since the muscarinic antagonist atropine suppresses FDM, the localization of mAChRs might provide insight into where atropine acts to block form-deprivation-induced ocular growth. Therefore, the first hypothesis tested was that different isoforms of mAChR are expressed in growth-regulating circuits. Indeed, mAChRs were detected in most tissues throughout the chick eye. At least 3 different isoforms of mAChR are expressed by bipolar, amacrine, or ganglion cells in the retina. Furthermore, mAChRs were detected in the RPE, choroid, and ciliary body.

These localizations of mAChRs are consistent with a role in visually guided ocular growth, since any of these tissues could, in theory, modify ocular growth.

Since the M1-selective antagonist pirenzepine prevents FDM in chicks (Stone et al., 1991; McBrien et al., 1993), activation of M1-type mAChRs is believed to promote excessive ocular growth during form-deprivation. However, an avian m1 homologue has not been cloned and characterized because it either does not exist or is expressed at extremely low levels (Neil Nathanson, personal communication). Regardless, pirenzepine has been shown to bind preferentially to cm2 and cm4 isoforms of the chick muscarinic receptor (Tietje et al., 1990; Tietje and Nathanson, 1991). This indicates not only that the pharmacological characteristics of chick mAChRs differ substantially from those of mammalian receptors, but also that cm1-mechanisms are *not* necessary for the pirenzepine-mediated suppression of FDM.

Atropine has been shown to elicit a number of effects upon retinal activity, including flattening of the pattern ERG (Schwahn et al., 1997) and increasing retinal dopamine content (Kaymak et al., 1997). Furthermore, atropine has been reported to block changes in the resting potential of the RPE elicited by muscarinic agonists *in vitro* (Schwahn and Schaeffel., 1998) and block muscarinic vasodilation and thereby cause vasoconstriction to decrease choroidal blood flow (Meriney and Pilar, 1987). It remains uncertain whether any of these effects itself suppresses FDM, and even whether these effects are elicited through mAChRs. However, evidence offered in this dissertation suggests that these atropine-mediated effects do not suppress FDM by antagonizing the action of ACh, released by amacrine cells in the retina.

Lind et al. (1997) and Marzani et al. (1994) have suggested that atropine and pirenzepine suppress FDM by directly modifying the activity of isolated, cultured scleral chondrocytes, because these muscarinic antagonists reduce their production of proteoglycans. However, it is unlikely that these affects are elicited through antagonism at muscarinic receptors. By definition, an antagonist blocks the actions of an agonist, and elicits no effect in the absence of agonist. However, in the studies showing that muscarinic antagonists affect the activity of chondrocytes, no muscarinic agonist was added to the preparation. Furthermore, cm2, cm3 and cm4 were not detected in scleral cells (Chapter 2; Fischer et al., 1998b). However, it is possible that an isoform of mAChR that was not assayed (i.e., cm5) is expressed by scleral chondrocytes. Regardless, action of atropine as an antagonist at cm5 expressed by chondrocytes remains an unlikely mechanism of FDM-suppression, since: (i) choroidal sources of ACh are not required for emmetropization or the development of FDM, and thereby seem unlikely to regulate scleral growth driven by visual cues (Wallman et al., 1981; Schaeffel et al., 1988; Troilo, 1990; Lin and Stone, 1991; Troilo and Wallman, 1991); (ii) atropine suppresses the production of proteoglycans by chondrocytes in the absence of an exogenous agonist (Marzani et al., 1994; Lind et al., 1997); and (iii) Ian Morgan (personal communication) was unable to detect a pirenzepine-induced decrease in proteoglycan synthesis using similar assays of scleral activity. The sclera remains a candidate for the site of atropine's FDM-suppressing affects, but it is unlikely that these affects are elicited through muscarinic receptors.

The second hypothesis tested in this dissertation was that a subset of amacrine

cells is required and sufficient for visual guidance of ocular growth. This hypothesis was tested by examining the cells destroyed by different amacrine cell-selective toxins that have different effects upon emmetropization and FDM. QA-induced excitotoxicity destroys many different subsets of amacrine cells (Chapter 3; Fischer et al., 1998f), while allowing visual guidance of ocular growth (Ehrlich et al., 1990; Chapter 4; Fischer et al., 1998c, d). Therefore, the cells destroyed by QA are not necessary for emmetropization or FDM. Surprisingly, QA destroyed many subsets of cells previously implicated as modulators of ocular growth by pharmacological studies. These cells include those that contain VIP, ENK, or ACh. In addition, QA depletes retinal stores of dopamine (Frank Schaeffel, personal communication), although it spares TH-immunoreactive cells (Chapter 3; Fischer et al., 1998f). These findings suggest that form-deprivation-induced reductions in retinal levels of ENK (Ali et al., 1993; Megaw et al., 1994), dopamine (Stone et al., 1989), and VIP polypeptide (Stone et al., 1988) and mRNA (McGlinn et al., 1998) are epiphenomena. In other words, these vision-dependent changes in levels of ENK, dopamine, and VIP are caused by form-deprivation but do not cause the excessive ocular growth that results from it. While form-deprivation-induced ocular growth does not necessarily arise from the same mechanisms as from normal visually guided ocular growth, the cells lost to QA-induced excitotoxicity can be considered non-essential to visually guided ocular growth because treated eyes remain emmetropic (Chapter 4; Fischer et al., 1998c, d) and remain susceptible to growth-modulation by plus or minus defocus (Frank Schaeffel, personal communication).

Like QA, NMDA destroyed many different subsets of amacrine cells (Chapter 3;

Fischer et al., 1998f), but unlike QA it caused a transient increase in the rate of ocular growth and subsequently prevented emmetropization and FDM (Fischer et al., 1997b, 1998g). The purpose of characterizing the cells destroyed by NMDA was to identify cells required for visually guided ocular growth. Unfortunately, the damage caused by NMDA-induced excitotoxicity was widespread and somewhat generalized, and overlapped substantially with the damage caused by QA, thereby making it impossible to identify a few, specific sets of cells required to regulate ocular growth. However, since NMDA-treatment abolishes and QA-treatment allows visually guided ocular growth, the subsets of cells destroyed by NMDA and *not* by QA are candidates for controlling ocular growth. Subsets of amacrine cells that appeared unaffected by QA, but were destroyed or damaged by NMDA include GAD-65- and GAT-1 immunoreactive cells (see Table 5.1), which are presumably GABAergic amacrine cells. A summary of the effects of different toxins upon visual guidance of ocular growth and histologically distinct subsets of amacrine cells is given in Table 5.1. Some of the histologically distinct subsets of amacrine cells identified immunocytochemically in this dissertation are illustrated in Figure 5.1.

Undoubtedly, only a fraction of the cells damaged or destroyed by NMDA and QA were identified by immunocytochemistry. It seems likely that many more subtypes of cells are present in the retina than those distinguished with a limited number of histological markers. This has two implications for understanding the roles of particular populations of cells in visual guidance of ocular growth. First, NMDA-induced excitotoxicity may prevent emmetropization and FDM by damaging or destroying

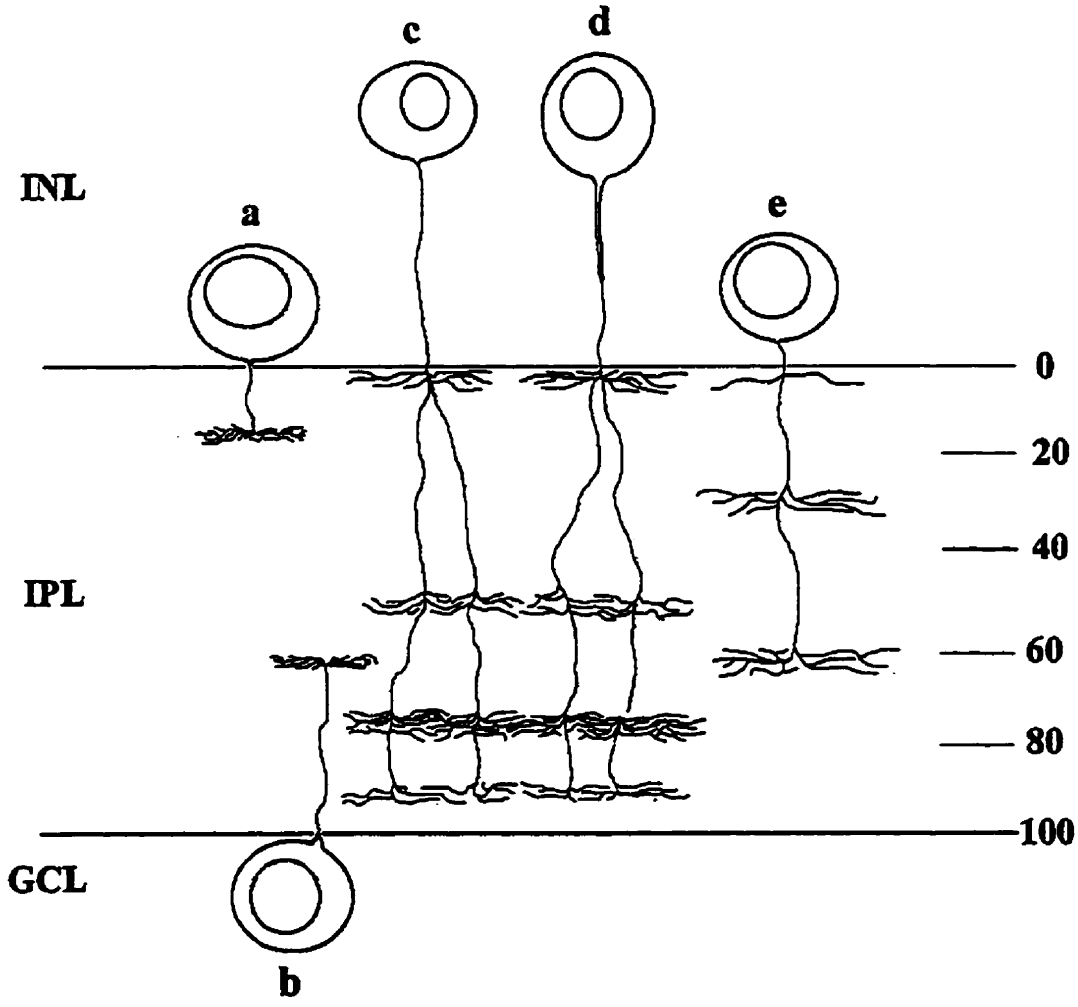
Table 5.1: Summary of the effects of toxins upon ocular growth and different subsets of retinal amacrine cells. Data on the damage caused by different toxins and effects of toxins upon ocular growth came from: (1) Chapter 3; Fischer et al., 1998f; and Frank Schaeffel (personal communication) for NMDA and QA; (2) Chapter 4 and Fischer et al., 1998c, d for ECMA; and (3) Fischer et al., 1998e and unpublished observations for colchicine.

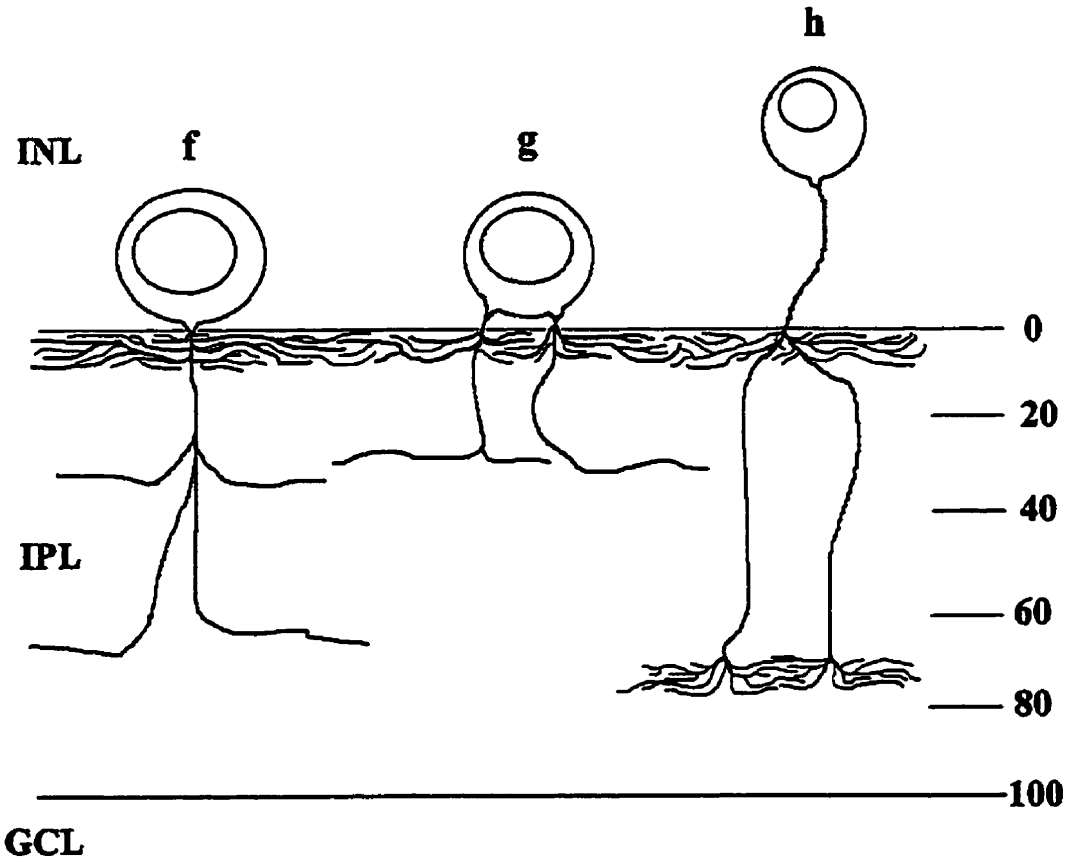
Table 5.1:

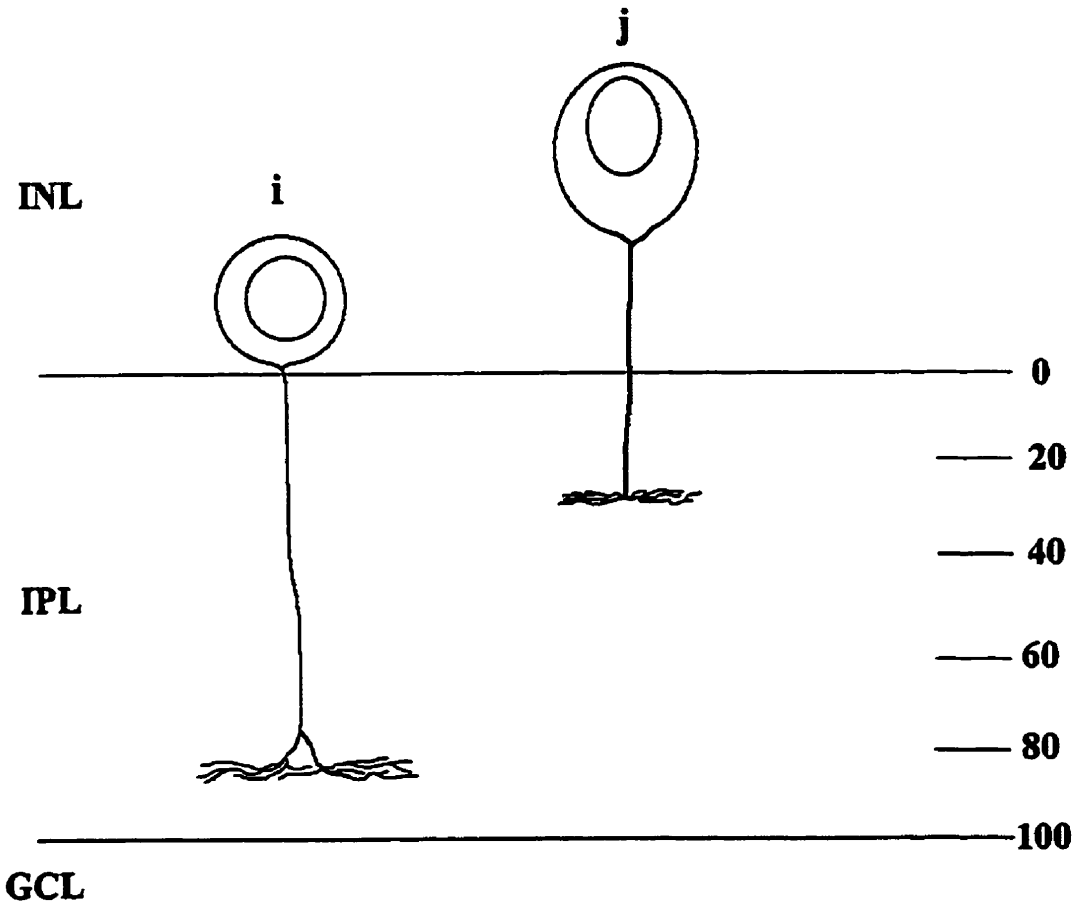
Amacrine cell type or growth-modulating stimuli	Toxin				Candidate for growth modulation
	NMDA	QA	ECMA	colchicine	
emmetropization	++++	-	-	++++	
minus lens	ND	-	ND	ND	
plus lens	ND	-	ND	ND	
form-deprivation	++++	-	ND	++++	
ChAT type-I	+	++++	++++	-	No
ChAT type-II	+++	++++	++++	-	No
ChAT type-III (non-ENSLI)	++++	++++	++	++	No
ChAT type-III (ENSLI)	+++	++++	++	++	No
VIP	+++	++++	-	++	No
TH	-	+	-	++++	No
Glucagon	-	-	-	++++	Yes
NOS type-1	ND	++++	ND	ND	No
NOS type-2	ND	++++	ND	ND	No
NOS type-3	ND	-	ND	ND	Yes
NOS type-4	ND	++++	ND	ND	No
Serotonin	+++	++++	-	ND	No
GABA	++	+	-	ND	Yes
GAD-65	++	+	-	ND	Yes
GAT-1	+++	+	-	ND	Yes
GluR1	++	++++	ND	ND	No
GluR2/3	+++	++	ND	ND	Yes
Parvalbumin	++	++++	-	+	No
PKC	+++	+++	-	-	No

(- little or no effect; + some loss of cells or affect on ocular growth; ++ moderate disruption; +++ substantial disruption; ++++ complete ablation; ND not done)

Figure 5.1: Illustrations of histologically and morphologically distinct amacrine cells that have been identified immunocytochemically. These amacrine cells include those described previously as (**a**) ChAT type-I, (**b**) ChAT type-II, (**c**) ChAT type-III non-ENSLI, (**d**) ChAT type-III ENSLI, (**e**) VIP, (**f**) TH, (**g**) glucagon, (**h**) serotonin, (**i**) parvalbumin, and (**j**) cm3.


c
d
INL
a
e
0
20
40
IPL
60
80
100
GCL
b


h
INL
f
g
0
20
40
IPL
60
80
100
GCL


j
i
INL
0
20
40
IPL
60
80
100
GCL

unidentified subsets of retinal cells. Second, some unidentified subsets of retinal cells that were not affected by QA-induced excitotoxicity might be responsible for visual guidance of ocular growth. Furthermore, immunolabelling was used to assay for the survival of histologically distinct cell-types following exposure to toxins, and this technique is not a test for viability. The persistence of immunoreactivity in a cell-type (eg. TH) in toxin-treated retinas does not necessarily indicate that the cell is fully functional, responsive to visual stimuli, or still capable of acting on target cells that are required for emmetropization (i.e., targets might be destroyed). For example, while I have shown that TH-immunoreactive cells survive QA-treatment, Frank Schaeffel (personal communication) has shown that retinal levels of dopamine are depleted by QA. Therefore, despite the persistance of TH-immunoreactivity in amacrine cells exposed to QA, dopamine synthesis and release could be severely impaired. Conversely, failure to detect cells by immunocytochemistry does not gaurantee that they are destroyed and/or non-functional.

Like NMDA, colchicine has recently been reported to induce excessive ocular growth, but to a much greater extent than that caused by NMDA (Fischer et al., 1998e). Colchicine was shown to destroy not only most ganglion cells, but also particular subsets of amacrine cells. It has been proposed that colchicine-induced ocular growth results from the destruction of TH- and/or glucagon-containing amacrine cells (Fischer et al., 1998e).

It could be argued that several redundant pathways control visually guided ocular growth, and that despite QA-induced destruction of opiate, VIPergic and cholinergic

retinal pathways (that putatively regulate ocular growth), other growth-controlling pathways in parallel with them remain intact. However, if this were true then a single pharmacological agent acting at one set of receptors in a growth-controlling pathway should *not* be able to suppress FDM. In theory, an antagonist or agonist that prevents FDM is acting upon only one signalling pathway.

Conclusions:

Currently, it is impossible to determine where in the eye atropine acts or to characterize all of the effects that it might elicit. Furthermore, it remains uncertain where and how muscarinic antagonists suppress FDM. Evidence offered in this dissertation has, however, indicated that retinal sources of ACh are not required for emmetropization or the development of FDM, and that atropine does not suppress FDM by antagonizing the actions of ACh in the retina. It is unlikely that FDM is suppressed by atropine's antagonism of ACh released in the choroid, because removal of cholinergic innervation arising from the ciliary ganglion has little effect upon normal or form-deprivation-induced ocular growth (Wallman et al., 1981; Schaeffel et al., 1988; Troilo, 1990; Lin and Stone, 1991; Troilo and Wallman, 1991). Therefore atropine may suppress FDM by acting directly upon scleral chondrocytes (via non-mAChRs) to reduce the synthesis of proteoglycans, or (more likely) by acting at non-muscarinic signalling pathways in the retina, RPE or choroid.

Evidence offered in this dissertation excludes a number of retinal amacrine cell types and the transmitters that they produce as being crucial components of growth-

controlling mechanisms. Subsets of amacrine cells that were previously thought to control ocular growth, but are no longer candidate growth-regulators in light of evidence offered in this dissertation, include those that produce ENK and neurotensin, ACh, and VIP. It remains uncertain which amacrine cells *are* critical for growth regulation. Amacrine cells that contribute to the regulation of ocular growth may include those that: (i) are responsive to NMDA (Fischer et al., 1998g); (ii) are resistant to QA and ECMA (Chapters 3 and 4; Fischer et al., 1998c, d, f); (iii) are destroyed by colchicine (Fischer et al., 1998e); and (iv) differentially accumulate or express Fos (McGuire and Stell, 1998), Egr-1 (Fischer et al., 1998a), cGMP (cyclic guanidine monophosphate; Fischer and Stell, 1998b), or phospho-ERK (Fischer and Stell, 1998c) in response to growth-regulating visual stimuli. The effects of growth-modulating visual stimuli and NMDA upon activity-dependent markers expressed or accumulated by amacrine cells are listed in Table 5.2. Potential growth-modulating pathways in the eye are summarized by the schematic diagram in Figure 5.2.

Glutamatergic transmission at NMDA, AMPA and metabotropic receptors is somehow involved in the retinal pathways that control ocular growth. We have recently reported that antagonists to NMDA receptors prevent FDM (Fischer et al., 1998g). Since NMDA receptors are expressed only by amacrine and ganglion cells in the chick retina (Fischer et al., 1998g), it seems likely that activation of NMDA receptors expressed by amacrine cells somehow plays a role in ocular growth control. Similarly, the OFF-channel glutamate receptor antagonist cis-2,3-piperdine-dicarboxylic acid (PDA), which is known to be selective for kainate receptors on chick OFF-bipolars (Morgan, 1987b),

Table 5.2: Affects of different growth-modulating visual stimuli upon rates of ocular growth and numbers of amacrine cells that express or accumulate different activity-dependent markers.

Table 5.2

Visual Stimuli	Affects on rates of ocular growth	Activity-dependent marker			
		Fos	Egr-1	pERK	cGMP
goggle-ON	⇑	⇑	⇓	⇑	ND
goggle-OFF	⇓	⇑	⇑	➡	ND
7 dioptres plus defocus	⇓	⇑	⇑	➡	⇓
7 dioptres minus defocus	⇑	ND	⇓	➡	ND

(⇑ increase, ⇓ decrease, ➡ no change, ND not done)

Figure 5.2: Schematic diagrams of growth-enhancing pathways in (**a**) open and (**b**) form-deprived eyes.

Fig. 5.2a: Growth-slowing pathways in open eyes

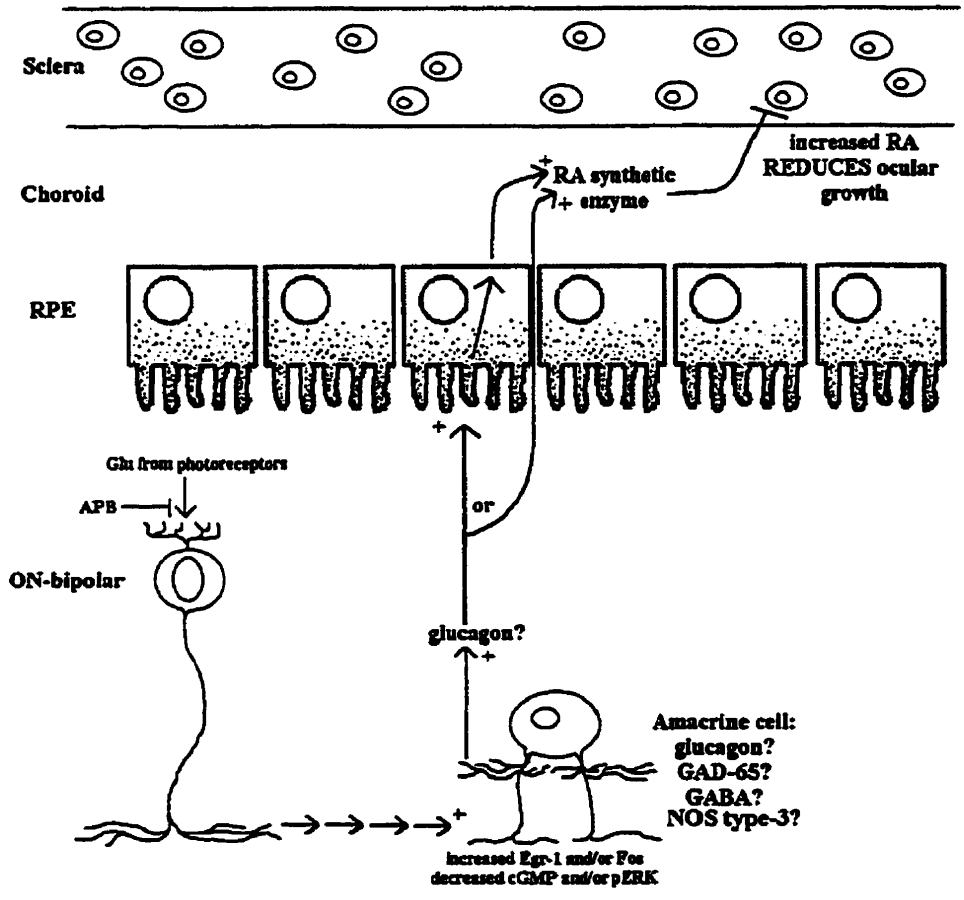
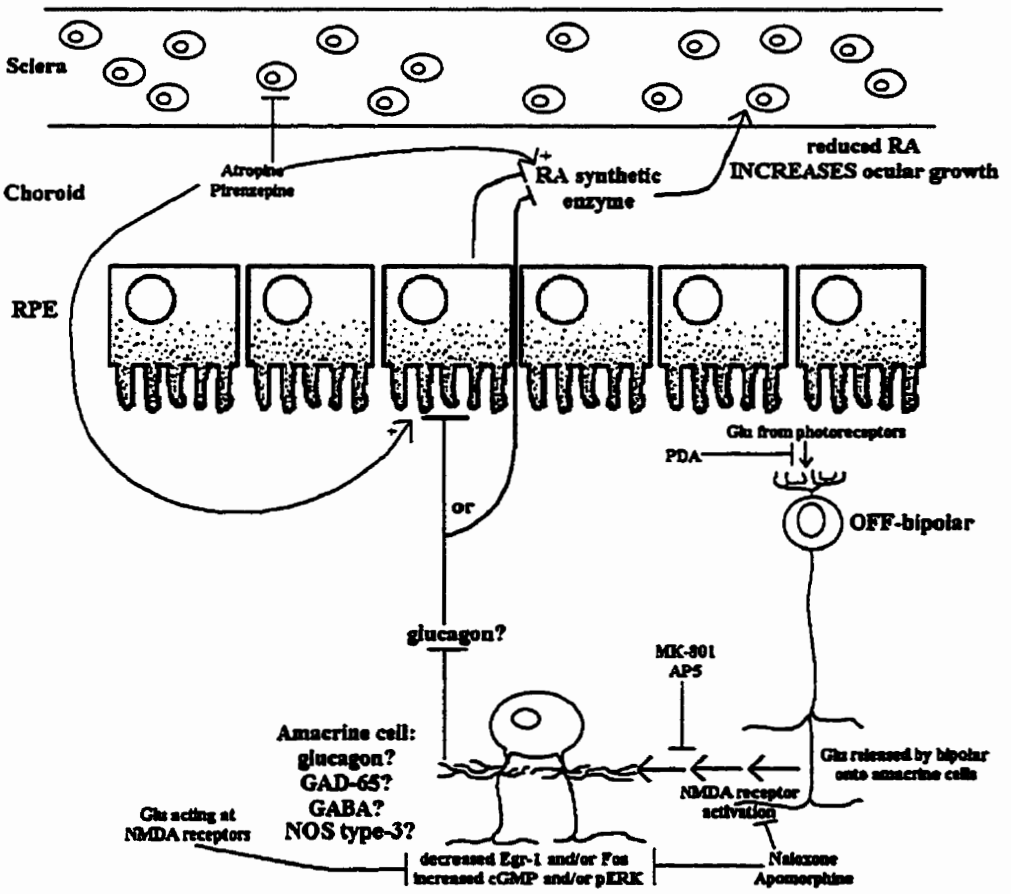


Fig. 5.2b: Growth-enhancing pathways in form-deprived eyes



has no effect on open eyes, but suppresses FDM (Xie et al., 1992; Crewther et al., 1996). In contrast, the ON-channel antagonist (mGluR6 agonist) L-2-amino-4-phosphonobutyrate (APB or L-AP4) causes hyperopic refractive error and decreased axial lengths in the open eyes of kittens (Smith et al., 1985) and chicks (Xie et al., 1992; Crewther et al., 1996), while having no effect on FDM. Taken together, these results might suggest that normal "grow" and form-deprivation "grow" signals originate independently from ON and OFF channels, respectively, in the retina (Fig. 5.2).

Glucagon-containing amacrine cells are promising candidates as modulators of ocular growth because these cells are destroyed by colchicine (Fischer et al., 1998e), are resistant to QA and ECMA (Chapters 3 and 4; Fischer et al., 1998c, d, f), accumulate phospho-ERK during form-deprivation (Fischer and Stell, 1998c), and up-regulate Egr-1 in response to NMDA or growth-slowing visual stimuli, but down-regulate Egr-1 in response to growth-enhancing visual stimuli (Fischer et al., 1998a). However, the ability of glucagon to modulate ocular growth has not been tested. It is possible that a neurotransmitter other than glucagon, also released by glucagon-immunoreactive cells, might regulate ocular growth. Further investigations are required to test whether glucagon-immunoreactive amacrine cells regulate ocular growth and whether vision-dependent changes in Egr-1 in these cells are crucial to visually guided ocular growth.

Alternatively, NOS type-3 cells may be candidate growth-modulators, since these cells are resistant to QA (Fischer and Stell, 1998a). The NOS inhibitor L-NAME has been reported to suppress FDM (Fujikado et al., 1997). However, recent studies in our lab have been unable to confirm these results using the NOS-inhibitors L-NAME, L-

NMMA, or L-NIO (Gudgeon et al., 1998). Therefore, NO seems unlikely to be a retina-derived growth modulator. However, the NOS type-3 cells that survive exposure to QA cannot be excluded as candidate growth-modulators, because a non-NO transmitter released by these cells could regulate ocular growth.

Future directions in myopia research:

Research directed toward identifying the retinal cells, circuitry and transmitters that regulate ocular growth will have to adopt new strategies and techniques (other than conventional pharmacological approaches) to obtain meaningful answers. Conventional pharmacological approaches have proven useful in demonstrating that drug therapies could be applied to control abnormal ocular growth, but have proven useless for identifying the endogenous transmitters and circuitry required for the visual guidance of ocular growth.

One technique that is producing promising data to identify candidate growth-modulating cells is activity-dependent labelling. In theory, growth-modulating visual stimuli (i.e., form-deprivation, plus or minus defocus) should alter the activity of many retinal cells, some of which should be crucial components in the control of growth. Recently, I have found that retinal levels of cGMP, phospho-ERK, and Egr-1 are modulated by growth-modulating stimuli (Fischer et al., 1998a, b; Fischer & Stell, 1998c).

Vision-dependent accumulation of cGMP:

Among the many characterized functions of cGMP is its action as the intracellular ligand to a subtype of ion channel (cGMP-gated cation channel) that modulates the electrical activity of photoreceptors and ON-bipolar cells. It remains uncertain whether similar mechanisms are used by other cell-types in the retina, but it seems likely that cGMP participates in other signalling pathways (e.g. cGMP-dependent protein kinase-mediated pathways). We have shown that cGMP-immunoreactivity is present in a few subsets of amacrine cells in the untreated chick retina (Table: 5.2; Fischer and Stell, 1998b). This labelling is abolished by two hours of plus-lens wear, while minus-lens wear has no effect. The effects of goggling and removal of a goggle on cGMP-immunolabelling in the retina remain to be determined.

cGMP is produced by the enzyme guanylate cyclase (GC) whose activity is enhanced directly by the labile free radical nitric oxide (NO; reviewed by Zhang & Snyder, 1995). Retinal levels of cGMP can be enhanced in different cell-types by the NO donor SNP (Gudgeon et al., 1998). Co-application of 40 nmol SNP with the PDE (phosphodiesterase) inhibitor IBMX (isobutylmethylxanthine) causes a dramatic increase in cGMP-immunoreactivity in bipolar cells 2 hours after treatment. These cGMP-immunoreactive bipolar cells do not contain M3 or calbindin-immunoreactivities. It seems likely that these cells are ON-bipolars, since ON-bipolars are known to express GC and accumulate cGMP in the light. Lower doses of SNP (4 nmol) cause a differential accumulation cGMP in at least 3 different subsets of amacrine cells, with only low levels of cGMP in bipolar cells. Since plus-lens wear (that slows ocular growth) reduces levels

of cGMP in amacrine cells, it is possible that SNP plus IBMX-induced accumulation of cGMP prevents FDM or lens-induced myopia. Chronic adminstration of toxic doses (1000 nmol) of SNP prevents FDM and results in severely flattened eyes with drastically shortened axial lengths (Gudgeon et al., 1998). Even lower doses of SNP, that cause cGMP to accumulate in amacrine cells, may suppress FDM, by damaging photoreceptors of other retinal cells (Gudgeon et al., 1998). Further studies are required to determine whether modulation of cGMP synthesis in amacrine cells plays a role in modulating ocular growth.

Vision-dependent accumulation of phospho-ERK:

The pERK (phospho-ERK or p44/42 MAP kinase) pathway is a protein kinase cascade that links growth and differentiation signals at the cell surface to transcriptional activation in the nucleus. Growth factor receptors (in particular receptors to FGF), increased concentration of intracellular calcium ions, and tyrosine kinases activate Ras, which in turn causes the sequential activation of c-Raf, MEK, and ERK (Sturgill et al., 1988; Payne et al., 1991; Cowley et al., 1994; Hunter, 1995; Hill & Triesman, 1995; Marshall, et al., 1995;). Once ERK has been phosphorylated by MEK, it is translocated to the nucleus and promotes transcription by phosphorylation of transactivators such as Elk-1 and Stat3. Recently, a selective and potent inhibitor of this MAP kinase cascade, PD98059, has been identified (Payne et al., 1991). This compound binds to MEK, thereby inactivating it by preventing phosphorylation by c-Raf.

In the untreated chick retina, pERK immunoreactivity is present in at least 5

different subtypes of amacrine cells (Fischer and Stell, 1998c). Two hours of form-deprivation or intravitreal NMDA cause a dramatic accumulation of pERK in the distal IPL and in the cell bodies of additional subsets of amacrine cells, including those that contain glucagon. It would be interesting to test whether ERK transduction cascades are activated in scleral chondrocytes following the onset of form-deprivation, since Rohrer et al. (1995) found that growth factors such as bFGF and TGF-β modulate ocular growth, possibly through direct actions on the sclera after intraorbital injection. It remains uncertain whether form-deprivation-induced increases in pERK result from decreased illumination imposed by the occluder or by the absence of sufficient contrast and required spatial frequencies. It is possible that increased excitatory stimulation through OFF-pathways in the distal IPL, resulting from reduction of illumination by goggles, could be responsible for activation of the ERK cascade. For example, in goldfish retina the distal IPL (or OFF-sublamina) contains the axon terminals of OFF-bipolar cells (Famiglietti & Kolb, 1976). Furthermore, it remains uncertain whether this vision-dependent accumulation of pERK contributes to the enhanced rates of form-deprivation-induced ocular growth, or is merely caused by the lack of visual stimuli without affecting rates of ocular growth. It would be of interest to test whether the ERK cascade-inhibitor PD98059 prevents FDM and the form-deprivation-induced increase in pERK.

Vision-dependent expression of Egr-1:

Recently, we have examined the vision-dependent expression of an immediate early gene, Egr-1, using affinity-purified antibodies applied to sections of retina (Fischer

et al., 1998a). The number of Egr-1-expressing amacrine cell nuclei was reduced by form-deprivation or minus-defocus, while such expression was increased by recovery from form-deprivation, plus-defocus, or intravitreal application of NMDA (Table 5.2). These vision-dependent changes in Egr-1 labelling were prevented by intravitreal application of the NMDA receptor antagonist MK-801. Among the amacrine cells that differentially expressed Egr-1 were those that contain glucagon. The proportion of glucagon-immunoreactive amacrine cells that expressed Egr-1 was decreased by minus-defocus, while plus-defocus had the opposite effect. Egr-1, an inducible nuclear protein with zinc-finger DNA-binding domains similar to those of the Sp1 transcription factor (Lemaire et al., 1988), may suppress genes whose expression is normally promoted by Sp1 by competing with Sp1 for binding at overlapping sequences of upstream regulatory elements. Visually guided ocular growth may be controlled by retinal changes in Egr-1 expression in amacrine cells, possibly in those that contain glucagon. However, it remains unknown whether increases in Egr-1 result in decreases glucagon gene transcription. Currently, studies are underway to test whether antisense-mediated knock-down of Egr-1 expression affects visual guidance of ocular growth.

Gene transfer and retinal control of ocular growth:

Virus-mediated gene transfer is another technique which holds great promise as a powerful experimental tool. Recombinant adenovirus has been used to transfer exogenous DNA into retinal cells including those of mouse (Bennet et al., 1994; Li et al., 1994; Bundenz et al., 1995), rabbit (Abraham et al., 1995), and embryonic chick

(Yamagata et al., 1994). In the embryonic chick retina, what appear to be Müller cells (but were reported to be midget bipolar cells) are efficiently transfected with exogenous DNA in adenoviral vectors (Yamagata et al., 1994). In hatched chicks, however, retinal cells become less susceptible to transfection by adenovirus than in embryos, and transgene expression is limited to RPE and Müller cells within 1 mm of the site of injection (Fischer et al., 1998h). Furthermore, adenovirus preferentially tranfects cells that express receptors for fibronectin and/or laminin (Belin and Boulanger, 1993), and, in the chick retina, these receptors are expressed only by Müller and RPE cells (Fischer et al., 1998h). These results suggest that, in the chick retina, gene transfer via adenoviral vectors may be limited to Müller cells and the RPE.

Recombinant sindbis virus is another vector system by which genetic manipulations might be elicited in many different cells (Xiong et al., 1989). However, preliminary results indicate that sindbis is incapable of transfering genes into cells of the chick retina (Fischer et al., 1998h). Since adenovirus and sindbis virus vectors will be of little use to transfect genes into post-mitotic neurons of the chick retina, retroviruses may be a successful alternative; but these will require *in ovo* manipulations since retroviruses only transfer genes into dividing cells (Morgan & Fekete, 1996). In theory, these genetic manipulations could allow for cell-specific changes in phenotype, such as suppressed expression of an enzyme or a receptor isoform. Future work in myopia research should include these new cell-specific manipulations to avoid the many problems of non-specificity and unknown side-effects associated commonly with pharmacological and toxicological techniques or whole-cell ablation. This work should also aspire to separate

neurochemical changes responsible for ocular growth-regulation from those changes that are casual by-products of growth-regulating visual stimuli.

References:

Abraham, N.G., da Silva, J.L. Lavorsky, Y., Stoltz, R.A., Kappas, A., Dunn, M.W. & Schwartzman, M.L. (1995). Adenovirus-mediated heme oxygenase-1 gene transfer into rabbit ocular tissues. Invest. Ophthalmol. Vis. Sci. 36: 2202-2210.

Ali, S.F., Hong, J.S. & Bondy, S.C. (1993). Response of neuropeptides and neurotransmitter binding sites in the retina and brain of the developing chick to reduced visual input. Int. J. Developmental Neurol. 1: 99-103.

Allen, Y.S., Marchbanks, R.M. & Sinden, J.D. (1988). Non-specific effects of the putative cholinergic neurotoxin ethylcholine aziridinium ion in the rat brain examined by autoradiography, immunocytochemistry, and gel electrophoresis. Neurosci. Lett. 95: 69-74.

Ankarcrona, M., Dypbupt, J.M., Bonofoco, E., Zhivotovsky, B., Orreninus, S., Lipton, S.A. & Nicotera, P. (1995). Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15: 961-973.

Arends, M.J. & Wyllie, A.H. (1991). Apoptosis: mechanisms and roles in pathology. Int. Rev. Exp. Path. 32: 223-254.

Ariel, M. & Daw, N.W. (1982a). Effects of cholinergic drugs on receptive field properties of rabbit retinal ganglion cells. J. Physiol. Lond. 324: 135-160.

Ariel, M. & Daw, N.W. (1982b). Pharmacological analysis of directionally sensitive rabbit retinal ganglion cells. J. Physiol. Lond. 324:161-186.

Barlow, P. & Marchbanks, R.M. (1984). Effect of ethylcholine mustard on choline dehydrogenase and other enzymes of choline metabolism. J. Neurochem. 43: 1568-1574.

Bartmann, M.L. & Schaeffel, F. (1994). A simple mechanism for emmetropization without cues from accommodation or colour. Vision Res. 34: 873-876.

Barrington, M., Sattayasai, J., Zappia, J. & Ehrlich, D. (1989). Excitatory amino acids interfere with normal eye growth in posthatch chicks. Curr. Eye Res. 8:781-792.

Baughman, R.W. & Bader, C.R. (1977). Biochemical characterization and cellular localization of the cholinergic system in the chicken retina. Brain Res. 138: 469-486.

Bedrossian, R.H. (1971). The effect of atropine on myopia. Ann. Ophthal. 3: 891-897.

Bedrossian, R.H. (1979). The effect of atropine on myopia. Am. J. Ophthal. 86: 713-717.

Belin, M.T. & Boulanger, P. (1993). Involvement of cellular adhesion sequences in the attachment of adenovirus to the HeLa cell surface. J. Gen. Virol. 74: 1485-1497.

Bennett, J., Wilson, J., Sun, D., Forbes, B. & Maguire, A. (1994). Adenovirus vector-mediated in vivo gene transfer into adult murine retina. Invest. Ophthal. Vis. Sci. 35: 2535-2542.

Bonner, T.I., Buckley, N.J., Young, A.C. & Brann, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. Science 237: 527-532.

Bonner, T.I., Young, A.C., Brann, M.R. & Buckley, N.J. (1988). Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. Neuron 1: 403-410.

Brecha, N. (1983). Retinal neurotransmitters: Histochemical and biochemical studies. In P.C Emson (ed): Chemical Neuroanatomy. New York: Raven Press, pp. 85-129.

Brecha, N., Karten, H.J. & Laverack, C. (1979). Enkephalin containing amacrine cells in the avian retina: immunohistological localization. Proc. Natl. Acad. Sci. USA 76: 3010-3014.

Buckley, N.J., Bonner, T.I., Buckley, C.M. & Brann, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. Mol. Pharmacol. 35: 469-476.

Bundenz, D.L., Bennet, J., Alonso, L. & Maguire, A. (1995). In vivo gene transfer into murine corneal endothelial and trabecular meshwork cells. Invest. Ophthal. Vis. Sci. 36: 2211-2215.

Campochiaro, P., Ferkany, J.W. & Coyle, J.T. (1985). Excitatory amino acid analogs evoke release of endogenous amino acids and acetylcholine from chick retina in vitro. Vision Res. 25: 1375-1378.

Choi, D.W. (1985). Glutamate neurotoxicity in cortical cell culture is calcium dependent. Neurosci. Letts. 58: 293-297.

Choi, D.W. (1988). Glutamate neurotoxicity and diseases of the nervous system. Neuron 1:623-634.

Choi, D.W. (1992). Excitotoxic cell death. J. Neurobiol. *23*: 1261-1276.

Conley, M., Fitzpatrick, D. & Lachica, E.A. (1986). Laminar asymmetry in the distribution of choline acetyltransferase-immunoreactive neurons in the retina of the tree shrew (*Tupaia belangeri*). Brain Res. 399:332-338.

Cowley, S., Paterson, H., Kemp, P. & Marshall, C.J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77: 841-852.

Coyle, J.T., Biziere, K. & Schwarcz, R. (1978). Neuro-toxicity of excitatory amino acids in the neural retina. In: Kainic Acid as a Tool in Neurobiology (eds. McGeer, E.G., McGeer, P & Olney, J.W.). Raven Press: New York, NY. pp. 177-188.

Creason, S., Tietje, K. & Nathanson, N.M. (1995). Characterization of a chick m5 muscarinic acetylcholine receptor. Soc. Neurosci. Abstracts 21: 2037.

Crewther, D.P., Crewther, S.G. & Xie, R.Z. (1996). Changes in eye growth by drugs which affect retinal On or OFF responses to light. J. Ocular Pharmacol. Therapeutics 12: 193-208.

Curtin, B.J. (1985). The Myopias: Basic Science and Clinical Management. Harper & Row: Philidelphia, PA.

D'Mello, S.R.,Galli, C., Ciotti, T. & Calissano, P. (1993). Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc. Natl. Acad. Sci.USA 90: 10989-10993.

Dorje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E. & Brann M.R. (1991). Antagonists binding profiles of five cloned human muscarinic receptor subtypes. J. Pharmacol. Exp. Ther. 256: 727-733.

Dvorak, D.R. & Morgan, I.G. (1983). Intravitreal kainic acid permanently eliminates OFF-pathways from chicken retina. Neurosci. Letts. 36: 249-253.

Dyer, J.A. (1979). Role of cycloplegics in progressive myopia: Ophthal. 86: 692-694.

Ebert, B., Thorklidsen, C., Andersen, S., Christrup, L.L. & Hjeds, H. (1998). Opiod analgesics as noncompetitive N-methyl-D-Aspartate (NMDA) antagonists. Biochem. Pharmacol. 56: 553-559.

Eckenstein, F., Baughman, R.W., Sofroniew, M.V. & Thibault, J. (1983). A comparison of the distribution of choline acetyltransferase and tyrosine hydroxylase immunoreactivities in the rat retina. Soc. Neurosci. Abs. 9:80.

Eckenstein, F. & Thoenen, H. (1982). Production of specific antisera and monoclonal antibodies to choline acetyltransferase: Characterization and use for identification of cholinergic neurons. EMBO J. 1: 363-368.

Ehrlich, D, Sattayasai, J., Zappia, J. & Barrington, M. (1990). Effects of selective neurotoxins on eye growth in the young chick. In: Myopia and the Control of Eye Growth (Bock, G.R. & Widdows, K., eds). Chichester: Wiley. Ciba Foundation Symposium. 155: 63-84.

Estrada, C., Triguero, D., Martin del Rio, R., & Gómez-Ramos, P. (1988). Biochemical and histological modifications of the cat retina induced by the cholinoselective neurotoxin AF64A. Brain Res. 439: 107-115.

Famiglietti, E.V. (1983). 'Starburst' amacrine cells and cholinergic neurons: mirror-symetric on and off amacrine cells of rabbit retina. Brain Res. 261: 138-144.

Famiglietti, E.V. & Kolb, H. (1976). Structural basis for ON- and OFF-center responses in retinal ganglion cells. Science 194:193-195.

Ferreira, I.L., Duarte, C.B., Santos, P.F., Carvalho, C.M. & Carvalho, A.P. (1994). Release of [^{3}H]GABA evoked by glutamate receptor agonists in cultured chick retina cells: effect of Ca^{2+}. Brain Res. 664: 252-256.

Fischer, A.J., McGuire, J.J., Schaeffel, F. & Stell, W.K. (1998a). Vision-dependent expression of Egr-1 in the chick retina. (in preparation).

Fischer, A.J., McKinnon, L.A., Nathanson, N.M. & Stell, W.K. (1997a). Identification and localization of muscarinic acetylcholine receptors (mAChRs) in ocular tissues of the chick. Invest. Ophthal. Vis. Sci. (Suppl). 38: 759.

Fischer, A.J., McKinnon, L.A., Nathanson, N.M. & Stell, W.K. (1988b). Localization and muscarinic acetylcholine receptors in the ocular tissues of the chick. J. Comp. Neurol. 392: 273-284.

Fischer, A.J., Miethke, P., Morgan, I.G. & Stell, W.K. (1998c). Cholinerigic amacrine cells are not required for the progression and atropine-mediated suppression of form-deprivation myopia. Brain Res. 794: 48-60.

Fischer, A.J., Miethke, P., Morgan, I.G. & Stell, W.K. (1998d). Retinal sources of acetylcholine (ACh) do not participate in visually guided ocular growth or atropine-mediated suppression of form-deprivation myopia (FDM). Invest. Ophthal. Vis. Sci. (Suppl). 39: 717.

Fischer, A.J., Morgan, I.G. & Stell, W.K. (1998e). Colchicine causes excessive ocular growth and myopia in chicks. Vision Res. 39: 685-697.

Fischer, A.J., Rohrer, B., Quong, R. & Stell, W.K. (1998h). Virus-mediated transfer of cell-specific genes into the retina of the chicks. (in preparation).

Fischer, A.J., Seltner, R.L.P., Poon, J. & Stell, W.K. (1998f). Immunocytochemical characterization of NMDA and QUIS-induced excitotoxicity in the retina of the chick. J. Comp. Neurol. 393: 1-15.

Fischer, A.J., Seltner, R.L.P. & Stell, W.K. (1997b). NMDA-induced excitotoxicity causes myopia in post-hatch chicks. Can. J. Ophthal. 32: 373-377.

Fischer, A.J. Seltner, R.L.P. & Stell, W.K. (1998g). Opiate and N-methyl-D-aspartate receptors in form-deprivation myopia. Vis. Neurosci. (in press).

Fischer, A.J. & Stell, W.K. (1998a). Nitric oxide-synthase-containing cells in the chick eye. J. Comp. Neurol. (accepted for publication).

Fischer, A.J., & Stell, W.K. (1998b). Vision-dependent accumulation of cGMP in neurons of the chick retina. (in preparation).

Fischer, A.J. & Stell, W.K. (1998c). Vision-dependent accumulation of phoso-ERK in amacrine cells of the chick retina. (in preparation).

Fisher, A., Mantione, C.R., Abraham, D.J., & Hanin, I. (1982). Long-term central cholinergic hypofunction induced in mice by ethylcholine aziridinium ion (AF64A). in vivo, J. Pharmacol. Exp. Therap. 222: 140-145.

Fujii, S., Honda, S., Sekiya, Y., Yamasaki, M., Yamamoto, M. & Saijoh, K. (1998). Differential expression of nitric oxide synthase isoforms in form-deprived chick eyes. Curr. Eye Res. 17: 586-593.

Fujikado, T., Kawasahi, Y., Fujii, J., Taniguchi, N., Okada, M., Suzuki, A., Ohmi, G., & Tano, Y. (1997). The effect of nitric oxide synthase inhibitors on form-deprivation myopia. Cur. Eye Res. 16: 992-996.

Furukawa, K, Abe, Y., Sorimachi, M. & Akaike, N. (1994). Nicotinic and muscarinic acetylcholine responses in the embryo chick ciliary ganglion cells. Brain Res. 657: 185-190.

Gadbut, A.P. & Galper, J.B. (1994). A novel M3 muscarinic acetylcholine receptor is expressed in chick atrium and ventricle. J. Biol. Chem. 269: 25823-25829.

Gibson, B.L. & Reif-Lehrer, L. (1984). In vitro effects of kainate on embryonic and posthatching chick retina. Dev. Brain Res. 15: 97-103.

Gimbel, H.V. (1973). The control of myopia with atropine. Can. J. Ophthal. 8: 527-532.

Golcich, M.A., Morgan, I.G. & Dvorak, D.R. (1990). Selective abolition of OFF responses in kainic acid-lesioned chicken retina. Brain Res. 535: 288-300.

Gómez-Ramos, P., Galea, E. & Estrada, C. (1990). Neuronal and microvascular alterations induced by the cholinotoxin AF64A in the rat retina. Brain Res. 520: 151-158.

Goss, D.A. (1982). Attempts to reduce the rate of increased myopia in young people -- a critical literature review. Amer. J. Optom. Physiol. 59: 828-841.

Gostin, S.B. (1962). Prophylactic management of progressive myopia. Southern Med. J. 55: 916.

Gottlieb, M., Fugate-Wentzek, L.A. & Wallman, J. (1987). Different visual deprivations produce different ametropias and different eye shapes. Invest. Ophthal. Vis. Sci. 28: 1225-1235.

Gottlieb, M. & Wallman, J. (1985). Retinal activity modulates eye growth: evidence from rearing in stroboscopic illumination. Soc. Neurosci. Abstract 13: 1297.

Gudgeon, J.H.R, Fischer, A.J. & Stell, W.K. (1998). Nitricoxidergic signalling pathways and the visual guidance of ocular growth. (in preparation).

Hamassaki-Britto, D.E., Brzozowska-Prechtl, A., Karten, H.J., Lindstrom, J.M. & Keyser, K.T. (1991). GABA-like immunoreactive cells containing nicotinic acetylcholine receptors in the chick retina. J. Comp. Neurol. 313: 394-408.

Hamassaki-Britto, D.E., Brzozowska-Prechtl, A., Karten, H.J. & Lindstrom, J.M. (1994). Bipolar cells of the chick retina containing α-bungarotoxin-sensitive nicotinic acetylcholine receptors. Vis. Neurosci. 11: 63-70.

Hanin, I. (1990). AF64A-induced cholinergic hypofunction. Prog. Brain Res. 84: 289-299.

Hanin, I. (1996). The AF64A model of cholinergic hypofunction: an update. Life Sciences 55: 1955-1964.

Haywood, J., Hambley, J. & Rose, S. (1975). Effects of exposure to imprinting stimulus on the activity of enzymes involved in acetylcholine metabolism on the chick brain. Brain Res. 92 : 219-225.

Hill, C.S. & Triesman, R. (1995). Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 80: 199-211.

Hodos, W. & Erichsen, J.T. (1990). Lower-field myopia in birds: an adaptation tha keeps the ground in focus. Vision Res. 30: 653-658.

Hodos, W. & Kuenzel, W.J. (1984). Retinal image degradation produces ocular enlargement in chicks. Invest. Opthal. Vis. Sci. 25: 652-659.

Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signalling. Cell 80: 225-236.

Hughes, T.E., Carey, R.G., Victorica, J., Dé Blas, A.L. & Karten, H.J. (1989). Immunohistochemical localization of $GABA_A$ receptors in the retina of the new world primate *Samiri sciureus*. Vis. Neurosci. 2: 565-581.

Hutchins, J.B. (1994). Development of muscarinic acetylcholine receptors in the ferret retina. Dev. Brain Res. 82: 45-61.

Hutchins, J.B. & Hollyfield, J.G. (1985). Acetylcholine receptors in the human retina. Invest. Ophthalmol. Vis. Sci. 11: 1550-1557.

Imperato, A., Porceddu, M.L. Morelli, M., Fossarello, M. & DiChiara, G. (1981). Benzodiazepines prevent kainate-induced loss of GABAergic and cholinergic neurons in the chick retina. Brain Res. 213: 205-210.

Ingham, C.A. & Morgan, I.G. (1983). Dose-dependant effects of intravitreal kainic acid on specific cell types in the chicken retina. Neurosci. 9: 165-181.

Irving, E.L., Sivak, J.G. & Callender, M.G. (1992). Refractive plasticity of the developing chick eye. Ophthal. Physiol. Optics 12: 448-456.

Jaffe, J.H. & Martina, W.R. (1985). Opioid analgesics and antagonists. In Goodman and Gilman's The Pharmacological Basis of Therapeutics 7th ed, ed, Goodman, A.G., Goodman, L.S., Rall, T. & Murad, F., pp. 491-531.

James, W.M. & Klein, W.L. (1985). Alpha-bungarotoxin receptors on neurons isolated from turtle retina: molecular heterogeneity of bipolar cells. J. Neurosci. 5: 352-361.

Kaymak, H., Hagel, G. & Schaeffel F. (1997). Intravitreal atropine suppresses deprivation myopia in chick more effeciently than dopamine agonists. Invest. Ophthal. Vis. Sci. (Suppl.) 38: S758.

Kerr, J.F.R., Wyllie, A.H. & Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. British J. Cancer 26: 239-257.

Keyser, K.T., Hughes, T.E., Whiting, P.J., Lindstrom, J.M. & Karten, H.J. (1988). Cholinoceptive neurons in the retina of the chick: an immunohistochemical study of the nicotinic acetylcholine receptors. Vis. Neurosci. 1: 349-366.

Kittila, C.A. & Massey, S.C. (1997). Pharmacology of directionally selective ganglion cells in the rabbit retina. J. Neurophysiol. 77: 675-689.

Kiyama, H., Katayama-Kumoi, Y., Kimmel, J., Steinbusch, H., Powell, J.F., Smith, A.D. & Tohyama, M. (1985). Three dimensional analysis of retinal neuropeptides and amines in the chick. Brain Res. Bull. 15: 155-165.

Kleinschmidt, J., Zucker, C.L. & Yazulla, S. (1986a). Neurotoxic action of kainic acid in the isolated toad and goldfish retina: I. Description of effects. J. Comp. Neurol. 254:184-195.

Kleinschmidt, J., Zucker, C.L. & S. Yazulla, S. (1986b). Neurotoxic action of kainic acid in the isolated toad and goldfish retina: II. Mechanism of action. J. Comp. Neurol. 254:196-208.

Lam, D.M.K. (1972). Biosynthesis of acetylcholine in turtle retina photoreceptors. Proc. Natl. Acad. Sci. USA. 69: 1987-1991.

Large, T.H., Rauh, J.J., De Mello, F.G. & Klein, W.L. (1985). Two molecular weight forms of muscarinic acetylcholine receptors in the avian central nervous system: Switch in predominant form during differentiation of synapses. Proc. Natl. Acad. Sci. USA. 82: 8785-8789.

Laties, A.M. & Stone, R.A. (1992). Treatment and control of ocular development. US Patent #5,122,522, June 16, 1992.

Lauber, J.K. & Oishi, T. (1990). Kainic acid and formoguanaime effects on environmentally-induced eye lesions in chicks. J. Ocular Pharm. 6: 151-156.

Leech, E.M., Cottriall, C.L. & McBrien, N.A. (1995). Pirenzepine prevents form deprivation myopia in a dose dependant manner. Ophthal. Physiol. Opt. 15: 351- 356.

Lemaire, P., Revelant, O., Bravo, R. & Charnay, P. (1988) Two genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc. Natl. Acad. Sci. USA 85: 4691-4695.

Li, T, Adamian, M., Roof, D.J., Eliot, L.B., Dryja, T.P., Roessler, B.J. & Davidson, B.L. (1994). In vivo transfer of a reporter gene to the retina mediated by an adenoviral vector. Invest. Ophthal. Vis. Sci. 24: 2543-2549.

Li, X.X, Schaeffel, F., Kohler, K. & Zrenner, E. (1992). Dose-dependent effects of 6-hydroxy-dopamine on deprivation myopia, electroretinograms & dopaminergic amacrine cells in chickens. Vis. Neurosci. 9: 483-492.

Lin, T. & Stone, R.A. (1991). Autonomic and visual interactions in the regulation of eye growth and refraction. Invest. Ophthal. Vis. Sci. (Suppl.). 32: 1202.

Lind, G.J., Chew, J.J., Marzani, D. & Wallman, J. (1997). M1-subtype muscarinic receptor antagonists reduce matrix production by scleral chondrocytes from chicks, Life Sciences 60: 13-14.

Lopez-Colome, A.M. & Somhano, F. (1984). Localization of L-glutamate and L-aspartate synaptic receptors in chick retinal neurons. Brain Res. 298: 159-162.

Luedde, W.H. (1932). Monocular cycloplegia for the control of myopia. Amer. J. Ophthal. 15: 603-610.

Ma, P.M. & Grant, P. (1984). Choline acetyltransferase and cholinesterases in the developing Xenopus retina. J. Neurochem. 42: 1328-1337.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signalling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80: 179-185.

Martin, W.R. & Sloan, J.W. (1977). Neuropharmacology and neurochemistry of subjective effects, analgesia, tolerance and dependence produced by narcotic analgesics. In: Handbook of experimental Pharmacology V45/I Drug Addiction I:Morphine, Sedative /Hypnotic and Alcohol Dependence, ed, Martin, W.R., pp. 43-158. Springer Verlag, Berlin.

Marwitt, R., Pilar, G. & Weakly, J.N. (1971). Characterization of two cell populations in the avian ciliary ganglion. Brain Res. 25: 317-334.

Marzani, D., Lind, G.J., Chew, S.J. & Wallman, J. (1994). The reduction of myopia by muscarinic antagonists may involve a direct effect on scleral cells. Invest. Ophthal. Vis. Sci. (Suppl.). 35: 1801.

Masland, R.H., Mills, J.W. & Cassidy, C. (1984). The functions of acetylcholine in the rabbit retina. Proc. R. Soc. Lond. B 223: 121-139.

McBrien, N.A., Moghaddam, H.O., Cottriall, C.L., Leech, E.M. & Cornell, L.M. (1995). The effects of blockade of retinal cell action potentials on ocular growth. emmetropization and form deprivation myopia in young chicks. Vision Res. 35: 1141-1152.

McBrien, N.A., Moghaddam, H.O. & Reeder, A.P. (1993). Atropine reduces experimental myopia and eye enlargement via a nonaccommodative mechanism. Invest. Ophthal. Vis. Sci. 34: 205-215.

McGlinn, A.M., Capehart, C., Wen, R., Macguire, M.G. & Stone, R.A. (1998). Altered retinal expression of VIP mRNA in chick myopia. Invest. Ophthal. Vis. Sci. (Suppl.) 39: S717.

McGuire, J.J. & Stell, W.K. (1998). Fos-labelling of retinal neurons activated by complex visual stimuli: relevance to form-deprivation myopia and ocular growth control. Soc. Neurosci. Abstracts (in press).

McKanna, J.A. & Casagrande, V.A. (1981). Atropine affects lid-suture myopia development: Experimental studies of chronic atropinization in tree shrews. Doc. Ophthal. Proc. Series. 28: 187-192.

McKinnon, L.A. & Nathanson, N.M. (1995). Tissue-specific regulation of muscarinic acetylcholine receptor expression during embryonic development. J. Biol. Chem. 270(35): 20363-20642.

Megaw, D., Cluff, P., Morgan, I.G. & Boelen, M.K. (1994). Deprivation of form vision and temporal contrast restores the levels of [leu] enkephalin in the chick retina. Proc. Australian Neurosc. Soc.5; 200.

Meriney, S.D. & Pilar, G. (1987). Cholinergic innervation of the smooth muscle cells in the choroid of the chick eye and its development. J. Neurosci. 7: 3827-3839.

Miles, F.A. & Wallman, J. (1990). Local ocular compensation for imposed local refractive error. Vision Res. 30: 339-349.

Millar, T.J., Ishimoto, I., Boelen, M.K., Epstein, M.L., Johnson, C.D. & Morgan, I.G. (1987a). Toxic effects of ethylcholine mustard arizidinium ion on cholinergic cells in the chicken retina, J. Neurosci. 7: 343-356.

Millar, T.J., Ishimoto, I., Chubb, I.W., Epstein, M.L., Johnson, C.D. & Morgan, I.G. (1987b). Cholinergic amacrine cells of the chicken: a light and electron microscope immunocytochemical study. Neurosci. 21: 725-742.

Millar, T.J., Ishimoto, I., Johnson, C.D., Epstein, M.L., Chubb, I.W. & Morgan, I.G. (1985). Cholinergic and acetylcholinesterase-containing neurons of the chicken retina. Neurosci. Letts. 61: 311-316.

Millar, T.J., Winder, C., Isimoto, I. & Morgan, I.G. (1988). Putative serotonergic bipolar and amacrine cells in the chick retina. Brain Res. 439: 77-87.

Mody, I. & MacDonald, J.F. (1995). NMDA receptor-dependent excitotoxicity: the role of intracellular Ca^{2+} release. Trends Pharmacol. Sci. 16: 356-359.

Morgan, B.A. & Fekete, D.M. (1996). Manipulating gene expression with replication-competent retroviruses. Meth. Cell Biol. 51: 185-209.

Morgan, I.G. (1983). The organization of amacrine cell types which use different transmitters in chicken retina. Prog. Brain Res. 2: 249-266

Morgan, I.G. (1987a). AMPA is a powerful neurotoxin in the chicken retina. Neurosci. Letts. 79:267-271.

Morgan, I.G. (1987b). Pharmacological properties of a kainic acid-prefering receptor on OFF-bipolar cells in the chicken retina. Soc. Neurosci. Abs. 13: 1553.

Morgan, I.G. & Ingham, C.A. (1981). Kainic acid affects both plexiform layers of the chicken retina. Neurosci. Letts. 21: 275-280.

Morgan, I.G., Wellard, J.M. & Boelen, M.K. (1994). A role for the enkephalin-immunoreactive amacrine cells of the chicken retina in adaptation to light and dark. Neurosci. Letts. 174:64-66.

Nathanson, N.M. (1987). Molecular properties of the muscarinic acetylcholine receptor. Annu. Rev. Neurosci. 10: 195-236.

Negishi, K., Kato, S. & Teranishi, T. (1988). Dopamine and rod bipolar cells contain protein kinase C-like immunoreactivity in some vertebrate retinas. Neurosci. Letts. 94: 247-252.

Norton, T.T., Essinger, J.A. & McBrien, N.A. (1994). Lid-suture myopia in tree shrews with retinal ganglion cell blockade. Vis. Neurosci. 11: 143-153.

Norton, T.T. & Siegwart, J.T. (1991). Local myopia produced by partial-field deprivation in tree shrew. Soc. Neurosci. Abstracts 17: 558.

Olney, J.W. (1986). Inciting excitotoxic cytocide among central neurons. Adv. Exp. Med. Biol. 203: 631-645.

Osbourne, N.N., FitzGibbon, F. & Schwartz, G. (1991). Muscarinic acetylcholine receptor-mediated phosphoinositide turnover in cultured human retinal pigment epithelium cells. Vision Res. 31: 1119-1127.

Parsons, J. (1923). Diseases of the eye (4th ed). London: Chruchill. p. 479.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J. & Sturgill, T.W. (1991). Indentification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. 10: 885-892.

Pendrak, K., Lin, T. & Stone, R.A. (1995). Ciliary ganglion choline acetyltransferase activity in avian macrophthalmos, Exp. Eye Res. 60: 237-243.

Peralta, E., Ashkenazi, A., Smith, D., Winslow, J., Ramachandran, J. & Capon, D. (1987). Distinct primary strucutres, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. EMBO J. 6: 3923-3929.

Peralta, E., Ashkenazi, A., Winslow, J., Ramachandran, J. & Capon, D. (1988). Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. Nature 334: 434-437.

Pickett-Seltner, R.L., Sivak, J.G. & Pasternak, J.J. (1988). Experimentally- induced myopia in chicks: morphometric and biochemical analysis during the first 14 days after hatching. Vision Res. 28: 323-328.

Pilar, G., Nunez, R., McLennan, I.S. & Meriney, S.D. (1987). Muscarinic and nicotinic synaptic activation of the developing chick iris. J. Neurosci. 7: 3813-3826.

Polans, A.S., Hutchins, J.B. & Werblin, F.S. (1985). Muscarinic cholinergic receptors in the retina of the larval tiger salamander. Brain Res. 148: 85-93.

Raviola, E. & Wiesel, T.N. (1985). An animal model of myopia. NEJ Med. 312: 1609-1615.

Reiner, A., Shih, Y-F. & Fitzgerald, M.E.C. (1995). The relationship of choroidal blood flow and accomodation to the control of ocular growth. Vision Res. 35: 1227-1245.

Rohrer, B., Spira, A. & Stell, W.K. (1993). Apomorphine blocks form-deprivation myopia in chickens by a dopamine D2-receptors mechanism acting in retina or retinal pigment epithelium. Vis. Neurosci. 10: 447-453.

Rohrer, B. & Stell, W.K. (1995). Stimulation of dopaminergic amacrine cells by stroboscopic illumination or fibroblast growth factor (bFGF, FGF-2). injections: possible roles in prevention of form-deprivation myopia in the chick. Brain Res. 686: 168-181.

Romano, C., Price, M.T. & Olney, J.W. (1995). Delayed excitotoxic neurodegeneration induced by excitatory amino acid agonists in isolate retina. J. Neurochem. 65: 59-67.

Ross, C.D. & McDougal, D.B.Jr. (1976). The distribution of choline acetyltransferase activity in vertebrate retina. J. Neurochem. 26: 521-526.

Rothman, S.M., Thurston, J.H. & Hauhart, R.E. (1997). Delayed neurotoxicity of excitatory amino acids in vitro. Neurosci. 22: 471-480.

Sakai, H.M. & Naka, K.-I. (1987). Signal transmission in catfish retina. IV. Transmission to ganglion cells. J. Neurophysiol. 58: 1307-1328.

Sakuranga, M. & Naka, K.-I. (1985a). Signal transmission in catfish retina. II Transmission to type-N cells. J. Neurophysiol. 53: 390-410.

Sakuranga, M. & Naka, K.-I. (1985b). Signal transmission in catfish retina. III Transmission to type-C cells. J. Neurophysiol. 53: 411-428.

Salceda, R. (1994). Muscarinic receptors in the retinal pigment epithelium during rat development. Neurochem. Res. 19: 1207-1209.

Sandberg, K., Schnaar, L., McKinney, H., Hanin, I., Fisher, A. & Coyle, J.T. (1985). AF64A: an active site directed irreversible inhibitor of choline acetyltransferase. J. Neurochem. 44: 439-445.

Sarthy, P.V. & Lam, D.M.K. (1979). Endogenous levels of neurotransmitter candidates in photoreceptor cells of the turtle retina. J. Neurochem. 32: 455- 461.

Sattayasai, J. & Ehrlich, D. (1987). Morphology of quisqualate-induced neurotoxicity in the chick retina. Invest. Ophthal. Vis. Sci. 28:106-117.

Schaeffel, F., Glasser, A. & Howland, H.C. (1988). Accommodation, refractive error and eye growth in chickens. Vision Res. 28: 639-657.

Schaeffel, F., Hagel, G., Bartmann, M., Kohler, K. & Zrenner, E. (1994). 6-Hydroxy dopamine does not affect lens-induced refractive errors but supresses deprivation myopia. Vision Res. 34: 143-149.

Schaeffel, F., Troilo, D., Wallman, J. & Howland, H.C. (1990). Developing eyes that lack accommodation to compensate for imposed defocus. Vis. Neurosci. 4: 177-183.

Schmid, K.L. & Wildsoet, C.F. (1993). Spatial frequency and contrast: Visual cues for the control of eye growth? Clin. & Exp. Optom. 76: 188.

Schmid, K.L. & Wildsoet, C.F. (1996). Effects of the compensatory responses to positive and negative lenses on intermittent lens wear and ciliary nerve section in chicks. Vision Res. 36: 1023-1036.

Schmidt, H.A. & Vijayaraghavan, S. (1992). Inhibition of the nicotinic acetylcholine response by serotonergic and muscarinic agents in chick ciliary ganglion neurones. Neuro. Pharm. 31: 1001-1008.

Schwahn, H.N. & Schaeffel, F. (1998). Studies on muscarinic receptors of retinal pigmented epithelium in a retina-RPE-choroid preparation of the chick. Invest. Ophthal. Vis. Sci. (Suppl.) 39: S716

Schwahn, H.N., Schaeffel, F. & Zrenner, E. (1997). Effects of atropine in the chick retina: in vitro studies on a retina-RPE-choroid preparation. Invest. Ophthal. Vis. Sci. (Suppl.) 38: S758.

Schwarcz, R. & Coyle, J.T. (1977). Kainic acid: neurotoxic effects after intraocular injection. Invest. Opthal. Vis. Sci. 16: 141-148.

Sheppard, A.M., Konopka, M., Robinson, S.R., Morgan, I.G. & Jeffrey, P.L. (1991). Thy-1 antigen is specific to ganglion cells in chicks. Neurosci. Letts. 123:87-90.

Seltner, R.L.P., Grant, V. & Stell, W.K. (1994). [Met^5]-enkephalin and form deprivation myopia. Invest. Ophthal. Vis Sci. (Suppl). 35: 2069.

Seltner, R.L.P., Rohrer, B., Grant, V. & Stell, W.K. (1997). Endogenous opiates in the chick retina and their role in form deprivation myopia. Vision Res.14: 801-809.

Seltner, R.L.P. & Stell, W.K. (1995a). The effect of vasoactive intestinal peptide on development of form deprivation myopia in the chick: a pharmacological and immunocytochemical study. Vision Res. 35: 1265-1270.

Seltner, R.L.P. & Stell, W.K. (1995b). Differential binding of a PNS and CNS vasoactive intestinal peptide (VIP). antagonist in the chick eye: possible dual role of VIP in form deprivation myopia. Invest. Ophthal. Vis. Sci. (Suppl). 36: S413.

Seltner, R.L.P. & Stell, W.K. (1996). NMDA receptors are obligatory in retinal pathways that control ocular growth in chicks. Proc. Austral. Neurosci. Soc. 7:56.

Sherman, S.M., Norton, T.T. & Casagrande, V.A. (1977). Myopia in lid-sutured tree shrew (*Tupia glis*). Brain Res. 124: 154-157.

Shukla, V.K. & Lemaire, S. (1994). Non-opioid effects of dynorphins: possible role of the NMDA receptor. Trends Pharmacol.Sci. 15: 420-424.

Simonian, N.A., R.L. Getz, J.C. Leveque, C. Konrad & J.T. Coyle (1996). Kainate induces apoptosis in neurons. Neurosci. 74: 675-683.

Slaughter, M.M., Mattler, J.A. & Gottlieb, D.I. (1985). Opiate binding sites in the chick, rabbit and goldfish retina. Brain Res. 339: 39-47.

Smith, E.L. Fox, D.A. & Duncan, G.C. (1985). Refractive error changes in kitten eyes produced by chronic ON-channel blockade. Invest. Ophthalmol. Vis. Sci. Abs. 26: 331.

Sormachi, M. (1993). Caffeine- and muscarinic receptor agonist-sensitive Ca^{2+} stores in chick ciliary ganglion cells. Brain Res. 627: 34-40.

Spira, A.W., Millar, T.J., Ishimoto, I., Epstein, M.L., Johnson, C.D., Dahl, J.L. & Morgan, I.G. (1987). Localization of choline acetyltransferase-like immunoreactivity in the embryonic chick retina. J. Comp. Neurol. 260: 526-538.

Stell, W.K., Miethke, P. & Morgan, I.G. (1997). Regulation of ocular growth in chickens is unaffected by ablation of cholinergic amacrine cells. Invest. Ophthal. Vis. Sci. (Suppl.). 38: 759.

Stone, R.A., Laties, A.M., Raviola, E. & Weisel, T.N. (1988). Increase in retinal vasoactive intestinal polypeptide after eyelid fusion in primates. Proc. Natl. Acad. Sci. USA. 85: 257- 260.

Stone, R.A., Lin, T. & Laties, A.M. (1991). Muscarinic antagonist effects on experimental chick myopia. Exp. Eye. Res. 52: 755-758.

Stone, R.A., Lin, T., Laties, A.M. & Iuvone, P.M. (1989). Retina dopamine and form-deprivation myopia. Proc. Natl. Acad. Sci. USA. 86: 704-706.

Sturgill, T.W , Ray, L.B., Erikson, E. & Maller, J.L. (1988). Insulin-stimulated MAP-2 kinase phosphorylates and acitivates ribosomal protein s6 kinase II. Nature 334: 715-718.

Su, Y.Y.T. & C.B. Watt (1987). Interaction between enkephalin and dopamine in the avian retina. Brain Res. 243:63-70.

Sugiyama, H., Daniels, M.P. & Nirenburg, M. (1977). Muscarinic acetylcholine receptors of the developing retina. Proc. Natl. Acad. Sci. 74: 5524-5528.

Tauchi, M. & Masland, R.H. (1984). The shape and arrangment of cholinergic neurons in the rabbit retina. Proc. Royal Soc. Lond. B 223: 101-119.

Tietje, K.M., Goldman, P.S. & Nathanson, N.M. (1990). Cloning and functional analysis of a gene encoding a novel muscarinic acetylcholine receptor expressed in chick heart and brain. J. Biol. Chem. 265(5): 2828-2834.

Tietje, K.M. & Nathanson, N.M. (1991). Embryonic chick heart expresses multiple muscarinic acetylcholine receptor subtypes. J. Biol. Chem. 266: 17382-17387.

Townes-Anderson, E. & Vogt, B.A. (1989). Distribution of muscarinic acetylcholine receptors on processes of isolated retinal cells. J. Comp. Neurol. 290: 369- 383.

Troilo, D. (1990). Experimental studies of emmetropization in the chick. In: Myopia and the Control of Eye Growth (Bock G.R. & Widdows, K., eds). Chichester: Wiley. Ciba Foundation Symposium. 155: 89-102.

Troilo, D., Gottlieb, M.D. & Wallman, J. (1987). Visual deprivation causes myopia in chicks with optic nerve section. Curr. Eye Res. 6: 993-999.

Troilo, D. & Wallman, J. (1991). The regulation of eye growth and refractive state: an experimental study of emmetropization. Vision Res. 252: 78-87.

Tumosa, N., Eckenstein, F. & Stell, W.K. (1984). Immunocytochemical localization of putative cholinergic neurons in the goldfish retina. Neurosci. Letts. 48: 255-259.

Tung, N.N., Morgan, I.G. & Ehrlich, D. (1990). A quantitative analysis of the effects of excitatory neurotoxins on retinal ganglion cells in the chick. Vis. Neurosci. 4: 217-223.

Vingrys, A.J., Squires, M.A., Napper, G.A., Barrington, M., Vessey, G.A. & Brennan, N.A. (1991). Prevention of form deprivation myopia in post-hatch chicks. Invest. Ophthal. Vis. Sci. (Suppl). 32: 1203.

Voigt, T. (1986). Cholinergic amacrine cells in the rat retina. J. Comp. Neurol. 248: 19-35.

Wallman, J. (1990). Retinal influences on sclera underlie visual deprivation myopia. In: Myopia and the Control of Eye Growth. (Bock G.R. & Widdows, K. eds). Chichester: Wiley. Ciba Foundation Symposium. 155: 126-141.

Wallman, J. (1993). Retinal control of eye growth and refraction. Prog. Retinal Res. 12: 133-153.

Wallman, J., Gottlieb, M.D., Rajaram, V. & Fugate-Wentzek, L.A. (1987). Local retinal regions control local eye growth and myopia. Science 237: 73-77.

Wallman J., Rosenthal, D., Adams, J.I. & Romagnano, L. (1981). Role of accommodation and developmental aspects of experimental myopia in chicks. In: Proceedings of the Third International Myopia Conference (Fledelius, H.C., Alsbirk, P.H. & Goldschmidt, E., eds). The Hague: Dr. W. Junk. Doc. Ophthal. Proc. Series. 28: 197-206.

Wallman, J., Turkel, J. & Trachtman, J. (1978). Extreme myopia produced by modest changes in early visual experience. Science 201: 1249-1251.

Wallman, J., Wildsoet, C., Xu, A., Gottlieb, M.D., Nickla, D.L., Marran, L., Krebs, W. & Christensen, A.M. (1995). Moving the retina: choroidal modulation of refractive state. Vision Res. 35: 37-50.

Wässle, H. & Riemann, H.J. (1978). The mosaic of nerve cells in the mammalian retina. Proc. R. Soc. Lond. 200: 441-461.

Watt, C.B. & Florack, W.J. (1994). A triple-label analysis demonstrating that enkephalin-, somatostatin- and neurotensin-like immunoreactivities are expressed in a single population of amacrine cells in the chicken retina. Brain Res. 634: 310-316.

Watt, C.B., Li, H.B. & Lam, D.M.K. (1985). The presence of three neuroactive peptides in putative glycinergic amacrine cells of an avian retina. Brain Res. 348:187-191.

Weisel, T.N. & Raviola, E. (1977). Myopia and eye enlargement after neonatal lid fusion in monkeys. Nature 266: 66-68.

Wildsoet, C.F., Clark, I.Q. & Teakle, E.M. (1995). Dose-dependent inhibitory effects of AMPA on form-deprivation myopia and lens defocus-induced changes in chicks. Invest. Ophthal. Vis. Sci. (Suppl) 36: S413.

Wildsoet, C.F. & Howland, H.C. (1991). Chromatic aberration and accomodation: their role in emmetropization in the chick, Invest. Ophthal. Vis. Sci. (Suppl.). 32: 1203.

Wildsoet, C.F., Howland, H.C., Falconer, S. & Dick, K. (1993). Chromatic abberation and accommodation: their role in emmetropization in the chick. Vision Res. 33: 1593-1603.

Wildsoet, C.F.& Pettigrew, J.D. (1988a). Kainic acid-induced eye enlargement in chickens: differential effects on anterior and posterior segments. Invest. Ophthal. Vis. Sci. 29: 311-319.

Wildsoet, C.F. & Pettigrew, J.D. (1988b). Experimental myopia and anomalous eye growth patterns unaffected by optic nerve section in chickens: Evidence for local control of eye growth. Clin. Vision Sci. 3: 99-107.

Wildsoet, C.F. & Wallman, J. (1995). Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks. Vision Res. 35: 1175-1194.

Witkowsky, P. & Schütte, M. (1991). The organization of dopaminergic neurons in vertebrate retinas. Vis. Neurosci. 7: 113-124.

Wong, E.H. & Kemp, J.A. (1991). Sites for antagomism of the N-methyl-D-aspartate receptor channel. Ann. Rev. Pharm. Toxicol. 31: 401-425.

Wylie, A.H., Kerr, J.F.R., & Currie, A.R. (1980). Cell death: the significance of apoptosis. Int. Rev. Cytol. 68:251-306.

Xie, R.Z., Crewther, S.G. & Crewther, D.P. (1992). The interaction between the ON and OFF responses to light and ocular growth in chicks. Proc. Austral. Neurosci. Soc. 3: 94.

Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C.M. & Huang, H.V. (1989). Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. Sci. 243: 1188-1191.

Yamagata, M., Jaye, D.L. & Sanes, J.R. (1994). Gene transfer to avian embruos with a recombinant adenovirus. Dev. Biol. 166: 355-359.

Yaqub, A. & Eldred, W.D. (1993). Effects of excitatory amino acids on immunocytochemically identified populations of neurons in turtle retina. J. Neurocytol. 22:644-662.

Yazulla, S. & Schmidt, J. (1977). Two types of receptors for alpha-bungarotoxin in the synaptic layers of the pigeon retina. Brain Res. 138: 45-57.

Zarbin, M.A., Wamsley, J.K., Palacios,J.M. & Kuhar, M.J. (1986). Autoradiographic localization of high affinity GABA, benzodiazepine, dopaminergic, adrenergic, and muscarinic cholinergic receptors in the rat, monkey, and human retina. Brain Res. 374: 75-92.

Zeevalk, G.D., Hyndman, A.G. & Nicklas, W.J. (1989). Excitatory amino acid-induced toxicity in chick retina: amino acid release, histology, and effects of chloride channel blockers. J. Neurochem. 53: 1610-1619.

Zeevalk, G.D. & Nicklas, W.J. (1990). Action of the anti-ishemic agent ifenprodil on N-methyl-D-aspartate and kainate-mediated excitotoxicity. Brain Res. 522: 135-139.

Zhang, J. & Snyder, S. (1995). Nitric oxide in the nervous system. Annu. Rev. Pharmacol. Toxicol. *35*: 213-233.