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Cell birth and fate in the teleost retina

by

David Julian

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Physiology

in the

GRADUATE DIVISION

of the

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When the time came to pick a thesis advisor at UCSF, I looked for the most rigorous and uncompromising scientist I could find. I doubt anyone could argue that Juan Korenbrot is not both of those. Juan is rarely satisfied that an experiment has been performed carefully enough, that the data have been analyzed accurately enough, or that the results have been presented clearly enough. He has a remarkable ability to understand complex problems, and to devise methods (albeit occasionally equally complex) to solve them. I have learned a great deal from him, and my science-education objectives have been completely met. But in addition, I think I have also learned from Juan something about how to be simultaneously a good person and a good scientist. Not an easy task at UCSF. In addition, I would like to thank the other two members of my thesis committee, Matt LaVail and David Copenhagen, for managing to find a delicate balance of exacting standards tempered by enthusiastic support. Their expectations of me were higher than my own, and I hope that wasn't too frustrating for them.

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Cell birth and fate in the teleost retina

by David Julian

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ABSTRACT

Teleost fish and some amphibia are unique among vertebrates because their eyes continue to grow throughout the life of the animal. The fish retina grows by two primary mechanisms: stretching of the mature retina and continuous addition of new retina from the peripheral margin of the retina in an area known as the peripheral germinal zone. Stretching of the mature retina causes a progressive decrease in the density of all cell types, except rods, which are maintained at constant density by the continual addition and differentiation of new photoreceptors from precursor cells located in the outer nuclear layer. These precursor cells are also thought to endow the teleost retina with its capacity to regenerate following surgical or neurotoxic injury. However, the cellular origin of these precursor cells and the mechanisms which control their proliferation are not clear. We have found that clusters of mitotically active cells exist in the inner nuclear layer of the mature retina of rainbow trout (*Oncorhyncus mykiss*) throughout the animals' life. Through a kinetic analysis using immunohistochemical double-labeling of thymidine analogs, we demonstrate that these cells migrate to the outer nuclear layer soon after their birth. We propose that these cells then give rise to rod precursor cells. These clusters had been identified previously in embryonic and larval fish, but were assumed to be

unimportant for older animals. In further examining the role of these clusters, we have found that their density is upregulated by light-induced injury to the retina. Our results suggest a revision of the model that explains the cellular origin of the rod precursors in the mature retina, and suggest that these proliferating inner nuclear layer cells may also provide a cellular mechanism for the teleost retina's remarkable ability to regenerate following injury.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **III**

ABSTRACT ..... **IV**

TABLE OF CONTENTS ..... **VI**

LIST OF TABLES ..... **VII**

LIST OF FIGURES ..... **VIII**

INTRODUCTION **1**

OVERVIEW OF CHAPTERS 15

CHAPTER ONE:
KINETICS OF GROWTH IN THE PERIPHERAL GERMINAL ZONE **22**

INTRODUCTION 22
MATERIALS AND METHODS 29
RESULTS 38
DISCUSSION 57

CHAPTER TWO:
CELL PROLIFERATION IN THE MATURE INNER NUCLEAR LAYER **68**

INTRODUCTION 68
MATERIALS AND METHODS 72
RESULTS 78
DISCUSSION 105

CHAPTER THREE:
RETINAL CELL PROLIFERATION IN RESPONSE TO LIGHT DAMAGE **111**

INTRODUCTION 111
MATERIALS AND METHODS 116
RESULTS 120
DISCUSSION 130

REFERENCES **136**

LIST OF TABLES

Table 1.2:
Differentiation time in PGZ 56

Table 1.3:
Comparison of PGZ and embryonic retinal differentiation 66

Table 3.1:
Distribution of body lengths 121

LIST OF FIGURES

Figure 1.1:
Relationship between eye diameter and growth rate in striped bass 39

Figure 1.2:
Size of neuroblast region in retinas from striped bass raised at different growth rates 41

Figure 1.3:
Distance from PGZ to first photoreceptor outer segments in striped bass raised at different growth rates.... 43

Figure 1.4:
Micrographs comparing PGZs of trout and zebrafish retinas 46

Figure 1.5:
Size of neuroblast region for different species of fish 47

Figure 1.6:
Distance to first cone outer segment for different species of fish 48

Figure 1.7:
Growth of trout under controlled conditions 50

Figure 1.8:
Relationship between time and distance in the PGZ 54

Figure 2.1:
Radial section of trout retina immunostained for PCNA 80

Figure 2.2:
Density of PCNA-positive INL cells in trout of different ages 82

Figure 2.3:
Radial distribution of BrdU-positive cells in trout retina 85

Figure 2.4:
Diurnal rhythm of PCNA-positive cells in trout retina 88

Figure 2.5:
Micrograph of trout retina double-labeled for IdU and BrdU 94

Figure 2.6:
Migration of IdU-labeled cells from the INL to the ONL 96

Figure 2.7:
Micrographs of PINC clusters crossing the outer plexiform layer 101

Figure 2.8:
Micrograph of fluorouracil-induced apoptosis in the PGZ 104

Figure 3.1:
PINC density in pigmented and albino trout after toxic light exposure 124

Figure 3.2:
PCNA-positive ONL cell density in pigmented and albino trout after toxic light exposure 126

Figure 3.3:
Comparison of PCNA density for outer and inner ONL 128

INTRODUCTION

"To suppose that the eye with all its inimitable contrivances for adjusting the focus to different distances, for admitting different amounts of light, and for the correction of spherical and chromatic aberration, could have been formed by natural selection, seems, I freely confess, absurd in the highest degree. When it was first said that the sun stood still and the world turned round, the common sense of mankind declared the doctrine false; but the old saying of *Vox populi, vox Dei*, as every philosopher knows, cannot be trusted in science. Reason tells me, that if numerous gradations from a simple and imperfect eye to one complex and perfect can be shown to exist, each grade being useful to its possessor, as is certainly the case; if further, the eye ever varies and the variations be inherited, as is likewise certainly the case and if such variations should be useful to any animal under changing conditions of life, then the difficulty of believing that a perfect and complex eye could be formed by natural selection, though insuperable by our imagination, should not be considered as subversive of the theory. How a nerve comes to be sensitive to light, hardly concerns us more than how life itself originated; but I may remark that, as some of the lowest organisms, in which nerves cannot be detected, are capable of perceiving light, it does not seem impossible that certain sensitive elements in their sarcode should become aggregated and developed into nerves, endowed with this special sensibility.

"In searching for the gradations through which an organ in any species has been perfected, we ought to look exclusively to its lineal progenitors; but this is scarcely ever possible, and we are forced to look to other species and genera of the same group, that is to the collateral descendants from the same parent-form, in order to see what gradations are possible, and for the chance of some gradations having been transmitted in an unaltered or little altered condition. But the state of the same organ in distinct classes may incidentally throw light on the steps by which it has been perfected."

So Charles Darwin begins the section titled "Organs of extreme Perfection and Complication" in his monumental work, *The Origin of Species*. Darwin proposed that the study of light-sensing organs in the lower animals might help us recognize how an organ of such complexity as the vertebrate eye could gradually evolve over time from its rudimentary progenitors. Furthermore, he suggested, correctly of course, that many of the phylogenic "gradations" might be passed along intact in their progenitor form, and that

identification of these ancestral remnants would help us track the evolutionary processes which formed the vertebrate eye. We now recognize that this is also true of the ontogenic processes by which complex tissues and organs develop from progenitor cells. Thus, it is likely that we can better understand the mechanisms that allow hundreds of specific cell types within the mammalian retina to develop from a few undifferentiated progenitor cells by studying this process in lower vertebrates. Furthermore, since it is an extension of the central nervous system, the retina, by virtue of its comparatively simple cellular architecture and well-defined function, is an attractive and accessible system in which to study the processes of development within the central nervous system as a whole (Dowling 1987).

Cell birth in the vertebrate retina

The eye develops from optic vesicles that are first generated as evaginations of the neural tube. Cells in the optic vesicle develop exclusively into an ocular phenotype (review in Reh 1992). Optic vesicles develop into optic cups consisting of two closely apposed layers of undifferentiated neuroepithelial cells. The cells in the posterior layer give rise to the pigmented epithelium, while the cells in the anterior layer are the progenitors of all the classes of neurons in the retina, as well as the Müller glial cells. Thus, during the development of the retina, multipotent stem cells from the anterior layer of the germinal epithelium ultimately produce a highly ordered structure composed of five major neuronal cell types: photoreceptors, horizontal cells, bipolar cells, amacrine cells and ganglion cells. These cell types are further subdivided into one hundred or more

specific cell subtypes. Retinal astrocytes are generated elsewhere and migrate into the retina (Watanabe and Raff 1988).

Retinal development in all vertebrates, except teleost fish and some amphibia, is completed during embryogenesis and early postnatal days. Almost all published reports of retinal development have used embryonic and early postnatal retinas in mammals, chicks, and frogs (Harris and Holt 1990 for review). In these animals, the retina develops from an apparently homogenous sheet of neuroepithelium. Proliferation and differentiation can take from a few days to over 100 days, depending on the species, and proceeds with a central-to-peripheral gradient which can be as short as 7.5 hours, as in Xenopus (Holt et al 1988), or as long as several days, as in the monkey (LaVail et al 1991). Within a species (and possibly more generally) the production of particular retinal cell types progresses through a consistent and predictable sequence, with certain cell types more likely to be generated early and others more likely to be generated later. In mice (Carter-Dawson and LaVail 1979 for review), monkeys (LaVail et al 1991) and cats (Zimmerman et al 1988), for example, ganglion and horizontal cells are born first, followed by cone photoreceptors, amacrine cells, rods, bipolar cells and, finally, Müller cells. The sequence of cell birth in lower vertebrates is similar, but not identical. In teleost fish (Hagedorn and Fernald 1992, Hollyfield 1972, Sharma and Ungar 1980), for example, during embryonic development ganglion cells and cone photoreceptors are born first, followed by bipolar, amacrine and horizontal cells. Rods photoreceptors are born last. However, even in very slowly developing retinas, there is generally significant overlap between the generation times of different cell types at any position in the retina

(LaVial et al 1991). As a result, different retinal cell types can be produced simultaneously in the same region.

From progenitors to photoreceptors: the making of a retina

The apparently homogenous neuroepithelum of the optic cup gives rise to all of the neuronal cell types of the retina. How does the neuroepithelium generate so many different cell types? A simple answer would be that individual neuroepithelial cells are specialized, each producing only one or a few types of progeny, which then develop into different types of mature retinal cells. However, a variety of experiments suggest that individual neuroepithelial cells are pluripotent, giving rise to many classes of retinal cells. Some of these studies were performed by tracing the lineage of progenitor cells by infecting the progenitors with a histologically identifiable retrovirus (Turner and Cepko 1987, Turner et al 1990), or by injecting the progenitors with horseradish peroxidase (Holt et al 1988) or a fluorescent dye (Wetts and Fraser 1988, Wetts et al 1989), such that all the progeny of an individual cell could ideally be recognized histologically. The daughters of an individual progenitor cell were virtually always found to comprise solid, radially oriented clusters of clones that could span each of the retinal laminae and contain every cell type. Infrequently, cells from a given clone were found to be tangentially displaced (Fekete et al 1994). In general, clusters derived from progenitors which were labeled earlier contained a greater number of cell types. However, the composition of the clusters was highly variable, even between clusters derived from progenitors labeled at the same time and within the same retina. Similar but even more striking results were obtained by Williams and Goldowitz (1992a, b). These authors constructed early mouse

embryos which were chimeras of domestic and wild-type mice. After the retinas were allowed to mature, retinal progeny of domestic and wild-type progenitor cells were distinguished histologically by identifying species-specific satellite DNA sequences. The clonally related clusters were invariably found to be solid, radially oriented, cylindrical columns which traversed every cell layer and contained every cell type. Furthermore, the cell types within each column were present in the same ratio as existed in the retina as a whole. These clones were thus described as "core samples" of the retina. These results provide strong evidence that a single, common stem cell can give rise to every retinal cell type through fate specification in successive cell divisions, and, further, that the various progeny of a particular progenitor remain in a single, radially oriented column which may can contain all retinal cell types.

Neurons in the visual cortex are grouped in functional columns (review in Miller 1994). The neurons are believed to proliferate and differentiate in the same columnar units, a concept described as the "radial unit hypothesis" (Rakic 1988). The retina may develop by a similar mechanism as an association of radial columns. This is supported by the observations in retinas of clonally related radial "cores," extending from the photoreceptor layer to the ganglion cell layer (Turner et al 1990, Williams and Goldowitz 1992a). In the embryonic chick retina, scanning electron microscope studies show a columnar organization that serves as scaffolding for the migration of neuron in a radial axis (Meller and Tezlaff 1976). This scaffolding is associated with Müller cells (Meller and Tezlaff 1976). In the rabbit retina, careful cell counts show a constant ratio of rods, bipolar and amacrine cells to Müller cells at various developmental stages, which has

been cited as evidence of "columnar units" of development (Reichenbach et al 1994). This columnar organization may also be evidenced by the mosaic arrays of cones seen in mature retina. While it is not yet clear whether cones within a mosaic "tile" are clonally related, the spacing of cones into 5 to 6 cell-diameter mosaics in vertebrates as diverse as the monkey and trout is suggestive of the 6.5 average cell-diameter columns seen by Williams and Goldowitz (1992a) in chimeric mouse retinas.

Individual, clonally related "columns" of retinal cells contain a variety of cell types: photoreceptors, bipolar cells, amacrine cells, horizontal cells and ganglion cells. How do the progeny of the seemingly pluripotent progenitor cells decide among their many developmental options? Specification of cell fate in the retina could occur in two ways: extrinsically and intrinsically (Cepko 1993, Harris 1993, Williams and Goldowitz 1993). With extrinsic regulation, the progenitor would be multipotential and cell fate determination would be dependent on extrinsic factors in the micro-environment of the neurons at the time of their birth. These factors include cell-signaling molecules (growth factors), receptor molecules, extracellular cell matrix molecules and diffusible factors. With intrinsic regulation, cell fate would be determined, or at least restricted, prior to the cell's birth. In this case, the cell fate would be determined by intrinsic factors (e.g., homeobox gene products in the precursor cell), alone or in combination with extrinsic factors. Although many authors have suggested that cell fate determination in the retina is probably due to a combination of intrinsic and extrinsic influences (Watanabe and Raff 1990 and 1992, LaVail et al 1991, Altshuler and Cepko 1992, Repka and Adler 1992a, Williams and Goldowitz 1992b), the nature of these influences is as yet unknown.

The results of some cell lineage experiments have been interpreted as indicating that cell fate is specified completely by extrinsic influences. The variability in the cluster composition observed in the precursor transfection and dyeing experiments described above (Turner and Cepko 1987, Turner et al 1990; Holt et al 1988; Wetts and Fraser 1988; Wetts et al 1989) was attributed to differences in microenvironmental cues sensed by different cells within a cluster. Furthermore, the variability was interpreted as evidence in support of precursor cells remaining uncommitted until or even after their birth. Thus, the progenitors were seen as always being multipotent for every retinal cell type, and the temporal separation of cell type cytogenesis was interpreted as being due to gradual net changes in extrinsic signals. On the other hand, Williams and Goldowitz (1992a, b) have proposed (on the basis of the very regular columns observed in their chimeric mouse experiments) that intrinsic signals in retinal progenitors restrict the progeny to one or a few cell types at different times during retinal development. The specific actions of extrinsic and intrinsic signals on cell specification in the vertebrate retina are, therefore, at best extremely unclear.

Of all the retinal cell types, the specification and differentiation of photoreceptors is perhaps the most studied. Cell-cell interaction (Watanabe and Raff 1990), diffusible factors that act over short distances (Altshuler and Cepko 1992, Watanabe and Raff 1992), growth factors (Bugra et al 1994, Kelley et al 1995, Fuhrmann 1995) and extracellular matrix molecules (Hunter et al 1992) have all been found necessary for complete development of photoreceptors from precursor cells. Interaction between rods and cones also appears necessary for their complete differentiation. In mammals, for

example, cones are born first, but the final stages of their differentiation, those necessary for phototransduction, occur at the same time as, and may depend on, that of rods (Van Ginkel and Hauswirth 1994, Usukura et al 1994). Interaction among cone subtypes also appears to provides cues necessary for their differentiation. In the macaque retina a subset of red and/or green cones generate outer segments (and presumably become functional) at least 3 weeks prior to the time the majority of cones mature (Wikler and Rakic 1994), and may provide the "protomap" foundation for the development of the cone mosaic array of the mature retina (Curcio and Hendrickson 1991, Wikler and Rakic 1994). The formation of a mosaic from a homogeneous population is suggestive of the mechanism described for the formation of ommatidia in Drosophila. In this system, however, the specification of R8, the first photoreceptor to differentiate, appears to be random.

Retina development in teleosts

Teleost fish and some amphibia are unique among vertebrates because their eyes continue to grow throughout the life of the animal (Hollyfield 1968, Straznicky and Gaze 1971, Johns 1977, Beach and Jacobson 1979). The fish retina grows by two primary mechanisms: First, the differentiated retina stretches, apparently as a result of the general expansion of the eye. This causes a progressive decrease in the density of all cell types, except rods (Müller 1952, Lyall 1957b). Rod density is maintained by the continual addition and differentiation of new photoreceptors from precursor cells located in the outer nuclear layer (review in Raymond 1985, Fernald 1991). Second, new retinal proliferation and differentiation occurs continually at the edge of the mature retina

(Müller 1952, Lyall 1957b) and in the embryonic fissure, which extends from the optic nerve head to the ciliary margin along the ventral aspect of the eye.

Peripheral Germinal Zone

In the "ring" of newly developing retina along the periphery of the retina, called the peripheral germinal zone (PGZ), and in the embryonic fissure, new retina is added concentrically from multipotent stem cells. Therefore, the PGZ represents a "timeline" of retinal development, proceeding from completely unspecified neuroepithelial cells at the margin, to completely mature retina more centrally. Although the PGZ is unique and is not found in higher vertebrates, it is likely that the overall mechanism and signals involved in retinal cell fate specification do not differ substantially from that of the mammalian retina. The unique structure of the PGZ means that all cell types are being continuously generated within a restricted area. If all precursors are born identical and multipotent, as suggested by some authors (Turner and Cepko 1987, Holt et al 1988, Wetts and Fraser 1988, Wetts et al 1989, Turner et al 1990), then, given the restricted volume of the PGZ, any extrinsic cell determination signals would have to act laterally at distances of less than a few cell diameters (less than 20 μm). This would seem to limit such signals to direct cell-cell contact.

However, several studies clearly demonstrate that retinal cell specification is sensitive to diffusible signals. For example, previous studies have demonstrated that ouabain- or 6-OHDA-induced neurotoxic destruction of specific cells in the mature retina can lead to upregulated production in the PGZ of specifically those cells (Negishi et al

1982, Reh and Tully 1986, Reh 1987). Furthermore, Altshuler and Cepko (1992) and Watanabe and Raff (1992) demonstrated that the specification of retinal precursors into either a rod photoreceptor or bipolar cell fate *in vitro* is dependent upon a diffusible factor which can act over a distance of at least 5000 μm. Since rods and bipolar cells are being continuously generated in the PGZ, then this diffusible factor must not be temporally regulated if a similar system exists for the PGZ. Additionally, since precursors destined for fates other than rods and bipolar cells are continuously present in the PGZ within less than 100 μm of differentiated rods and bipolar cells, the diffusible signal must not act on these precursors. Thus, the PGZ may present a unique setting in which to test these hypotheses of retinal specification and differentiation mechanisms.

Photoreceptors

In fish, both in embryonic development and in the development of the PGZ, cone outer segments are distinguished histologically well before rod outer segments can be recognized. Cone photoreceptors in differentiated trout retina (and, as discussed above, in many other vertebrates) are arranged in a regular pattern which is repeated in a two-dimensional mosaic array across the retina. In young trout, the cone mosaic consists of a square, with the four sides formed by four double cones and the corners formed by single UV-sensitive cones, surrounding a single cone (Lyall 1957, Hawryshyn and Harosi 1994). The UV-sensitive "corner cones" eventually disappear from the mature retina of the adult (Bowmaker and Kunz 1986, Hawryshyn et al 1989, Kunz 1987, Kunz et al 1994). When the cones are produced from the PGZ, there is no anatomic evidence of the future mosaic. Very quickly, however, the cones then apparently gradually differentiate and organize

into a regular pattern (Larison and BreMiller 1990, Kunz et al 1994). The remarkable fact, however, is that even in fish that lack UV-sensitive corner cones in the mature retina, the retina in the PGZ and embryonic fissure continues to differentiate with a normal cone mosaic that includes the corner UV-sensitive cone (Kunz 1987, Kunz et al 1994). These UV-sensitive cones then almost immediately disappear and are replaced by rods (Kunz et al 1994). This suggests that the particular mechanism of cell fate determination used in the PGZ cannot be abandoned in the adult, despite the apparent futility of generating the corner cones.

Cell Addition in Amphibian Retina

Early studies suggested that cells are also added to the mature retina of amphibians, but these studies have not been repeated using newer techniques. Throughout tadpole development, the inner nuclear layer at a given point in the retinas of both *Xenopus* and *Rana* becomes progressively thicker. Although the process by which this occurs is not understood, studies by Hollyfield (1968) suggested that new cells are added continuously throughout the inner nuclear layer in both animals. Even more interesting, however, was the conclusion that *Xenopus* and *Rana* appear to add cells by different mechanisms. In *Rana*, the inner nuclear layer near the fundus increases from 2 cells thick at stage 3, to 10-11 cells thick at the same location at stage 25. These new cells appeared to have come from cells which migrated in from the margin. After labeling the retina with ^{3}H-thymidine, Hollyfield (1968) found bands of labeled cells in the INL migrating toward the fundus. By 15 days after labeling, some cells had moved from the margin to a position in the inner nuclear layer immediately behind the lens.

Cells added to the ganglion cell layer and outer nuclear layer, however, remained in close proximity to the margin. The velocity of cell migration in the inner nuclear layer decreased with time after division from 16 μm/hr initially, to 9.1 μm/hr after 4 days. A similar mechanism was thought to account for the appearance of new rods throughout the retina (Johns 1977), but, as described above, it was soon found that that new rods are added from rod precursors situated throughout the retina (Fernald 1989). The results of experiments on *Xenopus* were more suggestive of a similar diffuse system of precursors, but such precursors were never found. Although the results of the *Rana* and *Xenopus* studies do not appear to have been repeated or expanded by other investigators, they have also not yet been disproved (Hollyfield, personal communication).

Injury Repair in the Fish Retina

Surgical or neurotoxic damage of the fish retina is repaired through the generation of new retina that arises from the remaining, undamaged tissue (review in Hitchcock and Raymond 1992). Surgically removing a quadrant of the retina in adult goldfish causes proliferation of cells both in the PGZ and along the cut edges of the remnant retina (Lombardo 1972). In time, new retina is regenerated. Ouabain-induced cytotoxic damage of the mature retina of goldfish and rainbow trout also stimulates cell proliferation in the PGZ and in the damaged retina (Kurz-Isler and Wolburg 1982, Maier and Wolburg 1979, Raymond et al 1988). Here, again, the retina eventually regenerates. Extensive experimentation (review in Hitchcock and Raymond 1992) has demonstrated that the regenerated retina derives not from cells in the PGZ, but locally from cells intrinsic to the mature retina. These progenitors of regeneration are proposed to be rod

precursors that have temporarily altered their fate in response to destruction of the retina (Raymond et al 1988, Braisted and Raymond 1993, Braisted et al 1994). Although newt, tadpole and embryonic chicken retinas can also regenerate after injury, it is through transdifferentiation of retinal pigment epithelial cells. Only the teleost retina regenerates from neuronal progenitor cells existing within the mature retina.

Experiments that utilize selective destruction of retinal layers with neurotoxic agents indicate that rod precursors can give rise to the regenerated retina (Raymond et al 1988, Braisted and Raymond 1993). Ouabain-treated retinas can be produced with one of the following patterns of destruction: 1. All retinal neurons destroyed, 2. All retinal neurons destroyed except horizontal cells, 3. All retinal neurons destroyed except horizontal cells, cones, and rods (Maier and Wolberg 1979, Kurz-Isler and Wolberg 1982, Raymond et al 1988). Raymond et al (1988) found that after treatment with ouabain, not only was new retina generated from the PGZ, but, in some cases, neural retina was also generated diffusely throughout the central retina. The source of this regeneration was apparently multipotent cells in the INL which normally produce only rod photoreceptors. These cells remain multipotent and may be regulated by the same mechanisms as the PGZ stem cells. Most importantly, these centrally located stem cells only regenerated neural retina when the extent of neurotoxic destruction included the cells of the photoreceptor layer. The presence of surviving cone photoreceptors was negatively correlated with the amount to which the rod precursors were induced to regenerate the retina, suggesting that the presence of mature cones inhibited their proliferation. These cells are still able to

produce rods, however, indicating that this particular pathway is not inhibited by cones, and that the postmitotic rod precursors are thus restricted in their fate.

In this scenario, the PGZ stem cells are always able to proliferate because the continuous expansion of the retina keeps the stem cells some distance from differentiated cone photoreceptors, while the centrally located stem cells are inhibited from proliferating into anything but rod photoreceptors by their close association with mature cone photoreceptors. In support of this hypothesis, Browman and Hawryshyn (1992) have demonstrated that UV-sensitive cones spontaneously regenerated into their correct position in mature trout retina several weeks after these cones were selectively eliminated by an injection of retinoic acid. Regardless of the extent of tissue injury, mitotic activity is transiently increased after ouabain injection. Although the duration of this increased proliferation has not been reported by previous authors, examination of their figures suggests that the effect is transient (Kurz-Isler and Wolberg 1982, figs 8 and 9; Raymond et al 1988, figs 5 and 9). Since the degree of proliferation did not appear to depend qualitatively on the degree of neuronal destruction, Raymond et al (1988) suggested that this may have been due to a direct effect of ouabain on the stem cells, or the effect of tissue injury. Thus this transient surge in PGZ proliferation would not necessarily indicate the presence of a general mitogenic feedback mechanism.

RETINA DEVELOPMENT IN RAINBOW TROUT

In the following studies, we have investigated the development of the retina in rainbow trout (*Oncorhyncus mykiss*). We have found that clusters of mitotically active

cells exist in the inner nuclear layer of the mature retina of rainbow trout throughout the animals' life. Through a kinetic analysis we demonstrate that these cells migrate to the outer nuclear layer soon after their birth, where they then likely give rise to rod precursor cells. These clusters had been identified previously in embryonic and larval fish, but were assumed to be unimportant for older animals (Johns 1982, Raymond and Rivlin 1987). In further examining the role of these clusters, we have found that their proliferation rate is sensitive to exogenous growth factors, and can be upregulated by light-induced injury to the retina. Our results suggest a revision of the model that explains the cellular origin of the rod precursors in the mature retina. Furthermore, we propose that these proliferating inner nuclear layer cells may also provide a cellular mechanism for the capacity of retinal regeneration known to exist in at least some fish.

OVERVIEW OF CHAPTERS

Chapter 1: Kinetics of growth in the peripheral germinal zone

While retinal development has been studied in teleost fish for many years, these studies have focused primarily on the development of the central retina in larval fish. Very few studies have characterized the development of retina in the PGZ. The first chapter of this study presents a series of experiments designed to characterize the overall nature and kinetics of development in the PGZ.

The first goal was to determine the relationship between the rate of body growth and the rate of PGZ proliferation and differentiation. We exposed striped bass (*Morone*

saxatilis) to feeding and temperature regimes designed to vary the rate of body growth and then examined the anatomical structure of the PGZ of these fish to determine whether proliferation and differentiation within the PGZ are sensitive to body growth. We observed that during rapid growth the rate of proliferation within the PGZ increases, but the rate of differentiation does not. The simplest explanation for this observation is that individual post-mitotic retinal neurons develop at a constant rate, irrespective of the rate of body growth. Both of these factors result in a significantly larger (or "stretched-out") PGZ in the more rapidly growing fish.

Based on the results of this experiment, it seemed likely that we would tend to find the largest PGZs in the most rapidly growing fish. Thus we surveyed five readily available teleost fish to find the most suitable animal model for further studies of retinal development: zebrafish (*Zebradanio rerio*), an African cichlid (*Haplochromis burtoni*), common goldfish (*Crassius auratus*), striped bass (*Morone saxatilis*), and rainbow trout (*Oncorhyncus mykiss,* formerly *Salmo gairdneri*). We found that the PGZ in trout fed under conditions that sustain rapid growth is about four times larger than that in the other four species, which are comparable to each other. We therefore selected the rainbow trout for further study.

As with the striped bass, we found that PGZ size in rainbow trout is sensitive to rate of body growth. To obtain reproducible developmental data we assumed it would be critical to experiment with fish whose life history was known and whose growth conditions were extremely well controlled. We thus developed a small-scale indoor

husbandry facility to raise the trout from fertilized eggs obtained from a local hatchery. With this facility we are able to grow trout at controlled and constant conditions.

With reliably consistent PGZ dimensions between different individuals, we explored the relationship between distance and time within the PGZ. As discussed above, the PGZ represents a time line of retinal development, with completely unspecified neuroepithelial cells at the margin, and completely mature retina more centrally. In these experiments we injected juvenile rainbow trout with the thymidine analog bromodeoxyuridine (BrdU) and allowed the fish to mature for a few days to several weeks. These fish were then sacrificed and the eyes were processed for immunolabeling of BrdU-positive cells. We then characterized the approximate time between birth in the proliferative zone and the acquisition of mature anatomical characteristics.

Chapter 2

During our investigations of PGZ proliferation utilizing the thymidine analog BrdU, we discovered a significant number of proliferating cells within the mature inner nuclear layer (INL). These cells form clusters that align vertically, reaching from the inner to the outer plexiform layers. These clusters had been identified previously in larval fish, and were presumed to migrate to the outer nuclear layer soon after their birth, where they then gave rise to rod precursor cells (Johns 1982, Raymond and Rivlin 1987). However, these cells were considered unimportant for older fish. We refer to these cells as proliferative inner nuclear layer cells (PINCs).

To investigate cell division in the growing retina of trout, we measured the cellular expression of the proliferating cell nuclear antigen (PCNA) in frozen sections of fixed retina. We found large numbers of PCNA-positive cells in the mature inner nuclear layer. To determine whether these cells were a phenomenon of only younger trout, we looked at proliferation in retinas of fish ranging in age from immediately after hatching to more than two years old (fully mature). We found that the density of PINCs declined as eye diameter increased, but that they exist in the retina of even in the oldest animals we tested. Furthermore, we found that the density of proliferating cells decreased progressively from either nasal or temporal pole towards the optic nerve in both the outer and inner nuclear layers. To determine whether these cells proliferated with a circadian rhythm, we assayed PCNA in retinas from fish sampled over a complete 24-hour cycle. We determined that the rate of mitosis in the ONL of trout retina, as in African cichlids (Chiu et al., 1995), has a diurnal rhythm being higher during the dark phase of the daily cycle. In contrast, we did not detect a significant rhythm in the rate of proliferation of the PINCs.

To improve optical resolution over that provided by frozen sections, we prepared 3 μm plastic sections from the retina of fish in which we sustained high levels of systemic BrdU through the use of indwelling subdermal cathethers. Additionally, by using BrdU in combination with a similar molecule, iodo-deoxyuridine (IdU), we were able to track any movement of PINCs from the INL to either the outer nuclear layer or the ganglion cell layer. Our results with double-labeled cells indicate that a fate of PINCs is to migrate into the ONL, where they likely replenish the pool of stem cells that give rise to rod

progenitors. In support of this, we found examples of clusters of BrdU-positive cells crossing the OPL.

If, in addition to migration, PINCs were to die a programmed death through apoptosis soon after birth, this event could be diagnosed through specific histochemical reactions. We used the TUNEL method to identify cells undergoing apoptosis, but could positively identify only very few cells by this method. Thus we have no evidence that PINCs disappear from the INL because of death by apoptosis.

Chapter 3

The anatomical features of the retina in pigmented trout exposed to natural summer sunlight are not significantly different from those found in comparable fish raised indoors (Allen and Hallows 1996). The retina of the albino trout, on the other hand, exhibits significant structural damage to the rod outer segments after exposure to toxic light levels (either natural sunlight in shallow raceways or constant illumination with tungsten lamps). However, there is no detectable structural damage of the rod inner segment nor of any part of the cone photoreceptors. Remarkably, the light-damaged retina of albino trout is fully repaired when fish are returned to light levels only about 1000-fold less intense than those of natural sunlight. After about 30 days indoors, both the density and size of rod outer segment in damaged albino trout are indistinguishable from those of pigmented fish. Teleost retinas, thus, must have remarkable repair mechanisms that both limit the extent of damage while exposed to toxic light levels and support the rapid repair

of rod photoreceptors. In this chapter we explore the possibility that PINCs are central to this repair mechanism.

In the experiments reported here we exposed trout to the same experimental conditions as did Allen and Hallows (1997). After treatment, we measured the density of neurogenic clusters in the retinas of these fish by assaying the cellular expression of PCNA, as described in detail in Chapter 2. In normal pigmented trout the density of PINCs is unaffected by any of the light conditions tested. This, of course, is what might be expected since their retinal structure was normal. In contrast, we observed a large increase in the counts of PCNA-positive cells in albino trout exposed to light levels that caused retinal damage. The elevated cell counts under toxic light levels were essentially the same, independent of the method of retinal damage. For albino trout in which the retina had recovered under dim illumination from structural damage, the cell counts also returned to the values we observed in normal, pigmented trout.

In contrast, the density of proliferative cells in the outer nuclear layer decreased after exposure to toxic light levels, when compared to retinas which had recovered from structural damage. This was true for retinas of both albino and pigmented trout. However, for both constant light and outdoor light, outer nuclear proliferation was higher in retinas from albino than from pigmented trout. It is possible that the decreased density of proliferating rod precursors after toxic light exposure results from inhibition or even direct injury. However, if these precursors are replenishing damaged rods, their decreased density is more likely the result of depletion from continued cell division. Our results demonstrate that PINCs upregulate either in number or in rate of proliferation in

response to light-induced retinal damage. In combination with the results from Chapter 2, we propose that these proliferating inner nuclear layer clusters are replenishing the depleted rod precursors in the outer nuclear layer and thereby endow the retina with the ability to recover from toxic light injury, even in albino animals.

CHAPTER ONE:

KINETICS OF GROWTH IN THE PERIPHERAL GERMINAL ZONE

INTRODUCTION

Background

The eyes of teleost fish and some amphibia continue to grow throughout the animal's life (Hollyfield 1968, Straznicky and Gaze 1971, Johns 1977, Beach and Jacobson 1979, Kock 1982). Although the retina of the embryonic fish develops in a laminar fashion, as does the retina of mammals and other higher vertebrates, the post-embryonic fish retina develops by an entirely separate process consisting of two distinct mechanisms: First, the differentiated retina stretches, causing a progressive decrease in the density of all cell types, except rods (Müller 1952, Lyall 1957b). Rod density is maintained by the continual differentiation and addition of new photoreceptors from precursor cells located in the outer nuclear layer (review in Raymond 1985, Fernald 1991). Second, new retinal differentiation occurs continually at the edge of the mature retina (Müller 1952, Lyall 1957b) and in the embryonic fissure, which extends from the optic nerve head to the ciliary margin along the ventral aspect of the eye. Throughout eye growth, however, visual acuity is maintained by adjusting the angular spacing of cones (and associated retinal neurons) through stretching, while visual sensitivity is maintained by the continual addition of new rods (Fernald 1991 for review). Thus, in the "ring" of newly developing retina along the periphery of the retina, called the peripheral germinal

zone (PGZ), and in the embryonic fissure, new retina is added concentrically from multipotent stem cells.

While retinal development has been studied in teleost fish for many years, these studies have focused primarily on the development of the central retina in embryonic fish, and very few studies have characterized the development of retina in the PGZ. Thus it is not clear to what extent the mechanisms of development in the PGZ are similar to, or even identical to, those of the embryonic retina. However, the PGZ provides an excellent system in which to test hypotheses about regulatory mechanisms of retinal cell specification and differentiation because the PGZ represents a "timeline" of retinal development, proceeding from completely unspecified neuroepithelial cells at the margin, to completely mature retina more centrally. This unique structure of the PGZ means that, unlike the laminar development characteristic of embryonic retina, all cell types in the PGZ are being synchronously and continuously generated within a restricted area. The experiments in this chapter are designed to answer some basic questions about retinal development in the PGZ, with the goal of providing a foundation for future research utilizing this unique system.

Is the PGZ sensitive to regulation?

The first question addressed is whether proliferation and differentiation in the PGZ are sensitive to regulatory influences. The mechanisms and signals that regulate retinal growth rates in vertebrates are not clear. Recent evidence demonstrates that when rainbow trout are fed reduced food rations, their body growth rate decreases as expected,

but their eye growth retina growth, does not decrease proportionally, resulting in the production of small fish with disproportionately large eyes (Pankhurst and Montgomery 1994). While these authors didn't specific measure retinal cell density, their histological studies indicated that retinal growth was proportional to eye growth. In support of this, Raymond, Hitchcock and Palopoli (1988) found that retinal proliferation in goldfish appears to be dependent on eye growth. These authors studied Black Moor goldfish, which have greatly enlarged eyes, presumably resulting from congenitally increased intraocular pressure. For fish in which the two eyes were of different sizes, the number of ganglion cells and dividing rod precursors was systematically higher in the larger eye.

In contrast, when microphthalmia is induced in embryonic chicks, presumably as a result of decreased intraocular pressure, retinal proliferation proceeds at a disproportionately high rate, resulting in folding of the retina (Coulombre 1956, Narbaitz and Marino 1988). Thus, these results indicate that retinal proliferation may be intrinsic and independent of eye growth. Additionally, Kröger and Fernald (1994) have suggested that eye growth in African cichlids is at least partially regulated by illumination history, particularly by light wavelengths that stimulate the green-sensitive cones, either alone or in concert with the blue-sensitive and yellow-sensitive cones.

Proliferation in the PGZ appears to locally regulated, since asymmetric growth in the PGZ is required to maintain the fovea in the central retina (Easter 1992, Cameron 1996). The mechanism for this local control has not been demonstrated. Furthermore, it is not known whether changes in intraocular pressure, body growth rate, or illumination history can alter the activity of the PGZ. To begin to address this question we focused on

effects of somatic growth and raised striped bass (*Morone saxatilis*) under conditions favoring high or low growth rates. We then assayed the retinal PGZs of these fish for differences in morphology indicating altered proliferation or differentiation rates. We found that proliferation rate, but not differentiation rate (as indicated by the appearance of photoreceptor outer segments), were increased in the fish subjected to high growth conditions.

Which fish is the best experimental model?

Having discovered that the PGZ is sensitive to growth rate, we sought to determine which teleost fish would possess the best experimental system for further study. The ideal PGZ would be very large, thereby providing good spatial resolution, and would be consistent between animals of the same age and raised under identical conditions. The ideal fish would be readily available year-round, and would be inexpensive and simple to raise. Thus the next task was to select the single most appropriate species for study of the PGZ.

The PGZ exists at the margin of the growing retina of all teleost fish studied to date. In fish, age is a poor predictor of developmental state, because development is linked to body size and body size is strongly influenced by nutritional composition of food, rate of feeding and water temperature (Piper et al 1982). Furthermore, as described above, this may not be strictly true of the central nervous system in fish, for which growth and development may be independent of body growth rate, within limits (Pankhurst and Montgomery 1994). Nonetheless, to obtain reproducible developmental data we assumed

that it would be critical to experiment with fish whose life history is known and whose growth conditions are extremely well controlled. We restricted our selection to one among five possible choices: the zebrafish (*Zebradanio rerio*), an African cichlid (*Haplochromis burtoni*), the common goldfish (*Crassius auratus*), the striped bass (*Morone saxatilis*), and the rainbow trout (*Oncorhyncus mykiss,* formerly *Salmo gairdneri*). To select among the species we carried out anatomical studies of the PGZ in fish of comparable developmental state. We found the PGZ in rainbow trout is several times larger than that of the other fish surveyed. Moreover, trout are hardy and inexpensive, and eggs are available year-round from a local hatchery.

How long does it take cells born in the PGZ to differentiate?

We reasoned that the explanation for the larger PGZ in rainbow trout was the species' rapid growth rate, as compared to the other fish surveyed. If, as suggested by our studies of growth rates in striped bass, proliferation rate, but not differentiation rate, are increased in rapidly growing fish, then this would suggest that the temporal resolution of the PGZ timeline was much higher that that of other fish. Rough calculations by Hitchcock and Easter (1986) suggested that the PGZ in goldfish takes from 6 to 16 months to mature. The width of the PGZ in the rainbow trout retina is at least twice that of the goldfish, and remains relatively constant with a constant rate of body growth. The purpose of this experiment is to determine the time required for neuronal cells born in the PGZ to acquire mature anatomical characteristics under normal growing conditions.

Since the PGZ represents a timeline of development, with the younger cells nearer the retinal margin and the older cells nearer the central retina, we reasoned that we could determine the approximate age of any retinal cell by its distance from the neuroblast region of the PGZ if we also knew the relationship between distance and time. Thus, we needed to determine this relationship. However, this assumes that the rate of retinal growth is constant. So we first developed methods for raising trout under controlled, constant growth rate conditions. This required construction of a recirculating aquarium with automatic feeders. Once this was accomplished, we were able to consistently raise rainbow trout from eggs under conditions that resulted in an approximately linear rate of eye growth.

Now with eyes growing at a constant rate, we determined the relationship between distance and time for the PGZ. This required us to be able to identify the birthdates of cells at different positions in the retina. When made available to cells, the thymidine analogs bromo-deoxyuridine (BrdU) and iodo-deoxyuridine (IdU) are taken up across the cell membranes and are incorporated into DNA during the "S" phase of the cell cycle. Cells containing BrdU or IdU can be identified histologically using specific immunohistochemical techniques. However, a simple single label pulse protocol could not be used in studies of the PGZ because both terminal and non-terminal cell divisions occur side by side. We developed an alternative protocol in which we use two different labeled deoxyuridine nucleotides, IdU and BrdU. The two markers can be selectively identified in histological sections with specific antibodies (see Methods) and can be used to precisely determine the birthdate of labeled cells (see Results). To determine the time-

distance calibration for each nuclear layer, we determined the distance between the neuroblast region of the PGZ and labeled cells in each of the nuclear layers, then divided these values by the post-birthdate age for each cell.

Is development of the PGZ similar to that of embryonic retina?

We next wanted to determine the similarities between retinal development in the PGZ with that of the characteristically laminar embryonic retinal development. The PGZ represents a timeline of development. Thus, if development in the PGZ is similar to that of the embryonic retina, the progression of anatomic changes along the peripheral-to-central developmental gradient in the PGZ should duplicate that found by serial observations of progressively more mature embryonic fish retina. Thus we compared the anatomy of the PGZ with published studies of retinal development in embryonic fish. We found that the overall changes in PGZ structure are nearly identical with those reported for the embryonic retina.

MATERIALS AND METHODS

Tissue processing for epoxy resin embedding

For retinas to be embedded in epoxy resin, the fish were deeply anesthetized in ice (warm-water fish) or in 0.05% tricaine methanesulfonate (trout) followed by decapitation and pithing. The eyes were then removed, bisected and placed in a mixed aldehyde fixative (1% paraformaldehyde and 2.5% glutaraldehyde in phosphate-buffered saline) for several hours or overnight at 4 °C. Fixed eyes were then rinsed four times in phosphate buffer and then post-fixed in 0.5% osmium tetroxide overnight. The eyes were then thoroughly rinsed, dehydrated in graded alcohols, and infiltrated with graded epoxy resin (Epon 812, Electron Microscopy Sciences) in propylene oxide. After infiltration in pure epoxy resin, the eyes were placed in BEEM capsules (Electron Microscopy Sciences) and the epoxy was polymerized at 80 °C. Blocks were sectioned on wet glass knives at 2 μm. Sections were stained with toluidine blue in borate buffer at 70 °C, cleared in water, and coverslipped under Permount (Fisher).

BrdU and IdU immunohistochemistry

In some of the experiments reported here, proliferating cells were identified following exposure to BrdU alone. In other experiments we followed the fate of cells by using two different markers of proliferation, BrdU and IdU, that can be selectively identified in the same section with specific antibodies and appropriate enzymatic amplification systems. We modified the double-label methods originally described by

Miller et al (1991). We used antibodies Bu1/75, a rat monoclonal antibody (mAb) which identifies BrdU specifically (SeraLab, Crawley Down, Sussex, supernatant obtained through Accurate Chemical Corp, Westbury, NY), and IU-4, a mouse mAb which recognizes both BrdU and IdU (Caltag, South San Francisco, CA),. We confirmed that in the trout retina Bu1/75 does specifically recognize BrdU, and not IdU, by finding that the antibody failed to label any cells in the retina of fish maintained for several days in water containing IdU.

Eyes were removed from fish anesthetized by immersion in an overdose of MS-222, decapitated and pithed. The intact eyes were fixed overnight at 4 °C in Bouins solution (Polysciences, Warrington, PA) They were then rinsed with water, the cornea removed and the lens extracted. The tissue was dehydrated through a series of alcohols and infiltrated overnight at 4 °C with glycol methacrylate (JB-4, Polysciences, Warrington, PA). The JB-4 was exchanged once again, polymerization initiated and embedding completed overnight at 4 °C. 3 mm thick sections were cut on glass knives, transferred to amino-silanized glass slides (Henderson 1989) and dried overnight at 37 °C.

To label with either of the two deoxyuridine retinal sections were rehydrated in distilled water for 10 min, incubated in 4 M HCl for 30 min and again rinsed with distilled water. Following 10 min immersion in 0.15 M TBS/T (0.15 M NaCl, 0.05 M Tris, 0.1% Tween-20, pH 7.4), slides were incubated in pronase E (1 mg/ml) in 0.15 M TBS/T for 30 min at room temperature. The enzyme was thoroughly rinsed with TBS/T and sections then incubated for 30 min in blocking solution. For Bu1/75, the blocking

solution consisted of 1:50 rabbit serum in 0.15 M TBS/T. This was followed by incubation in the primary Ab solution containing 1:13 Bu1/75 and 1:50 rabbit serum for 3 hrs. After rinsing with 0.15 M TBS/T sections were incubated in the biotinylated secondary Ab (rabbit anti-rat IgG) (1:100) with 1:50 rabbit serum in 0.15 M TBS/T for 2 hrs at room temperature or overnight at 4 °C. The slides were rinsed, incubated in casein blocking solution for 30 min and treated with the reagents of the ABC-AP kit. After thorough rinsing with 0.15 M TBS/T, alkaline phosphatase activity was detected with red AP substrate (SK-5100, Vector Labs, Burlingame, CA). This substrate rendered the cells red under transmitted light and fluorescent under epi-illumination. Sections were rinsed with water, air dried and mounted with Gel/Mount (Biomeda, Foster City, CA).

In double-labeled sections, BrdU was always immuno-labeled first as described above. After reacting with the Red AP substrate, sections were washed with distilled water and placed in 0.5 M TBS/T (0.5 M NaCl, 0.05 M Tris, 0.1% Tween-20, pH 7.4) for 30 min. They were next incubated for 30 min in blocking serum consisting of 1:50 horse serum, 2 drops/ml Avidin D blocking solution (Vector Labs) in 0.5 M TBS/T. This was followed by a 3 hr reaction with IU4-mAb (1:100) in a solution of 1:50 horse serum and 2 drops/ml biotin blocking solution (Vector Labs) in 0.5 M TBS/T. Sections were rinsed in 0.5 M TBS/T and then incubated for either 2 hr at room temperature or overnight at 4 °C in biotinylated secondary antibody (horse anti-mouse IgG) (1:100) with 1:50 horse serum in 0.5 M TBS/T. The slides were rinsed, incubated in casein blocking solution for 30 min and treated with the reagents of the ABC-AP kit. IdU was selectively recognized because the alkaline phosphatase activity in this reaction was detected with blue AP substrate

(SK-5300, Vector Labs). Slides were rinsed in water, air dried and mounted with Gel/Mount.

Measurements of Retinal Dimensions

In order to compare the dimensions of retinas from different fish, it was necessary to select structural features which were consistent, easily recognizable, and generally correlated with overall germinal zone size. The indicators used in this paper are the cross-sectional area of the neuroblast region, and the distance between the beginning of the germinal zone and the first identifiable cone outer segment. The neuroblast region is defined here as that region consisting of spindle-shaped neuroblast cells, which are characteristic of dividing neuronal precursors. These dimensions were measured in stained sections using a microscope with a camera lucida attachment and a computerized digitizing tablet, or with digitized images and image analysis software (NIH Image).

Effect of Body Growth on PGZ Dimensions

To examine the effects of somatic growth rate on PGZ proliferation, striped bass were raised at conditions favoring either high or low growth rates for three weeks. At the end of this period, the retinas were processed and analyzed for differences in gross morphology. Sixteen striped bass of the same age and approximately the same size were obtained from a commercial hatchery. Four of these were immediately fixed and processed for epoxy resin embedding. The remaining 12 were placed into recirculating aquaria and separated into either a high growth or low growth treatment group. For the

low growth group, 6 fish were maintained in 21 °C water and were fed commercial trout feed once per day to satiation (<5 min). For the high growth group, the remaining 6 fish were placed in 27 °C water and were given continuous access to food. After three weeks, the fish from both treatment groups were measured, anesthetized in ice water, decapitated, and the eyes were fixed for epoxy resin embedding. The cross-sectional area of the retina's neuroblast region, and the distance to the first cone outer segment were measured for each eye of each fish as described above.

Survey of PGZ

Juvenile zebrafish were obtained from a commercial hatchery (Ekk-Will Water-Life Resources, Gibsonton FL) via overnight air delivery. These fish were 3 weeks post-hatch upon arrival, with an average body length of 10.3 mm. They were placed in a recirculating aquarium in the lab at 27 °C, and were fed goldfish flakes, tropical fish food and spirulina. After three weeks their average body length was 28.8 mm (range 27-32 mm). The fish were then euthanized and their eyes were fixed and processed for epoxy resin embedding using the standard protocol. Goldfish were obtained from a local retail aquarist. The eyes from these fish were fixed and processed as above.

Juvenile striped bass were obtained from a local commercial hatchery (Professional Aquaculture, Chico CA). Average body length was 34.6 mm (range 25-53 mm). The eyes from these fish were fixed at the hatchery and stored on ice for return to the lab. The retinas were then processed for epoxy resin embedding as described above. Juvenile rainbow trout were obtained from a local commercial hatchery (Mt. Lassen

Trout Farms, Red Bluff CA). Fixation and processing was begun at the hatchery, as described above for striped bass. The average body length was 47 mm (range 23-93 mm).

Trout Husbandry

A high-intensity recirculating trout aquarium was constructed in the laboratory based on the principles outlined in Moe (1989) and Piper et al (1989). Fish were held in a 260 liter insulated fiberglass trough (3.3 m x 0.4 m x 0.3 m, length x width x depth) along which water circulated at 80 liters per min. The effluent water from the trough flowed by gravity into a sump tank with a 275 liter capacity (average water volume 200 liters). Water leaving the sump tank passed through a 2 cm thickness double-layer spiral (DLS) foam filter over a layer of crushed volcanic rock at the bottom of the tank. A magnetic drive pump circulated water from the sump tank through a UV-sterilizer, a water chiller and then into a manifold. Water from the manifold was diverted into either a two-stage protein skimmer, an aerobic "wet-dry" bioreactor containing 0.06 m^3 "bio-balls," or the trough. The effluent of the skimmer and the bioreactor drained into the trough. A blower pumped room air at low pressure (5 cm H_2O) directly into the bioreactor (5 liters per min) and through high capacity, low-resistance glass air diffusers into the trough (15 liters air per min). A diaphragm pump forced air at higher pressure (> 50 cm H_2O) through limewood or glass air diffusers into the protein skimmer (7 liters air per min total).

Water quality was maintained primarily by biological filtration (aerobic bacteria in the bioreactor and anaerobic bacteria in the volcanic rock), mechanical filtration (DLS filter) and fractionation (protein skimmer). The system was initially seeded with a

commercial bacterial culture (Fritz-zyme #7). UV-sterilization of the water was used to prevent infections of the fish and reduce algal growth. An in-line activated charcoal filter was also available, but was rarely needed.

Amines, nitrates and nitrites were measured with commercial kits. About 1/4 of the tank water was exchanged every other week with charcoal-filtered tap water. The entire system was kept indoors under artificial illumination designed to match the light spectrum of normal daylight (Vita-Lite, Duro-Test Corp.). Light cycled with 14 hr/10 hr light/dark periods. The chiller maintained the water at 15 °C and a programmable pH controller with an industrial electrode (Cole-Parmer Instruments) maintained water pH at 7.0 ± 0.1 by regulating a 5 M NaOH gravity-feed drip system.

Rainbow trout eyed eggs (approximately 4 weeks post-fertilization) were received year round from Mount Lassen Trout Farms (Red Bluff, CA). Batches of approximately 50 eyed eggs developed while laying on a plastic mesh that formed the floor of a acrylic box immersed in the water trough. The sides of the box facing the streaming water were perforated to allow continuous flow of water over the eggs, which were covered by about 1 cm of water. After hatching (about two weeks after receiving the eggs), the larvae were transferred to a deeper plastic box of similar construction in which the water depth was about 12 cm. The next developmental milestone, the appearance of fully developed fish capable of swimming (and also eating) followed by about two weeks. At this point, automatic feeders provided trout feed (Silver Cup Inc.) at a rate calculated from $F=4.92*(1.67*10^{5}*W)^{-.32}$, where F is feed weight per day and W is the fish weight

(derived from tables in Piper et al 1989). The calculated amount was delivered at 15 min intervals throughout the light hours.

BrdU and IdU Delivery

We continuously infused a solution containing BrdU or IdU to trout via an in-dwelling, subdermal catheter. The catheter consisted of about 15 cm long vinyl tubing 0.01"x0.03" (id x od) encased by a second, snug fitting vinyl tube. The small tube extended about 1 cm past the end of the larger tube. Prior to insertion, the catheter tips were dipped in ethanol and then in betadine and filled with the solution containing the labeled-dU. To insert the catheter, fish were first anesthetized in ice-cold MS-222 solution (7.5 mg/100 ml) and then placed on an aluminum block kept on ice. Under a dissection microscope, the catheter was inserted at the base of the tail and tunneled forward under the skin, alongside the dorsal ridge until its tip was approximately at the thorax. The outside vinyl tubing was used to secure the catheter by suturing it to the dorsal aspect of the tail. The catheter insertion wound and the suture points were dabbed with betadine and the fish returned to the aquaculture trough. Catheter insertion typically took about 1 min. In the trough, individual fish were maintained within a narrow cage that allowed them to swim, but did not allow them to "flip" and, thus, tangle the catheter line. The catheter was connected to a syringe pump that continuously infused nucleotide-containing solution at a rate of about 10 ml/hr. The infusion solution was 5% dextrose in water with 8 mg/ml BrdU and 3 ml/ml Tetracycline stock (50 mg/ml). To prevent infection of the wounds, individual fish were dipped after 3 days for 10-15 sec in a

RESULTS

Regulation of PGZ proliferation

To determine whether proliferation and differentiation in the PGZ are sensitive to regulatory influences, we raised striped bass under conditions favoring low growth (21 °C water with feeding once per day) or high growth (27 °C water with *ad lib* access to food). The retinas were then assayed for changes in cross-sectional area of the neuroblast region of the PGZ, or in the distance between the neuroblast region and the first outer segments of the cone and rod photoreceptors.

Although Pankhurst and Montgomery (1994) found that eye growth in rainbow trout could be independent of somatic growth, we found that eye diameter in the striped bass was approximately linearly correlated with body length regardless of growth rate (Fig 1). However, Pankhurst and Montgomery studied fish which had been on separate growth regimens for many months. Furthermore, they also suggested that the rate of eye growth probably begins to decrease when body growth rate reaches a lower limit. It is important to note that even on the low growth regimen, the striped bass in our experiments increased their body length by an impressive 20% on average over the three weeks of the experiment. Clearly, even this diet was well above a mere maintenance regimen and these fish were therefore unlikely to be starving or malnourished. The striped bass on the high growth regimen, on the other hand, increased their body length by 50% on average during the three weeks of the experiment.

Figure 1.1: Relationship between eye diameter and growth rate in striped bass

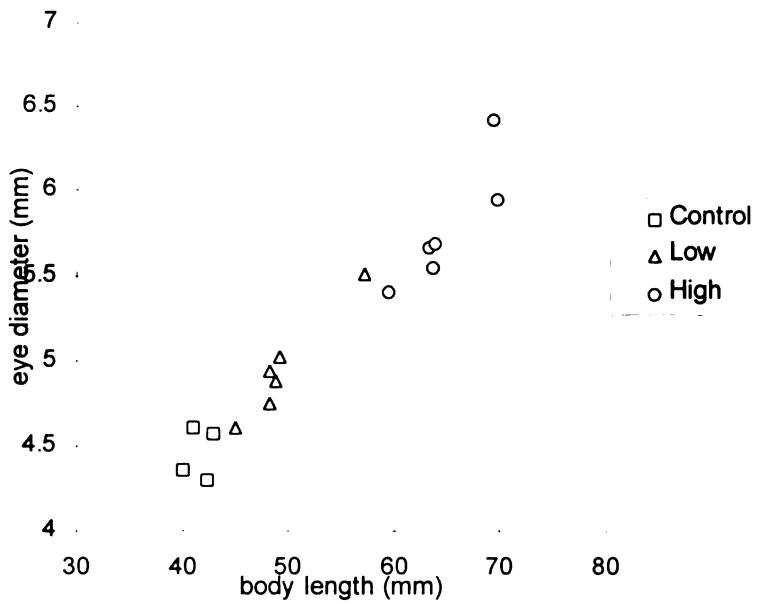


Figure 1. Relationship between eye diameter and body length for striped bass raised at different growth rates. Each data point represents the mean of 8 measurements from an individual (4 measurements for each eye). The control fish were sampled at the beginning of the experiment, and thus were smaller in body length and eye diameter.

The cross-sectional area of the neuroblast region was 50% larger in the high growth fish compared with the low growth fish (Fig 2). Since the neuroblast cells were not obviously larger, this indicates that the number of proliferating cells was increased by 50% as a result of the increased growth. Interestingly, the neuroblast region cross-sectional areas were virtually identical between the low growth and control fish. This might be expected since the control fish were sampled immediately upon arrival from the commercial hatchery, where the growth rates achieved are likely less than optimal due to cooler water and high competition for food.

Figure 1.2: Size of neuroblast region in retinas from striped bass raised at different growth rates

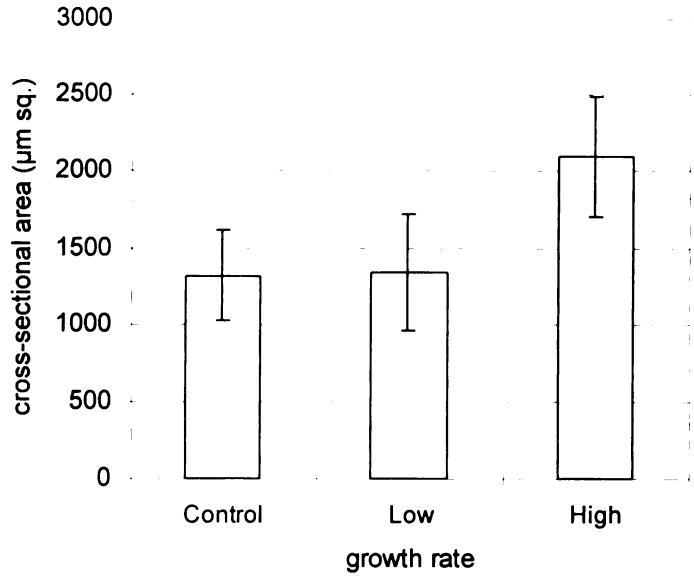


Figure 2. Cross-sectional area of neuroblast region in retinas from striped bass raised at different growth rates. Each data point represents the mean (± S.D.) of 8 measurements from an individual fish (4 measurements for each eye). The control fish were sampled at the beginning of the experiment, and thus are smaller in body length and eye diameter.

The distance between the beginning of the PGZ (the most distal portion of the PGZ) and the first rod and cone photoreceptor outer segment showed a trend similar to that of the neuroblast region cross-sectional area. Both the cone and the rod outer segments were 20% farther from PGZ in the high growth fish compared with the low growth fish (Fig 3). If we accept that the PGZ represents a timeline of retinal development, then the increased distance between the PGZ and the first cone outer segment suggests that the time required for complete cone differentiation (as indicated by the formation of an outer segment) did not decrease in the high growth fish. However, this conclusion is made less certain by two uncontrolled variables. First, the higher rate of retinal expansion in the high growth fish alters the distance-to-time relationship in the retina. This makes any comparisons of time, utilizing distance as an indicator, subject to error. Second, while it is clear that cone photoreceptors are born in the neuroblast region of the PGZ, the neuroblast region increases in size in the high growth fish. Thus some of the cones may be born farther away from the beginning of the PGZ in the high growth fish, which would result in differentiation also being farther from the beginning of the PGZ even if the differentiation rate itself was not affected. For a similar reason, we can not be certain that rod photoreceptor differentiation was also unaffected, since these cells are born outside of the neuroblast region.

Figure 1.3: Distance from PGZ to first photoreceptor outer segments in striped bass raised at different growth rates

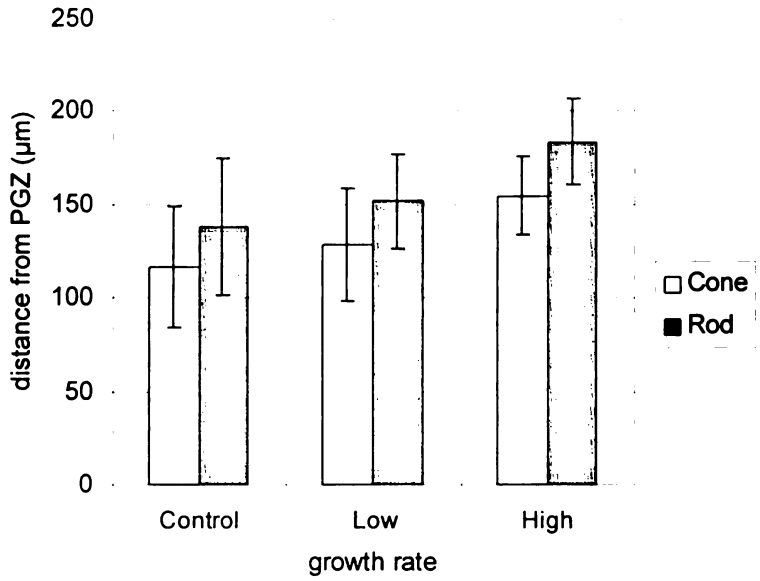
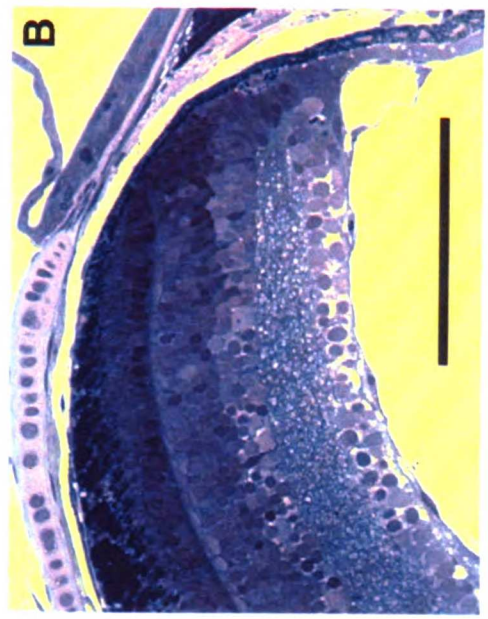


Figure 3. Distance from beginning of PGZ to first cone (open bars) or rod (shaded bars) photoreceptor outer segments in retinas from striped bass raised at different growth rates. Each data point represents the mean of 8 measurements from an individual (4 measurements for each eye). The control fish were sampled at the beginning of the experiment, and thus are smaller in body length and eye diameter.

Survey of Fish

We examined the retinas from five common and readily available fish, with all individuals at approximately the same level of development (early juvenile). The fish examined were the zebrafish, an African cichlid, the common goldfish, the striped bass, and the rainbow trout. With the exception of the zebrafish, which were maintained in the lab for several weeks after air delivery from the hatchery, the fish were processed at the hatchery, or immediately upon arrival to the lab. Representative sections from zebrafish and rainbow trout are presented in Figure 4. As is clear from these micrographs, the PGZ of trout is much larger than that of the zebrafish. We found that the PGZ from rainbow trout is about 5 times larger in area (Fig 5), and is 3 to 4 times longer (Fig 6) than the PGZ from any of the other fish studied.

Figure 4. Photomicrograph of a section of the retina of rainbow trout (A) or zebrafish (B). The sections shown are at the periphery of the retina and they encompasses the peripheral germinal zone (PGZ). This zone reaches from the single layered, undifferentiated neuroepithelium at the periphery (far right on the photographs) to the mature retina towards the center (far left on the photograph). The mature retina is easily identified by the typical layered arrangement of cell bodies and synaptic neuropils. The PGZ in the trout is about four times larger than that in zebrafish. Note the difference in magnification for the two images; the calibration bar in both micrographs is 100 μm.


B

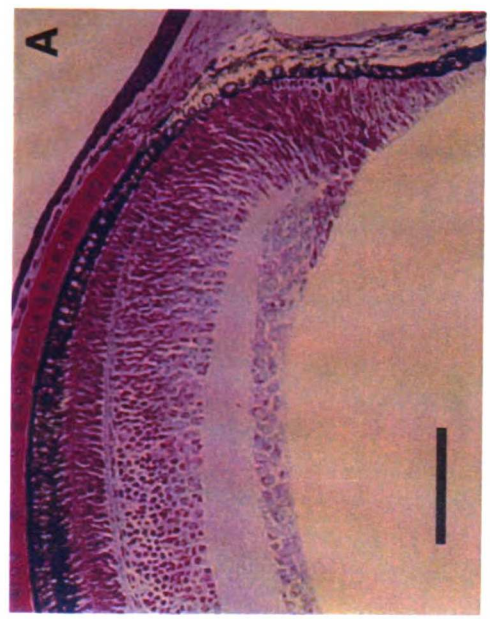

A

Figure 1.5: Size of neuroblast region for different species of fish

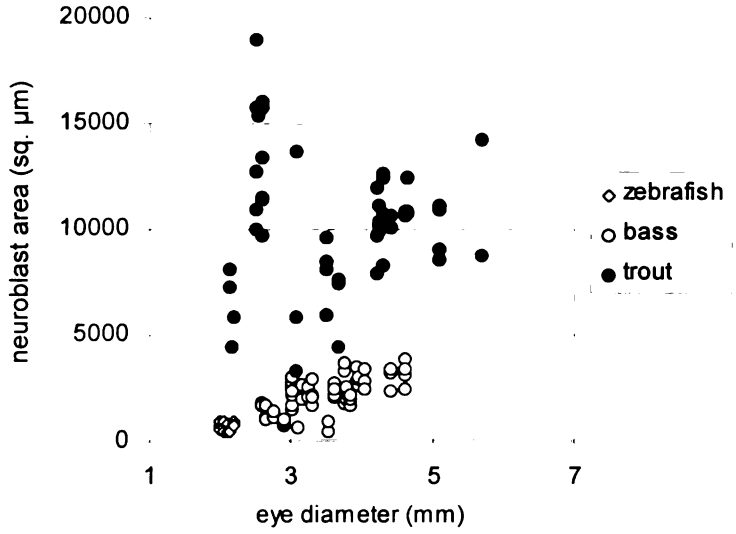


Figure 5. Cross-sectional area of neuroblast region in retinas from zebrafish (diamonds), striped bass (open circles), and rainbow trout (closed circles). The mean values are: zebrafish 714 μm^2; striped bass 2,116 μm^2; rainbow trout 10,325 μm^2. Goldfish and cichlid PGZ were generally intermediate to zebrafish and striped bass (data not shown).

Figure 1.6: Distance to first cone outer segment for different species of fish

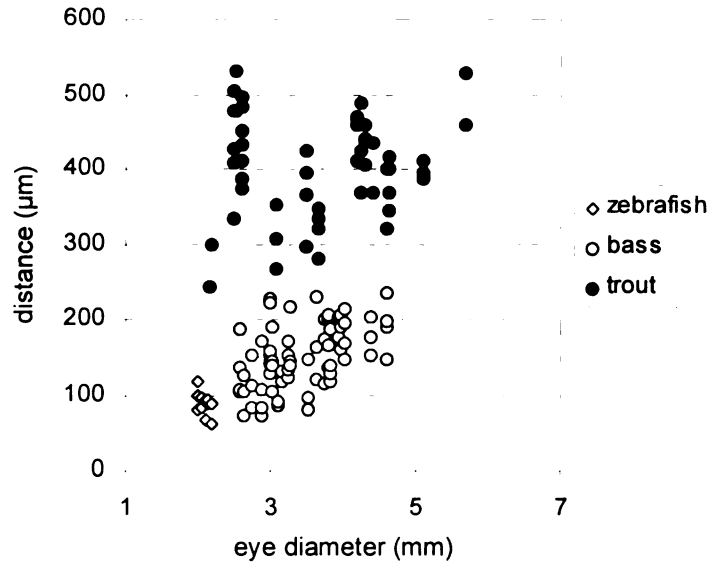


Figure 6. Distance from beginning of PGZ to the first cone photoreceptor outer segments in retinas from zebrafish (diamonds), striped bass (open circles), and rainbow trout (closed circles). The mean values are: zebrafish 91 µm; striped bass 150 µm; rainbow trout 401 µm. Goldfish and cichlid PGZ were generally intermediate to zebrafish and striped bass (data not shown).

Growth of rainbow trout

Rainbow trout is a particularly advantageous species in which to study retinal growth and development. Fertilized eggs are available on demand year-round, small scale aquaculture facilities can be constructed in the research lab, and the fish are sturdy and relatively easy to raise. A particular advantage of raising trout in the laboratory is that by maintaining a well controlled feeding schedule, the rate of eye growth can be defined and different batches of fish at comparable developmental stages can be studied. Figure 7 illustrates the average body weight of rainbow trout raised as described under Methods. Each point is the average weight of individual fish determined by weighing batches of animals, each consisting of 30 to 100 fish. Shown are data collected from 10 different batches raised at various times throughout the year and followed for 1-3 months. In trout there is a well characterized nomogram that relates body weight to body length (Piper et al 1982), and Lyall (1957) developed a nomogram relating body length to eye diameter. Using these nomograms, we converted body weight to eye diameter (Figure 7).

Figure 1.7: Growth of trout under controlled conditions

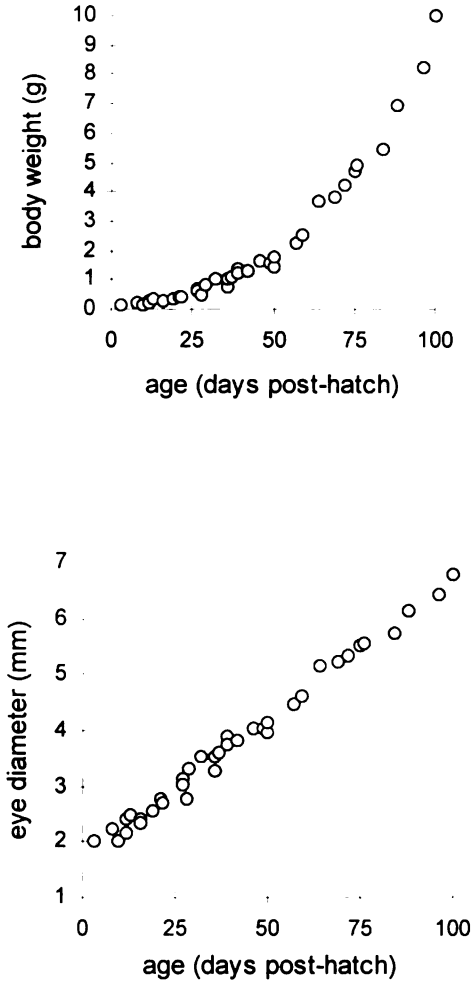


Figure 7. Growth of rainbow trout under laboratory conditions. Trout were raised from "eyed eggs" in a high-intensity recirculating aquarium. Water conditions were controlled to pH 7, 15 °C, and lighting was set to a 14:10 light:dark schedule. Animal care and feeding and water treatment procedures are described in the text. **Top panel**: Increase in body weight with time. Symbols represent average individual body weights for a given batch of 30-100 fish. Data shown represent 10 different batches of

fish raised at various times throughout the year and followed for 1-3 months. **Bottom panel**: Increase in eye diameter with time. Eye diameter was calculated from body length using nomogram provided by Lyall (1957), after first converting body weight data in left panel to body length using tables provided by Piper et al (1982).

Relationship between time and distance in the PGZ

We determined the relationship between distance and time for the PGZ by using the sequential administration of two different thymidine analogs to clearly identify the birthdate of cells in the PGZ. We first provided the fish with IdU for 36 hr, and then maintained a steady level of BrdU for up to the following 3 days. We then allowed the fish to grow for periods of a few hours to two weeks. Cells in terminal mitosis during the administration of IdU were labeled only with IdU. If the cells divided again, they were labeled both by IdU and BrdU. Cells that divided only after the IdU pulse were labeled with BrdU alone. To determine the time-distance calibration for each nuclear layer, we determined the distance between the neuroblast region of the PGZ and labeled cells in each of the nuclear layers and divided these values by the post-birthdate age for each cell.

The trout used in these experiments were raised under the same conditions as those for the fish represented in Fig 7. For each nuclear layer, the relationship between the age of a labeled cell and its distance from the neuroblast region was approximately linear (Fig 8). A least-squares linear regression (Table 1) was fit to each data set to estimate the rate of linear growth for each nuclear layer. We found that the growth rate for the inner nuclear layer, horizontal cell layer, and outer nuclear layer were all similar at approximately 35 μm per day. In contrast, the ganglion cell layer appeared to grow at only 21 μm per day.

Note that this slower growth rate for the ganglion cells might be expected because the more vitreally positioned ganglion cell layer curves sharply at the PGZ (see Fig 4A),

and therefore the ganglion cells grow over a smaller perimeter than do cells in the inner and outer nuclear layers. However, because ganglion cells are comparatively sparse and were often poorly labeled with IdU, we do not have reliable data for cells at late time points. Thus, this estimate for ganglion cell layer growth rate is likely to be less accurate than growth rate estimates for the other nuclear layers.

Figure 1.8: Relationship between time and distance in the PGZ

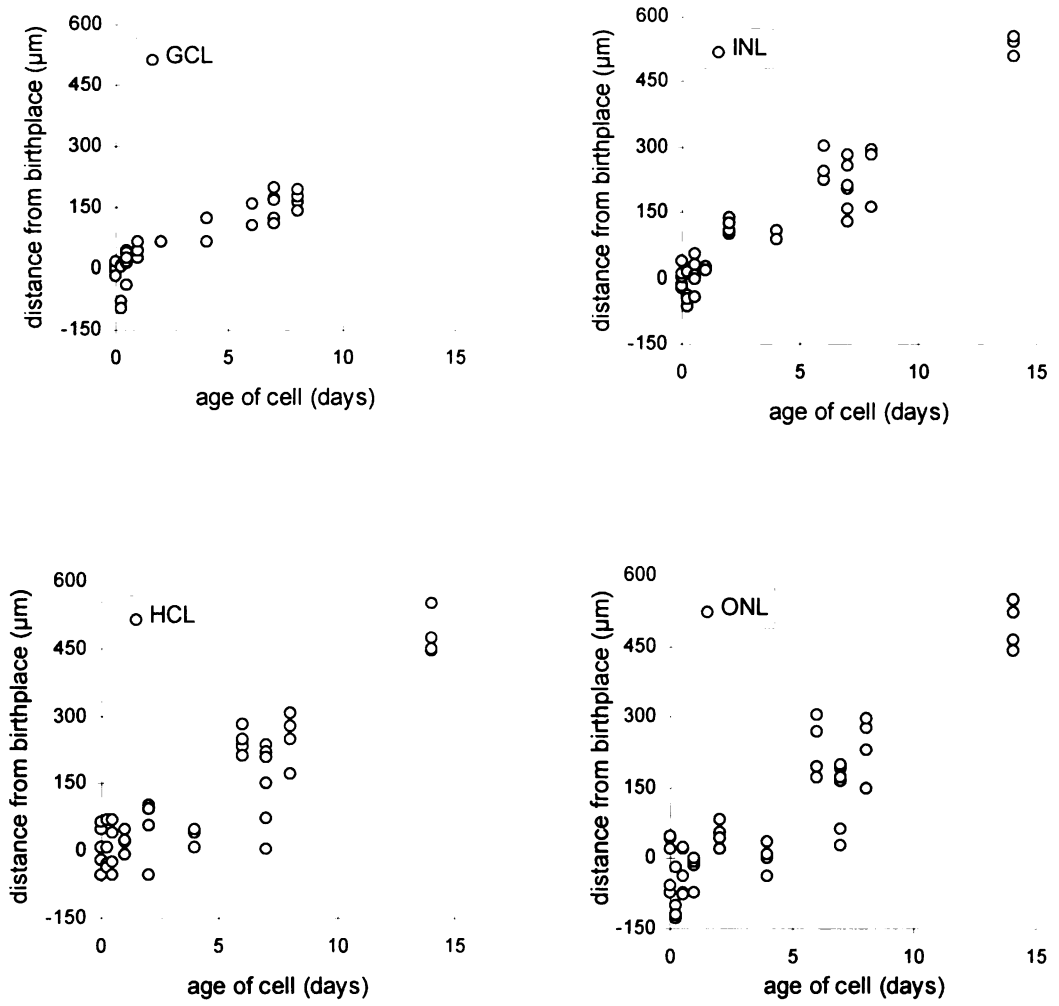


Figure 8. Relationship between time and distance for IdU-labeled cells in the rainbow trout retina. Distances are from the position at time 0 (at the switch from IdU to BrdU). Data represent 4 measurements from each of 10 different fish. Negative values at very early time points are due to variability in PGZ anatomy between histological sections of different fish, as opposed to "backwards" migration of cells.

Table 1.1: Growth rates near PGZ

Nuclear layer	GCL	INL	HCL	ONL
growth (μm/day)	21	37	33	36
correlation (r^2)	0.78	0.91	0.83	0.84

Table 1. Growth rates near PGZ for each retinal nuclear layer in rainbow trout. Least squares regressions (growth in μm per day) were fitted to the distance data presented in Fig 8. GCL is ganglion cell layer, INL is inner nuclear layer, HCL is horizontal cell layer, and ONL is outer nuclear layer. Growth rates for INL, HCL and ONL were not significantly different from each other.

Similarities between PGZ and embryonic development

Using the data from the previous section, we could estimate the time required for various characteristics of retinal differentiation to occur in the PGZ. These are listed in Table 2. These values are preliminary, but give a rough estimate of the rate of differentiation for the various parts of the neural retina.

Table 1.2: Differentiation time in PGZ

Characteristic	Time required
IPL	> 1 day
OPL	2 days
1st COS	4 days
1st ROS	5 days
Mature GCL	8 days
Mature INL	8 days
Mature ONL	9 days

Table 2. Approximate time required for retina derived from the PGZ to acquire characteristics of mature retina. "IPL and "OPL" represent the first appearance of the inner and outer plexiform layers, respectively. "Mature GCL" is time at which the ganglion cell layer coalesces into a layer one nucleus thick. "Mature INL" and "Mature ONL" are the times required for the inner and outer nuclear layers, respectively, to attain their mature thickness. "1st COS" and "1st ROS" represent the first appearance of an outer segment for the cone and rod photoreceptors, respectively.

DISCUSSION

Does rate of body growth affect rate of PGZ development?

In fish the eye does not grow in direct proportion to body size, and it displays negative allometry with body length (Lyall 1957b, Johns and Easter 1977, Pankhurst and Montgomery 1994). However, recent evidence demonstrates that ocular growth may also be uncoupled from somatic growth under certain circumstances (Pankhurst and Montgomery 1994, and for review). Pankhurst and Montgomery (1994) studied trout that were reared on high or low feed rations and found that up to a certain level of food deprivation, growth of the eye is maintained at the expense, or in spite, of low somatic growth. Thus trout fed low ration diets had proportionally larger eyes than trout raised on high ration diets. They further proposed that interspecific differences in ocular morphology, which had been assumed to be adaptive responses to the photic environment, may arise at least partially from the somatic growth profile of the fish. Thus, for example, fish found in the lower mesopelagic zones of the open ocean typically have very large eye sizes. But they also have very slow growth and grow to be very old. One explanation for this independence of retina growth on somatic growth may be that certain organ systems develop on a fixed growth program. Additionally, retinal proliferation in embryonic chick does not appear to be dependent on the rate of eye growth. Experimental induction of microphthalmia, either surgically (Coulombre 1956) or by intraocular injection of the cytotoxic drug cisplatin (Narbaitz and Marino 1988), did not cause in a proportional decrease in retinal proliferation, with the result that the retina

eventually began to fold back on itself. Thus the rate of growth of the neural layer appeared to be intrinsically regulated and not dependent on the rate of growth of the pigmented layer or sclera, or intraocular pressure.

In contrast, Kröger and Fernald (1994) raised cichlids under white light exposure with low feed rations and found that the lower food availability decreased somatic growth, as expected, and also eye growth. However, the dimensions of the eye were always presented as normalized to lens diameter, and no absolute measurements of the lens were presented. Thus, since we do not know the relationship between lens growth and somatic growth in these fish, it is not clear whether the results of Kröger and Fernald (1994) support or contradict those of Pankhurst and Montgomery (1994). It would be interesting to find out whether lens growth is dependent on somatic growth.

Our studies on striped bass have shown that the cross-sectional area of the neuroblast region increases with increased rate of body growth. One could imagine that increased body growth, which leads to increased eye diameter by an unknown mechanism, would "stretch out" the PGZ, unless there were some compensation. The only explanation for an increased cross-sectional area of the neuroblast region under these conditions, given that no large gaps are seen between cells and that individual neuroblast cells do not become larger, is that the neuroblasts are responding by increasing the rate of cell division. Furthermore, the increase in distance between the neuroblast region and the first cone outer segments suggests that the rate of differentiation for individual post-mitotic neurons does not change in response to alterations in somatic growth rate (although consideration must be made for the caveats presented in Results).

To our knowledge, this experiment provides the first description of exogenous influences on the rate of neuronal proliferation in the retina. However, the results are far from conclusive for the rate of differentiation. A specific test of our conclusion that the rate of retinal differentiation is independent of body growth rate would be to expose fish, ideally rainbow trout, to BrdU or IdU during an exposure otherwise identical to the one performed on here on striped bass. If the rate of post-mitotic development is constant, three specific findings should be observed. First, the labeled cells from the retinas of the faster-growing fish should be farther away from the ciliary margin than the labeled cells of the slower-growing fish. Second, all labeled cells should be at a similar stage of morphological development, irrespective of the rate of body growth. Third, the retinas of faster-growing fish should contain more labeled cells than those of slower-growing fish, since the rate of cell division at the margin would need to be increased to keep up with the demands imposed by increased retinal growth.

Rainbow trout have the best PGZ

Based on the results of the growth experiment, it seemed likely that we would tend to find the largest PGZs in the most rapidly growing fish. Thus we surveyed a number of readily available teleost fish to find the most suitable animal model for further studies of retinal development in our lab. We restricted our selection to one among five possible choices: zebrafish, an African cichlid (*Haplochromis burtoni*), the common goldfish, the striped bass and the rainbow trout. Retinal development has been studied to some extent in all these species and they are all abundant and readily available. To date, the vast majority of work on retinal development in fish has been performed in goldfish

or other closely related cyprinid species. However, their benefits of hardiness and low cost are outweighed by the difficulty of breeding them year round, since they are generally seasonal breeders. Thus their value for our studies would largely have been their historical significance. On the other hand, the increasing popularity of zebrafish among molecular biologists has resulted in an abundance of new experimental tools with which to study retinal development. Additionally, zebrafish are hardy, breed year round, and are very inexpensive to maintain. Although cichlids are currently used by only one laboratory to study retinal development (Fernald 1991 for review), they are relatively simple to breed and maintain in the laboratory throughout the year. Perhaps the most unusual candidate for our study was the rainbow trout. This species was selected for evaluation because it grows rapidly and is a strongly visual feeder. Trout are also naturally seasonal breeders, but long-standing commercial interests support an industry that can supply fertilized eggs year round. Additionally, while they are not generally raised as "hobby fish," as are the other candidate species, extensive information for commercial aquaculture and the commercial availability of high quality trout food makes it relatively easy to grow these fish in the lab under well controlled conditions (Piper et al 1982).

We found that the PGZ in trout fed under conditions that sustain rapid growth is about 4 times larger than that in the other 3 species, which are comparable to each other. For example, the distance between the point of transition from single to multicell epithelium at the margin of the PGZ and the first identifiable cone outer segment (Figure 1) is about 400 μm in trout, but only 90 μm in Zebrafish or goldfish. The larger PGZ

offers higher spatial (and therefore temporal) resolution, an important feature in our studies. Because of this significant size advantage, we elected to study the retina in rainbow trout. Also, extensive information and the commercial availability of optimized trout food makes it relatively easy to grow these fish in the lab under well controlled conditions (Piper et al 1982). The advantage of zebrafish, the potential use of genetic tools, is compromised by the small size of the PGZ and the fact that in these fish photoreceptors are organized in a two-tiered layer.

Retinal growth in trout

As with the striped bass, we found that PGZ size in rainbow trout is sensitive to rate of body growth (data not shown). To obtain reproducible developmental data we assumed it would be critical to experiment with fish whose life history was known and whose growth conditions were extremely well controlled. To allow us this ability we developed a small-scale indoor husbandry facility to raise the trout from fertilized eggs obtained from a local commercial supplier (Mt. Lassen Trout Farms, Red Bluff, CA) precisely 2 weeks after fertilization. Two weeks later fish hatch and a week later they start feeding. We sustain their growth at an optimal rate by following instruction of commercial trout feed suppliers (Silver Cup). Animals grow at a rapid pace, doubling their size every 20 days. We monitor growth rate daily. In four weeks time, the average fish weight is 1.2 gr. With this facility we are able to grow trout at controlled and constant conditions (Fig 7). We generally carry out all our studies in fish that are more than 4 and less than 6 weeks post-hatching. In this time window, the eyes grow from a

diameter of about 4.9 mm to 6 mm, but the histological characteristics of the PGZ do not change.

Over the first 3 months post-hatching, eye diameter increases linearly with time under our growth conditions. If we assume that the back of the eye is hemispherical and the retina covers all its surface, the observed change in eye diameter indicates that the retina grows at a rate of about 10% increase in surface area per week, a remarkable rate of growth. Using this same model, we can estimate that the retina expands radially from the center at a rate of 60 μm per day over this period. Kock (1982) calculated that retinal expansion accounted for 44% of retinal growth in the crucian carp inner nuclear layer, with the remaining 56% due to cell addition at the PGZ. Using these values, we would estimate that 56% of the 60 μm growth seen in the trout eye would be due to cell addition. The resulting figure of 34 μm is remarkably similar to the value of 35 μm determined from our measurements of retinal growth in the PGZ (Figure 8 and Table 1).

Similarities between PGZ and embryonic development

In fish, both in embryonic development and in the development of the PGZ, cone outer segments are distinguished histologically well before rod outer segments can be recognized. Cone photoreceptors in differentiated trout retina (and in many other vertebrates) are arranged in a regular pattern which is repeated in a two-dimensional mosaic array across the retina. In young trout, the cone mosaic consists of a square, with the 4 sides formed by 4 double cones and the corners formed by single UV-sensitive cones, all surrounding a single cone (Lyall 1957, Hawryshyn and Harosi 1994). The UV-

sensitive "corner cones" eventually disappear from the mature retina of the adult (Bowmaker and Kunz 1986, Hawryshyn et al 1989, Kunz 1987, Kunz et al 1994). As the cones are produced from the PGZ, there is no anatomic evidence of the future mosaic. Very quickly, however, the cones then apparently gradually differentiate and organize into a regular pattern (Larison and BreMiller 1990, Kunz et al 1994). Remarkably, the retina in the PGZ and embryonic fissure continues to differentiate with a normal cone mosaic that includes the corner UV-sensitive cone, even in fish that lack UV-sensitive corner cones in the mature retina (Kunz 1987, Kunz et al 1994). These UV-sensitive cones then almost immediately disappear and are replaced by rods (Kunz et al 1994). This suggests that the particular mechanism of cell fate determination used in the PGZ cannot be abandoned in the adult, despite the apparent futility of generating the corner cones.

Hagedorn and Fernald (1992) identified a second wave of neurogenesis, in addition to that in the neuroblast region of the PGZ. This second wave is restricted almost exclusively to the outer nuclear layer and produces a small number of cells destined to become rod photoreceptors. While the vast majority of dividing cells in the second wave are in the outer nuclear layer, they identified a small number of dividing cells (about 10 per eye) in the inner nuclear layer. Furthermore, the inner nuclear layer thickness decreases at a time coincident with the beginning of the second wave of neurogenesis. Hagedorn and Fernald concluded that a population of cells was moving out of the inner nuclear layer, presumably to become rod progenitors in the outer nuclear layer. They proposed that the integration of rod progenitors into the outer nuclear layer

from the inner nuclear layer may be a unique embryonic process, not repeated in the continuing adult development of the teleost retina. Thus, the precursors were thought to continue production of rods without any further migration from the inner nuclear layer, whereas pluripotent stem cells in the PGZ provide a continuing source of all retina cells types, including rods.

As the retina expands during growth, the relative position of all cells changes over time from near the margin to a steadily more central position. This forms two concentric zones of cell maturation in the retina. The central zone contains fully mature cells, while the PGZ contains immature cells. Hitchcock (1987) studied the distribution of a particular ganglion cell type in the goldfish, and found that, as in the mammalian retina, the cells were non-randomly distributed. He found that the dendritic fields of this cell type overlap such that every retinal point is covered by the dendritic field of at least one cell and often two. This coverage is established when the retina is small and ganglion cell density is high, and is maintained as the retina grows by stretching. This agreed with an earlier electrophysiological study showing that the size of the receptive field centers of goldfish ganglion cells increased with growth, maintaining a constant overlap (Macy and Easter 1981). Although immature ganglion cells at the retinal margin expand their dendritic fields by both adding new dendrites and by the interstitial growth of existing dendrites (Hitchcock and Easter 1986), as in the retina of the cat during retinal expansion (Mastronarde et al 1984), the mature ganglion cells in the goldfish increase dendritic field size only by passive towing of the dendrites by the expanding retina (Hitchcock and Easter 1986).

Since some new ganglion cells are added to the margin in addition to growth due to stretching, the visual angle subtended by a ganglion cell decreases with growth. However, dendritic field growth does match retinal growth due to stretch (Bloomfield and Hitchcock 1991). Both morphological and electrical modeling analyses indicate the small arbors of young fish and large arbors of older fish are virtually identical except for scaling differences (Bloomfield and Hitchcock 1991). In other words, the dendritic arbors of ganglion cells scale up in size as the retina stretches, while preserving the architecture established during the phase of dendritic outgrowth. Hitchcock and Easter (1986) found that ganglion cells in the PGZ of goldfish typically have fewer dendritic branches, shorter dendrites, and smaller dendritic field diameters than ganglion cells located more centrally. These authors estimated that ganglion cells took 6 months to mature in a 1 year-old goldfish, and 16 months to mature in a 4 year-old goldfish.

In contrast to the long period required for ganglion cells to acquire their mature dendritic morphology, the appearance of early markers of retinal differentiation, such as lamination of the neuronal layers and the production of outer segments by photoreceptors, occur very soon after the appearance of the optic primordia in embryonic fish. Hagedorn and Fernald (1992) described the morphological changes in the embryonic cichlid retina, and found that lamination was apparent by day 3, less than one day after the beginning of retinal cell proliferation. We have used their data to compare the timeline of retinal development in embryonic cichlids, when the retina develops in a laminar fashion, with that of the PGZ in trout (Table 3). The agreement between these two mechanisms, even

between different fish species, is surprisingly close, and suggests that cell differentiation in the PGZ and embryonic retina is identical.

Table 1.3: Comparison of PGZ and embryonic retinal differentiation

Characteristic	Embryonic cichlid	Trout PGZ
IPL	< 1 day	< 1 day
OPL	1-2 days	2 days
1st COS	3 days	4 days
1st ROS	6 days	5 days
Mature GCL	7 days	8 days
Mature INL	> 7 days	8 days
Mature ONL	> 7 days	9 days

Table 3. Time required for retinal differentiation in the embryonic retina of the African cichlid *Haplochromis burtoni* compared with the PGZ of rainbow trout. Data for the cichlid are from Hagedorn and Fernald (1992). These authors only presented data for the retina up to day 7, so all time points after that are not known. Data for trout and all abbreviations are from Table 2.

Questions for future research

This similarity between PGZ and laminar retinal development raises some interesting questions. For example, if all retinal precursors are born identical and multipotent, as has been suggested by some authors (Turner and Cepko 1987, Holt et al 1988, Wetts and Fraser 1988, Wetts et al 1989, Turner et al 1990), then any extrinsic cell determination signals would have to act laterally at distances of less than a few cell diameters (less than 20 μm) in the PGZ. This would seem to limit such signals to direct

cell-cell contact. However, several studies clearly demonstrate that retinal cell specification is sensitive to diffusible signals. For example, previous studies have demonstrated that neurotoxic destruction of specific cells in the mature retina can lead to upregulated production of specifically those cells in the PGZ (Negishi et al 1982, Reh and Tully 1986, Reh 1987). Furthermore, Altshuler and Cepko (1992) and Watanabe and Raff (1992) demonstrated that the specification of retinal precursors into either a rod photoreceptor or bipolar cell fate *in vitro* is dependent upon a diffusible factor which can act over a distance of at least 5000 μm. Since rods and bipolar cells are being continuously generated in the PGZ, then this diffusible factor must not be temporally regulated if a similar system exists for the PGZ. Additionally, since precursors destined for fates other than rods and bipolar cells are continuously present in the PGZ within less than 100 μm of differentiated rods and bipolar cells, the diffusible signal must not act on these precursors. Thus, the PGZ may present a unique setting in which to test these hypotheses of retinal specification and differentiation mechanisms.

CHAPTER TWO:

CELL PROLIFERATION IN THE MATURE INNER NUCLEAR LAYER

INTRODUCTION

The retina in the eye of teleost fish continues to grow throughout much of the animal's life (review in Fernald 1991) and has the capacity to regenerate when damaged by chemical or mechanical injury (Maier and Worburg 1979, Hitchcock et al 1992, Raymond et al 1988, Braisted and Raymond 1993). Investigation of retinal growth in teleosts, therefore, has served over time as a model system to study processes that regulate and modulate neuronal proliferation, specification and repair. This extends most recently, for example, to studies of the effects of growth factors on cell proliferation (Negishi and Shinagawa 1993, Mack et al 1995), investigation of genetic markers associated with neuronal regeneration (Hitchcock et al 1996) and the possible regulatory interaction among photoreceptors that may control the sequence of their birth (Stenkamp et al 1996). Lyall (1957) first described the growth of the retina in trout, extending the observations originally made by Müller (1952) in the retina of guppies. In very young trout, most of the retinal growth can be accounted for by the continuous development of new retinal tissue in a narrow ring (about 300 μm wide) at the periphery of the mature tissue. This ring of developing tissue is termed peripheral growth zone (PGZ). New retinal development also occurs in a fissure that extends along the ventral pole from the center of the retina to its margin named the embryonic or choroid fissure (Kunz 1987). In older fish, cell addition at the PGZ and choroid fissure continues, but a greater proportion

of growth reflects stretching of the mature retina. Retinal growth in other teleost fish is similar (goldfish: Johns and Easter 1977, Johns 1977, Meyer 1978, Wagner 1974, crucian carp: Kock 1982, carp: Negishi et al 1985)

Stretching of the mature retina as the eye grows causes a continuous decrease in the packing density of all retinal neurons, except rod photoreceptors. Nearly constant rod density reflects the continuous birth of new rods in the outer nuclear layer (review in Raymond 1985, Fernald 1991). In the growing retina of trout, while the density of rods is constant, the center-to-center distance between cones increases and a specific class of cones, those sensitive to UV light, disappear entirely. Thus, Lyall (1957) reported a 4:1 ratio of rods to cones in the mature retina of early post-embryonic fish, that increased to 7:1 in eyes 4 mm in diameter and 12:1 in 8 mm diameter eyes. In trout over 60 grams in weight the UV-sensitive cones disappear, apparently through programmed cell death (Bowmaker and Kunz 1987, Hawryshyn et al 1989). This specific cone loss can be induced prematurely by systemic elevation of thyroxin (Browman and Hawryshyn 1992, 1994). Interestingly, UV-sensitive cones continue to be generated at the peripheral growth zone and choroid fissure even when they have disappeared from the mature retina (Kunz 1987). Sandy and Blaxter (1980) and Johns and Fernald (1981) (see Johns 1982 for complete details) were the first to demonstrate that the new rods continuously added to the differentiated retina develop from precursor cells embedded in the outer nuclear layer.

Johns (1982) described in detail the anatomical sequence of events underlying post-embryonic retinal growth in the goldfish retina, with particular attention to the

features of photoreceptors. Using pulse injection of ^{3}H-thymidine to label proliferating cells, Johns observed in larval fish (those within 1 week of hatching) the expected mitotic activity in the PGZ. Surprisingly, she also observed labeled cells in the ostensibly mature inner and outer nuclear layers adjacent to the germinal zone. The mitotically active cells of the inner nuclear layer formed clusters and, occasionally, individual cell bodies of a cluster were observed in the outer plexiform layer (Raymond and Rivlin 1987). In juvenile fish (>180 days post-hatch), injected ^{3}H-thymidine did not label cells in the inner nuclear layer at all. Johns (1982) proposed that the clusters of proliferating cells observed in the inner nuclear layer at the larval stage, which she called neurogenic clusters, were undifferentiated cells that, within the lifetime of the larval stage, migrated across the outer plexiform layer and into the outer nuclear layer. In the outer nuclear layer, the neurogenic cells became the progenitors of rods, which are born throughout the fish's lifetime. After larval development, new rod precursors would be inserted into the developing outer nuclear layer at the peripheral growth zone by a similar mechanism. Raymond and Rivlin (1987) supported this proposal through the anatomical reconstruction of neurogenic clusters from serial electron-microscopic sections of the retina. This study established that neurogenic clusters in larval goldfish consisted of 3 to 4 cells wrapped by branches of the Müller cells. One of the reconstructed clusters included cell bodies clearly located in the outer plexiform layer.

We report here on a study of growth in the mature retina of trout reared in the laboratory under conditions optimized to favor rapid development. We measured cellular proliferation in the retina with the use of labeled deoxy nucleotides made available not

through the usual pulse injection, but through their sustained presence in the metabolic pool over many days. We have uncovered that mitotically active cells exist in the inner nuclear layer of the mature retina throughout the animals' life, and not just during its larval stage, as data previously available in goldfish would have predicted (see above). These proliferative cells in the inner nuclear layer appear anatomically analogous to the neurogenic clusters first described by Raymond (1982). We describe some of the anatomical features of the clusters of proliferating cells and through a kinetic analysis we demonstrate that they migrate to the outer nuclear layer soon after their birth. These cells are likely to give rise to the rod precursor cells. Our results suggest a revision of the model that explains the cellular origin of the rod precursors in the mature retina. Moreover, the proliferating inner nuclear layer cells may also provide a cellular mechanism for the retinal regeneration known to occur in response to chemical or mechanical injury.

MATERIALS AND METHODS

Continuous provision of labeled deoxy-uridine

We used either brominated (BrdU) or iodinated (IdU) nucleotide and two different methods to sustain elevated systemic levels of labeled deoxy-uridine in laboratory-reared trout (see Chapter 1 for details of animal husbandry). In the first method, we continuously infused a solution via an in-dwelling catheter. This protocol is described in detail in Chapter 1. Briefly, 1-2 g fish were first anesthetized in ice-cold MS-222 solution (7.5 mg/100 ml) and then placed on an aluminum block kept on ice. The catheter was then inserted into the base of the tail and sutured to the skin. The catheter insertion wound and the suture points were dabbed with betadine and the fish returned to the aquaculture trough. In the trough, individual fish were maintained within a narrow cage that allowed them to swim, but did not allow them to "flip" and, thus, tangle the catheter line. The catheter was connected to a syringe pump that continuously infused nucleotide-containing solution at a rate of about 10 ml/hr. The infusion solution was 5% dextrose in water with 8 mg/ml BrdU and 3 ml/ml Tetracycline stock (50 mg/ml). To prevent infection of the wounds, individual fish were dipped every 3 days for 10-15 sec in a solution of malachite green (66 mg/liter). We infused fish for periods ranging from 1 to 8 days.

In a second method, 1-1.5 g fish numbering up to 25 were kept at 15 °C in 0.5 liter volume of water containing either 2 g/l IdU or 4 g/l BrdU. In this small container water contained neutral regulator (0.08 g/l, SeaChem Labs) and was continuously aerated, but

there were no provisions to maintain water quality by biological means. Therefore, water was exchanged at least every other day. We maintained fish in the labeled water for periods ranging from 1 to 10 days. This second method was much simpler, but yielded less intensely labeled cells, presumably because the concentration of labeled nucleotide in the metabolic pool was smaller than by infusion.

PCNA immunohistochemistry

Eyes were removed from fish anesthetized by immersion in an overdose of MS-222, decapitated and pithed. The intact eyes were fixed overnight with continuous agitation at 4 °C in alcoholic formaldehyde (9:1 95% ethanol:40% formaldehyde) (Bancroft and Stevens 1990). The eyes were rinsed with water, the cornea removed and the lens extracted. The eyecup was immersed in 30% sucrose for several hours. The sucrose was replaced overnight at 4 °C with 1:1 30% sucrose:Tissue-Tek O.C.T compound (Miles Inc.). The tissue was freeze-embedded in the O.C.T. compound and 16 μm sections cut with a freezing microtome. The sections were transferred to gelatin-subbed slides and dried.

Sections were rehydrated in PBS/T for 10 minutes, incubated for 1 hr in blocking serum (20 μl/ml horse serum) and then incubated overnight at 4 °C in a mixture consisting of 1:50 horse serum and 1:100 of mouse mAb against PCNA (PC10, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS with 0.3% Triton-X and 1% DMSO. Following this incubation sections were rinsed with PBS/T and incubated for 2 hours in 1:50 horse serum and 1:100 biotinylated horse anti-mouse Ab. To reduce nonspecific

background staining, the sections were again rinsed in TBS/T and incubated for 30 min in casein blocking solution (0.5% casein in Dulbecco's PBS with 0.05% Tween-20 and 0.5 g/l Na azide). After rinsing with PBS/T the sections were incubated for 1 hr with reagents of the Vectastain ABC-AP kit (Vector Labs, Burlingame, CA). After thorough rinsing with PBS/T, alkaline phosphatase activity was detected with red AP substrate (SK-5100, Vector Labs, Burlingame, CA), thus rendering the BrdU containing cells red in color under transmitted light. Sections were rinsed with water, dehydrated through graded alcohols followed by xylene and mounted in Cytoseal 60 (Stephens Scientific, Camden, NJ).

BrdU and IdU immunohistochemistry

In some of the experiments reported here, proliferating cells were identified following incubation with BrdU alone. In other experiments we followed the fate of cells by using two different markers of proliferation, BrdU and IdU, that can be selectively identified in the same section with specific antibodies and appropriate enzymatic amplification systems. We modified the double-label methods originally described by Miller et al (1991). The immunohistochemical techniques for identifying BrdU and IdU are described in detail in Chapter 1. We utilized the antibodies Bu1/75, a rat mAb which identifies BrdU specifically (SeraLab, Crawley Down, Sussex, supernatant obtained through Accurate Chemical Corp, Westbury, NY), and IU-4, a mouse mAb which recognizes both BrdU and IdU (Caltag, South San Francisco, CA). Antibodies were recognized using an alkaline phosphatase-linked ABC reaction (Vector Labs, South San Francisco, CA), as described in Chapter 1.

Histochemical detection of cells in apoptosis

Fish were anesthetized in ice-cold MS-222, decapitated and pithed. The eyes were then removed, and eyecups produced by removing the cornea and lens. Eyecups were fixed overnight with gentle agitation at room temperature in 4% paraformaldehyde in phosphate buffer. They were then embedded in paraffin and 7 μm sections cut and transferred onto gelatin-subbed slides (Bancroft and Stevens 1990). The presence of short stretches of DNA in cell nuclei, characteristic of apoptosis, was identified by the TUNEL method (Gavrielli et al 1992) using a commercial kit (Boehringer-Mannheim, with alkaline phosphatase converter). To confirm that our method could detect apoptotic cells in retinal sections (positive control) we used: a) DNAase I-treated sections prepared from normal animals and b) sections prepared from animals exposed to 5-fluorouracil (5-FU). This cytotoxic agent is known to cause apoptosis in dividing cells (Sakaguchi et al 1994). We determined independently that single intraperitoneal injections of 5-FU (0.02-0.2 mg 5-FU /g fish weight in 5% dextrose) cause apoptosis in the proliferative zone of the trout retina within 2 days of injection.

Cell counting

We counted cells following the methods and recommendations suggested by Saper (1996). To determine the number of PCNA-positive cells in the inner and outer nuclear layers (INL and ONL) of retinas collected from trout of various ages, we counted under the microscope every identifiable cell profile in sections that extended from nasal to temporal peripheral growth zone (PGZ) along the equatorial plane. In each section we

also measured the distance between the nasal and temporal PGZs along the photoreceptor margin using software tools on digital image files (NIH-Image video acquisition and analysis software). To compare data among various animals and stages of growth, we expressed our results in units of cell density, not as absolute numbers. This method is valid because as the eye grows, PCNA-positive cells did not change in size or shape, only in prevalence.

We determined the distribution of the density of INL and ONL proliferating cells along the equatorial plane from nasal to temporal pole. In these experiments we measured 4 radial sections 3 μm thick prepared from the retina of one eye of each of 6 fish, all of approximately the same weight (range 3.5 to 4.5 g). To compare data among the various experimental animals, we defined 9 equal size fields along the retina from nasal to temporal poles, each 720 μm in length, excluding the peripheral growth zone. We defined the optic nerve as the origin. Because of the asymmetry in optic nerve location, there were 5 counting fields on the nasal side of the sections and 4 on the temporal side. We counted under the microscope BrdU-positive cell profiles in the INL and ONL and expressed results simply as the density of cells in each counting field. Again, this ratioing method is valid because the size of the sampling field was constant and the cells did not change in size, shape or orientation.

To analyze the number of BrdU- and IdU-positive cells in the INL and ONL of animals exposed to both labels, we used the following strategy. Equatorial sections running from nasal to temporal PGZ were photographed on color reversal film, both under brightfield and epifluorescence illumination, and all analyses were conducted on

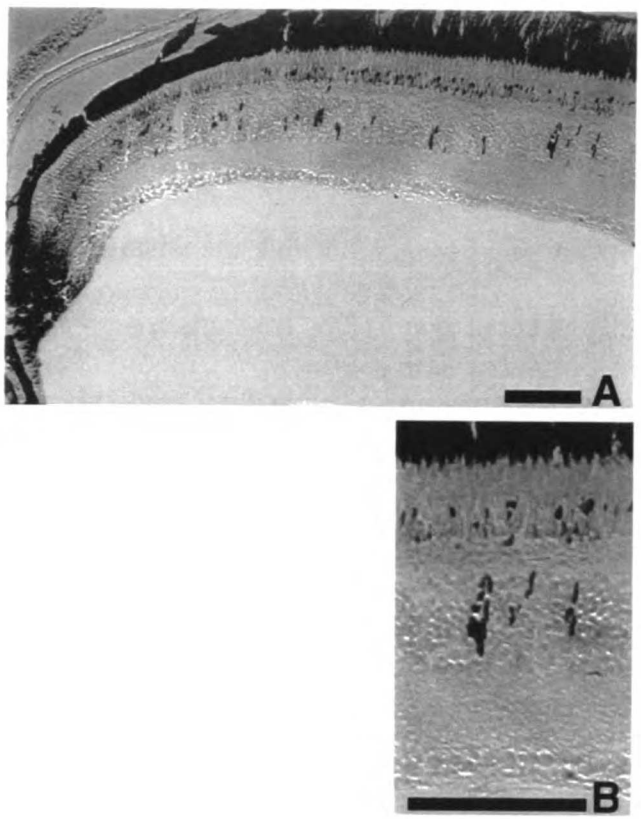
the photographic images. Because all fish in these experiments were of similar weight (1.06 ± 0.08 g, mean ± SD, n=19, range=0.99 - 1.23 g) their eye diameters and the size of each histological section were also similar. BrdU or IdU-positive cells were easily identified by their distinct color. In each image, we defined a field, 1800 μm in length starting just inside the PGZ in the nasal pole. Nucleotide-labeled cell profiles were counted within that field in the INL and ONL of each and every section. We also measured the total number of cell profiles in the INL within a field 180 μm in length. The number of labeled cell profiles in each section, then, could be normalized by an unvarying feature because the density, size, shape and orientation of the INL cells in the mature retina of fish of nearly the same size does not change significantly over a period of 8 days, the maximum length of our experiments. Therefore, while we cannot provide an absolute number of deoxynucleotide-positive cells, we can compare the numbers measured in the many sections investigated, because each number is a ratio where the denominator is constant among the sections.

RESULTS

Proliferative cells in the inner nuclear layer of the retina

To investigate cell division in the growing retina of trout, we measured the cellular expression of the proliferating cell nuclear antigen (PCNA). This nuclear protein is synthesized in early G1 and S phases of the cell cycle and serves as an excellent marker to identify cells undergoing mitosis (Yu et al 1992). PCNA is highly conserved among vertebrates and Negishi et al (1990) first reported the successful use of a PCNA antibody in fish retina. A typical micrograph of trout retina immunoreacted with an antibody against PCNA is shown in Figure 1. As could be expected, we found PCNA-positive cells in the proliferative growth zone (PGZ) at the margin of the retina and in the outer nuclear layer. The former are cells dividing in the process of retinal development, whereas the latter are progenitors of rod photoreceptors. We were surprised, however, to discover large numbers of PCNA-positive cells in the mature inner nuclear layer. These cells, which we will refer to as proliferating inner nuclear cells (PINCs) frequently existed as clusters that aligned in vertical columns spanning the nuclear layer from inner to outer plexiform layers (Figure 1).

Figure 1. **A**: Micrograph of a radial section of trout retina immunolabeled with PCNA antibody. The 16 μm cryosection was cut from nasal to temporal pole along the equator of the eyeball of a 2 g trout. PCNA-expressing cells were identified with a secondary antibody linked to alkaline phosphatase, and appear as darkly stained cell bodies in the section. The section includes the nasal PGZ. PCNA-positive cells are found in the PGZ, the outer and the inner nuclear layer. In the mature inner nuclear layer, PCNA-positive cells form clusters that align vertically and reach from the inner to the outer plexiform layers. **1B**: Magnified view of representative clusters in 1A. The scale bars in each figure are 100 μm.


A
B

Prevalence of PINCs in older trout

As discussed above, Johns (1982) first observed proliferating cells in the mature INL of goldfish, but only at their larval stage, not in older animals. We explored the prevalence of PINC clusters in trout of various ages. For smaller fish (eye diameter ≤ 4 mm) we investigated animals reared in our laboratory. These animals were up to 2 months post-hatch (about 2 g weight). Older animals were collected at the trout farm that provided us with the fertilized eggs. The largest eye we studied (16 mm diameter) was collected from a sexually mature fish, 24 months post-hatch, that weighted 3 Kg. Cell counts as a function of eye diameter are shown in Figure 2. To compare among individual fish, cell counts were normalized with respect to the length of the retinal section, as detailed under Methods. Each point is the average of values measured in 4 to 6 sections of a single fish. Data were collected in a total of 26 animals. We detected PCNA-positive cells in the INL of every one of the fish we studied. The density of PINCs, however, declined as eye diameter increased. This decline is approximately exponential ($r^2 = 0.90$, standard error = 0.15). Thus, the prevalence of PINCs declines with animal age, but they exist in the retina of even in the oldest animals we tested.

Figure 2.2: Density of PCNA-positive INL cells in trout of different ages

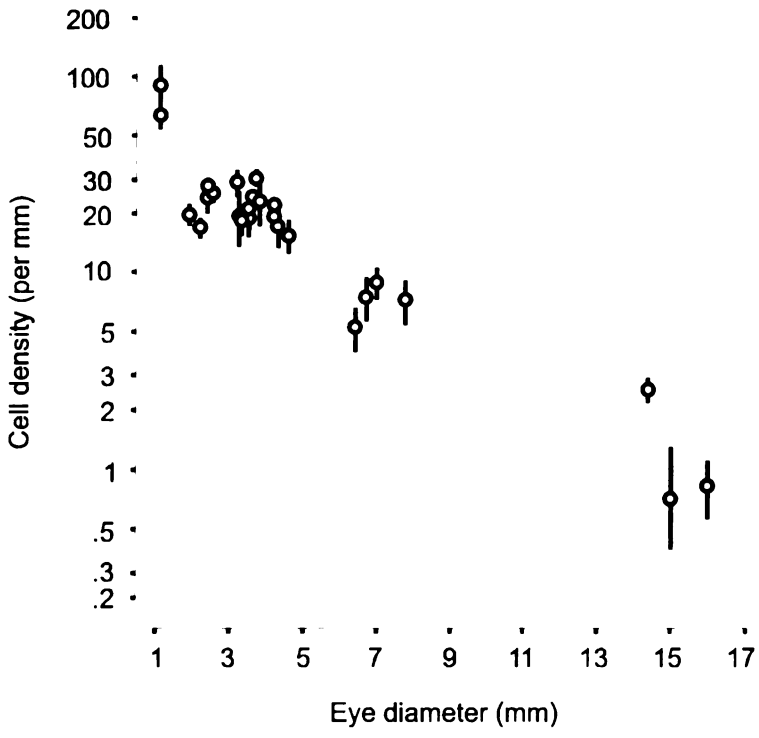


Figure 2: Average density of PCNA-immunoreactive cells in the mature inner nuclear layer of retinas from rainbow trout of various ages between 1 day and 2 years post-hatch. Density was measured as the number of PCNA-positive INL cells per linear mm in 16 μm thick equatorial radial cryosections cut from nasal to temporal poles. Cells counts do not include immunoreactive cells in, or immediately adjacent to, the proliferative growth zone. Arrows indicate approximate body weights at the ages indicated. Vertical lines on the data points mark ± SD.

Spatial distribution of clusters of PINCs

We investigated the distribution density of the PINCs in the equatorial plane of the eye, between the nasal and temporal poles. To improve optical resolution over that provided by frozen sections, we prepared 3 μm plastic sections from the retina of fish in which we sustained high levels of systemic BrdU through the use of indwelling subdermal cathethers. Single peritoneal injections of the nucleotide yielded far fewer clusters of PINCs than we observed following labeling with sustained levels. This result likely reflects the slow rate of proliferation of the cells in the INL clusters. In this experiment we continuously delivered BrdU through the indwelling catheter for 2 to 4 days and immediately sacrificed the animals. BrdU-positive cells, that is, cells that were in "S" phase at some time during the peritoneal infusion, were present both in the ONL and the INL. The cells labeled in the ONL presumably differentiated into rods and they appeared as single labeled nuclei located in either of two distinct bands on the sclerad and vitread limits of the ONL. The labeled INL cells, as was the case with PCNA label, appeared in clusters aligned vertically frequently reaching from the scleral to the vitreal ends of the nuclear layer (Figure 1b).

To measure the distribution of BrdU-positive cells, we counted labeled cells in radial sections cut at the equator from nasal to temporal poles. To compare among various animals we normalized cell density as described under Methods. Figure 3 illustrates the distribution of newly born cells across the retina, both in the ONL and the INL. The height of the bar is the mean of values measured in 6 fish at the same

developmental stage (approximately 6 weeks post-hatch), in 4 to 6 sections from each fish. In both nuclear layers the density of proliferating cells decreased progressively from either nasal or temporal pole towards the optic nerve. At any location, however, the density of BrdU-positive cells was higher in the ONL than the INL. Also, in both nuclear layers, the density of BrdU-positive cells was larger on the nasal than the temporal side of the retina. On both temporal and nasal sides, the density of PINCs was largest in the section immediately adjacent to the PGZ. These findings are similar to those for rod progenitors in *Haplochromis burtoni* (Kwan et al 1996).

Figure 2.3: Radial distribution of BrdU-positive cells in trout retina

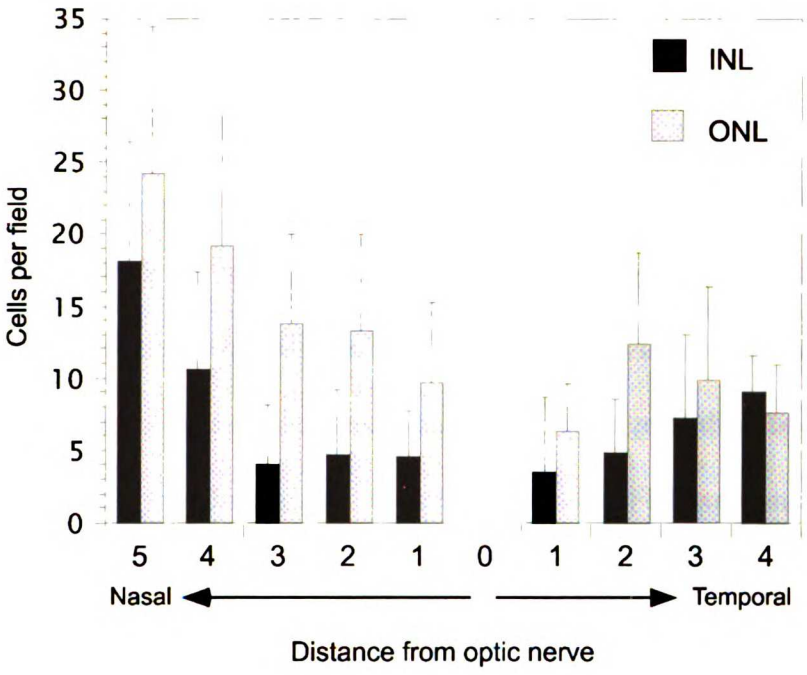


Figure 3: Density distribution of BrdU-positive cells along the equatorial plane from nasal to temporal poles in the retina of rainbow trout. The number of cells in each of 9 fields, 720 μm-wide, was counted in both outer and inner nuclear layers. Each bar represents the mean (± SD) from 4 radial sections from one eye of each of 6 fish.

Diurnal rhythm in the rate of cell proliferation in the INL and the ONL

The rate of cell division in the ONL exhibits a diurnal rhythm in African cichlid fish, being higher during the night phase of a 12 hr dark/light cycle (Chiu, Mack and Fernald 1995). Before undertaking a detailed study of the fate and function of the PINCs we thought it important to determine whether their rate of birth exhibited a daily rhythm. We conducted these measurements in mid-summer with fish raised indoors at the Mount Lassen Trout Farm. We studied animals at the same stage of development as our previous laboratory studies (about 40 days post-hatch). Fish were maintained under normal seasonal light supplemented with timed fluorescent lighting. Sunrise was at 6:00 AM and sunset at 8:30 PM. Over a 24 hr period, we collected and sacrificed animals at 3 hr intervals commencing and ending at 9:30 AM. In darkness, we collected animals under infrared illumination with a viewing device equipped with an image intensifier. To assay the rate of proliferation, we determined the number of PCNA-positive cells along the equatorial plane of the retina from nasal to temporal poles. We collected data from the retina of 3 to 4 fish at every time point and we counted cells in 4 equatorial retinal sections from one eye of each fish. To compare data among the many fish investigated, we defined the average cell density measured at 6:30 AM as unity.

The rate of mitosis in the ONL in trout, as in African cichlids (Chiu et al 1995), has a diurnal rhythm, being higher during the dark phase of the daily cycle. We found that the PCNA-positive cell density in the ONL increased steadily and significantly during the early morning hours, peaking just before sunrise (Figure 4).. In contrast, a

similar trend was not seen for PCNA-positive cells in the INL, although we did detect a significantly decreased density in the INL at 15:30. However, given the abruptness of this density change, we cannot be certain it is physiologically relevant.

Figure 2.4: Diurnal rhythm of PCNA-positive cells in trout retina

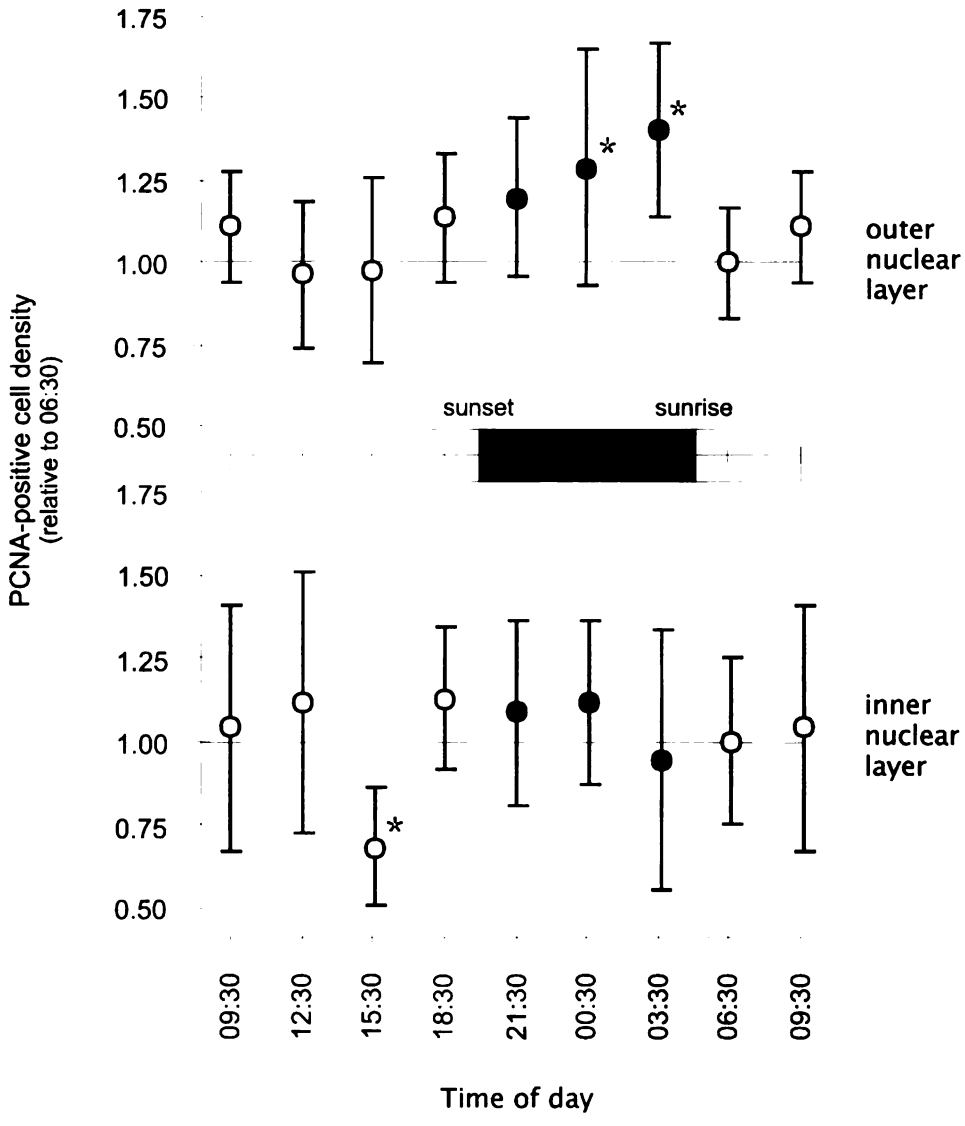


Figure 4: Number of PCNA-immunoreactive cells in the outer (top) and inner (bottom) nuclear layers of rainbow trout retina measured at selected times throughout a single 14.5/9.5 light/dark summertime diurnal cycle. Animals of the same age were sampled at 3 hr intervals from 09:30 to 09:30. Data points are the average (± SD) of the number of PCNA-immunoreactive cells from the nasal pole to the temporal pole (excluding the peripheral growth zones). Cells were counted in 4 sections from each of 3

to 4 fish at each time point. The beginning and end time points were also averaged. To compare data throughout the diurnal cycle, cell counts were normalized with respect to the counts measured at 06:30 AM (shortly after sunrise). Data points indicated by an asterisk are significantly different from the control density (at 06:30, $p < 0.05$; ANOVA with Tukey multiple comparisons test).

Fate of the PINCs

We hoped to determine the fate of the proliferating cells in the INL by labeling them with BrdU sustained for 48 hrs, and then allowing them to reach maturity in the absence of label. In fish sacrificed immediately at the end of BrdU delivery, we observed numerous clusters of PINCs as well as labeled nuclei in the ONL. In contrast, in fish sacrificed 30 days after the end of BrdU delivery there were few, if any, BrdU-labeled cells in the INL. The ONL, of course, had numerous labeled nuclei, presumably all in mature photoreceptors (Johns and Fernald 1981). The loss of labeled cells in the INL could arise from one or a combination of several possible mechanisms: 1) dilution of the label because of continuing cell division, 2) migration of the cells away from the INL, either to other layers in the retina or to other tissues, and 3) programmed cell death. Dilution of the label is an unlikely mechanism. If PINCs divided continuously and their progeny resided in the INL, labeled cells would disappear, but the density of INL cells would also increase with time. It is well known, however, than in all teleosts, including trout, the density of cells in the inner nuclear layer decreases continuously as the retina ages (Johns and Easter 1977, Johns 1977, Kock 1982).

Migration of PINCs to the ONL

The clusters of PINCs in trout of all ages are anatomically similar to the "neurogenic clusters" described by Johns (1982) in the retina of larval goldfish. She proposed that these clusters served as the source of the stem cells that give rise to the rod progenitors in the ONL, and showed that cells of a cluster can occasionally be found in

the outer plexiform layer (Raymond and Rivlin 1987). We tested the thesis that in the retina of adult trout, PINCs serve the same function as the "neurogenic clusters" in larval goldfish and, therefore, migrate from the INL to the ONL. We took advantage of the technical possibility of recognizing the history of repeated cell divisions by labeling proliferating cells with IdU or BrdU provided in sequence. Cells labeled with one or the other nucleotide can be specifically identified with immunohistochemistry.

We executed the following experiment. Thirty trout raised from the same batch of eggs and all of nearly the same size and weight (1.06 ± 0.08 g, mean ± SD, range=0.99 - 1.23 g) were transferred to 0.5 l water with IdU (2 g/liter), fed as usual and kept at 15 °C for a total of 43 hrs. Water was exchanged after 24 hrs with fresh IdU solution. After 43 hrs, 3 fish were removed and their eyes processed as described under Methods. These animals defined the conditions at time zero. The remaining animals were rinsed in water and then transferred to 0.5 liter of water with BrdU (4 g/liter) maintained at 15 °C. The water was exchanged for fresh BrdU solution every 24 hrs. Two or 3 animals were removed from the BrdU-containing water at selected time points up to 196 hrs after transfer to BrdU. Immediately upon removal fish were sacrificed and their eyes processed as described under Methods.

Figure 5 shows typical micrographs of retinal sections immunoreacted to selectively identify cells labeled with IdU alone, and those labeled with BrdU alone or with both nucleotides. It was critically important to distinguish cells labeled with IdU alone from those labeled with BrdU only or a combination of labels. This task was made easier by the fact that the BrdU reaction product was fluorescent, but the IdU reaction

product was not. To count cells we photographed the same microscopic field successively under brightfield and under epifluorescence. We counted cells in the photographic images and normalized data as described under Methods.

Figure 5: Micrograph of radial sections of trout retina immunolabeled with BrdU and IdU-specific antibodies. The 3 mm sections were cut from temporal to nasal poles along the equator. Shown are the nasal poles, including the PGZ. Fish were incubated in 2 g/L IdU for 43 hours, and then transferred to 4 g/L BrdU. Panels A and B are the same section, obtained from a fish sacrificed 6 hours after its transfer to BrdU. Panels C and D are the same section, obtained from a fish sacrificed 196 hours after its transfer to BrdU. Panels A and C were photographed under transmitted light, where IdU-labeled cells appeared blue, while BrdU-labeled cells appeared red. Panels B and D were photographed under epi-illumination, where only the BrdU labeled cells appeared fluorescent. It was thus possible to unequivocally determine whether cells contained IdU only or both labels. The extensive fluorescent labeling of section D, when compared with section B, confirms that it was obtained from a fish exposed to BrdU for a longer time period than that in section B. Scale bar is 100 μm.

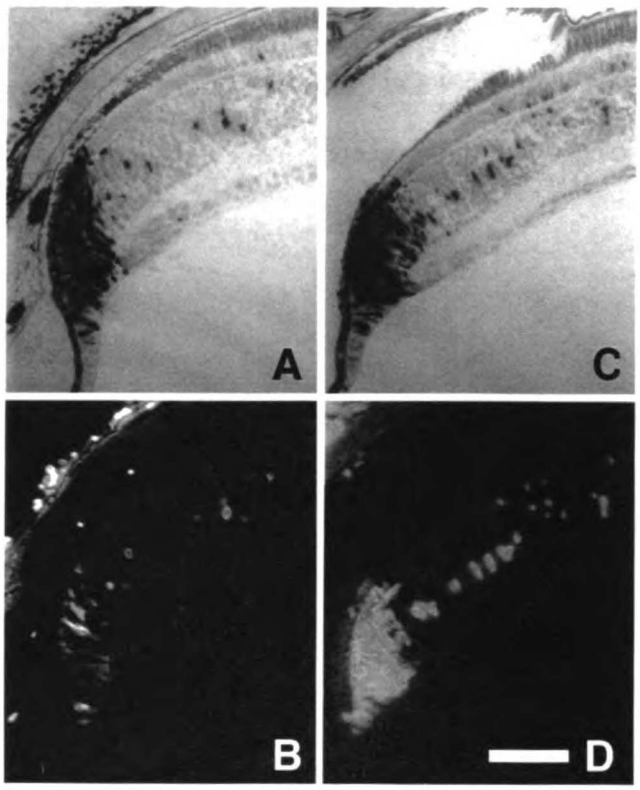

A
C
B
D

Figure 6 illustrates cell counts in the INL and ONL. Each data point is the average (± SD) of counts made in 4 equatorial sections from each of 2 to 3 fish collected at the time points indicated in the abscissa. Counts of cells labeled with IdU alone are presented in the left panels, whereas the right panels illustrate counts of cells labeled with BrdU or a mixture of BrdU and IdU. Cells labeled with IdU alone are cells that were in "S" phase sometime in the 43 hr period when only IdU was available and did not then divide again. That is, they were in their terminal mitosis sometime in the 43 hr period. Cells labeled with both labels were in "S" phase when IdU was available, and then again when BrdU was available. BrdU-only labeled cells were in "S" phase, perhaps more than once, exclusively when BrdU was available.

Figure 2.6: Migration of IdU-labeled cells from the INL to the ONL

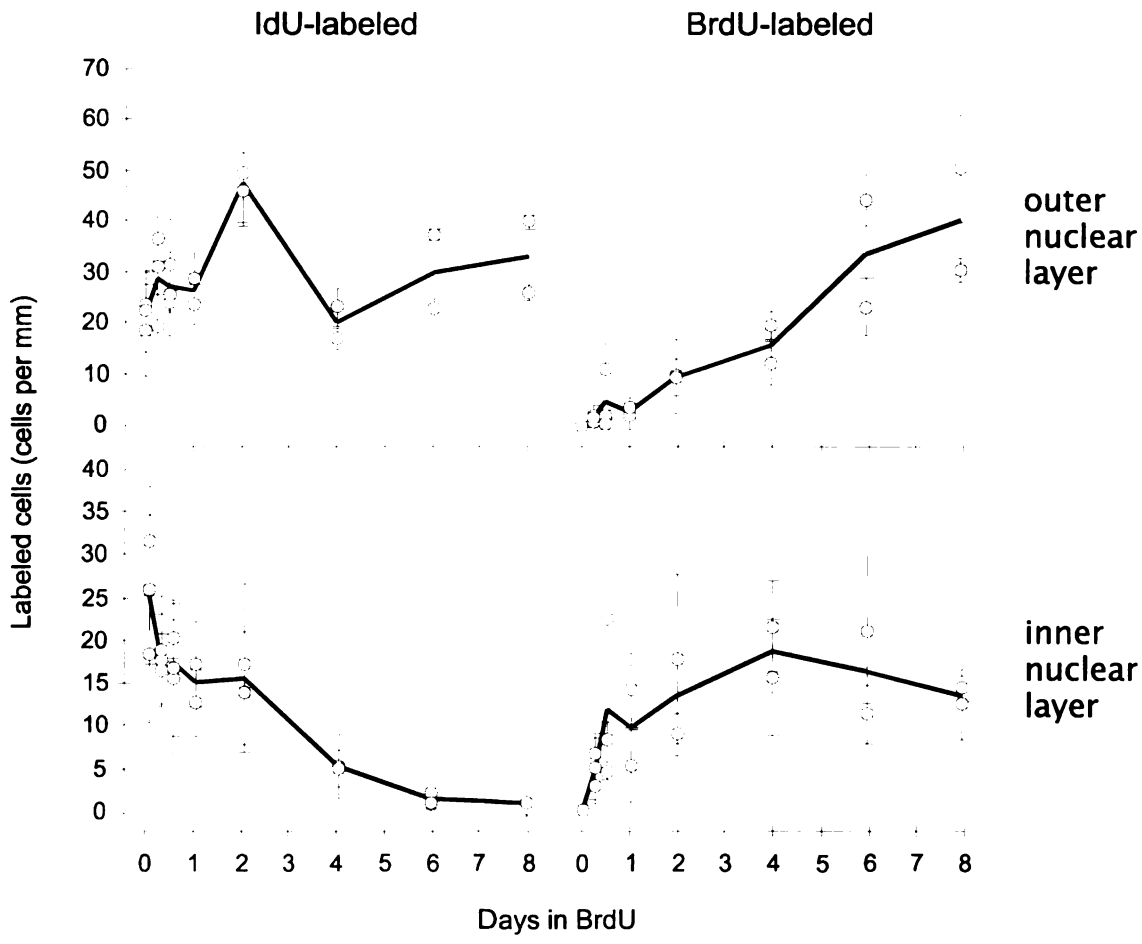


Figure 6: Counts of labeled cells in the outer and inner nuclear layers of rainbow trout exposed to IdU for 43 hours, and then exposed to BrdU starting at time indicated as zero in the abscissa. Fish were sacrificed at various times after first being exposed to BrdU. The density of IdU- and BrdU-labeled cells in the ONL (top panels) and INL (bottom panels) was determined in the equatorial plane, in 1.8 mm length of retina adjacent to the peripheral growth zone at the nasal pole. Each point is the average (± SD) of counts in 4 to 6 sections of a single fish. The IdU-labeled cells can be seen to

IdU-only labeled cells continuously disappeared from the INL. The loss of the cells had a roughly exponential time course with a half-time of about 3 days following the switch to BrdU. At the same time that these cells were disappearing, BrdU-only and double-labeled cells appeared and reached a steady state level about 4 days after the switch to BrdU. These results confirm that the loss of IdU label is not simply due to dilution due to continued cell division. If this were the case, then the BrdU-only and double-labeled cells would have increased linearly in time. That a steady state is reached indicates that in the INL there exists a kinetic balance between the birth of new cells and either their migration away from the INL or their death.

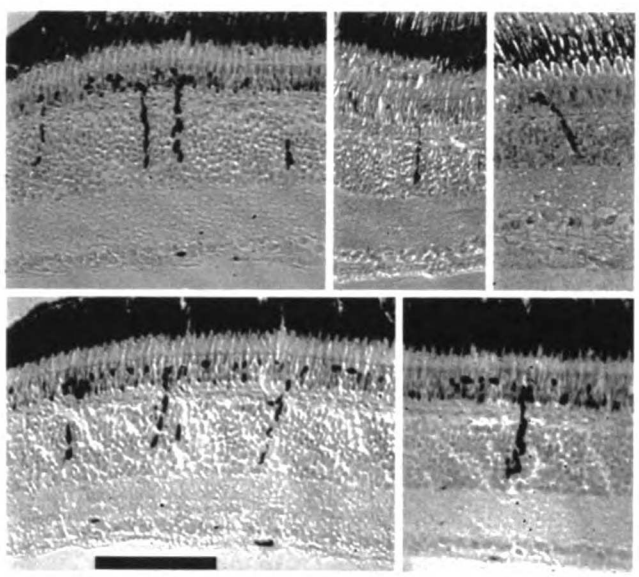
The experimental results also demonstrate that IdU-only labeled cells actually migrate out of the INL and appear in the ONL The data shown in Figure 6 indicates that at the time fish were switched from IdU to BrdU, there already existed a steady number of IdU-only cells in the ONL. These cells were either born in the ONL or migrated from the INL during the incubation in IdU. About 1 day after switching to BrdU there occurred a large and transient increase in the number of IdU-only cells in the ONL. These added cells can only have come from the INL since they cannot arise from cell division in the ONL. Any cell that divides in the ONL after switching to BrdU would be labeled with BrdU-only or a mixture of the nucleotides. Also, the added IdU-only cells that migrated from the INL likely divided again in the ONL, thus explaining why the rise in cell count was transient. That continuous cell division occurs in the ONL without loss of cells is apparent because the number of BrdU-only or mixed label cells increased linearly with

time. Contrast this linear rise with the saturation in labeled cell number observed in the INL.

Cells newly born in the INL cross the outer plexiform layer

Our results with double-labeled cells indicate that a fate of clusters of newly born cells in the INL is to migrate into the ONL and likely replenish the pool of stem cells that give rise to rod progenitors. Direct observation of cells in these clusters crossing from the INL to the ONL would make the evidence presented above even more compelling. We searched under the microscope for evidence of this migration in sections immunostained with anti-BrdU antibody. We searched in retinal sections of fish exposed for 36 hrs to BrdU added to tank water. In Figure 7 we show selected examples of clusters of BrdU-positive cells crossing the OPL. The frequency of these events was small and we could not use an unbiased method to calculate this frequency since we specifically searched sections to find examples of this event. Nonetheless, Figure 7 demonstrates that bodies of newly born cells can be seen to cross the outer plexiform layer. Since these bodies are elements in a cluster of cells and clusters exist in the INL and not the ONL, then these cells must be migrating from the INL to the ONL and not the other way around.

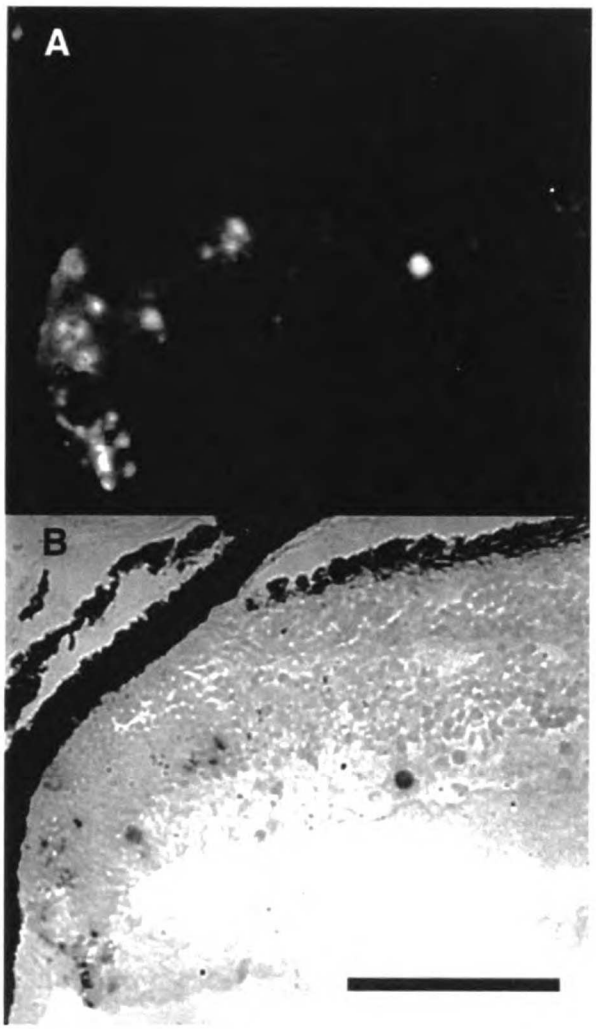
Figure 7. Micrographs of radial retinal sections immunoreacted to identify PCNA-positive cells. Sections from different fish were selected to demonstrate PCNA-positive cells that form parts of clusters and are localized in the outer plexiform layer. The scale bar is 100 μm.



There are no detectable apoptotic cells in the mature INL

If in addition to migration, PINCs were to die a programmed death through apoptosis, this event could be diagnosed through specific histochemical reactions. We used the TUNEL method to identify cells undergoing apoptosis (Gavrieli et al 1992). Few cells were positively identified as apoptotic by this method. In 32 histologic sections from 5 fish, a total of 15 apoptotic cells were identified in the neural retina, and almost all of these were in or near the PGZ. Figure 8 illustrates a positive control that establishes that our failure to detect apoptotic cells in the mature INL was not due to the inadequacy of our histochemical method. We applied the TUNEL method to retinal sections of 5-FU treated fish. In these animals TUNEL detected apoptotic cells in the PGZ, as expected, since 5-FU causes apoptosis of proliferating cells. Indeed, in 5-FU treated fish, the PINC cells could be identified as dividing cells because they were apoptotic (data not shown). Thus, we have no evidence that PINCs disappear from the INL because of death by apoptosis.

Figure 8. Micrograph of a retinal section of a trout exposed to 5-fluorouracil. The same section was photographed under epi-illumination (panel A) or brightfield illumination (panel B). The section was processed with the TUNEL method to detect cells in apoptosis. Apoptotic cells, those that were undergoing cell division during the exposure to 5-FU appear darkly stained under transmitted light (panel B) and fluorescent under epi-illumination (panel A). The scale bar is 100 μm.


A
B

DISCUSSION

We have discovered in the retina of growing trout, cells that divide and proliferate in the mature inner nuclear layer. The cells form clusters that align vertically, reaching from the inner to the outer plexiform layers, and we refer to them as proliferative inner nuclear layer cells (PINCs). The INL is primarily composed of the nuclei of fully differentiated neurons (horizontal, bipolar and amacrine cells) that are not expected to undergo cell division. PINCs could be dividing Müller (glial) cells, but they are almost certainly not because, as we have shown, the nuclei of the progeny of the dividing cells do not accumulate in the INL. Indeed, double-label experiments have allowed us to demonstrate that PINCs migrate from the inner to the outer nuclear layer with a time constant of about 3 days after their birth. We have no evidence that PINCs undergo apoptosis in the INL. However, we have not quantitatively accounted for all the PINCs. It is possible that some of them may still undergo a program of cell death which is not characterized by the production of small fragments of nuclear DNA, the deterministic feature tested by our method of assay.

The PINCs, once in the outer nuclear layer continue to divide and they most likely then differentiate into rod precursors. The precursors give rise to rod photoreceptors that are continuously added to the ONL of the growing fish retina (review Fernald 1991). In this respect, what we call PINCs in the mature trout retina are almost certainly the same cells identified by Raymond (1982) in the retina of the larval goldfish and referred to as "neurogenic clusters." Those cells also form vertically aligned clusters and migrate from

the inner to the outer nuclear layer (Raymond and Rivlin 1987). The normal function of the PINC, to replenish the pool of rod precursors in the ONL, must be regulated to achieve a balance in which the rate of replacement of precursors is matched to the rate of rod differentiation. The rate of rod differentiation, on the other hand, must be controlled to match the rate of retinal growth. Trout grow much more rapidly than goldfish. For example, goldfish take about 2 years to reach a body length of about 8 cm (Johns 1982), but it only takes about 8 weeks post-hatch for trout to reach this size. Since eye size and body size are proportional, it follows that the eye in the trout grows more rapidly than in goldfish and, therefore, the rate of rod proliferation is higher in trout than in goldfish. It is not surprising, therefore, that PINC cells are readily observed in trout at least over their first 2 years of life, but they have been previously observed only in larval goldfish, when retinal growth must be substantially faster than in adults.

There are also technical aspects to consider in explaining the experimental detection of PINCs in the retina of adult trout but only in larval goldfish and not at all in other teleosts before. Previous studies have used single intraperitoneal or intraocular injections of labeled deoxynucleotides to discover mitotic cells in the retina (Sandy and Blaxter 1980, Johns 1982, Hagedorn and Fernald 1992). This method only detects cells that divide within a time window defined by the metabolic half-life of injected nucleotide (about 2 hrs after intraperitoneal injection, Hagedorn and Fernald 1992). The methods we used (continuous intraperitoneal infusion or continuous immersion) created longer-lasting labeling windows. Indeed, we have observed PINCs in both juvenile goldfish and zebrafish after immersion in BrdU-containing water for 24 to 36 hrs. Even under these

experimental conditions, however, clusters of PINCs are infrequent in these species. Thus, extended exposure to labeled deoxynucleotides favors detection of infrequent events of cell division.

Although PINCs may be difficult to detect in slow growing retinas because they divide infrequently, the cells are likely to exist in all teleost retina. Their prevalence, and thus the ability to detect them, is simply a reflection of the physiological state of the retina. They reproduce at a rate just sufficient to meet the demands of tissue growth. This possibility is made strikingly apparent by the experimental results of Negishi and Shinagawa (1993) and Hitchcock et al (1996). Negishi and Shinagawa (1993) found that in 5-6 cm long goldfish, intraocular injection of several growth factors, control proteins and even saline alone resulted in the appearance of clusters of PCNA-positive cells in the INL. These clusters align radially from the inner to the outer plexiform layers and appear identical to the PINCs we have described here. Hitchcock et al (1996) found that in goldfish injected with BrdU, excision of small pieces of retina lead to the appearance of BrdU-labeled cells in the mature INL of the retina near the area of damage. These cells were arranged in clusters that spanned the INL from the inner to the outer plexiform layers. These clusters, again, appear identical to the PINC clusters we have described here. The experiments of Negishi and Shinagawa (1993) and Hitchcock et al (1996) both measure the cellular response of the retina to injury. We propose that this response includes signals that upregulate the rate of cell division of PINCs that are otherwise present, but dividing infrequently, preceding injury.

Teleost retina regenerates when damaged mechanically (Hitchcock et al 1992, Cameron and Easter 1995) or chemically (Maier and Wolburg 1979, Raymond et al 1988, Braisted and Raymond 1993). The tissue regenerates from stem cells located within the retina itself. The hypothesis has been advanced that these stem cells are located in the ONL and are the same cells that generate rods under normal conditions (Raymond et al 1988, Hitchcock et al 1992). This hypothesis is supported by the findings that retinal damage causes an increase in the rate of cell division in the ONL and that regeneration occurs only if the ONL is damaged. Damage limited to the INL only does not activate tissue regeneration (Raymond et al 1988, Hitchcock et al 1992). In light of this thesis, it is surprising that an antibody against *Pax6*, an early developmental regulatory gene, does not label rod precursors in the normal retina, but does label the neural progenitor cells that give raise to the regenerated tissue (Hitchcock et al 1996). The surprise arises from the fact that *Pax6* appears to be transiently expressed in retinal cells at a developmental stage that precedes rod specification: *Pax 6* antibody labels all cells in the PGZ and the earliest embryonic retina. Thus, for the thesis to be correct it would be necessary for rod precursors, which do not express *pax6*, to dedifferentiate to an earlier, *pax6* expressing stage. The identification of PINCs as the cells that generate the rod precursor cells may explain this experimental paradigm. It may simply be that the ONL has two types of undifferentiated cells: PINCs, which are pluripotential cells able to regenerate every one of the retinal neurons, and the rod precursor cells, which are descendants of PINCs and whose developmental fate has become restricted to photoreceptors.

Recent findings in the developing *Xenopus* retina further suggest that in embryonic *Xenopus* where, like in fish, retinal development occurs in a PGZ (Beach and Jacobson 1979) there also exist radially aligned clusters of dividing cells embedded in the otherwise mature INL (Dorsky et al 1997). Anatomically, these clusters resemble those of PINCs we have described here. The clusters of INL cells in *Xenopus* transiently express Delta and Notch gene products with the same pattern and at the same time as the cells in the PGZ (Dorsky et al 1997). In Xenopus, these gene products are theorized to enable differentiating retinal cells to respond to inductive factors that specify cell fate. If the same were the case in fish retina, these data would support the proposition that PINC clusters may be in a developmental stage that precedes that of rod precursors and is analogous to that of the neuroepithelial cells in the PGZ.

Raymond (1982) first proposed that rod precursors are left in place in the ONL during embryogenesis through the migration of undifferentiated cells from the INL to the ONL. These undifferentiated cells form the neurogenic clusters in the INL. The thesis was extended to the growing eye, where it was proposed that rod precursors are generated by the migration from the INL to the ONL of cells from neurogenic clusters that exist only within the peripheral growth zone (Raymond and Rivlin 1987). Hagedorn and Fernald (1992) also proposed that rod precursors are stem cells left behind during the wave of neurogenesis that creates the retina. They considered the theoretical possibility that these stems cells might be initially located in the INL, but argued that it was more parsimonious to expect them to be located in the ONL from the very beginning. Our findings support and extend Raymond's (1982) thesis. Cells from the neurogenic

clusters, the cells we call PINCs, migrate from the INL to the ONL and generate the rod precursors. This migration, however, is not restricted to a larval period or to the peripheral germinal zone. It occurs throughout the retina, although it is more prevalent near the PGZ, and continues in the growing eye of sexually mature fish. Moreover, PINCs may be in a state of differentiation that precedes that of rod precursors and is less restricted in its developmental fate. These cells may be the ones that respond to signals generated by mechanical or chemical injury and regenerate the entire retinal tissue.

CHAPTER THREE:

RETINAL CELL PROLIFERATION IN RESPONSE TO LIGHT DAMAGE

INTRODUCTION

Rod photoreceptors in the retinas of all vertebrates are exposed to high intensity light levels while operating in an environment high in oxygen tension. These factors, in combination with the high content of polyunsaturated fatty acids in the rod outer segment, create conditions under which the potential for cell injury is high. Yet, in normal pigmented eyes there is no apparent rod damage under normal conditions of luminance. The mechanisms responsible for rod maintenance in a seemingly unfavorable environment have been recently reviewed (Organisciak and Winkler 1994). Under normal conditions, damage of rod outer segments is limited both because the membranes in the outer segment are continuously renewed and because specific mechanisms of cell repair or cell protection exist in these cells (Penn and Williams 1986, Penn and Anderson 1991). However, abnormal conditions such as prolonged exposure to bright light, cause photoreceptor damage and, in the extreme, cell death (Noell 1980, Kremers and van Norren 1989).

The retina of rodents is damaged by exposure to toxic light levels and has, therefore, served as a prototypical model to study the mechanisms of light damage in the human retina (Organisciak and Winkler 1994). Rodents, however, are not representative of all forms of light-dependent retinal damage among vertebrates. A case in point is the difference between rats and teleost fish. Albino rats raised in dim light (6 lux) exhibit

significant retinal damage when exposed to light levels in the range between 65 and 135 lux (Semple-Rowland and Dawson 1987). In these animals, the rod photoreceptors are severely damaged and their outer segments disappear, with rods being injured much more extensively than cones (Cicerone 1976, LaVail 1976). In contrast, albino rainbow trout exposed to natural summertime sunlight (~100,000 lux) exhibit only partial destruction of the rod outer segment, while the rod inner segments and the entire cone photoreceptors are unaffected (Allen and Hallows 1997).

Allen and Hallows (1997) have described in detail the retinal morphology and visual sensitivity of pigmented and albino rainbow trout (*Oncorhyncus mykiss*) exposed to natural summer sunlight. The anatomical features of the retina in pigmented trout are not significantly different from those found in comparable fish raised indoors. The retina of the albino trout raised outdoors, on the other hand, exhibits structural damage. Exposure of albino trout to about 4 months of natural summertime light causes a decrease both in the number (about 1/3) and the length (about 1/6) of rod outer segments when compared with size-matched pigmented fish exposed to the same luminance. However, there is no detectable structural damage of the rod inner segment nor of any part of the cone photoreceptors. In studies of visual performance, albino and pigmented fish reared outdoors display similar scotopic visual sensitivity immediately after being placed in darkness, and therefore prior to dark adaptation (as expected since cones are unaffected). On the other hand, pigmented fish increase their sensitivity by about 2.5 log units upon full dark-adaptation, whereas the dark-adapted sensitivity of albino trout increases only about 0.5 log units.

Remarkably, the light-damaged retina of albino trout is fully repaired when fish are returned to light levels about 1000-fold less intense than those of natural sunlight. After about 30 days indoors, both the number and size of rod outer segments in damaged albino trout are indistinguishable from those of pigmented fish. Teleost retinas, thus, are endowed with remarkable repair mechanisms that both limit the extent of damage while exposed to toxic light levels and support the rapid repair of rod photoreceptors. The cellular basis of such mechanisms are presently unknown.

Unlike other vertebrates, the retina in teleost fish continues to grow throughout much of the animal's life (review in Fernald 1991) and has the capacity to regenerate when some of its mass is destroyed by chemical or mechanical injury (review in Hitchcock and Raymond 1992). Normal retinal growth reflects both a stretching of the mature retina and the ongoing development of new tissue in a narrow band (about 300 μm wide) at the peripheral margin of the mature retina termed the peripheral germinal zone, or PGZ (Lyall 1957, Müller 1952, Johns and Easter 1977). Cell addition also occurs along a fissure that extends from nearly the center of the eye to the margin along the ventral pole of the eye (named embryonic or choroid. Fissure; Kunz 1987). As the mature retina stretches, the density of all cell types declines, except that of rods. This is due to the continuous and specific addition of new rods to the photoreceptor layer. The added rods originate from mitotically active progenitor cells embedded in the outer nuclear layer (ONL) of the differentiated retina (Sandy and Blaxter 1980, Johns and Fernald 1981, Johns 1982). We have suggested (Chapter 2) that in the retina of rainbow trout the rod progenitors in the outer nuclear layer are continuously replenished by

undifferentiated cells that are born in the inner nuclear layer (INL) and then migrate into the ONL. The newly born cells in the INL form clusters scattered throughout the retina and are referred to as "neurogenic clusters." Individual cells migrate from the neurogenic clusters and across the outer plexiform layer about 2 to 3 days after their birth.

Whereas rod photoreceptor nuclei decrease in number in light-damaged rodent retina, rod photoreceptor nuclei in light-damaged albino trout retina remain at a constant level. In a biological steady state, the rate of cell birth and migration from the neurogenic clusters to the ONL must be in dynamic balance with the rate of rod differentiation. If this were not the case, the number of nuclei in the ONL and, therefore, its thickness would either increase or decrease continuously. This, of course, does not happen. The existence of neurogenic clusters in trout may provide the retina with the ability to adjust the rate of creation of rod progenitors, and this ability may, in turn, endow the retina with mechanisms to maintain and/or repair its rod photoreceptors in the face of biological challenges that might damage these cells, such as, for example, the damaging effects of bright light on rod photoreceptors of albino trout (Allen and Hallows 1996).

If the neurogenic clusters provide the retina with a cellular mechanism to repair and maintain its rod population under toxic conditions, then it may be expected that the number of neurogenic clusters or the rate of transfer of cells from the INL to the ONL will change in response to the toxic challenge. In collaboration with Don Allen at the University of Texas, Permian Basin, we have tested this hypothesis by determining the density of neurogenic clusters and rod progenitors in pigmented and albino trout exposed to toxic light levels. Our results indicate that the density of neurogenic clusters is indeed

under regulation and it does increase in response to the toxic challenge. These results are consistent with our hypothesis that, rather than being repaired, at least some damaged rod photoreceptors are being wholly replaced.

MATERIALS AND METHODS

Experimental animals.

Experimental animals were raised and maintained at the Kamas State Fish Hatchery in Summit County, UT. Normal, pigmented rainbow trout and tyrosinase-negative albino rainbow trout were received as embryos in January from the Egan Hatchery (Wayne County, UT). Both pigmented and albino trout were raised indoors in dim, cycling light (shaded sunlight) until April, at which time they were transferred to outdoor raceways where they were maintained until mid May. At that time, both pigmented and albino fish were separated into three experimental groups: Group "O" remained in the outdoor raceway, which was constructed of unpainted concrete. Group "L" was brought indoors on May 9th, but fish were then kept under constant light (Tungsten sources). Group "S" was brought indoors on May 19th and, using neutral density shades, the indoors "S" group fish were exposed to natural sunlight that was 1/1000 of the intensity measured outdoors. All fish in the three groups were sacrificed on June 5th. As described before (Allen and Hallows 1997), the nearly 1 month interval under dim light was sufficient for the damaged retina of albino animals to be repaired to an anatomical state essentially indistinguishable from that of fish that had never been damaged by light. Thus, the "S" group serves as a measure of the features of both "undamaged" and "repaired" retinas.

Retinal histological sections

Fish were killed by decapitation and the eyes enucleated, slit along the sclera and placed in cold (8-12 °C) fixative (2% paraformaldehyde-2% glutaraldehyde in 0.087 M phosphate buffer, pH 7.3, with 3% added sucrose). After 30 min, eyes were hemisected and the posterior pole was fixed in the cold for at least 24 hrs. Dark-adapted eyes were processed under deep red light during the first hour. Following fixation, the eyecups were washed in buffer, post fixed in 1% osmium tetroxide to enhance outer segment staining, and embedded in a mixture of Epon 812/Araldite 502. Radial sections of eyecup were cut across the posterior pole to include at least 1 mm of retina to either side of the optic nerve exit point. Sections were stained with toluidine blue. Rod outer segment length and number as well as nuclear density in the ONL were measured with the use of a calibrated ocular reticle.

PCNA immunohistochemistry

Fish were killed by decapitation and then pithed. Eyes were removed, a small incision made in their sclera and then fixed overnight at 4 °C in alcoholic formaldehyde (9:1 95% ethanol:40% formaldehyde) (Bancroft and Stevens 1990). The eyes were rinsed with water and immersed in 30% sucrose for 12 hrs. They were then hemisected and the anterior segment discarded. The eyecups were placed in 1:1 v/v 30% sucrose:Tissue-Tek O.C.T. compound (Miles Inc.) and kept at 4 °C until further processed.

Eyecups were transferred to O.C.T. compound, frozen and 16 μm thick sections cut in a freezing microtome. The sections were cut along the equator between the nasal and temporal poles. Sections were rehydrated in PBS/T for 10 minutes, incubated for 1 hr in blocking serum (20 μl/ml horse serum) and then incubated overnight at 4 °C in a mixture consisting of 1:50 horse serum and 1:100 of mouse mAb against PCNA (PC10, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS with 0.3% Triton-X and 1% DMSO (diluent solution). Following this incubation sections were rinsed with PBS/T and incubated for 2 hours in 1:50 Horse serum and 1:100 biotinylated horse anti-mouse Ab in the diluent solution. To reduce nonspecific background staining, the sections were again rinsed in TBS/T and incubated for 30 min in casein blocking solution (0.5% casein in Dulbecco's PBS with 0.05% Tween-20 and 0.5 g/liter Na azide). After rinsing with PBS/T the sections were incubated for 1 hr with reagents of the Vectastain ABC-AP kit (Vector Labs, Burlingame, CA). After thorough rinsing with PBS/T, alkaline phosphatase activity was detected with Red AP substrate (SK-5100, Vector Labs, Burlingame, CA). Sections were rinsed with water, blot dried and mounted in Gel/Mount (Biomeda, Foster City, CA).

Cell counts

Immunostained, 16 μm frozen sections were observed at 160x and every PCNA-positive cell in the INL between the nasal and temporal margins was manually counted. Abundant PCNA-positive cells in the marginal growth zones were carefully excluded from the counts. For the counts of PCNA-positive cells in the ONL, the slides were

coded so that the person counting was "blind" to the treatment protocol. Next, a low magnification image of the entire section was captured with an imaging system (Sony color CCD camera with a Nikon Micro lens and Macintosh 8100 computer under the control of "NIH-image" software). Analytical tools in the software package were used to determine the length of the section from one margin to the other. In order to compare among the various experimental groups, cell counts were normalized by the length of the sections.

RESULTS

In the experiments reported here we investigated the density of "neurogenic clusters" in pigmented and albino trout both exposed to toxic light levels and upon return to less intense light. "Neurogenic clusters" are clusters of newly born cells found in the inner nuclear layer of trout of all sizes (at least up to 3 kg), but the density of neurogenic clusters in trout decreases in larger, more mature trout (Chapter 2). Therefore, valid comparisons among various animal groups can only be made if the size and age of the animals in each group is similar. In these experiments each of the six experimental groups investigated consisted of four individual fish. Table 1 details the mean, standard deviation and range of lengths of animals in each group. As intended, the size of fish in each experimental group are not statistically different.

Table 3.1: Distribution of body lengths

Experimental Group	Mean	SD	Range
Outdoors Albino (OA)	6.22	0.55	5.7-7.0
Outdoors Normal (ON)	5.90	0.87	5.1-7.0
Continuous Light Albino (LA)	7.40	0.42	7.0-8.0
Continuous Light Normal (LN)	6.87	0.37	6.5-7.2
Shade Indoor Albino (SA)	6.37	0.47	6.0-7.0
Shade Indoor Normal (SN)	6.50	0.45	6.1-7.1

Table 1. Statistical distribution of body length in the experimental groups (length in cm, measured from tip of mouth to base of tail). Groups were not significantly different in length (ANOVA, $p > 0.05$).

Histological damage of the retina in fish exposed to toxic light levels.

Allen and Hallows (1997) have shown that exposure to natural summertime light damages the retina of albino, but not pigmented trout. This damage, a loss of rod outer segments, is reversed upon placing the fish indoors at light levels 1/1000 of the those measured outdoors. In the experiments reported here we exposed trout to the same experimental conditions of Allen and Hallows (1997) and confirmed their findings. Trout exposed to natural light outdoors are identified as group "O" and those subsequently returned to shade indoors are identified as group "S". We added a new experimental group to attempt to obtain more severe retinal damage. These fish, identified as group

"L", were exposed to the natural light outdoors along with the other groups, but they were then brought indoors and exposed continuously to bright tungsten light. While the luminance of the tungsten lamps was less than that measured outdoors, the light is continuous and lacks the diurnal dark-light cycle of the outdoors. In rodents, continuous light causes more damage than cyclic light of comparable intensity.

As expected, the retina of albino trout exposed to continuous light (group "L") trout was structurally damaged, while that of pigmented fish was normal. Histological analyses carried out by D. Allen demonstrated that the outer segment volume (number x length taking the rod outer segment diameter to be constant at 2.8 μm) in the damaged retina was about 1/6 that of the pigmented control eye. The extent of damage in the albino trout was not significantly different between the animals maintained outdoors with normal solar illumination and those maintained inside with reduced intensity continuous light (Allen and Hallows 1997). That is, continuous light was not more damaging than light with diurnal dark-light cycles of intensity.

Density of neurogenic clusters in the INL

We measured the density of neurogenic clusters by assaying the cellular expression of the proliferating cell nuclear antibody (PCNA) antigen in retinal sections. This nuclear protein is a cyclin synthesized in early G1 and S phases of the cell cycle and it serves as an excellent marker to identify cells that are undergoing DNA replication. Negishi et al (1990) first reported the successful use of PCNA antibody in fish retina. We have previously shown that this marker identifies clusters of newly born cells in the INL

of trout (Chapter 2). Some of these cells are destined to migrate across the outer plexiform layer into the ONL.

Figure 1 illustrates the average density of PCNA-positive cells forming neurogenic clusters in the INL in an equatorial retinal section from nasal to temporal margins. This counting method does not include the PGZ and noes not analyze the regional distribution of clusters within each section. We have previously reported that the density of neurogenic clusters is higher nearer the PGZ than in the center of the retina (Chapter 2). The data indicate that in normal pigmented trout the density of cells in neurogenic clusters is unaffected by any of the light conditions tested. The small differences in mean values are not statistically significant (ANOVA with Tukey multiple comparisons test, $p > 0.05$). This, of course, is what might be expected since their retinal structure was normal. In contrast, we observed a large increment in the counts of PCNA-positive cells in albino trout exposed to light levels that caused retinal damage ($p < 0.05$). The elevated cell counts under toxic light levels were essentially the same, independent of the method of retinal damage: cycling outdoor light ("O") or continuous tungsten light in the indoors ("L"). In albino trout in which the retina had recovered from structural damage (group "S"), the cell counts also returned to the values we observed in normal, pigmented trout.

Figure 3.1: PINC density in pigmented and albino trout after toxic light exposure

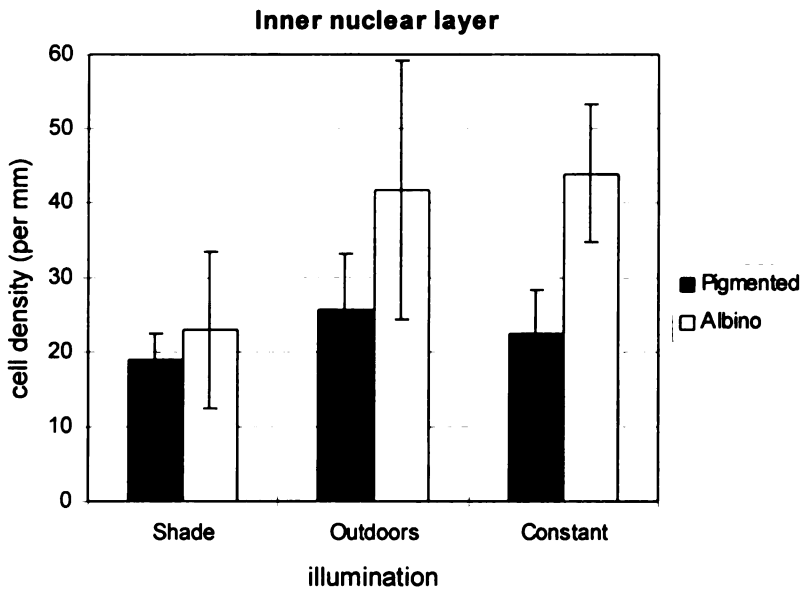


Figure 1. Change in density of PCNA-positive cells in the INL of pigmented (shaded bars) and albino (open bars) rainbow trout exposed to varying illumination treatments. Bars represent average density (± SD) in cells per radial mm of retina. Each bar is the average of measurements in 3 sections in each of 8 eyes (4 experimental animals). To compare between animals, cell counts in each section were normalized by the length of the section. See text for explanation of illumination treatments.

Density of PCNA-positive cells in the ONL

Due the high level of PCNA expression in the ONL, we were worried that bias might be introduced unintentionally to the counts by the person quantifying the cell counts. Thus, we coded the slides so that the person counting was blind to the treatment protocol. However, since the difference between retinas from pigmented and albino trout was clearly apparent during counting by the presence or absence of pigment in the "pigmented" epithelium, we could not make the person counting blind to this effect. As with the PCNA-positive cells in the INL, we counted all cells within an equatorial retinal section from nasal to temporal margins, and normalized the count to the length of the retinal section.

As with the INL, the density of PCNA-positive cells in the ONL of albino trout was significantly higher than the density in pigmented trout for both the constant light and outdoor light treatment groups (Fig 2). In albino trout in which the retina had recovered from structural damage, the cell counts were similar to the values we observed in normal, pigmented trout. However, although exposure to toxic light levels consistently increased the density of PCNA-positive cells in the ONL of albino trout when compared with the same treatment in pigmented trout, exposure to toxic light resulted in a significantly *decreased* density of PCNA-positive cells in the ONL of pigmented trout. This finding was robust and unexpected, and suggests that exposure to normal sunlight causes a change in the proliferative activity of cells in the ONL of pigmented trout.

Figure 3.2: PCNA-positive ONL cell density in pigmented and albino trout after toxic light exposure

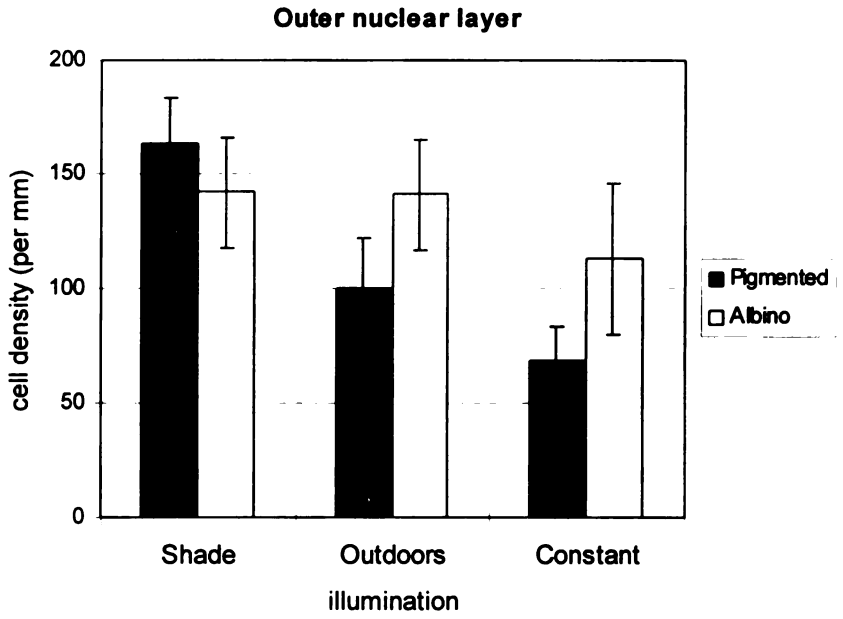


Figure 2. Change in density of PCNA-positive cells in the ONL of pigmented (shaded bars) and albino (open bars) rainbow trout exposed to varying illumination treatments. Bars represent average density (± SD) in cells per radial mm of retina. Each bar is the average of measurements in 3 sections in each of 8 eyes (4 experimental animals). To compare between animals, cell counts in each section were normalized by the length of the section. See text for explanation of illumination treatments.

In the ONL, PCNA-positive nuclei are present both along the inner (vitread) and outer (sclerad) border of the nuclear layer. Mack and Fernald (1995) (see also Chapter 2) have shown that mitosis occurs at the outer border of the ONL, and that the newly generated nuclei then take a final position along the inner border of the ONL. In contrast, we have found that PCNA-positive cells are located throughout the ONL (Chapter 2). However, the density of PCNA-positive cells is slightly higher along the inner than the outer border of the ONL in normal trout exposed to normal, cycling room light. We did not know whether the difference in location of PCNA-positive cells might be a factor in the responsiveness of these cells to photoreceptor light-damage. Therefore, when counting PCNA-positive cells in the ONL, we distinguished between nuclei found in the inner (vitread) one-half of the ONL from those found in the outer (sclerad) one-half.

Figure 3 illustrates the effect of light exposure on the density of PCNA-positive cells in the inner and outer ONL for pigmented and albino trout. The PCNA-positive cell density was consistently higher in the inner ONL than the outer ONL in albino trout, regardless of light exposure. However, toxic light exposure caused a significant decrease in the density of PCNA-positive cells in both the inner ONL and the outer ONL in pigmented trout, and eliminated the differential between the two halves of the ONL.

Figure 3.3: Comparison of PCNA density for outer and inner ONL

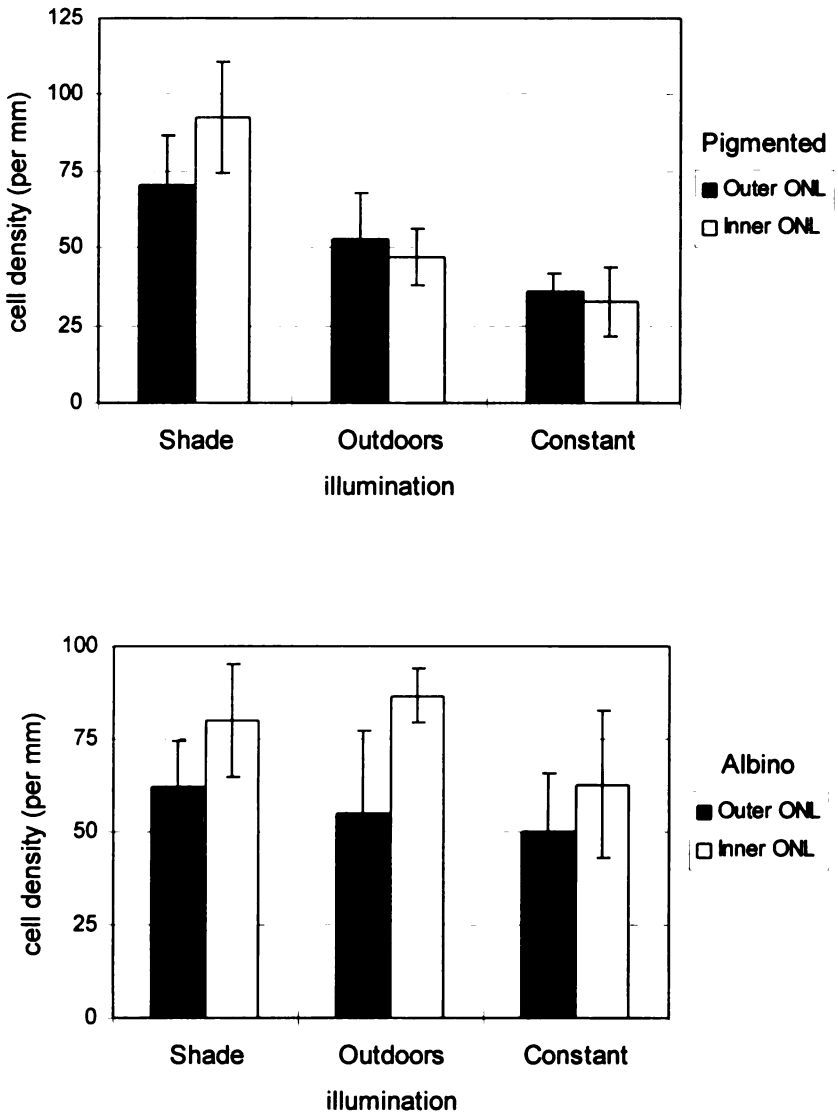


Figure 3. Comparison of PCNA density for outer ONL (shaded bars) and inner ONL (open bars). **Top**: pigmented trout. **Bottom**: albino trout. Bars represent average density (± SD) in cells per radial mm of retina. Each bar is the average of measurements in 3 sections in each of 8 eyes (4 experimental animals). To compare between animals,

cell counts in each section were normalized by the length of the section. See text for explanation of illumination treatments.

DISCUSSION

Previous studies have demonstrated that the teleost fish retina is sensitive to light damage. Fish raised under constant light exposure at 1240 lux for 14 days (Penn 1985) or 340 lux for one year (Raymond et al 1988a) suffered a partial loss of rod photoreceptor outer segments and decreased density of rod photoreceptor nuclei. Recently, Allen and Hallows (1997) demonstrated that albino rainbow trout exposed to normal summertime solar illumination can suffer increased retinal damage compared with pigmented animals. The damage in albino trout, however, is limited to a loss in the length and number of rod photoreceptor outer segments. This loss is reversed within several weeks of the animals' return to light about 1/1000 less intense than the toxic levels. Despite the damage to rod photoreceptor outer segments caused by solar illumination, cone photoreceptors do not appear to be damaged at all and the number of photoreceptor nuclei (both rod and cone) is unchanged. Since the number of cone outer segments is unchanged, the constant number of ONL nuclei indicates that there is no net loss of rod nuclei in the ONL of light-damaged albino fish.

An unaltered number of rod nuclei in the ONL could reflect a mechanism that prevents the loss of the rod nuclei present in the retina before the onset of bright light. However, an alternative explanation is that light causes loss of rod nuclei (as occurs in rodents exposed to bright light), but these rods are rapidly replaced by neurogenesis to maintain the original steady state nuclear density. An enhanced replacement rate of damaged rod nuclei in response to light damage is plausible because teleost retina do

continuously add new rods to the ONL from mitotically active rod progenitor cells located in the ONL. In trout, these rod progenitors are continuously replenished by mitotically active cells that migrate from the inner to the outer nuclear layer (Chapter 2). These cells form clusters in the INL that are named "neurogenic clusters".

If the reason that toxic light levels do not change the density of rod nuclei is that the rate of rod photoreceptor neurogenesis from rod progenitors is enhanced, then the retinas of light-damaged albino trout might display evidence of an upregulation of rod progenitor proliferation in the ONL and a decreased average age of rod photoreceptor nuclei. Furthermore, previous authors (Hagedorn and Fernald 1992) have suggested that rod progenitors, unlike typical stem cells, have an intrinsic limit to their quantity of cell divisions. If this is true, then the proposed increased rate of rod production after light damage would create an increased need for more rod progenitors. We have suggested (Chapter 2), that these new rod progenitors come from the neurogenic clusters in the INL. Thus, in addition to an upregulation of rod progenitor proliferation in the ONL and a decreased average age of rod photoreceptor nuclei, light damaged trout retina may also provide evidence of an increased transfer rate of cells from the INL to the ONL and upregulation of neurogenic cluster proliferation in the INL.

As a test of this hypothesis, we measured the density of PCNA-positive cells in the INL and ONL of normal, light-damaged, and repaired retina of albino trout. As a control for each experimental condition, we also measured the density of neurogenic clusters in pigmented fish exposed to identical luminance. As expected from our hypothesis, we found that exposure to toxic light levels increased the density of PCNA-

positive cells in the INL of albino trout by 60-90%, compared with pigmented trout exposed to the same illumination protocol. These results for albino trout support our hypothesis that the rate of neurogenic cluster cell proliferation would be enhanced in light-damaged retina, suggesting that the maintenance of rod photoreceptor inner segments during toxic light exposure, and the eventual recovery of rod photoreceptor outer segments after recovery in shade, is supported by proliferation in the INL.

In contrast to the results for INL proliferation, our results for ONL proliferation were somewhat ambiguous. As in the INL, increased PCNA-staining after toxic light exposure was also seen in the ONL, where the PCNA-positive cell density was increased in albino trout exposed to toxic light levels, when compared with pigmented trout. However, we were surprised to find that, unlike the results for the INL, toxic light exposure caused a decrease in the PCNA-positive cell density in the ONL for pigmented trout, and possibly also albino trout, when compared with similarly pigmented trout that had been returned to shade. This might seem to indicate that the rate of proliferation was decreased as a result of toxic light exposure. However, it is important to recognize that by counting PCNA-positive cells, we are actually getting an index of the number of cells that are in the process of proliferation, rather than the rate of their proliferation.

Thus, the decrease in PCNA-positive cell density could be at least partially influenced by a depletion of rod progenitors. However, this appears to be inconsistent with our finding that the PCNA-positive cell density was elevated in the ONL of albino trout when compared with pigmented trout, since we would expect even more depletion in this case. Recognizing that light damage in pigmented trout retina is not sufficient to

cause upregulation of proliferation in the INL (Fig 1), one explanation for this apparent discrepancy would be that the number of "replacement" rod progenitor cells available in the INL is insufficient to completely replenish the partially depleted rod progenitor pool in the ONL. In albino trout, however, where light damage does cause upregulation of proliferative activity in the INL, the rod progenitor pool in the ONL can be maintained at the same density as is found in trout raised in the shade.

Allen and Hallows (1997) found that the anatomical features of the retina in pigmented trout raised outdoors are not significantly different from those found in comparable fish raised indoors. However, as described above, our findings provide evidence that exposure to sunlight causes damage to photoreceptors in normal, pigmented trout. However, whether in albino or pigmented trout, we propose that light-induced damage leads to the death of at least some rod photoreceptors, presumably by apoptosis (Organisciak et al 1995, Remé et al 1995). Allen and Hallows (1997) did not identify an increase in the number of pycnotic nuclei in light-damaged retina, although they did see a four-fold increase in the number of macrophages in albino trout exposed to sunlight, compared with pigmented trout exposed to the same illumination. Cells that die by apoptosis can be rapidly scavenged by macrophages, and may be difficult to identify as pycnotic nuclei, since this morphological phase is transient and short-lived. However, the use of TUNEL labeling would increase the probability of positively identifying this event (see Chapter 2). Further experiments will be required to confirm that light-damaged trout retina contains more apoptotic nuclei in the ONL.

Previous studies have clearly demonstrated that surgical or neurotoxic damage of the fish retina is repaired through the generation of new retina that arises from the remaining, undamaged tissue (review in Hitchcock and Raymond 1992). For example, surgically removing a quadrant of the retina in adult goldfish causes proliferation of cells both in the PGZ and along the cut edges of the remnant retina (Lombardo 1972), leading to compete regeneration of the retina. Ouabain-induced cytotoxic damage of the mature retina of goldfish and rainbow trout also stimulates cell proliferation in the PGZ and in the damaged retina (Kurz-Isler and Wolburg 1982, Maier and Wolburg 1979, Raymond et al 1988). Here, again, the retina eventually regenerates.

Extensive experimentation (review in Hitchcock and Raymond 1992) has demonstrated that the regenerated retina derives not from cells in the PGZ, but locally from cells intrinsic to the mature retina. These progenitors of regeneration have been proposed to be rod precursors that have temporarily altered their fate in response to destruction of the retina (Raymond et al 1988, Braisted et al 1994). However, we have suggested (Chapter 2) that the proliferating INL cells, which are abundant in rainbow trout, are the ultimate source of this regenerative capacity, whether by supporting increased proliferation of rod progenitors, or directly by acting as multipotent stem cells. In support of this hypothesis, we would found increased proliferative activity of neurogenic cluster cells in the INL of light-damaged trout retina.

However, we have not conclusively demonstrated that the progeny of this increased proliferative activity in the INL eventually replace damaged rod photoreceptors. An alternative explanation is that light-damage causes the release of growth factors or

other signals associated with an injury response, and that these signals enhance neurogenic cluster proliferation. However, in this case there would be no need for the progeny of this excess proliferative activity, and these cells would need to be eliminated from the retina, either by apoptosis or migration. Finally, since the PCNA antigen is elevated in all phases of the cell cycle except the rest phase (G_0), it is also possible that the increased density of PCNA-positive cells in the INL does not reflect an increased rate of proliferation, but rather an increase in the time the neurogenic cells remain out of G_0. However, to our knowledge such a phenomenon has not previously been reported for the PCNA antigen.

Further studies will be required to demonstrate conclusively that light damage in albino trout, and possibly also pigmented trout, causes the death and eventual regeneration of rod photoreceptors. For example, utilizing the thymidine analogs bromodeoxyuridine and iododeoxyuridine to identify cell birthdates, as described in Chapters 1 and 2, would allow us the accurate quantification of photoreceptor renewal rates both before and after light damage. If light damage increases rod replacement, then this should be evident from an increased rate of rod photoreceptor renewal during and/or immediately following exposure of albino trout to full solar illumination. If, however, the maintenance of a constant density of rod photoreceptor nuclei reflects photoreceptor protection rather than photoreceptor replacement, then the rate of rod photoreceptor turnover should not be enhanced in light-damaged retina from albino trout, compared with pigmented trout or albino trout raised in shade.

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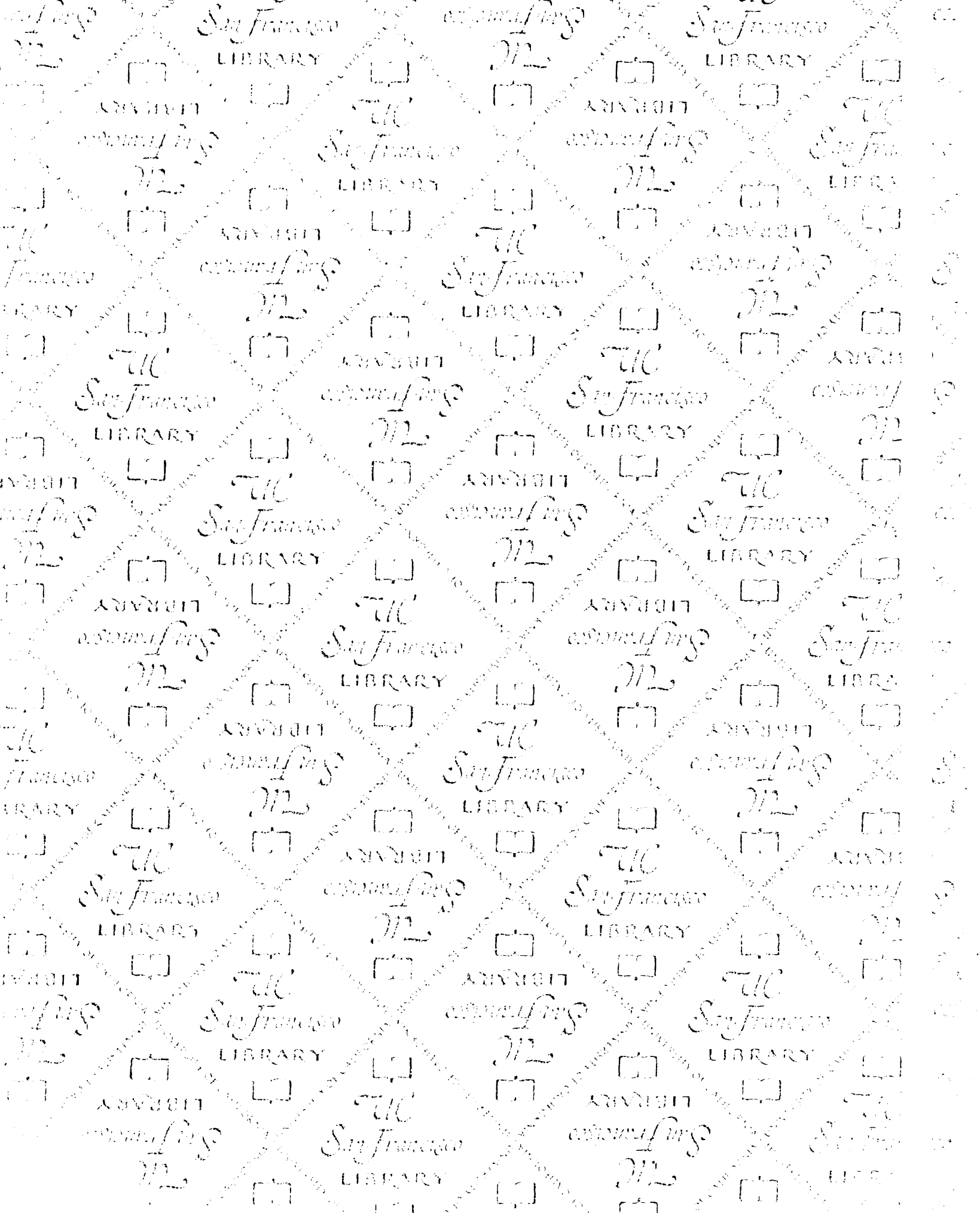
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